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A complex phenotype in a child with familial HDL deficiency due to a novel frameshift mutation in *APOA1* gene (apoA-I$_{Guastalla}$)

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KEYWORDS:
APOA1 mutation;
Truncated apoA-I;
Hypertriglyceridemia;
Metabolic syndrome;
β-thalassemia trait

BACKGROUND: We describe a kindred with high-density lipoprotein (HDL) deficiency due to *APOA1* gene mutation in which comorbidities affected the phenotypic expression of the disorder.

METHODS: An overweight boy with hypertriglyceridemia (HTG) and HDL deficiency (HDL cholesterol 0.39 mmol/L, apoA-I 40 mg/dL) was investigated. We sequenced the candidate genes for HTG (*LPL, APOC2*, *APOA5, GPIHBP1, LMF1*) and HDL deficiency (*LCAT, ABCA1* and *APOA1*), analyzed HDL subpopulations, measured cholesterol efflux capacity (CEC) of sera and constructed a model of the mutant apoA-I.

RESULTS: No mutations in HTG-related genes, *ABCA1* and *LCAT* were found. *APOA1* sequence showed that the proband, his mother and maternal grandfather were heterozygous of a novel frameshift mutation (c.546_547delGC), which generated a truncated protein (p.[L159Afs*20]) containing 177 amino acids with an abnormal C-terminal tail of 19 amino acids. Trace amounts of this protein were detectable in plasma. Mutation carriers had reduced levels of LpA-I, preβ-HDL and large HDL and no detectable HDL-2 in their plasma; their sera had a reduced CEC specifically the ABCA1-mediated CEC. Metabolic syndrome in the proband explains the extremely low HDL cholesterol level (0.31 mmol/L), which was half of that found in the other carriers. The proband's mother and grandfather, both presenting low plasma low-density lipoprotein cholesterol, were carriers of the β-thalassemic trait, a condition known to be associated with a reduced low-density lipoprotein cholesterol and a reduced prevalence of cardiovascular disease. This trait might have delayed the development of atherosclerosis related to HDL deficiency.

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CONCLUSIONS: In these heterozygotes for apoA-I truncation, the metabolic syndrome has deleterious effect on HDL system, whereas β-thalassemia trait may delay the onset of cardiovascular disease.

Introduction

High-density lipoprotein (HDL) deficiency is currently defined as an HDL cholesterol (HDL-C) level below 40 mg/dL (1.04 mmol/L) for men or below 50 mg/dL (1.29 mmol/L) for women (corresponding approximately to the bottom tertile in Caucasian population).[1] In primary HDL deficiency due to monogenic disorders, HDL-C level is usually below the fifth percentile: 29 mg/dL (0.75 mmol/L) in males and 36 mg/dL (0.93 mmol/L) in females.[2] Severe HDL deficiency occurs when the levels of HDL-C are below the first percentile: 20 mg/dL (0.52 mmol/L).[3,4] These extreme values are often observed in patients with hypertriglyceridemia (HTG; triglyceride [TG] > 5.7 mmol/L) or, in the absence of HTG, in patients with malignancy or treated with anabolic steroids and in patients with rare monogenic disorders affecting the metabolism of HDL (primary HDL deficiency).[1,2,4] The most frequent primary HDL deficiencies are caused by mutations in any of 3 major HDL-related genes such as *ABCA1*, *LCAT*, and *APOA1*.

The presence of 2 defective *ABCA1* alleles is the cause of Tangier disease[2,4]; heterozygous carriers of *ABCA1* mutation have reduced plasma HDL-C and may be more susceptible to premature atherosclerosis and coronary heart disease. Genetic deficiency of *LCAT* due to 2 defective alleles is the cause of 2 conditions known as LCAT deficiency and fish-eye disease. Heterozygous carries of defective *LCAT* alleles are asymptomatic but show a variable reduction of plasma HDL-C levels.[2,4]

APOA1 gene encodes apoA-I, the major protein constituent of HDL, which provides the structure of HDL particles, mediates the initial steps in HDL assembly (lipidation by ABCA1 transporter), and promotes LCAT activation. The presence of 2 defective alleles, which abolish or greatly reduce the synthesis of apoA-I (asynthetic mutations) is associated with extremely low or undetectable level of apoA-I and systemic manifestations, such as cutaneous xanthomas, corneal opacity, and premature cardiovascular disease (CVD). Heterozygous carriers of mutant alleles have reduced plasma HDL and apoA-I and appear to be more susceptible to develop premature CVD.[2,4] Missense mutations of apoA-I (usually found in heterozygous condition) affect the structure of apoA-I, often leading to altered function and/or increased catabolism. The low HDL-C level is not necessarily associated with cardiovascular risk (as demonstrated by the case of apoA-I Milano). Some apoA-I missense mutations are associated with hereditary amyloidosis due to tissue accumulation of apoA-I fragments as amyloid deposits (Supplementary Table S1 and Supplementary references).

