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Identification of novel *APOB* mutations by targeted next-generation sequencing for the molecular diagnosis of familial hypobetalipoproteinemia



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ABSTRACT

Background and aims: Familial hypobetalipoproteinemia (FHBL) is a co-dominant disorder characterized by decreased plasma levels of LDL-cholesterol and apolipoprotein B (ApoB). Currently, genetic diagnosis in FHBL relies largely on Sanger sequencing to identify *APOB* and *PCSK9* gene mutations and on western blotting to detect truncated ApoB species.

Methods: Here, we applied targeted enrichment and next-generation sequencing (NGS) on a panel of three FHBL genes and two abetalipoproteinemia genes (*APOB*, *PCSK9*, *ANGPTL3*, *MTTP* and *SAR1B*).

Results: In this study, we identified five likely pathogenic heterozygous rare variants. These include four novel nonsense mutations in *APOB* (p.Gln845*, p.Gln2571*, p.Cys2933* and p.Ser3718*) and a rare variant in *PCSK9* (Minor Allele Frequency <0.1%). The affected family members tested were shown to be carriers, suggesting co-segregation with low LDL-C.

Conclusions: Our study further demonstrates that NGS is a reliable and practical approach for the molecular screening of FHBL-causative genes that may provide a mean for deciphering the genetic basis in FHBL.

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1. Introduction

Familial hypobetalipoproteinemia (FHBL) belongs to a heterogeneous group of monogenic disorders characterized by reduced plasma levels of low-density lipoprotein cholesterol (LDL-C) and apolipoprotein B (ApoB) below the fifth percentile for age and sex in the population. FHBL is a co-dominant disorder whose frequency

in the heterozygous form is estimated to be 1:1000–1:3000 [1,2]. FHBL heterozygotes may be asymptomatic or have some clinical manifestations such as liver steatosis. Furthermore, FHBL has been associated with a longevity syndrome linked to cardiovascular protection [1,2]. FHBL is genetically heterogeneous and may be due to loss-of-function mutations in *APOB* [1,2], or less frequently in *PCSK9*, which encodes the proprotein convertase subtilisin/kexin type 9 [3–5]. In addition, familial combined hypolipidemia - which is characterized by low levels of LDL-C, high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) can be caused by mutations in the angiopoietin-like 3 gene (*ANGPTL3*) [6,7]. Despite previous gene discoveries, the genetic etiology of FHBL remains to be determined in approximately 50% of cases [1,8].

Currently, genetic diagnosis in FHBL relies largely on Sanger

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sequencing of *APOB*. Because *APOB* is a large gene composed of 29 exons covering 14,121 bp, Western-blotting may be used to detect truncated protein species that are >30% of full-length protein size [1,2]. When Western blotting is negative, sequencing the 25 first exons of *APOB* gene (exons 1 to 25) - a time consuming and costly approach - is mandatory [9,10]. Next-generation sequencing (NGS) technologies enable rapid and cost-effective sequencing of targeted genomic regions. NGS is thus a powerful approach for genetic diagnostics in inherited Mendelian disorders [11,12]. In the present study, we developed a targeted resequencing panel to screen for coding variations amongst three known FHBL and two abetalipoproteinemia (ABL) genes. Re-sequencing these genes in eight unrelated individuals with FHBL revealed four novel missense mutations in *APOB* that segregate with the low LDL-C trait in related individuals as well as one likely pathogenic mutation in *PCSK9*.

2. Patients and methods

2.1. Study approval

The study (NCT02354079) was conducted in compliance with current Good Clinical Practice standards. We obtained informed consent from individuals involved in the study and approval from local institutional ethics committees (CPP OUEST IV 49/14).

2.2. Patient recruitment and clinical evaluation

Eight unrelated individuals routinely followed for FHBL in the department of endocrinology at Nantes University Hospital have been recruited. In the probands, FHBL diagnosis was based on low levels of fasting calculated plasma LDL-C < 50 mg/dL without lipid lowering therapy. Fasting lipid profile (total cholesterol, HDL-C, LDL-C and TG) and ApoB levels were also determined in some of their relatives. Affected relatives were diagnosed by LDL-C < 80 mg/dL and/or ApoB < 50 mg/dL. Anthropometric data, biochemical tests and abdominal ultrasonography were recorded.

