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Arteriosclerosis, Thrombosis, and Vascular Biology

CLINICAL AND POPULATION STUDIES

Contribution of *APOE* Genetic Variants to Dyslipidemia

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BACKGROUND: apo (apolipoprotein) E has crucial role in lipid metabolism. The genetic variation in *APOE* gene is associated with monogenic disorders and contributes to polygenic hypercholesterolemia and to interindividual variability in cholesterol. *APOE* rare variants may be involved in the phenotype of genetic hyperlipidemias.

METHODS: Exon 4 of *APOE* were sequenced in all consecutive unrelated subjects with primary hyperlipidemia from a Lipid Unit (n=3667) and 822 random subjects from the Aragon Workers Health Study. Binding affinity of VLDL (very low-density lipoprotein) to LDL receptor of pathogenic predicted apoE variants was analyzed in vitro. Lipoprotein particle number, size, and composition were studied by nuclear magnetic resonance.

RESULTS: In addition to common polymorphisms giving rise to APOE2 and APOE4, 14 gene variants were found in exon 4 of *APOE* in 65 subjects. p.(Leu167del) in 8 patients with isolated hypercholesterolemia and in 8 patients with combined hyperlipidemia. Subjects with p.(Arg121Trp), p.(Gly145Asp), p.(Arg154Ser), p.(Arg163Cys), p.(Arg165Trp), and p.(Arg168His) variants met dysbetalipoproteinemia lipid criteria and were confirmed by nuclear magnetic resonance. VLDL affinity for the LDL receptor of p.(Arg163Cys) and p.(Arg165Trp) heterozygous carriers had intermedium affinity between APOE2/2 and APOE3/3. p.(Gly145Asp) and p.(Pro220Leu) variants had higher affinity than APOE3/3.

CONCLUSIONS: *APOE* genetic variation contributes to the development of combined hyperlipidemia, usually dysbetalipoproteinemia, and familial hypercholesterolemia. The lipid phenotype in heterozygous for dysbetalipoproteinemia-associated mutations is milder than the homozygous APOE2/2-associated phenotype. Subjects with dysbetalipoproteinemia and absence of APOE2/2 are good candidates for the study of pathogenic variants in *APOE*. However, more investigation is required to elucidate the significance of rarer variants of apoE.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: apolipoprotein ■ exon ■ mutation ■ lipid metabolism ■ phenotype

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Many hyperlipidemias have a substantial genetic component. Traditionally, this genetic component has been assigned as monogenic, polygenic, and complex or multifactorial, depending on whether the phenotype is mainly due to a major and rare gene defect with Mendelian transmission, to a combination of multiple common risk alleles with minor effects, or if is the result of the interaction of the genetic variation in multiple genes with environmental factors, such as overweight or obesity, respectively.[1] Although the genes responsible of many genetic diseases causing hyperlipidemia have already been described,[2] there are still many individuals with the suspicion of genetic defects but without the genetic cause known.

Mature apo (apolipoprotein) E is a glycoprotein of 299 amino acids, synthetized in many tissues, including liver, brain, and tissue macrophages with a crucial role in lipid metabolism.[3] ApoE is a component of chylomicrons, very low-density (VLDL) and HDL (high-density lipoproteins); through interaction with different receptors including

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Nonstandard Abbreviations and Acronyms

Apo	apolipoprotein
AWHS	Aragon Workers Health Study
CHLP	combined hyperlipidemia
FH	familial hypercholesterolemia
HDL	high-density lipoprotein
HDLc	HDL cholesterol
HUMS	Hospital Universitario Miguel Servet
LDL	low-density lipoprotein
LDLr	low-density lipoprotein receptor
Lp(a)	lipoprotein(a)
LRP	LDL-related protein
NMR	nuclear magnetic resonance
VLDL	very low-density lipoprotein

the LDL (low-density lipoprotein) receptor (LDLr), LRPs (LDL-related proteins) and heparan sulphate proteoglycans promotes the clearance of remnants of chylomicrons and VLDL by the liver[4]; facilitates cholesterol-efflux to HDL from macrophages incorporating free cholesterol and phospholipid from ABCA1[5]; and may stimulate adipogenesis from triglyceride-rich lipoproteins.[6]

In humans, there are 3 common genetically determined isoforms of apoE, named apoE2, apoE3, and apoE4 are under the control of 3 APOE alleles APOE2, APOE3, and APOE4. The isoforms differ in primary structure at 2 sites: residues 130 (single nucleotide variation [SNV] rs429358) and 176 (SNV rs7412). In concordance with this critical role in lipid metabolism, the genetic variation in APOE gene is associated with monogenic disorders including familial dysbetalipoproteinemia[7] and familial hypercholesterolemia (FH)[8] but also contributes to polygenic hypercholesterolemia[9] and to the interindividual variability in blood cholesterol of normolipidemic subjects in the general population.[10] Subsequently, common and rare genetic variations in *APOE* are good candidates to explain, at least in part, the phenotype of some genetic hyperlipidemias of uncertain etiology. The *APOE* gene has 4 exons, with >85% of the mature protein coded by exon 4, where the vast majority of mutations are located.[11]

To establish the role of *APOE* gene variation in the etiology of genetic hyperlipidemias, we have sequenced the main *APOE* coding region in a large group of subjects from a lipid clinic and from the general population, and analyzed lipid phenotype, lipoprotein size, number and composition by nuclear magnetic resonance (NMR) and "in vitro" LDLr binding of the genetic variants found.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Highlights