Here, we describe a child with HTG, in whom the extremely low level of HDL was initially thought to be linked to a disorder of TG metabolism. However, further investigations revealed that HDL deficiency was largely due to presence of a novel frame-shift mutation in *APOA1* gene, which causes the formation of a truncated apoA-I.

Methods

Patient MG

The proband was a 13-year-old overweight boy who was referred to our Lipid Clinic for HTG associated with severe HDL deficiency. Since the age of 7, he has been gaining weight reaching a body mass index (BMI) value of 23.0 kg/m^2 (>97th percentile for age- and gender-matched Italian children).[5] At the age of 12, when determined for the first time, his plasma lipid profile was the following: total cholesterol (TC) 4.65, TGs 4.16, HDL-C 0.48, non-HDL-cholesterol (non-HDL-C) 4.17 mmol/L. On admission to the Lipid Clinic, clinical, biochemical, and genetic investigations of the proband and all available family members were performed.

Informed consent was obtained from all subjects investigated and in the case of the proband from his parents. The study protocol was approved by the institutional human investigation committee of each participating institution.

Biochemical analyses

Plasma lipids

Plasma levels of TC, HDL-C, and TG were determined by standard enzymatic techniques (Roche Diagnostics GmbH, Mannheim, Germany). Serum non-HDL-C concentration was calculated by subtracting HDL-C concentration from TC concentration. Low-density lipoprotein cholesterol (LDL-C) was calculated using the method suggested by Martin et al.[6] Plasma apoA-I and apoB were measured by nephelometry (Siemens AG Healthcare Diagnostics, München, Germany).

HDL subpopulations

Plasma levels of HDL particles containing only apoA-I (LpA-I) and both apoA-I and apoA-II (LpA-I:A-II) were

determined by electroimmunodiffusion in agarose gel.[7] HDL subclass distribution according to particle size was determined by non-denaturing polyacrylamide gradient gel electrophoresis of the d < 1.21 g/mL total plasma lipoprotein fraction.[7,8] HDL particles were divided into 3 size intervals: small (diameter 7.2–8.2 nm), medium (diameter 8.2–8.8 nm), and large (diameter 8.8–12.7 nm), and the percentage of band corresponding to each size interval was reported.[7,8] HDL subclasses were also analyzed by nondenaturing 2-dimensional electrophoresis followed by immunodetection against human apoA-I (Calbiochem, San Diego, CA), and serum content of preβ-HDL was expressed as a percentage of total apoA-I.[8] LCAT ability to esterify cholesterol within endogenous lipoproteins (cholesterol esterification rate, CER) was determined as previously described.[9]

Sequence of candidate genes

Genomic DNA was extracted from peripheral blood leukocytes by a standard procedure. The exons and flanking regions of the major candidate genes for HTG (*LPL*, *APOC2*, *APOA5*, *GPIHBP1*, and *LMF1*) and for hypoalphalipoproteinemia (*APOA1*, *ABCA1*, and *LCAT*) were amplified and sequenced as reported previously.[10,11] For *APOA1* sequencing, see Supplementary Methods for details. The mutation in *APOA1* gene was designated according to the Human Genome Variation Society, 2013 version (http://www.hgvs.org/mutnomen/recs-DNA.html). ApoA-I protein sequence variants were designated according to http://www.hgvs.org/mutnomen/recs-prot.html.

Genotyping for *APOE*, *APOC3*, and *GCKR* gene polymorphisms

The proband and family members were also genotyped for the *APOE* (rs7412 and rs420358), *APOC3* –482 C/T (rs2854117) and *GCKR* c.1337 C/T (rs1260326) single nucleotide polymorphisms (SNPs), known to affect plasma TGs.[12]

Sequence of beta-globin gene (*HBB*)

In some members of the family carrying the β-thalassemic trait, the *HBB* gene was sequenced according to a previously reported method.[13]

Detection of truncated apoA-I in plasma and molecular modeling of the mutant apoA-I

Plasma samples from some members of the proband's family were run on 16% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The membrane was probed with a rabbit anti-human apoA-I antibody (Calbiochem, San Diego, CA).[14]

Molecular modeling of mutant apoA-I

The tridimensional structure coordinates file of apoA-I (PDB code: 2A01), which was downloaded from the Protein Data Bank (PDB; http://www.rcsb.org/pdb/). The apoA-I mutant model was obtained by the residue rotamer explorer function implemented in the MOE Sequence Editor (MOE: Chemical Computing Group Inc, Montreal, Canada [http://www.chemcomp.com/MOE_Protein_and_Antibody_Modeling.htm] (see Supplementary Methods for details).

Serum cholesterol efflux capacity

The cholesterol efflux capacity (CEC) of the whole serum and of apoB-depleted serum from family members carrying *APOA1* mutation and from 6 control subjects was evaluated in cell models expressing ABCA1, ABCG1, or SR-BI. Cells were labeled with [1,2-^{3}H]-cholesterol that was used as a tracer for cholesterol efflux assay. Details of the methods used in our laboratory were previously reported[15] and are summarized in Supplementary Methods.