2.3. Capture design and library preparation

We developed a re-sequencing custom design based on the HaloPlex™ Target Enrichment system (Agilent Technologies) to perform high-throughput sequencing of the coding regions of genes previously linked to FHBL (*APOB*, *PCSK9*, *ANGPTL3*) and ABL (*MTTP*, *SAR1B*) encoding the Microsomal Triglyceride Transfer Protein and the Secretion Associated, Ras related GTPase 1B, respectively). Targeted coding regions (exons + about 10-bp non coding flanking sequences) correspond to 22.83 kb of genomic sequence. Sequencing (100-bp paired-end reads) was performed on Illumina HiSeq 2500 station.

2.4. Variant calling and filtering

Raw sequence reads were aligned to the human reference genome (GRCh37) using BWA-MEM (version 0.7.5a) after removing sequences corresponding to Illumina adapters with Cutadapt (v1.2). Variants were called for each sample separately using GATK UnifiedGenotyper (version 2.8) and Samtools mpileup (version 0.1.19); they were considered for further analyses when found by both algorithms. Variants frequency was determined using the Exome Aggregation Consortium database (Exac.broadinstitute.org/); they were considered as rare when the minor allele frequency (MAF) was lower than 0.1% in the non-Finnish European population. The potential effect of variants was determined using Variant Effect Predictor annotations (Ensembl). Filtering was performed using Knime4Bio [13].

2.5. Validation (visualization and Sanger sequencing)

All relevant variants identified in the eight patients were reviewed by visual inspection of sequence reads using the Integrative Genomics Viewer [14]. Sanger sequencing was performed for validation and co-segregation analysis among family members. Regions of interest were amplified by polymerase chain reaction and sequenced with the dye-terminator chemistry (ABI PRISM 3730, Applied Biosystems, Foster City, CA).

2.6. Dosage of lipoproteins in plasma

Plasma levels of ApoA-I, ApoA-II, ApoB-100, ApoC-II, ApoC-III, ApoE and Lp(a) were measured using the multiplexed LC/MS/MS method as previously described [15].

2.7. Lipoprotein separation and ApoB western blotting

The LDL plasmatic fractions were obtained by ultracentrifugation separation protocol [16]. 50 µg of extracted proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblots probed with polyclonal antibody ApoB-100 (AB742) (1:1000) and HRP-conjugated antibodies (rabbit anti-goat IgG-HRP: sc-2768) (1:10,000) were purchased from Merck Millipore and Santa-Cruz biotechnology respectively.

3. Results

3.1. Targeted resequencing

Eight patients followed for FHBL in our clinical department have been included in this study. Their plasma lipid panels are listed in the Supplemental Table 1. Five genes were sequenced using the custom capture design that we developed based on the HaloPlex™ technology. The mean coverage depth was 201X (±90) per sample with 98% of the target regions being covered at least 10 times (96.1% covered at least 20 times). Mean coverage depths throughout the five target genes are shown in Supplemental Fig. 1.

We identified five heterozygous rare variants (MAF < 0.1%) that alter ApoB or PCSK9 protein sequence. The four *APOB* variants (p.Gln845*, p.Gln2571*, p.Cys2933* and p.Ser3718*) are likely pathogenic nonsense variants (SO: 0001587 stop gained) that are absent from the ExAC database.

The *PCSK9*-c.523 G > A variant was reported twice in the ExAC database (65,770 non-Finnish European population alleles). Predicted functional consequences of this variant either lead to: -a *PCSK9* p.Asp175Asn missense variant (SO: 0001583) respectively annotated as tolerated or benign by SIFT/PolyPhen algorithms and displaying low conservation scores (CADD score = -0.226944 and GERP score = -1.33) or to -a splice variant (SO: 0001630), that leads to a frameshift followed by a premature stop codon *PCSK9*-p.Asp175Serfs*13.

No variant was identified in the three other targeted genes according to our filtering criteria.

3.2. Identified variants co-segregate with FHBL

Segregation analysis of the *APOB* variants were performed in proband's family members when available (Fig. 1). Patients' clinical and biological characteristics are reported in Table 1.

The first *APOB* variant (Chr2:21,246,468; c.2533G > A; *APOB*-p.Gln845*) was identified in a 49 years-old woman (II:2/Family I) presenting low LDL-C (20 mg/dL). The proband's sons (III:1 and III:2) who had low LDL-C levels harbor the mutation. The youngest boy (III:2) also exhibited hepatic steatosis without increased liver

