Heterozygosity for apolipoprotein A-I(R160L)$_{Oslo}$ is associated with low levels of high density lipoprotein cholesterol and HDL-subclass LpA-I/A-II but normal levels of HDL-subclass LpA-I

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Abstract We studied a Norwegian patient and his family, who presented with low HDL-cholesterol. DNA sequence analysis of the apoA-I gene revealed heterozygosity for a mutation in the apoA-I gene that causes a leucine for arginine replacement at residue 160. Compared to unaffected family members, heterozygous carriers of apoA-I(R160L)$_{Oslo}$ had 60–70% lower mean levels of HDL-cholesterol, 50–60% lower mean levels of apoA-I and 70–80% lower levels of apoA-II. Moreover, the serum concentration of the apoA-II-containing HDL-subclass LpA-I/A-II was decreased by 70% whereas the concentration of the apoA-II-free HDL-subclass LpA-I did not differ from that in unaffected family members. The decrease of LpA-I/A-II was associated with the lack of large LpA-I/A-II. ApoA-I(R160L)$_{Oslo}$ was present at increased concentrations relative to normal apoA-I in plasma, HDL$_3$, and LpA-I. However, only trace amounts of the variant isoform were detectable in immunopurified LpA-I/A-II. Preβ_1-LpA-I contained normal and variant apoA-I isoforms. We conclude that the failure of apoA-I(R160L)$_{Oslo}$ to form LpA-I/A-II causes low HDL-cholesterol in heterozygous carriers of this apoA-I variant.—**Leren, T. P., K. S. Bakken, U. Daum, L. Ose, K. Berg, G. Assmann, and A. von Eckardstein.** Heterozygosity for apolipoprotein A-I(R160L)$_{Oslo}$ is associated with low levels of high density lipoprotein cholesterol and HDL-subclass LpA-I/A-II but normal levels of HDL-subclass LpA-I. *J. Lipid Res.* 1997. **38:** 121–131.

Supplementary key words familial hypoalphalipoproteinemia • apoA-I variants • cardiovascular risk factors • HDL-subclasses

The concentration of high density lipoprotein (HDL) cholesterol is inversely related to the risk of myocardial infarction (reviewed in ref. 1). Outcomes of family and twin studies suggest that genes control 35–50% of the interindividual variability in HDL-cholesterol levels (2). Moreover, 20% of patients with myocardial infarction below age 60 years have several first-degree relatives with low levels of HDL-cholesterol (3). The best understood conditions of familial low levels of HDL-cholesterol are caused by rare mutations in the genes encoding apolipoprotein (apo) A-I, which is the major protein component in HDL, and the cholesterol esterifying enzyme lecithin:cholesterol acyltransferase (LCAT) (reviewed in refs. 4–6). To date, approximately 40 different mutations in the apoA-I gene have been described. Only null alleles and a minor proportion of nonsynonymous mutations led to decreased levels of HDL-cholesterol (7–24). Thus apoA-I gene defects do not account for the high prevalence of familial hypoalphalipoproteinemia in survivors of myocardial infarction. Moreover, in most cases heterozygous carriers did not appear to be at increased risk of coronary heart disease although they frequently had HDL-cholesterol levels below the threshold values for increased cardiovascular risk. Nevertheless, natural apoA-I variants can serve as invaluable tools to elucidate structure–function relationships that help this multifunctional protein play its pivotal role in reverse cholesterol transport (reviewed in ref. 25). To date, the domains of apoA-I necessary to form distinct HDL-subclasses (26–28), to activate

Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; IEF, isoelectric focusing; LDL, low density lipoprotein; LpA-I, lipoproteins containing apoA-I but not apoA-II: LpA-I/A-II, lipoproteins containing both apoA-I and apoA-II: PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.

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LCAT (review in refs. 25, 29), to bind to cell-surface receptors (30, 31), and to promote cholesterol efflux from cells (26, 27, 30, 32, 33) are unknown. The most informative apoA-I variants in this regard will be those that cause low levels of HDL-cholesterol. We here describe a novel apoA-I variant, apoA-I(R160L)$_{Oslo}$, which is associated with low levels of HDL-cholesterol in heterozygous carriers.

MATERIALS AND METHODS

Subjects

The male proband was born in 1950. He was diagnosed with mild hypertension in his early twenties. Otherwise, he had been healthy until the age of 32 when he experienced dizziness followed by slight paresis of the left half of the body. Physical examination revealed left-sided hemiparesis. At two occasions cerebral computer tomographies with or without contrast fluid revealed no pathological findings. No cerebral vessel anomaly was found by cerebral angiography. Electroencephalograms showed slight generalized dysrhythmia without focal changes. Echocardiography revealed slight hypertrophy of the left ventricle. Electrocardiograms were normal. Due to these negative findings it was concluded that the patient had suffered from a cerebral infarction presumably due to a thromboembolic event. The hemiparesis lasted for about 1 year.