Capacity of serum to remove cholesterol from cholesterol-loaded macrophages

Murine peritoneal macrophages cells preloaded with cholesterol by exposure to acetylated low-density lipoprotein (LDL) were incubated in the presence of 2.5% of serum from carriers of *APOA1* mutation and from 3 control subjects for 7 hours. Cell cholesterol mass was determined before and after the incubation with serum[16] (see Supplementary Methods for details).

Results

Proband's kindred

On admission to our Lipid Clinic, the 13-year-old proband (III.1 in Fig. 1) had a BMI of 28.2 kg/m^2 and a waist circumference of 91.5 cm (both above the 95th percentile), whereas systolic and diastolic blood pressures were close to the median levels for gender and age. Biochemical evaluation confirmed the previously documented HTG (plasma TG 7.13 mmol/L) associated with very low levels of HDL-C and apoA-I (Table 1) and showed a fasting plasma glucose of 4.28 mmol/L, plasma insulin of 27.8 μU/mL and homeostatic model assessment of insulin resistance (HOMA-IR) of 5.29 (the latter 2 parameters were above the 99th percentile), indicating insulin resistance and the presence of metabolic syndrome.[17,18] Additional laboratory data are reported in Supplementary Results. Carotid ultrasound examination showed a max-intima-media thickness (IMT) of 0.65 mm, a value >75th percentile for comparable age

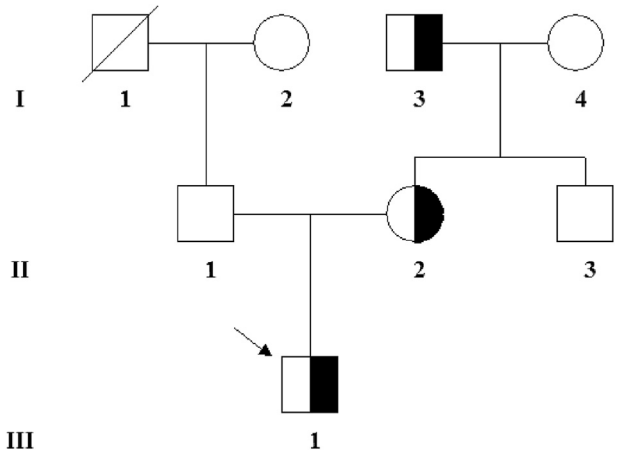

Figure 1 Pedigree of the proband's kindred. The proband is indicated by an arrow. Half-shaded symbol indicates the carriers of the apoA-I mutation.

and gender.[19] In view of these findings, the patient was put on hypocaloric diet supplemented with n-3 fatty acids (850 mg/d). After 6 months of treatment, his BMI decreased to 26.6 kg/m^2 and plasma TG to 2.81 mmol/L, whereas the extremely low level of HDL-C was unaffected (Table 1). The proband's 53-year-old father (II.1 in Fig. 1) was overweight, had a mixed hyperlipidemia (Table 1) and an increased IMT (max IMT 1.3 mm) in the external carotid arteries. The proband's 52-year-old mother (II.2 in Fig. 1) was also overweight and had a reduced HDL-C level (<5th percentile for gender and age; Table 1) and LDL-C of 2.98 mmol/L (<25th percentile). She was a carrier of the β-thalassemic trait, due to heterozygosity for the beta-globin (*HBB*) gene mutation c.118C>T, p (Q40*). The 54-year-old maternal uncle (II.3 in Fig. 1), affected by severe obesity, had a mild elevation of plasma TG, a moderate reduction of HDL-C (Table 1), hyperuricemia (547 μmol/L), and normal glucose tolerance (HbA1c 38 mmol/mol); carotid ultrasound showed a max IMT of 1.2 mm in the external carotid arteries. The 75-year-old paternal grandmother (I.2 in Fig. 1) was affected by type 2 diabetes mellitus. The 86-year-old maternal grandfather (I.3 in Fig. 1) was a carrier of the β-thalassemic trait (*HBB* c.118C>T) and in the past had been a heavy smoker. Since the age of 74, he had suffered from peripheral arterial disease at lower limbs (grade IIb of Leriche-Fontaine), mainly due to a severe atherosclerosis with 70% stenosis of the left external iliac artery and 60% stenosis of the left superficial femoral artery. He also had sub-renal fusiform aortic aneurysm with diameter of ~35 mm, bilateral carotid atherosclerosis with fibrocalcific plaques, causing respectively 30% and 40% stenosis of the external and internal carotid arteries and hypertensive heart disease; at the age of 84, he had an episode of atrial flutter treated with cooled-tip radiofrequency catheter ablation. Repeated evaluations of his plasma lipid levels over the last 15 years had documented a stable reduction of HDL-C (HDL-C 0.68 ± 0.09 mmol/L), with LDL-C below the 15th percentile (3.23 ± 0.13 mmol/L) and normal TG levels (0.96 ± 0.13 mmol/L). The 82-year-old maternal grandmother (I.4 in Fig. 1) was apparently healthy and had normal lipid levels.