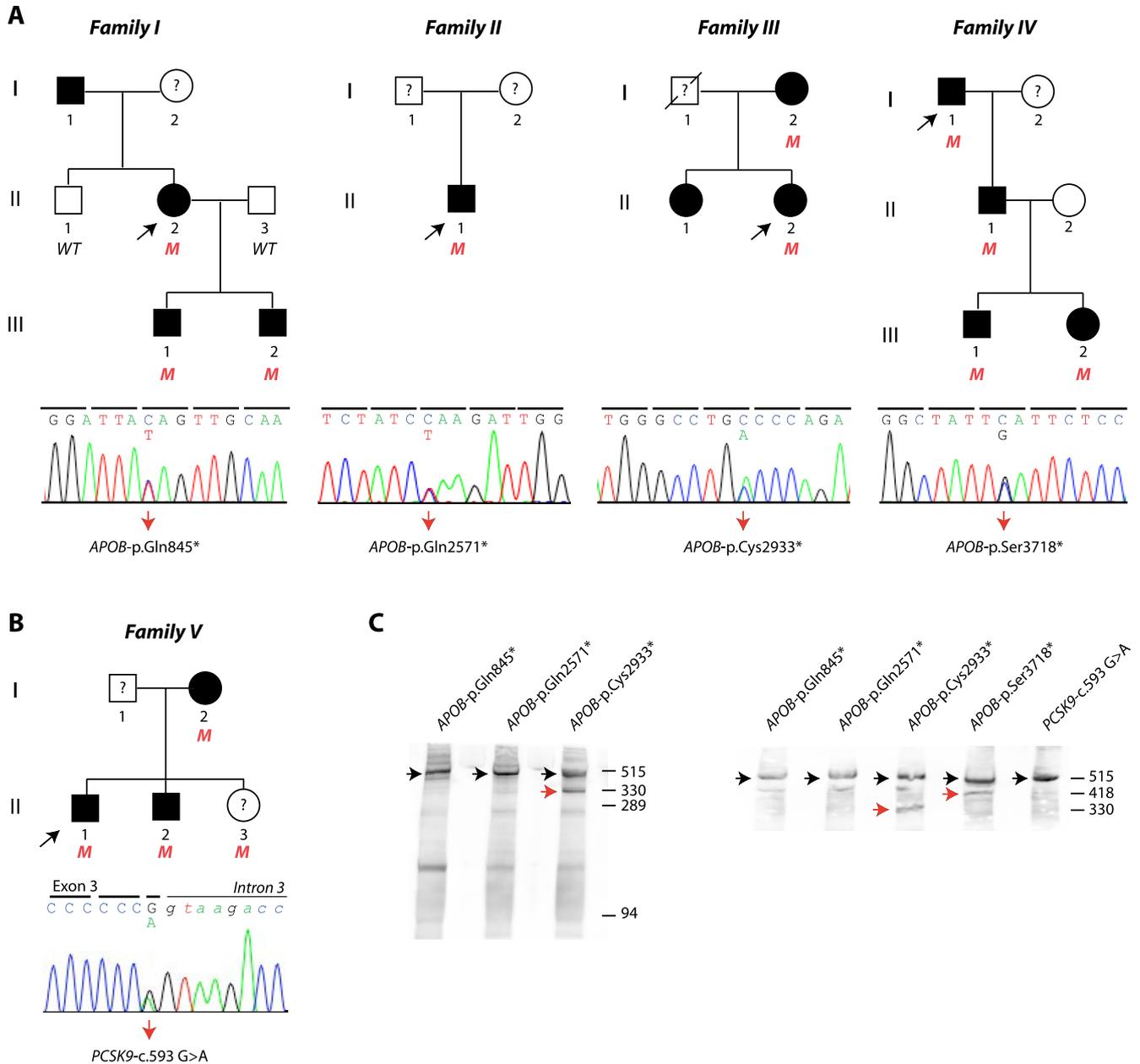


Fig. 1. Pedigrees and mutations. Upper panels: Pedigrees. Arrows indicate index cases. Female are identified by circles and males by squares. FHBL affected individuals are indicated by filled symbols, unaffected individuals by empty symbols and individuals with unknown cholesterol phenotype by (?). Heterozygous mutations are designated M, and the wild-type sequence WT. Lower panels: Sanger sequencing chromatograms showing the heterozygous *APOB* mutations (A) and the *PCSK9* variant (B). C-*APOB* p.Cys2933* and p.Ser3718* mutations generate circulating truncated proteins. Western-blot was performed with lipoprotein plasmatic fractions from *APOB*-mutated patients on a 6% acrylamide gel with a short migration time (left panel) or on a 5% acrylamide gel with a long migration time to discriminate high molecular forms (right panel). All samples present a secreted WT-ApoB form (ApoB-100: 515 kDa) in the plasma (black arrows) as seen in the patient carrying the *PCSK9* variant (*PCSK9*-c.593 G > A) taken as a control. No truncated forms of ApoB were identifiable for ApoB-18 (p.Gln845*:94 kDa) and ApoB-56 (p.Gln2571*: 289 kDa). The truncated forms ApoB-64 (p.Cys2933*: 330 kDa) and ApoB-81 (p.Ser3718*: 418 kDa) are visible (red arrow) at the expected molecular weights.

enzymes. The two family members who did not carry the variant presented normal LDL-C levels. Interestingly, the proband's paternal grandfather died late in life (>95 years), potentially suggesting a longevity syndrome as previously described in FHBL [1,2].