The patient came to our attention at the age of 41 years because routine laboratory analysis revealed abnormally low levels of HDL cholesterol (0.45 mmol/l) and apoA-I (52 mg/dl). Clinical examination did not reveal anything abnormal except hyperreflexia in the left half of the body. Specifically, there were no corneal cloudings, no planar xanthomas; and no tonsil anomalies.

The proband is the oldest of three siblings. His father underwent surgery at the age of 68 because of aortic aneurysm. Otherwise family history was not indicative for the occurrence of premature cardiovascular disease in the family. All studies were performed with the subjects' informed consent.

Blood samples

Venous blood samples were taken after at least 12 h fasting. After centrifugation at 4°C for 15 min at 2,000 *g*, sera and EDTA-plasmas were either analyzed directly or aliquoted and immediately frozen at −70°C. Serum samples were used for the quantification of lipids, lipoproteins, and apolipoproteins. Plasma samples were used for the determination of LCAT activity and the separation of lipoproteins by either ultracentrifugation, nondenaturing two-dimensional polyacrylamide gradient gel electrophoresis (2D-PAGGE), or immunoaffinity chromatography and of apolipoproteins by isoelectric focusing (IEF).

DNA extraction

DNA was extracted from white blood cells using a Model 340A Nucleic Acid Extractor (Applied Biosystems Inc., Foster City, CA).

Haplotype analysis at the apoA-I locus

Four restriction fragment length polymorphisms (RFLPs) within or closely linked to the apoA-I gene were used to construct haplotypes at the apoA-I locus. These RFLPs were the MspI polymorphism in intron 3 (34), the PstI polymorphism in the intergenic region between the apoA-I and apoC-III genes (35), the SacI polymorphism in the apoC-III gene (36), and the PvuII polymorphism in the apoC-III gene (37). PCR-based methods were developed to analyze these RFLPs. Briefly, amplicons containing the polymorphic sites were cut with the relevant restriction enzymes and analyzed by gel electrophoresis. Haplotypes were constructed under the assumption that there had been no recombination within the apoA-I locus.

Analysis of single-strand conformation polymorphisms of the apoA-I gene

Analysis of single-strand conformation polymorphisms (SSCP) (38) was performed to screen for mutations in the apoA-I gene. The mixtures prepared for the PCRs contained 400 ng DNA, 20 pmol of each of the two primers, 200 μM dNTPs, 3.3 nM ^{32}P-dCTP (3000 Ci/mmol) and the buffer supplied with the Taq DNA polymerase (Perkin Elmer, Foster City, CA). The reaction volume was 100 μl. Eight μl of the loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol) were added to 2 μl of radiolabeled PCR products and heated for 2 min at 94°C. Two μl of this mixture was loaded in each well of a 6% nondenaturing sequencing gel (acrylamide: bisacrylamide 100: 1.5). Electrophoresis was performed under four different conditions in a cold room. These conditions were 15 W, 40 W, or 50 W in the absence of glycerol, and 50 W in the presence of 10% glycerol. The gels were transferred to Whatman 3 MM papers, dried, and autoradiographed for 5–7 days at −80°C.

DNA sequencing

Symmetric PCR products were used as templates for DNA sequencing. One of the primers contained the sequence for the −21M13 universal primer at the 5′ end to allow the sequencing reactions to be performed with

dye-labeled −21M13 as the sequencing primer. The PCR products were purified by ultrafiltration using Centricon-100 columns (Amicon Inc., Beverly, MA) prior to the sequencing reactions. DNA sequencing was performed on a 373A DNA sequencer (Applied Biosystems Inc., Foster City, CA) using Thermo Sequenase according to the instructions by the manufacturer (Amersham International plc., UK). The first nucleotide of exon 1 was given position +1.

Quantification of lipids, lipoproteins, apolipoproteins, and LCAT activity

Levels of cholesterol and triglycerides were determined by automated, enzymatic techniques (Boehringer Mannheim, Germany). HDL-cholesterol was also measured enzymatically after precipitation of apoB-containing lipoproteins by phosphotungstic acid/magnesium chloride (Boehringer Mannheim). LDL-cholesterol was quantified by the formula of Friedewald, Levy, and Fredrickson (39). Apolipoproteins A-I, A-II, and B were measured by immunoturbidimetric assays from Boehringer Mannheim (Germany). LpA-I was determined by immunoelectrophoresis using ready-to-use plates from Sebia (Paris, France). LpA-I/A-II levels were calculated as the difference between the concentrations of apoA-I and LpA-I. The activity of plasma to esterify cholesterol was assayed either as the esterification of [^{3}H]cholesterol (New England Nuclear, Boston, MA) that was incorporated into reconstituted, apoA-I-containing particles (i.e., LCAT activity, for details see refs. 40, 41) or after equilibration of [^{3}H]cholesterol with endogenous lipoproteins of plasma (i.e., plasma cholesterol esterification rate, for details see refs. 41, 42) or apoB-depleted plasma (i.e., HDL cholesterol esterification rate, for details see refs. 41, 43).