- Exon 4 of APOE were sequenced in all consecutive unrelated subjects with primary hyperlipidemia from a lipid unit.
- Binding affinity of VLDL (very low-density lipoprotein) to LDL receptor, and lipoprotein particle number, size, and composition of pathogenic apoE variants was analyzed.
- Over 2% of subjects with combined hyperlipidemia or isolated hypertriglyceridemia carry pathogenic variants in APOE.
- Subjects with dysbetalipoproteinemia and absence of *APOE2/2* are good candidates for the study of pathogenic variants in *APOE.*

Subjects

Patients From the Lipid Unit (HUMS Cohort)

All consecutive unrelated subjects studied at the Lipid Unit of the Hospital Universitario Miguel Servet (HUMS), Zaragoza, Spain from January 2008 to July 2021 were included in this study after the exclusion of a secondary cause and following a lipid-lowering diet (n=3667). Secondary hyperlipidemia was considered in presence of poorly controlled type 2 diabetes (HbA1c ≥8%), renal disease with glomerular filtration rate <30 mL/min and macroalbuminuria, cholestasis (direct bilirubin >1 mg/dL), hypothyroidism (thyroid stimulating hormone >6 mIU/L), pregnancy, autoimmune diseases, treatment with protease inhibitors, high doses of steroids, retinoids or cyclophosphamide or alcohol consumption >30 g/day.

Subjects were divided according to their lipid phenotype as follows: 1673 patients were diagnosed with isolated hypercholesterolemia (isolated HC) if triglycerides (TG) <150 mg/dL and LDL cholesterol ≥130 mg/dL or apoB ≥120 mg/dL; 1646 were diagnosed of combined hyperlipidemia (CHLP) when TG ≥150 mg/dL and non-HDL cholesterol (HDLc) ≥160 mg/dL or apoB ≥120 mg/dL; 129 patients with isolated hypertriglyceridemia (isolated HTG) when ≥150 mg/dL and non-HDLc <160 mg/dL and apoB <120 mg/dL; and 219 subjects were considered normolipidemic (TG <150 mg/dL and LDL cholesterol <130 mg/dL and apoB <120 mg/dL).[1] Dysbetalipoproteinemia (DBL) was considered when non-HDLc/apoB≥1.7 plus TG/(apoB) ≥1.35, in mg/dL.[12] There were 210 subjects with DBL criteria, all included in the combined hyperlipidemia group.

AWHS Cohort

We also studied 822 random subjects from the AWHS (Aragon Workers Health Study) as a control and validation population for the *APOE* variants. The AWHS is a longitudinal cohort study of cardiovascular risk factors and subclinical atherosclerosis performed among 5331 workers at the Opel Spain factory in Figueruelas (Zaragoza, Spain), who have been followed since 2009. The baseline study included a lipid and apolipoprotein profile.[13]

AWHS Subjects With Dysbetalipoproteinemia Phenotype

In addition to the randomly selected volunteers from AWHS, all subjects with hyperlipidemia, as defined above, and dysbetalipoproteinemia criteria (non-HDLc/apoB≥1.7 plus TG/

(apoB)≥1.35, in mg/dL; n=392) at baseline[12] were selected from the total AWHS cohort for *APOE* gene variant analysis. Among these, 43 subjects had been already studied because they had been included in the previous random cohort study.

All patients from HUMS and all controls from AWHS signed informed consent to a protocol previously approved by Comité Ético de Investigación Clínica of Aragón (CEICA). Samples from patients included in this study were provided by the Biobank of the Aragon Health System (PT17/0015/0039), integrated into the Spanish National Biobanks Network and they were processed following standard operating procedures with the appropriate approval of the Ethics and Scientific Committees.

A flow chart showing the subject selection in the study is presented in Figure 1.

Lipid Analysis

Lipid and lipoprotein analyses were performed on EDTA plasma samples collected after at least 10 hours overnight fast, following a lipid-lowering diet and without lipid-lowering drugs for at least 4 weeks in the absence of cardiovascular disease or very high risk. In those subjects under lipid-lowering drug, concentrations of total cholesterol and triglycerides were estimated multiplied by 1.43 corresponding to a mean reduction of 30% in patients receiving statins or fibrates, respectively.[14,15] Total cholesterol and TG levels were determined by standard enzymatic methods. HDLc was measured directly by an enzymatic reaction using cholesterol oxidase (UniCel DxC 800; Beckman Coulter Inc, Brea, CA). LDL cholesterol was calculated by Friedewald's formula if TG <400 mg/dL. Apo B and Lp(a) (lipoprotein[a]) were determined by IMMAGE kinetic nephelometry (Beckman-Coulter Inc).

Genetic Analysis

Whole blood genomic DNA was isolated using standard methods. Exon 4 of the *APOE* gene (NM_000041.4) was amplified by polymerase chain reaction, as previously described[16] and purified by ExoSap-IT (USB). Amplified fragments were sequenced by the Sanger method using the BigDye 3.1 sequencing kit (Applied Biosystems) in an automated ABI 3500xL sequencer (Applied Biosystems). DNA sequences were analyzed using VariantReporter software (Applied Biosystems).

To evaluate the pathogenicity of identified variants, we used SIFT,[17] PolyPhen-2,[18] and PredictSNP.[19] The effect of variants in potential splicing sites was predicted with FruitFly.[20] To predict changes in protein stability upon point mutations, we used the CUPSAT tool.[21] To compare the frequency of identified variants in the studied subjects with that of the general population, we compiled the allele frequencies of the identified variants from the 1000 Genomes Project[22] and GnomAD.[23] Finally, the ClinVar database was used to aggregate information about genomic variation and its relationship to human health.[24]

Lipoprotein Particle Analysis

Lipoprotein particle concentrations were measured by NMR, based on the LipoScale test in subjects with *APOE* genetic variants. NMR analysis determines the lipid composition (ie, cholesterol and TG) and the mean size of VLDL, LDL, and HDL, as well as the particle number of 9 lipoprotein subclasses (large, medium, and small VLDL, LDL, and HDL).[25] Briefly, particle concentration and diffusion coefficients were obtained from the measured amplitudes, as well as attenuation of their spectroscopically distinct lipid methyl group NMR signals, using the 2D diffusion-ordered 1H NMR spectrometry (DSTE) pulse.