The second *APOB* variant (Chr2:21,232,029; c.7711G > A; *APOB*-p.Gln2571*) was identified in a 41 years-old man (II:1/Family II) who had low LDL-C (38 mg/dL), pre-diabetes and liver steatosis. DNA from his family was not available for segregation analyses.

The third *APOB* variant (Chr2:21,230,941; c.8799 G > T; *APOB*-p.Cys2933*) was identified in a 24 years-old woman (II:2/Family III) who showed low LDL-C (28 mg/dL). Her mother (I:2) and sister

(II:1) also had low LDL-C. The proband's mother harbor the *APOB*-p.Cys2933* variant.

The fourth *APOB* variant (Chr2:21,228,587; c.11153 G > C; *APOB*-p.Ser3718*) was identified in an 84 years-old man (I:1/Family IV) who showed a low LDL-C level (33 mg/dL). The three other affected members of the family, his son (II:1) and great-children (III:1 and III:2) with low LDL-C levels harbor the *APOB*-p.Ser3718* variant. Subject II-1 also had type 2 diabetes and severe non-alcoholic steatohepatitis (NASH) with precirrhotic lesions and well-differentiated hepatocellular carcinoma (HCC) diagnosed on liver biopsy.

Table 1
Main biological features and mutations of FHBL family members.

	Age	Gender	BMI	Diabetes	TC (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	TG (mg/dL)	FPG (mmol/L)	HbA1C (%)	AST (ULN)	ALT (ULN)	GGT (ULN)	ALP (ULN)	Liver steatosis	Molecular alteration
Family I																
I:1	83	M	n.a.	n.a.	n.a.	58	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
II:1	51	M	n.a.	n.a.	207	135	51	102	n.a.	n.a.	0.52	0.64	0.35	0.71	n.a.	WT
II:2	49	F	20	No	143	20	120	18	4.7	5.0	0.66	0.74	0.38	0.39	N	<i>APOB</i> -p.Gln845*
II:3	48	M	n.a.	n.a.	230	155	52	117	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	WT
III:1	17	M	18	No	98	14	79	25	n.a.	n.a.	0.40	0.32	0.42	0.37	n.a.	<i>APOB</i> -p.Gln845*
III:2	15	M	18	No	104	7	91	31	n.a.	n.a.	0.55	0.46	0.30	0.63	Y	<i>APOB</i> -p.Gln845*
Family II																
II:1	40	M	34	Prediabetes	113	38	69	32	7.2	5.9	0.8	1.34	0.47	0.36	Y	<i>APOB</i> -p.Gln2571*
Family III																
I:2	61	F	24	No	145	55	81	45	4.9	5.9	1.08	1.03	0.55	0.54	n.a.	<i>APOB</i> -p.Cys2933*
II:1	39	F	n.a.	n.a.	166	17	147	12	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
II:2	24	F	27	No	143	28	110	26	4.2	n.a.	0.88	1.00	0.88	1.01	n.a.	<i>APOB</i> -p.Cys2933*
Family IV																
I:1	84	M	26	No	106	33	60	60	4.7	n.a.	0.62	0.42	1.44	0.50	Y	<i>APOB</i> -p.Ser3718*
II:1	58	M	34	Yes	123	40	68	75	7.2	6.5	2.34	2.26	11.73	0.47	Y ^a	<i>APOB</i> -p.Ser3718*
II:2	58	F	n.a.	No	250	142	98	50	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
III:1	26	M	21	No	102	36	57	46	n.a.	5.4	0.46	0.74	0.34	0.23	n.a.	<i>APOB</i> -p.Ser3718*
III:2	18	F	19	No	124	49	62	65	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	N	<i>APOB</i> -p.Ser3718*
Family V																
I:2	52	F	22	No	132	63	55	72	n.a.	5.5	0.54	0.37	0.33	0.56	n.a.	<i>PCSK9</i> -c.523G > A
II:1	23	M	22	No	120	50	55	89	4.5	n.a.	0.53	0.29	0.18	n.a.	n.a.	<i>PCSK9</i> -c.523G > A
II:2	16	M	20	No	141	74	52	76	4.3	n.a.	1.05	0.54	0.41	0.32	n.a.	<i>PCSK9</i> -c.523G > A
II:3	13	F	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	<i>PCSK9</i> -c.523G > A