Table 1 Plasma lipids and apolipoproteins and *APOE*, *APOC3*, *GCKR* and *HBB* genotype in MG kindred

Subject	I.2	I.3	I.4	II.1	II.2	II.3	III.1	III.1 on Rx
APOA1 alleles	W/W	M/W	W/W	W/W	M/W	W/W	M/W	M/W
Age (y)	75	86	82	53	52	54	13	13.5
Gender	F	M	F	M	F	M	M	M
BMI (kg/m^2)	25.3	27.0	24.0	25.9	25.8	38.7	28.2	26.6
TC	4.00	3.93	4.32	8.27	4.32	5.14	4.78	3.67
HDL-C	1.00	0.62	1.19	0.90	0.80	1.00	0.39	0.31
Non-HDL-C	3.00	3.31	3.13	7.37	3.51	4.13	4.39	3.36
LDL-C	2.31	2.83	2.53	5.42	2.98	3.35	2.38	2.44
TG	1.90	1.03	1.44	4.94	1.17	1.89	7.13	2.81
ApoA-I	120	64	136	132	83	102	40	—
ApoB	90	91	82	178	87	108	85	—
APOE genotype	ε3ε3	ε3ε3	ε2ε3	ε3ε3	ε2ε3	ε3ε3	ε3ε3	—
APOC3-482C>T rs2854117	CT	CC	CC	TT	CC	CC	CT	—
GCKR c.1337C>T rs1260326	CT	CT	CC	CT	CC	CC	CT	—
HBB genotype and β-globin protein	c.118CC W/W	c.118CT W/p.(Q40*)	c.118CC W/W	c.118CC W/W	c.118CT W/p.(Q40*)	c.118CC W/W	c.118CC W/W	—

W, wild type allele or protein; M, mutant allele.
Rx represents data on treatment with hypocaloric diet and n-3 fatty acids 850 mg/d; lipid values (mmol/L); apolipoprotein values (mg/dL).

Analysis of TG-related genes

As summarized in Table 1, the index patient presented with overweight associated with marked HTG and low HDL-C. We firstly thought that HTG was related to the presence of some rare variants in one of the major genes (*LPL*, *APOC2*, *APOA5*, *GPIHBP1*, and *LMF1*), which control the intravascular lipolysis of TG-rich lipoproteins (very low–density lipoprotein [VLDL] and chylomicrons). In view of the absence of rare variants in these genes, a polygenic HTG was taken into consideration[20] in the light of: (1) the HTG in proband's father; (2) the finding that the proband and his father were carriers of rare *APOC3* and *GCKR* alleles, known to be associated with higher plasma TG levels[12]; and (3) the presence in the proband of one *APOE* ε2 allele (Table 1). HTG could also have resulted from an increased secretion or a defective catabolism of VLDL and chylomicrons, as reported to occur in obesity and insulin resistance conditions.[20,21] The reduction of BMI and plasma TG, after 6 months of a strict hypocaloric diet and treatment with n-3 fatty acids, suggested that overweight and insulin resistance were the major contributors to HTG.

Sequence of HDL-related genes

The observation that the reduction of plasma TG level was not associated with the expected increase in plasma HDL-C raised the possibility that the proband had a primary HDL deficiency. This hypothesis was supported by the presence of low levels of HDL-C and apoA-I in proband's mother and maternal grandfather in the absence of HTG (Table 1).

For this reason, we sequenced *ABCA1*, *LCAT*, and *APOA1* genes. Although the sequence of *ABCA1* and *LCAT* was negative, that of *APOA1* gene revealed that the proband was heterozygous for a dinucleotide deletion in exon 4 (c.546_547delGC), resulting in a shift of the reading frame with the insertion of a premature termination codon in messenger RNA (mRNA; Fig. 2). The predicted translation product of this abnormal mRNA is a truncated protein p.(Leu159Alafs*20) of 177 amino acids containing a string of 19 novel amino acids downstream from the alanine residue at position 158 of the mature protein. The proband's mother and the maternal grandfather, but not the maternal uncle, were found to carry this mutation (Table 1).

Mutant apoA-I in plasma

The immunoblot of plasma apoA-I showed the presence in the proband's plasma of a major 28 kDa band corresponding to wild type (WT) apoA-I and a 20.8 kDa band consistent with the size of the predicted truncated protein (Fig. 3). The 20.8 kDa truncated protein was also detectable in plasma of proband's mother and maternal grandfather. The WT and mutant apoA-I were also evaluated by Western blotting after the ultracentrifugation of plasma at density 1.210 g/mL. The WT/mutant apoA-I ratio in 1.210 g/mL top and bottom was the same.