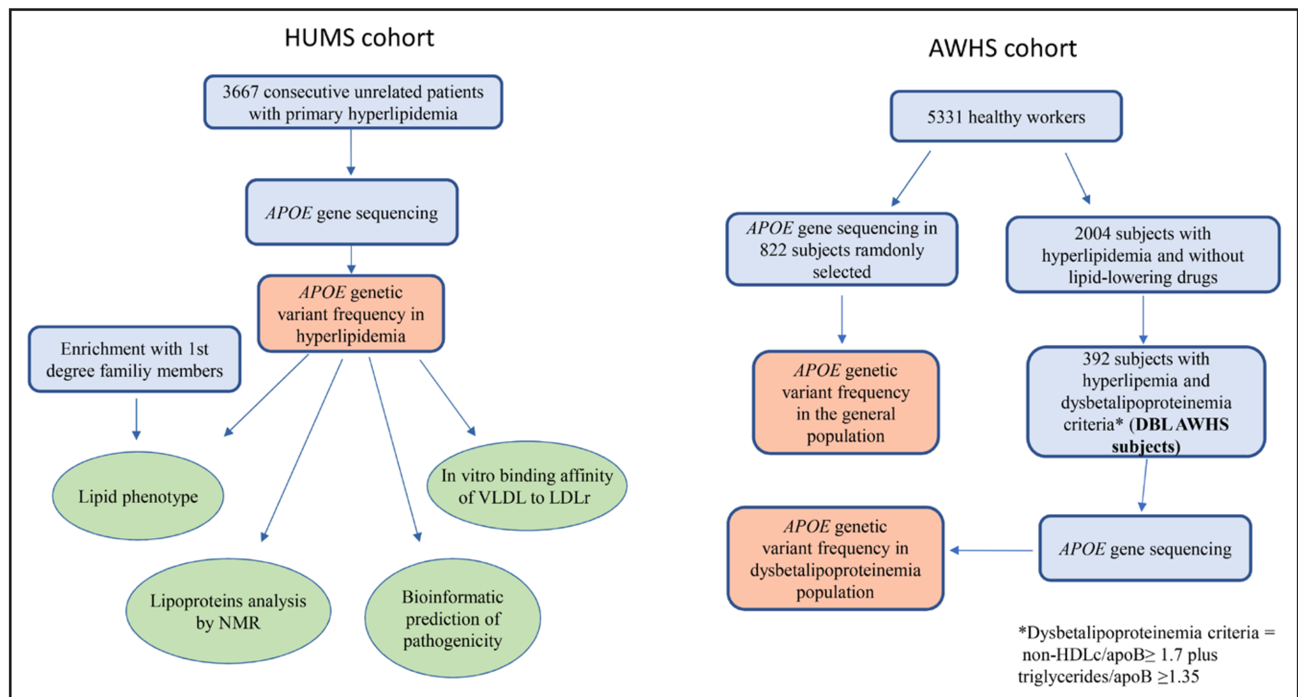

Figure 1. Flow chart of the study.
AWHS indicates Aragon Workers Heath Study; HUMS denotes Hospital Universitario Miguel Servet; LDLr, low-density lipoprotein receptor; NMR, nuclear magnetic resonance; and VLDL, very low-density lipoprotein.

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The methyl signal was surface-fitted with 9 Lorentzian functions associated with each lipoprotein subclass—large, medium, and small. The area of each Lorentzian function was related to the lipid concentration of each lipoprotein subclass, and the size was calculated from their diffusion coefficient.

Each subclass particle concentration was calculated by dividing the lipid volume by the particle volume of a given class. Lipid volumes were determined by using common conversion factors to convert concentration units into volume units. The weighted average of VLDL, LDL, and HDL particle sizes were calculated from various subclass concentrations by summing the known diameter of each subclass, multiplied by its relative percentage of subclass particle number.

Binding Affinity of VLDL to LDLr

To obtain the binding affinity curves of VLDL to LDLr, 96-well plates were coated with 0.4 mg/mL of LDLr ectodomain at pH 7.4 overnight. The next day, plates were blocked for 1 hour, and serial dilutions of different VLDL isolated by ultracentrifugation with *APOE* variants were done, starting from 20 mg/mL cholesterol. After the plates were washed, primary antibodies against ApoB were added for 1 hour, and secondary ones were conjugated to HRP for another hour. Finally, the HRP substrate was added to measure the absorbance (405 nm).[8] Plotting a graph with added VLDL cholesterol µg/mL versus absorbance at 405 nm, affinity curves were obtained and SigmaPlot software was used to analyze them. Applying a 5-parameter logistic curve (5PL) and a Macro created by us, EC_{50} values were obtained. All values were standardized to VLDL from APOE3/3 donor subjects.

Statistical Analysis

Analyses were performed using the statistical computing software IBM SPSS Statistics 21.0. Quantitative variables are expressed as mean±SD for variables with normal distribution. Variables with a skewed distribution are expressed as median and interquartile ranges. The Kruskal–Wallis test was used to assess differences among the groups. Quantitative categorical variables are expressed as n (%) and statistical differences were assessed by Chi-squared test. The level of significance was set at $P<0.05$.

RESULTS

Subjects From the HUMS Cohort

Clinical, anthropometric, and biochemical characteristics of the 3667 HUMS patients divided according to their lipid phenotype, showed differences in all lipid and apolipoprotein variables, as expected due to classification, but also in the percentage of men and prevalence of diabetes and cardiovascular disease (Table 1).