Age is expressed in years; body mass index (BMI) in kg/m², total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), triglycerides (TG) in mg/dL; fasting plasma glucose (FPG) in mmol/L; glycosylated hemoglobin (HbA1c) in %; transaminases (aspartate aminotransferase (AST) and alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT), are expressed in upper limits of normal (ULN). Amino acid designation begins with "1" at translation start codon 129 of *APOB* cDNA (Genbank entry NM_000384.2); F, female; M, male; WT, wild-type sequence; n.a., not available.

^a Hepatocellular carcinoma.

The *PCSK9* variant (Chr1:55,512,319; c.523 G > A; *PCSK9*) was identified in a 23 years-old man (II:1/Family V) with low LDL-C level (50 mg/dL). The proband's mother (I:2) and brother (II-2) who also had low LDL-C (63 mg/dL and 74 mg/dL respectively) harbor the variant. The proband's sister (II:3) with unknown phenotype also harbor the variant.

3.3. *APOB*-p.Cys2933* and p.Ser3718* mutations generate circulating truncated proteins

APOB-p.Gln845* and p.Gln2571* mutations can potentially generate truncated proteins with expected weight of 94 kDa and 289 kDa respectively. These truncated forms were not visible in plasma (Fig. 1C). *APOB*-p.Cys2933* and p.Ser3718* mutations lead to the formation of expected truncated ApoB forms (ApoB-64 and ApoB-81) of 330 kDa and 418 kDa (Fig. 1C, right panel) that can be secreted by the liver and lipidated.

4. Discussion

We have developed a targeted NGS approach to improve molecular diagnosis for FHBL. By testing this approach on a series of eight FHBL probands, we identified four heterozygous likely

disease-causing *APOB* nonsense mutations. Our 50% detection rate for *APOB* mutation is in line with previously reported *APOB* mutation prevalence in FHBL patients [8,1]. Liver steatosis was diagnosed in four patients from three families with *APOB* mutations, including one with severe NASH complicated by a HCC, reinforcing the hypothesis that some *APOB* mutations could lead to FHBL, liver cirrhosis and HCC [17]. We also identified a rare *PCSK9*-c.523 G > A variant that co-segregates with FHBL. Although *in silico* analyses predict a potential *PCSK9* splice variant, further functional experiments are needed to determine whether this variant is associated with a *PCSK9* loss-of-function.

APOB encodes a product containing 4563 amino acids and encompasses 29 exons. Over 90 mutations have been reported in subjects with FHBL, mostly nonsense and frameshift mutations and novel mutations are continually being identified. Moreover, missense mutations have been described in FHBL [18,19] and could be missed by current genetic testing using western-blotting as a first-line approach. Because most unrelated FHBL kindreds carry private mutations and because there is no evidence for FHBL mutation hotspots, further development of reliable, high throughput and cost effective molecular screening approaches are needed. In the context of genetically heterogeneous diseases involving large genes, targeted resequencing technology has become an accurate,

fast and cost effective approach, which is applicable in routine molecular clinical diagnosis [20,21]. Johansen et al. recently designed a large targeted resequencing panel to investigate 73 genes in patients presenting various forms of dyslipidemia and related metabolic disorders [12]. They demonstrated that - despite some limitations due to lower coverage depth for particular genomic regions and to the nondetection of genome structural variation - this panel allows the detection of most known mutations based on Sanger sequencing as well as high detection rates for coding mutations in previously unsequenced samples.

Interestingly, the patients carrying no disease-relevant variant in the known FHBL-susceptibility genes could be subjected to whole exome sequencing combined with exhaustive familial investigation, in order to identify new disease genes. The demonstration that PCSK9 loss-of-function mutations are associated with reduced cardiovascular events [22] has been the accelerator for the development of PCSK9 inhibitors. More recently, monoclonal antibodies directed against ANGPTL3 have demonstrated lipid-lowering efficacy in monkeys [23]. In this context, comprehensive genetic resequencing ranging from exome to whole genome but also RNA sequencing could represent the first step towards identifying new drug targets in hypercholesterolemia.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.atherosclerosis.2016.04.010>.

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