Isolation of plasma lipoproteins

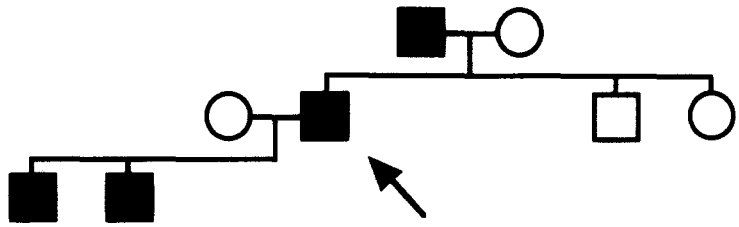
HDL_2 and HDL_3 were isolated from plasma by sequential ultracentrifugation at densities of $1.061 < d < 1.125$ g/ml and $1.125 < d < 1.21$ g/ml, respectively, using a Beckman ultracentrifuge and a Ti50.4 rotor (44). HDL-subfractions containing apoA-II (i.e., LpA-I/A-II) and lacking apoA-II (i.e., LpA-I) were separated by anti-apoA-II-immunoaffinity chromatography. Briefly, 100 mg of γ-globulin fraction of a sheep anti-apoA-II antiserum (Boehringer Mannheim) was coupled to 20 ml CNBr-activated Sepharose 4B (Pharmacia, Sweden) following the recommendations of the manufacturer. Aliquots of 50 µl plasma in 1 ml PBS were circulated through the column for 18 h at 4°C. The bound fraction was eluted with 200 mM sodium acetate buffer (pH 3.0). Both bound and unbound fractions were dialyzed against 1 mM PBS and subsequently lyophilized to be used for isoelectric focusing (see below).

Electrophoretic demonstration of apoA-I-isoforms

Normal and variant isoforms of apoA-I were separated by isoelectric focusing (IEF) as described previously (45). As samples we used either 2 µl plasma, 10 µg HDL_2, 10 µg HDL_3 or equivalents of 5 µl plasma from the unbound and bound fractions of plasma that were passed through an anti-apoA-II-Immunoaffinity chromatography column. After separation, IEF strips were either stained directly with Coomassie Blue or used for electroblotting or two-dimensional (2D-) immunoelectrophoresis (46). After electroblotting onto nitrocellulose sheets, apoA-I or apoA-II were visualized using polyclonal antibodies from Boehringer Mannheim against human apoA-I and apoA-II, respectively. For 2D-immunoelectrophoresis, IEF strips were transferred onto agarose gels that contained 4% (v/v) of the γ-globulin fraction of a polyclonal sheep anti-apoA-I-antiserum (Boehringer Mannheim, Germany) (46). The relative concentrations of normal and variant apoA-I isoforms were determined by scanning densitometry of either the Coomassie Blue-stained gels or the anti-apoA-I immunoblots using the Ultrascan of Pharmacia (Bromma, Sweden). As the normal isoform apoA-I_2 and the variant isoform apoA-I_1 have identical isoelectric points, the concentration of the variant apoA-I was corrected by a factor that was obtained by multiplication of the ratio apoA-I_2/normal apoA-I_1 in a control sample with the concentration of normal apoA-I_1 in the patient sample. This value was subtracted from the concentration of normal apoA-I_2 + variant apoA-I_1.

Nondenaturing two-dimensional electrophoresis

The distribution of apoA-I- and apoA-II-containing lipoproteins was analyzed by nondenaturing two-dimensional polyacrylamide gradient gel electrophoresis (2D-PAGGE) of plasma where agarose gel electrophoresis was followed by polyacrylamide gradient gel electrophoresis (47). Briefly, in the first dimension, 20 µl plasma was separated by electrophoresis at 4°C in a 0.75% agarose gel using a 50 mM merbital buffer (pH 8.7, Serva, Heidelberg, Germany). Bromphenolblue was added to a standard sample to visualize albumin in the native gel. The electrophoresis was stopped when the albumin/bromphenolblue marker had migrated 6 cm. Agarose gel strips containing the preseparated lipoproteins were then transferred to a 3–20% polyacrylamide gradient gel. Separation in the second dimension was performed at 40 mA for 4–5 h at 10°C. During this time, the endogenous plasma albumin, which because of bromphenolblue added to the cathodic buffer (300 µl per liter buffer) was visible in the native gel as a faint blue band, had migrated 10 cm. The proteins separated in the PAGGE gel were electroblotted onto a nitrocellulose



Total serum cholesterol (mmol/l)	3.1	4.3	5.7	5.0	5.3	9.5	5.7	4.9
Triglycerides (mmol/l)	2.04	2.39	0.49	1.85	1.51	1.57	2.31	1.27
HDL cholesterol (mmol/l)	0.33	0.41	1.74	0.45	0.65	1.38	1.06	1.50
ApoA-I (mg/dl)	63	47	126	52	86	130	142	169
Haplotype markers								
MspI	-/+	-/+	+/+	-/+	-/+	+/+	+/+	+/+
PstI	+/+	+/-	+/-	+/-	+/+	+/-	+/-	+/-
SacI	+/-	+/-	-/-	+/-	+/-	+/-	-/-	-/-
PvuII	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
SSCP pattern of exon 4	A	A	B	A	A	B	B	B
Nucleotide 1561	G/T	G/T	G/G	G/T	G/T	G/G	G/G	G/G
SSCP pattern of exons 1-3	C	D	C	D	C	D	C	C
Nucleotide 319	C/T	T/T	C/T	T/T	C/T	T/T	C/T	C/T