Distribution of APOE Variants

The identified *APOE* variants in subjects from HUMS and AWHS groups and their comparison with those reported in the general population are shown in Table 2. In addition to common polymorphisms giving rise to APOE2 and APOE4, 14 gene variants were found in exon 4 of *APOE* in 65 subjects. Ten of them were found only in the HUMS subject group, two variants were found only in the AWHS cohort, and two variants were identified in both groups. Five variants were found in more than one subject: p.(Gly145Asp) in two CHLP patients from HUMS; p.(Arg154Ser) in one patient with isolated HC and 14 patients with CHLP from HUMS and 3 subjects from AWHS (all three with CHLP); p.(Arg163Cys) in 5 patients with CHLP and 1 patient with isolated HTG from HUMS; p.(Leu167del) in 8 patients with isolated HC and 8 patients with CHLP from HUMS; and the synonymous

Table 1. Clinical and Biochemical Characteristics According to Hyperlipidemia Phenotype in Subjects From HUMS

	HUMS (n=3667)				
	Normolipemia (n=219)	Isolated HC (n=1673)	CHLP (n=1646)	Isolated HTG (n=129)	*P*
Age, years	38.0 (26.2–50.0)	51.0 (39.0–59.0)	51.0 (42.0–58.0)	45.0 (35.0–55.0)	<0.001
Men, n (%)	106 (48.6)	674 (40.4)	1059 (64.4)	99 (76.7)	<0.001
Body mass index, kg/m^2	24.0 (21.3–27.7)	25.1 (22.6–27.8)	28.1 (25.7–30.8)	28.0 (25.6–30.8)	<0.001
Total cholesterol, mg/dL	180 (162–194)	284 (253–319)	287 (252–327)	178 (167–193)	<0.001
Non-HDL cholesterol, mg/dL	127 (110–138)	221 (192–255)	240 (208–278)	141 (129–152)	<0.001
HDL cholesterol, mg/dL	52.0 (43.0–64.0)	60.0 (50.0–72.0)	45.0 (37.0–54.0)	37.0 (31.0–46.5)	<0.001
Triglycerides, mg/dL	74.0 (52.0–108)	97.0 (75.5–119)	248 (188–382)	246 (190–343)	<0.001
Lipoprotein(a), mg/dL	20.0 (7.40–46.4)	33.0 (12.5–78.3)	21.4 (6.89–61.2)	14.3 (4.58–30.5)	<0.001
Apolipoprotein A1, mg/dL	143 (125–171)	158 (139–181)	139 (123–159)	128 (113–152)	<0.001
Apolipoprotein B, mg/dL	86.7 (74.9–96.0)	142 (123–165)	152 (130–179)	92.8 (80.0–103)	<0.001
Hb1Ac, %	5.20 (5.00–5.40)	5.40 (5.20–5.60)	5.60 (5.30–5.90)	5.50 (5.10–5.80)	<0.001
Diabetes, n (%)	6 (2.70)	36 (2.20)	181 (11.0)	17 (13.2)	<0.001
Cardiovascular disease, n (%)	17 (8.00)	128 (8.20)	205 (12.9)	6 (4.70)	<0.001

Quantitative variables are expressed as median (percentile 25–75). The Kruskal–Wallis test was used to assess differences among the groups. Quantitative categorical variables are expressed as n (%), and statistical differences were assessed by Chi-squared test. CHLP indicates combined hyperlipidemia; HC, hypercholesterolemia; HDL, high-density lipoprotein; HTG, hypertriglyceridemia; and HUMS, Hospital Universitario Miguel Servet.

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Table 2. ***APOE*** **Variant Distribution in HUMS and AWHS Cohorts and Their Allele Frequency Reported in International Genome Databases**

Predicted amino acid change	Nucleotide change (RefSNP)	Normo-lipemia (n=219)	Isolated HC (n=1673)	CHLP (n=1646)	Isolated HTG (n=129)	Any HLP (n=3448)	AWHS (n=822)	Allele frequency in international genome databases	
								1000 Genomes Project	Gno-mAD
	c.237-33C>G, n	0	0	1	0	1	0	…	…
p.(Met82Ile), n	c.246G>T (rs557845700)	0	0	0	1	1	0	<0.001	<0.001
p.(Ala104=), n	c.312G>C	0	0	0	1	1	0	…	…
p.(Arg108Trp), n	c.322C>T (rs1050106163)	0	0	0	0	0	1	…	<0.001
p.(Tyr136His), n	c.406T>C	0	0	0	0	0	1	…	…
p.(Gly145Asp), n	c.434G>A (rs267606664)	0	0	2	0	2	0	…	<0.001
p.(Arg154Ser), n	c.460C>A (rs121918393)	0	1	14	0	15	3	…	<0.001
p.(Arg163Cys), n	c.487C>T (rs769455)	0	0	5	1	6	0	0.007	0.007
p.(Arg165Trp), n	c.493C>T (rs1402219759)	0	0	1	0	1	0	…	…
p.(Leu167del), n	c.500_502delTCC (rs515726148)	0	8	8	0	16	0	…	<0.001
p.(Arg168His), n	c.503G>A (rs376170967)	0	0	1	0	1	0	…	<0.001
p.(Gly191Cys), n	c.571G>T	0	1	0	0	1	0	…	…
p.(Ala217=), n	c.651C>T (rs72654468)	1	6	3	0	9	4	0.001	0.001
p.(Pro220Leu), n	c.659C>T (rs1265743589)	0	0	1	0	1	0	…	<0.001
Total, n (%)	…	1 (0.45)	16 (0.96)	36 (2.19)	3 (2.32)	55 (1.60)	9 (1.09)		

CHLP indicates combined hyperlipidemia; GnomAD, Genome Aggregation Database; HC, hypercholesterolemia; HTG, hypertriglyceridemia; and n, number of subjects.