Plasma HDL subpopulations

We next looked more specifically at the HDL subpopulations present in proband's plasma and in the plasma of other family members. Table 2 summarizes that mutation carriers had a reduced level of LpA-I, undetectable HDL-2,

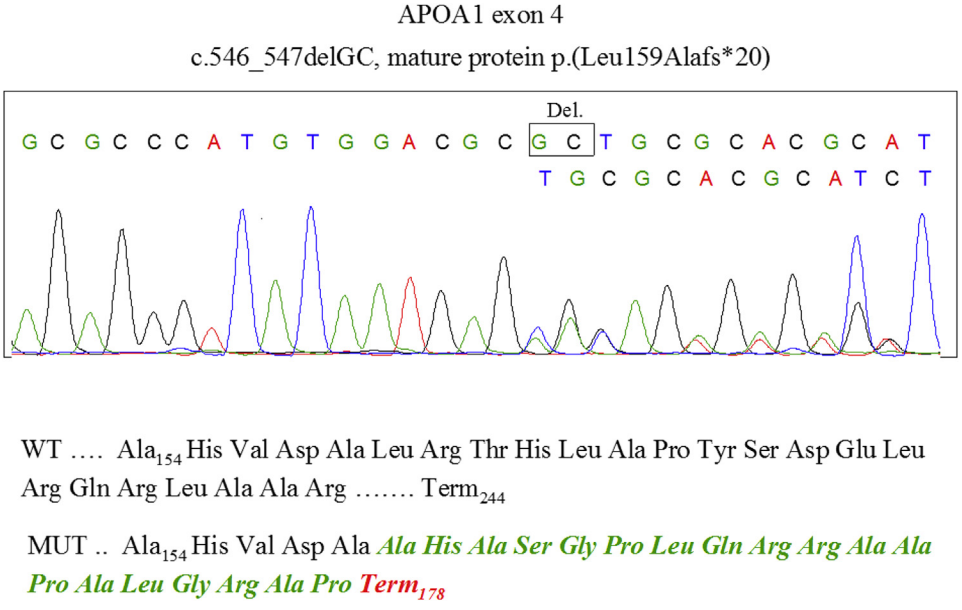

Figure 2 Partial sequence of exon 4 of *APOA1* gene. The top panel shows the nucleotide sequence in the proband; the deleted nucleotides are boxed. The lower panels show the corresponding amino acid sequence of wild type and mutant apoA-I, respectively. The stretch of the 19 novel amino acids (in italics) of the C-terminal end of the truncated protein is shown.

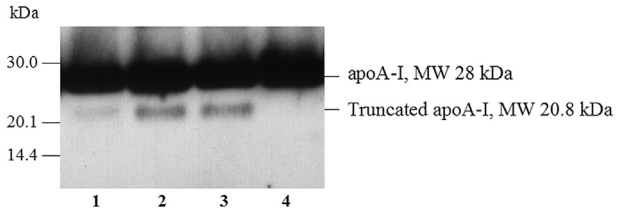

Figure 3 Immunoblot of plasma apoA-I. The plasma of mutation carriers contains the wild type apoA-I (28 kDa) and a 20.8 kDa band consistent with the size of the predicted truncated protein. Lane 1: proband; lane 2: proband's mother; lane 3: proband's maternal grandfather; lane 4: proband's maternal uncle.

and a reduced level of preβ-HDL. The content of large HDL was also reduced, whereas that of small HDL was increased. CER was slightly reduced in the proband but within, or close to, the normal range in the other two carriers. The maternal uncle, in spite of the moderately reduced level of HDL-C and LpA-I, had detectable HDL-2 (with a size within the normal range) and a normal distribution of HDL subpopulations.

Cholesterol efflux capacity of whole sera and apoB-depleted sera

First, we evaluated the total CEC of whole serum and apoB-depleted serum (B [-] serum) using J774 murine macrophages treated with cAMP. In such conditions, total release of cholesterol from these cells occurs through the major pathways of known relevance in cholesterol efflux from macrophages (ie, aqueous diffusion, ABCA1, ABCG1, and SR-BI mediated efflux, respectively). As summarized in Table 3, TC efflux to B(-) sera of the 3 apoA-I mutation carriers was significantly reduced, as compared with the efflux to B(-) sera of the 6 controls. Similar results were obtained when cAMP stimulated J774 murine macrophages were incubated with whole serum (Table 3).

Aqueous diffusion process was evaluated in J774 murine macrophages, which under basal conditions express negligible levels of ABCA1, ABCG1, and SR-BI. Under these conditions, the efflux to B(-) sera of the 3 apoA-I mutation carriers was significantly lower than the efflux to B(-) sera of controls (Table 3). Similar results were obtained when J774 were incubated with whole serum (Table 3).

ABCA1-mediated efflux to B(-) sera of apoA-I mutation carriers was markedly reduced as compared with B(-) sera of controls. ABCA1-mediated cholesterol efflux to whole sera of apoA-I mutation carriers showed a similar trend, although the difference with respect to control sera was less pronounced (Table 3).

ABCG1-mediated cholesterol efflux to B(-) sera of the 3 apoA-I mutation carriers was reduced as compared with (B-) control sera, especially in the index case (3.52 ± 0.22 vs 4.48 ± 0.39 in the proband's mother and 4.19 ± 0.59 in the maternal grandfather). No difference was found in CEC values to whole serum (Table 3).

SR-BI–mediated cholesterol efflux to B(-) sera of mutation carriers was lower than the efflux to B(-) sera of controls. A similar finding was observed for the CEC to whole sera (Table 3).