Fig. 1. Pedigree and laboratory findings of a Norwegian kindred with familial hypoalphalipoproteinemia. Filled symbols are used to indicate those with abnormally low levels of HDL-cholesterol and apoA-I. The RFLPs used as haplotype markers at the apoA-I locus are indicated by the restriction enzymes. Presence and absence of restriction sites are indicated by + and −, respectively. SSCP patterns A and B of exon 4 and the nucleotide at position 1561 relate to the findings described in Fig. 2. R indicates arginine, and L indicates leucine. The SSCP patterns C and D of exons 1–3 relate to the polymorphism at position 319.

membrane. ApoA-I- and apoA-II-containing lipoproteins were immunodetected by the use of sheep antibodies against human apoA-I or human apoA-II (Boehringer Mannheim). Antigen–antibody compexes were visualized by the use of horseradish peroxidase-conjugated antibodies from rabbit to sheep-IgG (DAKO, Denmark).

IEF of preβ$_1$-LpA-I

Nondenaturing 2D-PAGGE was modified as a three-step electrophoresis in the sequence agarose gel electrophoresis → PAGGE → IEF to determine the relative concentration of normal and variant apoA-I in HDL-subclass preβ$_1$-LpA-I. Twenty-μl samples of plasmas from two heterozygotes for the apoA-I variant and from two unaffected members as well as several standard plasmas were separated by agarose gel electrophoresis as described before. Thereafter one lane with the separated lipoproteins of a standard sample was removed and stained with oil-red. This gel strip was then used to localize preβ-migrating lipoproteins in the unstained gel. The proportion of the unstained gel containing the preβ-migrating lipoproteins of all adjacent samples was cut perpendicular to the electrophoretic axis, removed, and transferred to a PAGGE-gel for electrophoresis as previously described. After completion of this electro-

TABLE 1. List of oligonucleotides used as primers for PCR

Oligonucleotide	DNA Sequence	5′ Position [b]
A	5′-GGAGGCCAGCCCTCAACCCTTCTGT-3′[a]	1174
B	5′-GCACCCCCCTCTATTCTCCCAAAAG-3′	1907
C	5′-AAGAGAAGCTGAGCCCACTGGGCGA-3′[a]	1495
D	5′-CGCGCGCGCCCATGTGGACGCGCTGCT-3′	1537
E	5′-CTGCAGACATAAATAGGCCCTGCAA-3′[a]	−38
F	5′-GCCCTCAACCCCAGGCTGGGTCCTT-3′[a]	649

[a]Indicates that the primer was synthesized with the 18 bp-21M13 universal primer as a 5′ tail.
[b]The first nucleotide of exon 1 was given position +1.

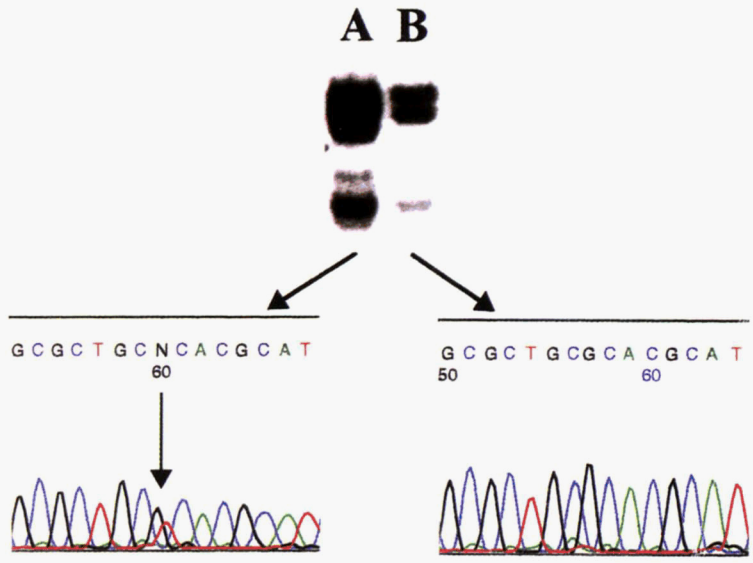


Fig. 2. SSCP analysis of exon 4 of the apoA-I gene. SSCP analysis of exon 4 of the apoA-I gene revealed two different patterns (A and B) when the nondenaturing gel electrophoresis was performed at 50 W in the presence of 10% glycerol. The DNA sequences underlying the two patterns were characterized by DNA sequencing. Whereas SSCP pattern B represented the wild type sequence, SSCP pattern A was due to heterozygosity (G/T) at nucleotide 1561.

phoresis, a gel strip with the preβ-migrating proteins of a single standard sample was removed. Its proteins were electroblotted onto a nitrocellulose sheet for subsequent visualization of preβ$_1$-LpA-I with anti-apoA-I antibodies (see above). This immunoblot was then used to localize preβ$_1$-LpA-I in the native gel, which meanwhile had been stored at 4°C. A 1-cm-broad gel strip containing preβ-$_1$-LpA-I of adjacent samples was cut perpendicularly to the running axis and transferred to an IEF gel for the separation of apoA-I isoforms as described before. After this run, proteins were electroblotted onto nitrocellulose sheets for detection of apoA-I isoforms with anti-apoA-I antibodies (see above). The staining intensity of the bands was measured by scanning densitometry.