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variant p.(Ala217=) in 14 subjects homogeneously distributed among groups including subjects with normolipidemia and subjects from AWHS. The frequency of subjects with an identified variant in the *APOE* gene was significantly different when comparing the CHLP (2.19%), isolated HTG (2.32%) and whole dyslipidemia groups (1.60%), each at a time, with either the normolipidemic group (0.45%; $P<0.05$, in all comparisons) or with AWHS (1.09%; $P<0.05$, in all comparisons). The intron variant detected (c.237-33C>G), not previously described, was identified in a subject from HUMS with CHLP phenotype and APOE2/2 genotype.

Lipid Profile Associated With APOE Variants

To establish the possible impact of the identified *APOE* variants in the lipid profile, we analyzed them independently, and according to the *APOE* genotype of the carrier subject. First-degree relatives of index cases carrying an *APOE* variant were included to increase the number of carriers. Non-HDLc/apoB and TG/apoB ratios were calculated for the diagnosis of DBL (Table 3). Homozygous subjects for p.(Cys130Arg), that is, the 35 subjects with APOE2/2 genotype, had a mean non-HDLc/apoB ratio of 2.76 (±0.78) and a mean TG/apoB ratio of 6.11 (±3.08). As expected, these E2/E2 subjects met both ratios, as most of the homozygous APOE2/2 subjects exhibit DBL. Subjects carrying the c.237-33C>G variant fulfilled DBL criteria only when they were APOE2/2 homozygous but not in presence of other *APOE* genotypes. Subjects with p.(Arg121Trp), p.(Gly145Asp), p.(Arg154Ser), p.(Arg163Cys), p.(Arg165Trp), and p.(Arg168His) variants met DBL criteria, and the variants: p.(Met82Ile), p.(Ala104=), p.(Arg108Trp), p.(Tyr136His), p.(Leu167del), p.(Gly191Cys), and p.(Pro220Leu) did not.

Lipoprotein Particle Analysis by NMR

Individual results of lipoprotein particle composition (Table 4), mean size, and particle number of VLDL (Table S1), LDL (Table S2), and HDL (Table S3) by NMR confirmed the results of the lipid profile and the association of certain *APOE* variants with DBL. VLDLc and VLDLc/VLDL-TG ratio were much higher in p.(Arg163Cys) and p.(Arg165Trp) carriers than in APOE3/3 subjects, with values in the range of that of APOE2/2 subjects. Carriers of the p.(Arg154Ser) variant showed moderately high VLDLc and VLDLc/VLDL-TG ratio, with higher values than APOE3/3 subjects. VLDLc and VLDLc/VLDL-TG ratio of p.(Met82Ile), p.(Ala104=), p.(Arg108Trp), p.(Tyr136His), p.(Gly145Asp) p.(Leu167del), p.(Gly191Cys), and p.(Pro220Leu) were in the normal range with no differences from APOE3/3 subjects (range 0.18-0.38). The same pattern was observed for the number of small VLDL particles that discriminated quite well with those *APOE* variants associated with DBL.

Table 3. Lipid Profile According to *APOE* Variant and Classic *APOE* Genotype

Nucleotide change Ref SNP n	Predicted amino acid change	*APOE* Geno-type	Age, y	TC, mg/dL	Non-HDLc, mg/dL	HDLc, mg/dL	TG, mg/dL	ApoB, mg/dL	Non-HDLc/apoB	TG/apoB	DBL criteria*	ASCVD, N
n=35		E2/2	48.9±12.2	327±149	279±144	48.7±13.8	520±467	102±43.8	2.76	6.11	yes	9
c.237-33C>G n=1	-	E3/2	68.0	250	206	44.0	284	132	1.56	2.15	no	0
c.237-33C>G n=1	-	E2/4	49.0	265	208	57.0	255	140	1.49	1.82	no	0
c.237-33C>G n=2	-	E2/2	40.0±1.41	487±4.24	444±4.24	43.0±8.49	981±202	120±7.78	3.72	8.29	yes	0
c.246G>T rs557845700 n=1	p.(Met82 Ile)	E3/3	39.0	184	122	62.0	211	90.0	1.35	2.34	no	0
c.312G>C n=1	p.(Ala104=)	E3/3	41.0	135	103	32.0	292	68.6	1.50	4.26	no	0
c.322C>T rs1050106163 n=1	p.(Arg108 Trp)	E3/3	32.0	191	118	73.0	52.0	80	1.48	0.65	no	0
c.361C>T rs11542037 n=1	p.(Arg121 Trp)	E3/3	49.0	248	207	41.0	234	100	2.07	2.34	yes	0
c.406T>C n=1	p.(Tyr136 His)	E3/2	42.0	191	148	43.0	140	94.0	1.57	1.49	no	0
c.434G>A rs267606664 n=2	p.(Gly145 Asp)	E3/2	67.0±0	344±125	299±114	45.0±11.3	355±79.9	136±19.8	2.16	2.66	yes	1
c.434G>A rs267606664 n=1	p.(Gly145 Asp)	E2/2	43.0	162	116	46.0	151	55.0	2.11	2.75	yes	1
c.460C>A rs121918393 n=19	p.(Arg154 Ser)	E3/3	48.8±11.9	330±170	282±173	48.0±11.3	512±759	125±36.6	2.03	2.89	yes	2
c.460C>A rs121918393 n=3	p.(Arg154 Ser)	E3/2	59.0±12.7	271±29.7	229±39.9	42.3±12.3	277±77.5	108±24.0	1.98	2.25	yes	1
c.460C>A rs121918393 n=5	p.(Arg154 Ser)	E3/4	46.6±12.4	285±34.8	224±31.1	62.0±29.4	206±68.4	133±18.6	1.70	1.53	yes	2
c.487C>T rs769455 n=5	p.(Arg163 Cys)	E3/3	40.0±5.93	265±57.5	220±50.9	44.5±12.2	406±193	120±26.6	1.84	3.43	yes	0
c.493C>T n=1	p.(Arg165 Trp)	E3/4	53.0	329	284	45.0	643	126	2.25	5.10	yes	0
c.500_502delTCC rs515726148 n=36	p.(Leu167del)	E3/3	39.6±16.2	326±81.8	265±72.7	60.6±20.4	207±186	156±43.8	1.73	1.26	no	2
c.503G>A rs376170967 n=1	p.(Arg168 His)	E2/2	52.0	294	229	65.0	152	50.1	4.57	3.03	yes	0
c.571G>T n=1	p.(Gly191 Cys)	E3/3	56.0	308	224	84.0	88.0	133	1.68	0.66	no	0
c.651C>T	p.(Ala217=)	E3/3	49.4±14.7	280±88.0	225±86.5	54.7±11.8	132±77.0	127±46.7	1.85	0.76	no	0