Capacity of serum to remove cholesterol from cholesterol-loaded macrophages

We next measured the capacity of serum to remove cholesterol from cholesterol-loaded macrophages. All tested sera had a cholesterol removal capacity comparable to that of control sera, with the exception of the serum of the proband, whose removal capacity was almost half of that observed in the other carriers of the apoA-I mutation (Supplementary Table S3).

Model of mutant apoA-I

As specified previously, the dinucleotide deletion in exon 4 results in a C-terminal truncated protein of 177 amino acids (73% of the size of the 243 amino acids of the WT mature apoA-I). In addition, mutant apoA-I contained a string of 19 novel amino acids at the C-terminal end.

With the aim of exploring at a molecular level, the pathological impact caused by apoA-I amino acidic mutations (in the truncated apoA-I), in the absence of experimental X-ray for the mutant protein, a 3D-model was built and used to analyze any difference concerning the steric and electrostatic profile of the WT and mutant apoA-I proteins.

On the basis of our calculations, the derived mutant apoA-I model proved to be characterized by a quite different hydrophilic and hydrophobic profile, if compared with the

Table 2 HDL subpopulations in MG kindred

Subject	*APOA1* gene alleles	LpA-I (mg/dL)	LpA-I:A-II (mg/dL)	HDL-2 (nm)	HDL-3 (nm)	preβ-HDL (%)	Large (%)	Medium (%)	Small (%)	CER (nmol/mL/h)
I.3	M/W	30	61	ND	8.6	4.3	41	22	37	29.1
II.2	M/W	30	53	ND	8.6	4.0	42	20	38	35.7
II.3	W/W	38	57	10.3	8.5	11.4	51	22	27	45.9
III.1	M/W	20	28	ND	8.6	4.5	41	21	38	24.1
Control	W/W	52	60	10.8	8.5	10.6	56	20	24	34.0
Ref. values		>42	>58	9.0–12.7	7.8–9.0	10–14	50–75	13–30	6–24	30–60

M, mutant allele; ND, not detectable; W, wild-type allele.

Table 3 Cholesterol efflux capacity (CEC) to apoB-depleted sera and to whole sera in controls and in apoA-I mutation carriers

	ApoB-depleted sera			Whole sera		
CEC	Controls	Carriers	P^*	Controls	Carriers	P^*
Total diffusion	7.92 ± 0.33	4.15 ± 0.64	<.001	10.58 ± 0.41	7.87 ± 0.77	<.001
Aqueous diffusion	5.71 ± 0.34	3.37 ± 0.42	<.001	6.90 ± 0.28	5.43 ± 0.62	<.001
ABCA1-CEC	2.21 ± 0.27	0.78 ± 0.32	<.001	3.68 ± 0.50	2.44 ± 0.51	<.001
ABCG1-CEC	5.41 ± 0.44	4.06 ± 0.56	<.001	6.10 ± 0.74	6.12 ± 0.84	NS
SR-BI-CEC	2.35 ± 0.22	1.05 ± 0.36	<.001	3.07 ± 0.30	1.92 ± 0.38	<.001

NS, not significant.
*Mann-Whitney test.

WT XR data (see Supplementary Table S4). More specifically, the WT protein was folded bearing 2 highly polar regions properly oriented outside the helix domain, including the negative charged motif Asp168 and Glu169 (NCM) and the positive charged motif (PCM) Arg 171, Gln 172 and Arg 173, as highlighted by the contact preference depicted in Figure 4. In sharp contrast, the mutant apoA-I model displayed positive charged and hydrophobic residues (Arg168 and Ala169) and mild polar or hydrophobic amino acids (Pro171, Ala172, Leu173) at the corresponding WT NCM and PCM positions. With regard to the lipophilic protein profiles, the WT apoA-I showed numerous bulky residues, such as Leu159, Leu163, Tyr166, Leu170, and Leu174, which were projected inside the helix core domain. The corresponding positions of the mutant apoA-I model were occupied by smaller or charged amino acids (Ala159, Gly163, Gln166, Ala170, Gly174; Fig. 4).

Discussion

In the present work, we describe a novel frameshift mutation in *APOA1* gene (c.546_547delGC), which causes the production of a truncated protein of 177 amino acids (p.[L159Afs*20]). The mutation was found in heterozygous form in a 13-year-old overweight boy with HTG and severe HDL deficiency and in 2 family members, the proband's mother and maternal grandfather. The truncated protein of 20.8 kDa was detectable in plasma in minute amounts, with respect to the WT apoA-I of 28 kDa encoded by the normal allele. The abnormal protein is devoid of the C-terminal domain (helix 7-10),[22,23] which is crucial for the ABCA1-mediated efflux of cholesterol and phospholipids from cell membrane and for the initial lipid binding to lipid-free apoA-I and lipid-poor apoA-I (pre-β1) and the formation of nascent discoidal HDL particle.[24–27]

To date, several mutations of *APOA1* gene have been identified: 53 of them (in homozygous, compound heterozygous, or heterozygous forms) were associated with HDL deficiency; 21 other mutations (in heterozygous form) were involved in the development of amyloidosis (Supplementary Table S1). Among the previously described mutations causing HDL deficiency, 18 were nonsense or frameshift mutations causing truncated proteins. Six of these mutations eliminated a region spanning from helix 6 to helix 10, resulting in proteins containing a number of amino acids ranging from 177 to 229 (Supplementary Table S2).