RESULTS

Identification of the genetic defect

A study of the propositus' family (pedigree in **Fig. 1**) identified several members affected with levels of HDL-cholesterol and apoA-I below the 5th percentile of sex-matched controls from the Caucasian population (48),

TABLE 2. Effect of apoA-I(L160R)$_{Oslo}$ on parameters of lipid metabolism

Genotype	Wild Type + ApoA-I(R160L)	Two Wild Type Alleles
Subjects (n males/n females)	4M/0F	1M/3F
Total cholesterol (mmol/L)	4.42 ± 0.98	6.45 ± 2.07
Triglycerides (mmol/L)	1.95 ± 0.37	1.41 ± 0.75
HDL cholesterol (mmol/L)	0.46 ± 0.14[a]	1.40 ± 0.28
LDL cholesterol (mmol/L)	2.56 ± 0.88	4.41 ± 2.03
Apolipoprotein A-I (g/L)	0.62 ± 0.17[a]	1.42 ± 0.19
Apolipoprotein A-II (g/L)	0.16 ± 0.03[a]	0.58 ± 0.16
Apolipoprotein B (g/L)	1.30 ± 0.30	1.43 ± 0.57
LpA-I (g/L)	0.39 ± 0.07	0.37 ± 0.04
LpA-I/A-II (g/L)	0.39 ± 0.23[a]	1.26 ± 0.22
LCAT (nmol/h/mL)	11.9 ± 1.7[a]	17.3 ± 1.3
Fractional cholesterol esterification rate		
In plasma (%)	8.08 ± 1.47	10.5 ± 1.98
In HDL (%)	6.15 ± 4.39[b]	13.70 ± 3.14

M, male; F, female.
[a]$P < 0.001$; [b]$P < 0.05$, Student's *t*-test.