(*Continued*)

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Table 3. Continued

Nucleotide change Ref SNP n	Predicted amino acid change	*APOE* Geno-type	Age, y	TC, mg/dL	Non-HDLc, mg/dL	HDLc, mg/dL	TG, mg/dL	ApoB, mg/dL	Non-HDLc/apoB	TG/apoB	DBL criteria*	ASCVD, N
rs72654468												
n=14												
c.651C>T	p.(Ala217=)	E3/4	43.2±18.2	272±63.2	208±59.5	63.4±11.9	96.0±70.2	134±39.2	1.61	0.81	no	0
rs72654468												
n=5												
c.659C>T	p.(Pro220 Leu)	E3/3	46.3±14.6	259±38.0	202±52.3	57.0±16.6	218±138	135±46.2	1.52	1.64	no	0
rs1265743589												
n=3												

Quantitative variables are expressed as mean±SD. apoB indicates apolipoprotein B; ASCVD, atherosclerotic cardiovascular disease; DBL, dysbetalipoproteinemia; HDLc, high-density lipoprotein cholesterol; n, number of subjects; TC, total cholesterol; and TG, triglyceride.

*Dysbetalipoproteinemia criteria: non-HDLc/apoB) ≥1.7 and TG/apoB ≥1.35.

Pathogenicity Prediction of APOE Variants

ClinVar database information was available for p.(Gly145Asp): uncertain significance, p.(Arg154Ser): pathogenic, p.(Arg163Cys): likely pathogenic, p.(Leu167del): pathogenic, and p.(Ala217=): benign. p.(Arg154Ser) and p.(Arg163Cys) were associated with DBL, p.(Leu167del) with isolated HC and CHLP and p.(Ala217=) without clinical significance. Thus, there was an excellent correlation between ClinVar information and our biochemical findings.

The c.237-33C>G and p.(Ala104=) variants were predicted as neutral for PredictSNP2, in agreement with the lipid profile and carrier distribution in our studied groups. The p.(Arg121Trp), p.(Tyr136His), p.(Arg165Trp), p.(Arg168His), and p.(Pro220Leu) variants were considered as deleterious with at least 3 out of 4 predictors. The information from p.(Met82Ile), p.(Arg108Trp), and p.(Gly191Cys) was not consistent among the different predictors. A detailed description of bioinformatic prediction analysis is presented in Table S4.

Binding Affinity of VLDL to LDLr

EC_{50} values for VLDL binding to LDLr for those apoE variants that were not previously reported are shown in Figure 2A. The obtained EC_{50} values for each variant were standardized to that of VLDL from APOE3/3 donor subjects (Figure 2B). VLDL from subjects with APOE2/2 genotype had a much higher EC_{50} value (198.9%), indicating lower affinity, while p.(Arg163Cys) and p.(Arg165Trp) from heterozygous subjects had intermedium affinity between APOE2/2 and APOE3/3. p.(Gly145Asp), and p.(Pro220Leu) variants had lower EC_{50} values (higher affinity than APOE3/3). The remaining variants did not differ from APOE3/3 in terms of binding affinity to LDLr.

APOE Gene Sequencing Analysis of AWHS Participants With DBL Lipid Criteria

Among 2004 AWHS participants with hyperlipidemia, 392 fulfilled DBL criteria with the following sequencing results: 12 *APOE2* homozygous subjects and 6 heterozygous carriers of other *APOE* variants: p.(Arg121Trp) 1 subject, p.(Gly145Asp) 1 subject, p.(Arg154Ser) 2 subjects, p.(Leu167del) 1 subject, and p.(Ala217=) 1 subject.

DISCUSSION

The present work studies the *APOE* gene in different types of primary hyperlipidemias among patients attended in a Lipid Unit. To our knowledge, this is the first study in which the exon 4 of the *APOE* gene is systematically screened for the presence of mutations, the lipid phenotype is analyzed by NMR and the in vitro binding affinity to LDLr of the VLDL-carrying apoE variants is studied. The fact that apoE plays an important role in lipid metabolism and that it is found in all plasma lipoproteins would suggest that mutations in different sites of the protein could be associated with different lipid disorders, not only with familial dysbetalipoproteinemia. A lipid disorder characterized by the accumulation of cholesterol-enriched VLDL is usually caused by APOE2/2 homozygosity.[7] The results of this study confirm that rare *APOE* variants in the population, other than the APOE2 and APOE4 alleles, play an important role in the development of isolated hypercholesterolemia[8] and mixed hyperlipidemia, with and without familial dysbetalipoproteinemia.[11,28] Twelve gene variants were found in exon 4 of *APOE* in 56 subjects with hyperlipidemia, 4 of them not previously described. We also found another 2 different *APOE* variants in the AWHS cohort, p.(Arg108Trp), previously described and reported as benign,[29] and p.(Tyr136His) not previously described. Finally, a last variant was identified in DLP subjects from AWHS, p.(Arg121Trp); it has been previously described but there is no information about its possible pathogenicity in databases.