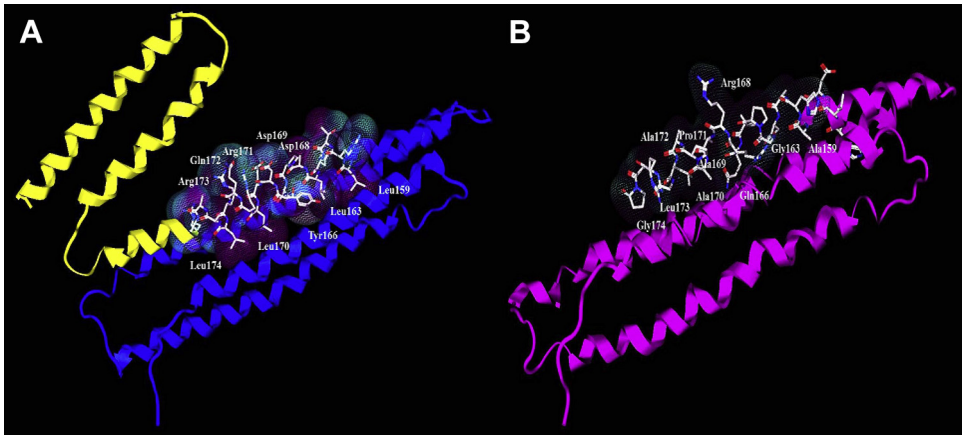

Figure 4 (A) Molecular model of wild type apoA-I. The contact preference areas involving residues 159 to 177 is depicted (H-bonding and hydrophobic regions are colored in cyan and purple, respectively). The protein backbone is shown by ribbon (residues 1–158 in blue, 178–243 in yellow). (B) Molecular model of truncated apoA-I. The contact preference areas of the truncated apoA-I (residues 159–177) are depicted. H-bonding and hydrophobic regions are colored in cyan and purple, respectively. The protein backbone is shown by ribbon, colored in magenta.

The extremely low level of the truncated apoA-I found in the plasma of the 3 mutation carriers may be due to several factors such as: (1) a low production rate by liver and intestine due to a rapid degradation of the corresponding mRNA containing a premature termination codon (nonsense-mediated mRNA decay), as documented for another truncated apoA-I[28]; (2) a rapid degradation of a protein recognized as structurally abnormal by endoplasmic reticulum degradation machinery; and (3) a poor lipidation of the protein by ABCA1-mediated pathway due to the lack of the normal C-terminal domain (see previous section) and the presence of an abnormal novel C-terminal domain. Defective lipidation may promote a rapid removal of the protein from the circulation, as documented by the kinetic analysis of labeled C-terminal truncated apoA-I in normolipemic rabbits.[29] Whether the plasma of the mutation carriers contains a small subset of HDL particles containing exclusively the mutant apoA-I or the latter is incorporated in HDL containing the WT apoA-I is an open question. In principle, it is difficult to envisage the belt dimerization of truncated with the WT apoA-I, as the truncation eliminates the arginine residue at position 173 (R173), which is involved in formation of salt bridges D89/E92-R173 crucial for dimer formation of WT apoA-I.[30] It is, however, possible that a formation of salt bridges occurs between D89/E92 residues and the arginine at position 175 (R175) of the novel C-terminal amino acid tail of the truncated protein (Fig. 2). Regardless of the mechanisms of belt formation, it is possible that the coexistence of both WT and truncated apoA-I or the presence of dimers of truncated apoA-I in the same particle, may render the latter more unstable and more prone to a rapid removal from the circulation.

The domain involved in LCAT activation (residues 134–145), which in discoidal HDL forms an amphipatic tunnel, allowing the presentation of phospholipids and free cholesterol to LCAT active site,[31] is maintained in the truncated apoA-I. However, the elimination of the C-terminal domain involved in the lipid binding would abolish the function of the LCAT activation domain for lack of lipid substrates. The CER found in the apoA-I truncation carriers (Table 2) was slightly below or close to the lower limits of the normal range. This might be the result of a reduced availability of WT apoA-I containing discoidal particles, which maintain a full LCAT activation capacity.

The plasma concentration of WT apoA-I was very low in the proband and was approximately 40% to 50% of the control values (136.7 ± 22.0 and 146.0 ± 23.0 in a sample of 750 males and 550 females randomly selected from Italian population) in the other 2 carriers. The reduced pool of WT apoA-I explains the reduced level of LpA-I particles and preβ-HDL. The reason underlying the absence in the carriers of HDL-2 and the reduction of large HDL with the concomitant increase in small HDL, which are common findings in both primary and secondary forms of HDL deficiencies, is poorly understood. It is possible that this change in particle size distribution is caused by some impairment of the intravascular remodeling of HDL mediated by LCAT or a more rapid removal of the large HDL from the plasma compartment.