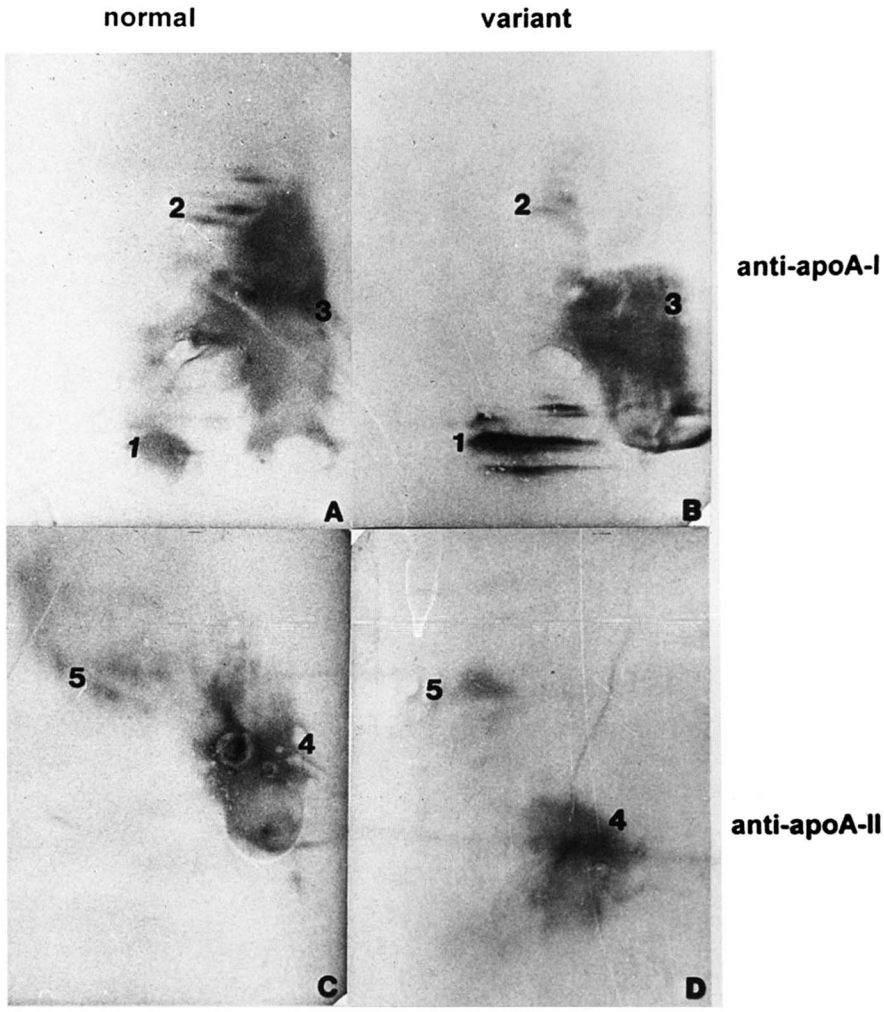


Fig. 3. 2D-PAGGE and immunoblotting of apoA-I- and apoA-II-containing lipoproteins in plasmas of a normoalphalipoproteinemic individual and a hypoalphalipoproteinemic carrier of apoA-I(R160L)$_{Oslo}$. Nondenaturing 2D-electrophoresis was performed in the order agarose gel electrophoresis → nondenaturing polyacrylamide gradient gel electrophoresis. After electroblotting onto nitrocellulose membranes, apoA-I- (A,B) and apoA-II-containing lipoproteins (C,D) were detected using polyclonal sheep antibodies against either human apoA-I or human apoA-II. Note the presence of preβ_1-LpA-I (spots denoted with 1) and preβ_2-LpA-I (spots denoted with 2) in plasmas of both the propositus (A) and his unaffected wife (B). However, proportions with α-LpA-I or α-LpA-II of larger size present in normal plasma (A: spots denoted with 3, C: spots denoted with 4) lacked in the plasma of the propositus (B: spots denoted with 3, D: spots denoted with 4).

namely his father and his two children. The mean levels of HDL cholesterol and apoA-I among these four subjects were 0.46 mmol/l and 62 mg/dl, respectively. The corresponding values among the four other subjects in the family were 1.40 mmol/l and 142 mg/dl, respectively. These results were consistent with low levels of HDL cholesterol and apoA-I being inherited in an autosomal dominant fashion.

Haplotype analyses at the apoA-I locus using four haplotype markers showed that low levels of HDL cholesterol and apoA-I segregated with haplotype: MspI: −, PstI: +, SacI: + and PvuII:− at the apoA-I locus (Fig. 1). SSCP analysis of exon 4 of the apoA-I gene was performed for the eight family members. Primers A and B (**Table 1**) were used to create a 752 bp fragment that was cut with restriction enzymes BglI and MboII to create fragments of 260 bp, 254 bp, and 238 bp. When gel electrophoresis was performed at 50 W in the presence of 10% glycerol, two different SSCP patterns were observed (**Fig. 2**). Hypoalphalipoproteinemic subjects had pattern A and normoalphalipoproteinemic subjects had pattern B (Fig. 1). These data suggested the presence of a mutation in exon 4 of the apoA-I gene that segregates with low levels of HDL cholesterol. DNA sequencing of exon 4 of the apoA-I gene using primers A or C as 5′ primers and primer B as 3′ primer (Table

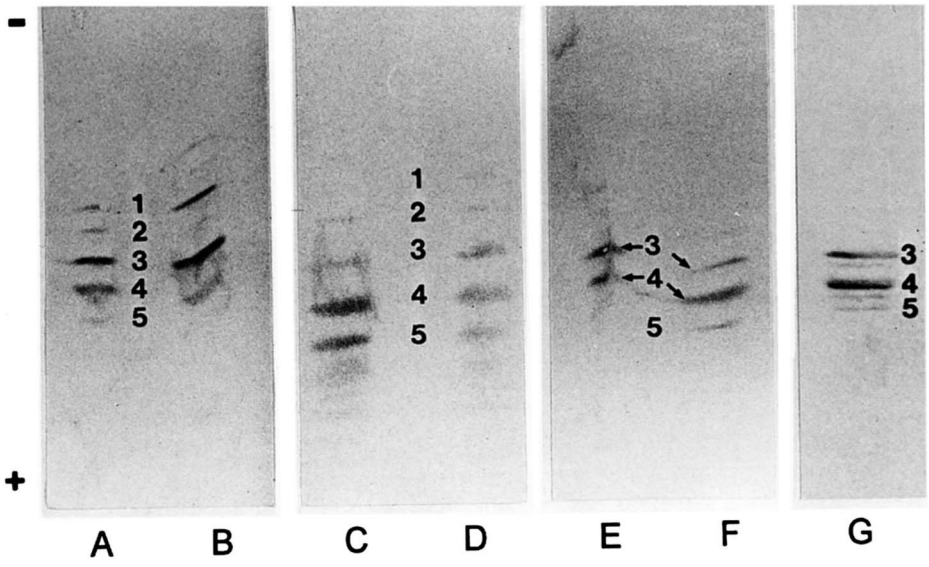


Fig. 4. Demonstration of apoA-I by IEF of plasma and HDL-subclasses. IEF was performed as reported previously (44) on 2 µl plasma (A,B), 10 µg HDL_3 (C), 10 µg HDL_2 (D), LpA-I/A-II (E), and LpA-I (F) of 5 µl plasma, and $preβ_1$-LpA-I (G) of 40 µl plasma. Lanes A and C through G contain samples of the propositus, lane B a sample of his unaffected wife (control). Band 1 corresponds to normal proapoA-I, band 2 to variant proapoA-I, band 3 to normal apoA-I_1, and band 5 to variant apoA-I_2. In normal plasma (lane B), band 4 corresponds to normal apoA-I_2. In samples of apoA-I variant carriers (lanes A, C–G), band 4 contains both normal apoA-I_2 and variant apoA-I_1. Note that HDL_3 (C) and LpA-I (F) contain variant apoA-I isoforms (bands 4 and 5) at higher concentrations than normal apoA-I isoforms (band 3). By contrast, LpA-I/A-II contain only traces of the apoA-I variant (E, band 4).