The main conclusions to be drawn from our study include:

The *APOE* variant p.(Leu167del) is not uncommon in our population. Carriers represent approximately

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Table 4. Lipoprotein Particle Composition by NMR According to the Presence of an *APOE* Variant*

APOE VARIANT	AGE	*APOE* GENOTYPE	VLDLc, mg/dL	IDLc, mg/dL	LDLc, mg/dL	HDLc, mg/dL	VLDL-TG, mg/dL	IDL-TG, mg/dL	LDL-TG, mg/dL	HDL-TG, mg/dL	VLDLc/VLDL-TG
None	50	E2/2	104	27.8	164	52.7	249	25.2	44.2	54.3	0.42
None	47	E2/2	94	24.0	81.7	46.3	253	22.5	31.4	57.7	0.37
None	35	E2/2	121	24.9	151	61.2	263	19.7	39.7	54.5	0.46
None	18	E2/2	156	22.5	89.7	35.9	647	22.0	20.6	34.3	0.24
None	46	E2/2	109	55.2	181	65.9	395	33.1	48.3	55.1	0.28
None	39	E2/2	133	64.6	101	27.5	383	53.3	59.6	86.1	0.35
c.237-33C>G	39	E2/2	155	18.5	146	20.0	658	13.4	22.9	48.6	0.24
c.237-33C>G	41	E2/2	196	37.8	6.26	74.4	505	36.8	65.1	106	0.39
c.237-33C>G	49	E2/4	41.8	19.1	145	57.4	148	17.5	24.9	25.7	0.28
c.237-33C>G	68	E3/2	47.1	22.8	89.5	51.6	152	22.1	25.1	39.7	0.31
p.(Met82Ile)	39	E3/3	30.3	27.7	88.6	99.4	111	25.6	23.4	49.5	0.27
p.(Ala104=)	41	E3/3	49.1	10.8	28.1	44.4	187	14.8	18.2	50.0	0.26
p.(Arg108Trp)	52	E3/3	3.13	5.30	123	80.1	25.8	7.95	13.4	15.9	0.12
p.(Tyr136His)	26	E3/2	33.4	12.1	109	42.9	116	11.8	16.1	24.2	0.29
p.(Gly145Asp)	67	E3/2	37.3	26.9	164	53.6	95.5	23.1	34.9	30.9	0.39
p.(Gly145Asp)	67	E3/2	35.0	19.0	129	41.1	157	18.4	21.6	28.3	0.22
p.(Arg154Ser)	69	E3/2	24.6	19.1	101	63.9	61.3	17.9	19.4	27.1	0.40
p.(Arg154Ser)	48	E3/3	67.5	24.6	147	39.6	200	25.3	34.9	37.9	0.34
p.(Arg154Ser)	24	E3/3	53.9	19.5	95.4	35.3	268	19.0	19.9	25.1	0.20
p.(Arg154Ser)	57	E3/3	63.7	18.2	179	41.3	174	15.6	29.8	27.5	0.37
p.(Arg154Ser)	37	E3/4	64.2	24.4	156	43.6	162	20.0	33.1	34.6	0.40
p.(Arg163Cys)	35	E3/3	80.7	17.6	91.8	53.2	224	19.4	34.1	54.5	0.36
p.(Arg163Cys)	47	E3/3	144	34.1	6.26	79.5	414	36.7	54.9	114	0.35
p.(Arg165Trp)	53	E3/4	126	28.2	6.26	67.2	371	32.8	50.5	96.0	0.34
p.(Leu167del)	61	E3/3	19.2	20.3	102	31.0	85.8	15.5	28.0	30.5	0.22
p.(Leu167del)	53	E3/3	46.9	26.4	119	36.8	236	19.1	36.4	38.9	0.20
p.(Leu167del)	18	E3/3	15.1	17.1	250	72.6	22.4	11.6	33.9	18.7	0.67
p.(Ala217=)	76	E3/3	5.99	6.40	127	65.5	30.8	7.20	13.8	13.3	0.19
p.(Ala217=)	27	E3/4	3.70	5.74	203	70.4	22.0	5.59	21.8	7.51	0.17
p.(Pro220Leu)	36	E3/3	33.0	17.4	180	52.2	130	14.9	24.7	15.7	0.25
p.(Pro220Leu)	63	E3/3	65.3	22.8	112	51.7	219	21.2	30.7	42.5	0.29
p.(Pro220Leu)	40	E3/3	7.08	10.7	136	80.4	49.7	12.1	15.3	24.9	0.14
None	29	E3/3	6.88	6.40	60.2	44.1	24.3	6.41	8.07	13.0	0.28
None	74	E3/3	14.3	11.7	78.3	74.2	37.2	11.9	12.8	25.0	0.38
None	44	E3/3	4.49	4.95	95.8	43.4	25.0	5.21	10.4	14.0	0.18
None	41	E3/3	7.91	5.49	106	61.0	26.0	7.23	12.8	10.8	0.30

(*Continued*)