The quantitative and qualitative changes of plasma HDL prompted us to investigate CEC of whole serum and apoB-depleted serum, a well-established function of the HDL system. By using experimental settings designed to explore the various cell pathways involved in cell cholesterol removal, we show that overall, the CEC was greatly reduced in the carries of truncated apoA-I. This finding is likely to reflect the changes in HDL subpopulations, specifically of preβ-HDL particles, which are involved in the ABCA1-mediated cholesterol efflux. This implies that the early stages of reverse cholesterol transport are greatly impaired in mutation carriers, rendering them more susceptible to develop atherosclerotic plaques.

When compared with the other mutation carriers, the proband showed much lower levels of HDL-C and apoA-I, which remained unchanged after a substantial reduction of plasma TG after a hypocaloric diet. In this subject, the severe HDL deficiency is likely to ensue from the combined effect of several factors: (1) the apoA-I mutation inherited from his mother; (2) the presence of metabolic syndrome, which is known to affect HDL metabolism by increasing hepatic lipase–mediated catabolism of TG-loaded HDL particles resulting from an enhanced CETP activity, which occurs in HTG[32]; and (3) the inheritance of some additional genetic factors from his father who has combined hyperlipidemia with low HDL.[33]

The very low level of HDL observed in the proband may also explain the reduced capacity of his serum to remove cholesterol from cholesterol-enriched macrophages, as shown in Supplementary Table S3.

The geographical origin of the proband's kindred was Guastalla, a small town in the North-East of Italy close to the Po river. In this region, there is a relatively high prevalence of carriers of β-thalassemia trait due to the presence of a few mutations of the *HBB* gene.[34] Indeed, we found that the proband's mother and maternal grandfather were carriers of the p.(Q40*) mutation of the β-globin chain. Studies conducted in a large number of subjects carrying the β-thalassemia trait have shown that the presence of this trait has a lowering effect on plasma concentration of TC, LDL-C, and apoB.[35,36] In vivo kinetics study documented an increased fractional clearance rate of LDL in carriers of the β-thalassemia trait,[37] possibly due to an increased receptor-mediated uptake of LDL by the bone marrow to provide cholesterol for the increased proliferation of erythroid precursors. Moreover, the LDL lowering effect associated with the β-thalassemia trait may be also related to the activation of the monocyte/macrophage system in spleen, liver, and bone marrow with the generation of several inflammatory cytokines, which at least in vitro in HepG2 cells, increase LDLR gene expression and reduce the assembly of apoB-containing lipoproteins.[38] In addition, a recent meta-analysis of 8 case-control studies (involving 9479 individuals) showed that the β-thalassemia

trait was associated with a reduced risk of arterial cardiovascular and cerebrovascular events in male patients (odds ratio 0.39; confidence interval 0.24–0.62).[39] In agreement with the aforementioned findings, the LDL-C level was $<25^{th}$ percentile in proband's mother and $<15^{th}$ percentile in proband's grandfather with respect to the distribution of LDL-C level in gender- and age-matched normal individuals of the Italian population. The β-thalassemia trait does not appear to reduce HDL-C level, although a reduced plasma apoA-I level was observed in males carrying this trait.[36] In this context, it is likely that in the proband's grandfather, the β-thalassemic trait, by maintaining a low level of atherogenic apoB-containing lipoproteins, has attenuated the "combined deleterious" effect of the low plasma HDL-C (due to apoA-I mutation) and of the heavy smoking habits on the development of atherosclerosis, the manifestations of which occurred late in life.

Needless to say that the proband, being at high risk of developing CVD, requires therapeutic interventions and a strict follow-up schedule. We have recommended life style modifications, which include a hypocaloric diet with restriction of saturated fat and carbohydrate intake (specifically simple sugars) supplemented with n-3-fatty acids (3 g/d),[40–42] and an increased physical activity. These modifications are expected to reduce body weight, insulin resistance, and plasma TG levels. If no normalization of plasma TG is obtained, fibrates, either alone or in combination with statins (or ezetimide), will be given to reduce non-HDL cholesterol level as much as possible.[42] In the future, we may consider new therapies such as apoC-III-targeted antisense oligonucleotide to reduce the secretion of VLDL, promote LPL-mediated VLDL, and chylomicron hydrolysis and reduce the apoC-III–induced arterial inflammation.[42,43]

Conclusions

In conclusion, we describe a novel frameshift mutation of the *APOA1* gene found in an Italian kindred. The mutant allele generates a truncated protein, which is devoid of the C-terminal domain and is detectable in plasma in minute amounts. In 2 carriers of the apoA-I mutation, additional factors, namely the metabolic syndrome in the proband and the β-thalassemic trait in the maternal grandfather, seem to play a role in modifying the biochemical and clinical phenotypic expression of the main genetic disorder.

Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jacl.2015.09.001.

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