1) identified heterozygosity for a G→T mutation at nucleotide 1561 (Fig. 2) in all family members with low levels of HDL cholesterol and apoA-I and SSCP pattern A (Fig. 1). This transversion in codon 160 of the apoA-I gene leads to a leucine for arginine substitution in the encoded protein which we term apoA-I(R160L)$_{Oslo}$. The presence of this defect was confirmed by a PCR-based assay for which primer D spanning nucleotides 1537 to 1561 with T at its 3′ end, was used as the 5′ primer (Table 1). The 3′ primer was primer B (Table 1). This assay with an annealing temperature of 60°C, gave a PCR product of 106 bp only in those with apoA-I(R160L)$_{Oslo}$ (data not shown).

To rule out other defects in the apoA-I gene that may segregate with low levels of HDL cholesterol and apoA-I, SSCP analysis was also performed on exons 1–3. This was done by amplifying a 685 bp fragment spanning exon 1 to exon 3 as well as the intervening introns, using primer E as 5′ primer and primer F as 3′ primer (Table 1). This fragment was cut with HindIII to create fragments of 252 bp and 433 bp prior to the nondenaturing gel electrophoresis. Two different patterns were observed which, however, did not segregate with levels of HDL cholesterol and apoA-I (Fig. 1). DNA sequencing of this 685 bp fragment was performed in all eight subjects in order to characterize the DNA sequences underlying SSCP patterns C and D. Whereas those with SSCP pattern D had the wild-type sequence (34), those with SSCP pattern C were heterozygous for a C → T mutation at nucleotide 319 (position +41 of intron 2) (Fig. 1).

Effects of apoA-I(R160L)$_{Oslo}$ on lipids, lipoproteins, and apolipoproteins as well as LCAT activity

In **Table 2** we have compared mean values of lipids, lipoproteins, apolipoproteins, and LCAT activity in heterozygotes for apoA-I(R160L)$_{Oslo}$ and in unaffected family members. Compared to unaffected family members, heterozygotes for apoA-I(R160L)$_{Oslo}$ had significant decreases in HDL cholesterol (−67%), apoA-I (−56%), apoA-II (−70%), LpA-I/A-II (−70%), LCAT activity (−30%), and HDL cholesterol esterification rate (−55%). Interestingly, plasma levels of the HDL-subclass LpA-I were not decreased.

To analyze possible effects of apoA-I(R160L)$_{Oslo}$ on the distribution of apoA-I-containing particles in more detail, we separated plasmas by nondenaturing 2D-PAGGE and visualized apoA-I- and apoA-II-containing lipoproteins by immunoblotting (**Fig. 3**). Both unaffected family members and carriers of apoA-I(R160L)$_{Oslo}$ had $preβ_1$-HDL and $preβ_2$-HDL (Fig. 3A and B). The α-migrating particles with either anti-apoA-I or anti-apoA-II immunoreactivity of apoA-I(R160L)$_{Oslo}$ heterozygotes lacked proportions with larger size found in normal plasma (Fig. 3B and D).

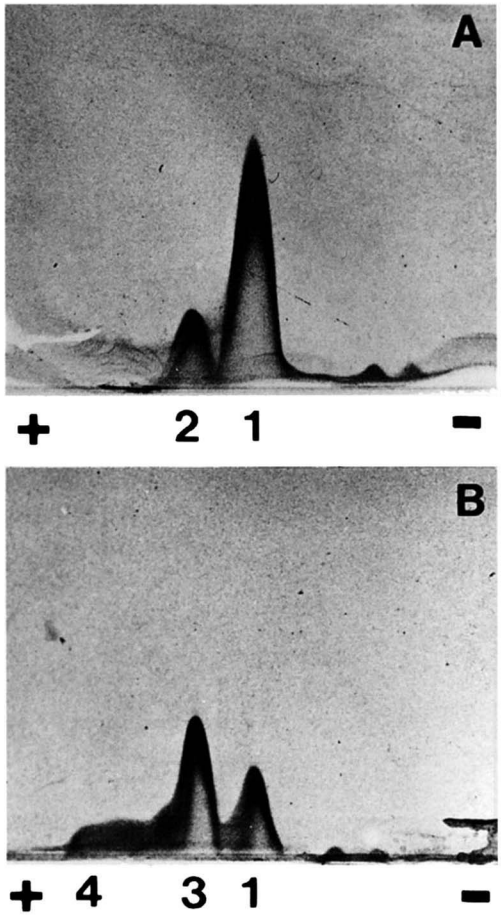


Fig. 5. Demonstration of apoA-I by two-dimensional immunoelectrophoresis. Immunoelectrophoresis was performed perpendicular to the IEF-axis in a 1% agarose gel that contained 4% (v/v) of the γ-globulin fraction of an anti-apoA-I-antiserum (46). Part A demonstrates apoA-I isoforms in normal plasma, part B apoA-I isoforms in the propositus' plasma. In A, apoA-I_1 is marked 1; apoA-I_2 is marked 2. In B, 1 denotes normal apoA-I_1; 3 denotes the sum of normal apoA-I_2 and variant apoA-I_1; and 4 denotes variant apoA-I_2.

To analyze the relative distribution of normal and mutant allele products, we separated plasmas and lipoproteins of the apoA-I variant carriers by IEF. All hypoalphalipoproteinemic plasmas contained two additional bands anodic from normal mature apoA-I and proapoA-I, respectively (**Fig. 4**, lane A). Scanning densitometry of IEF gels as well as two-dimensional immunoelectrophoresis of plasma in the sequence IEF → immunoelectrophoresis (**Fig. 5**) demonstrated that the variant apoA-I isoform is present in plasma at slightly elevated concentration relative to normal apoA-I (60% instead of expected 50%). HDL_3 contained significantly more variant apoA-I isoforms than normal apoA-I isoforms (Fig. 4, lane C), whereas HDL_2 contained normal and variant apoA-I at about identical concentrations (Fig. 4, lane D). The plasma fraction adsorbed to an anti-apoA-II immunoaffinity chromatography column, i.e., LpA-I/A-II contained normal apoA-I but only traces of the variant apoA-I isoform (Fig. 4, lane E) whereas the apoA-II-free unbound fraction, i.e., LpA-I, contained the variant apoA-I isoforms at larger amounts than normal apoA-I isoforms (Fig. 4, lane F). IEF of apoA-I in preβ$_1$-LpA-I that was pre-separated by agarose gel electrophoresis and nondenaturing PAGGE revealed that preβ$_1$-LpA-I contains normal and variant apoA-I isoforms at equal proportions (Fig. 4, lane G).

DISCUSSION

We have identified a novel apoA-I variant, apoA-I(R160L)$_{Oslo}$, that causes in heterozygous carriers a pronounced decrease of HDL cholesterol and apoA-I. As only this defect and no other polymorphisms in the apoA-l gene segregated with low HDL cholesterol in the affected Norwegian family, a causal relationship is very likely. Further support for such a causal relationship can be derived from the disproportionate distribution of normal and variant apoA-I isoforms among HDL subclasses (Fig. 5). Thus, in addition to apoA-I(G26R)$_{Iowa}$ (20), apoA-I(Δ107K) at least in men (22, 23), apoA-I(L141R)$_{Pisa}$ (17, 49), apoA-I(R151C)$_{Paris}$ (50), apoA-I(L159P)$_{Zavalla}$ (24), apoA-I(P165R) (19), and apoA-I(R173C)$_{Milano}$ (18), this variant provides another example of a single amino acid substitution in apoA-I that severely reduces HDL cholesterol levels in heterozygous carriers. This in contrast to most other single amino acid substitutions in apoA-I that have no effect on HDL cholesterol (4, 51). Most single amino acid substitutions in apoA-I that exert pronounced effects on HDL cholesterol cluster between amino acid residues 140 and 173. The deletion of amino residues 143–165 in apoA-$I_{Seattle}$ even led to near absence of HDL in a heterozygous carrier (21). These observations point to an essential role of this domain for the regular function and metabolism of HDL. Interestingly, monoclonal antibodies with epitopes residing in this region were found to react with apoA-I only in specific HDL subclasses and to decrease the capacity of apoA-I, HDL, and serum to promote cholesterol efflux from cells (26, 27, 32).

Its severely reduced concentration in LpA-I/A-II but enrichment in LpA-I points to the disturbed association of apoA-I(R160L)$_{Oslo}$ with distinct HDL subclasses. One may speculate that apoA-I(R160L)$_{Oslo}$ is disturbed in its interaction with apoA-II. Probably as a consequence of absent apoA-I(R160L)$_{Oslo}$ in apoA-II-containing HDL, low HDL cholesterol in heterozygotes for apoA-I(R160L)$_{Oslo}$ was almost exclusively caused by a decrease in HDL subclass LpA-I/A-II while some other apoA-I defects cause decreases of both LpA-I and LpA-I/A-II (20, 49, 50, 52). A predominant decrease of LpA-I/A-II has

also been observed in heterozygotes for apoA-I(Δ107K) (23) and patients with familial LCAT deficiency (53). As apoA-I(Δ107K) is associated with decreased LCAT-cofactor activity (29) and as LCAT activity was reduced in heterozygotes for apoA-I(R160L)$_{Oslo}$, one may speculate that deficiency of LpA-I/A-II is due to impaired LCAT activity. However, other apoA-I variants with disturbed LCAT activity were associated with more prominent decreases of LpA-I than LpA-I/A-II (49, 52, 54) so that this is a less likely explanation.

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