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Table 4. Continued

APOE VARIANT	AGE	*APOE* GENOTYPE	VLDLc, mg/dL	IDLc, mg/dL	LDLc, mg/dL	HDLc, mg/dL	VLDL-TG, mg/dL	IDL-TG, mg/dL	LDL-TG, mg/dL	HDL-TG, mg/dL	VLDLc/VLDL-TG
None	53	E3/3	9.42	6.15	85.5	46.9	38.2	6.71	7.77	15.5	0.25
None	49	E3/3	14.0	9.99	88.3	50.8	54.8	10.2	11.0	20.5	0.26

Normal values (median [25th–75th] for NMR lipids are: VLDLc: 8.59 (4.66–13.8), IDLc: 10.1 (6.14–14.06), LDLc: 128 (111–149), HDLc: 56.6 (48.4–66.7), VLDL-TG: 51.1 (36.4–71.4), IDL-TG: 10.7 (7.45–13.95), LDL-TG: 16.0 (12.6–19.5), HDL-TG: 12.6 (10.3–15.4), VLDLc/VLDL-TG: 0.17 (0.15–0.19). From references.[26,27] HDLc indicates high-density lipoprotein cholesterol; IDLc, intermediate-density lipoprotein cholesterol; LDLc, low-density lipoprotein cholesterol; NMR, nuclear magnetic resonance; TG, triglyceride; and VLDLc, very low-density lipoprotein cholesterol.

*Each file corresponds to a different subject.

1/200 subjects with hyperlipidemia. Its accompanying phenotype is either isolated hypercholesterolemia, similar to FH, or combined hyperlipidemia as initially described[30]; 1 subject with this variant showed a high VLDLc/VLDL-TG ratio in the NMR analysis, however, with normal VLDLc. The mechanism by which the p.(Leu167del) *APOE* mutation produces hypercholesterolemia is the fact that the VLDL carrying the mutant apo E produces LDLr downregulation.[8] A second *APOE* variant, p.(Gly191Cys), was also found in a 56-year-old patient with isolated hypercholesterolemia and without DBL phenotype. The study of the canonical FH genes: *LDLR, APOB,* and *PCSK9*, did not show any pathogenic mutation. The in vitro binding affinity of her isolated VLDLs for the LDLr showed a significant reduction. None of the studied relatives presented the mutation or hypercholesterolemia. Polyphen2 showed a prediction of probably damaging (1000 scored). Whether this rare variant is the cause of the increase in LDL cholesterol levels in this patient will require further studies.

The *APOE* variants p.(Arg154Ser), p.(Arg163Cys), and p.(Arg165Trp), located in the *APOE* receptor-binding site for the LDLr, are mostly present in subjects with CHLP. Their phenotype includes high VLDLc, high VLDLc/apoB ratio, DBL criteria, and reduced VLDL affinity for the LDL receptor. Hence, these variants produce a dominant form of DBL, although with a milder phenotype than APOE2/2 subjects. The variants p.(Arg154Ser) and p.(Arg163Cys) have been previously associated with dominant DBL,[31] and in this report, we confirm the affected binding affinity for p.(Arg163Cys). The variant p.(Arg165Trp) has been previously identified in combination with p.(Lys164Asn) in the same allele in a single family.[32] The variant p.(Arg165Trp) in the subject in our study was present on a normal APOE3 allele, suggesting its pathogenicity by itself.

The DBL criteria non-HDLc/apoB ≥1.7 plus TG/apoB ≥1.35 not only may help to identify APOE2/2 subjects with a sensitivity of 100% as previously described[13] but also the *APOE* variants associated with dominant familial dysbetalipoproteinemia. In this study, all carriers of variants p.(Arg154Ser), p.(Arg163Cys) and p.(Arg165Trp) from the Lipid Unit or AWHS study fulfilled these criteria. However, the number of subjects without pathogenic mutations in *APOE* associated with DBL is high, so they should be considered a screening method, and newly discovered mutations, there has to be a defining compositional or functional analysis of lipoproteins.

The genetic analysis of the *APOE* gene identified different rare variants whose pathogenicity could not be demonstrated clinically, in vitro or with current bioinformatic software. The assignment of pathogenicity of rare gene variants is an important medical problem nowadays.[33] *LDLR* gene-specific software improves predicting capacity for assessing the pathogenicity of *LDLR* variants in FH.[34] Given the large number of *APOE* genetic variants described so far,[11] and probably more in the next future, the implementation of such a tool would constitute a valuable approach to help to solve the problem.

Our study has several limitations: (1) The identified variants in *APOE* gene are rare mutations, and therefore, they explain a small percentage of hyperlipidemias in our population. (2) DBL is a highly fluctuating phenotype, due to changes in body weight, changes in diet, alcohol consumption, etc This phenotype variability makes diagnosis difficult and therefore, the association between the presence of some *APOE* variants and the presence of DBL may not always be unequivocal. (3) Some identified variants, c.237-33C>G or p.(Arg168His), are located on an APOE2 allele, and in these cases, it is difficult to know their effect, as they are located on a defective allele itself. (4) The study of the *APOE* sequence in our study is limited to exon 4. Although *APOE* exon 4 encodes for most of the mature apoE and the vast majority of mutations have been described in exon 4,[11] we cannot rule out that we have missed some pathogenic variants outside of exon 4 of *APOE*. (5) The diagnosis of DBL by NMR is not sufficiently validated with respect to ultracentrifugation, and could explain why the VLDLc/VLDL-TG ratio is somewhat lower in APOE2/2 subjects than expected by ultracentrifugation as has been suggested by a recent UK biobank study, in which individuals with DBL and APOE2/2 genotype, lipid measurement by NMR may be discordant with biochemistry.[35]

In summary, *APOE* genetic variation contributes to the development of DBL, CHLP, and FH. The phenotype in

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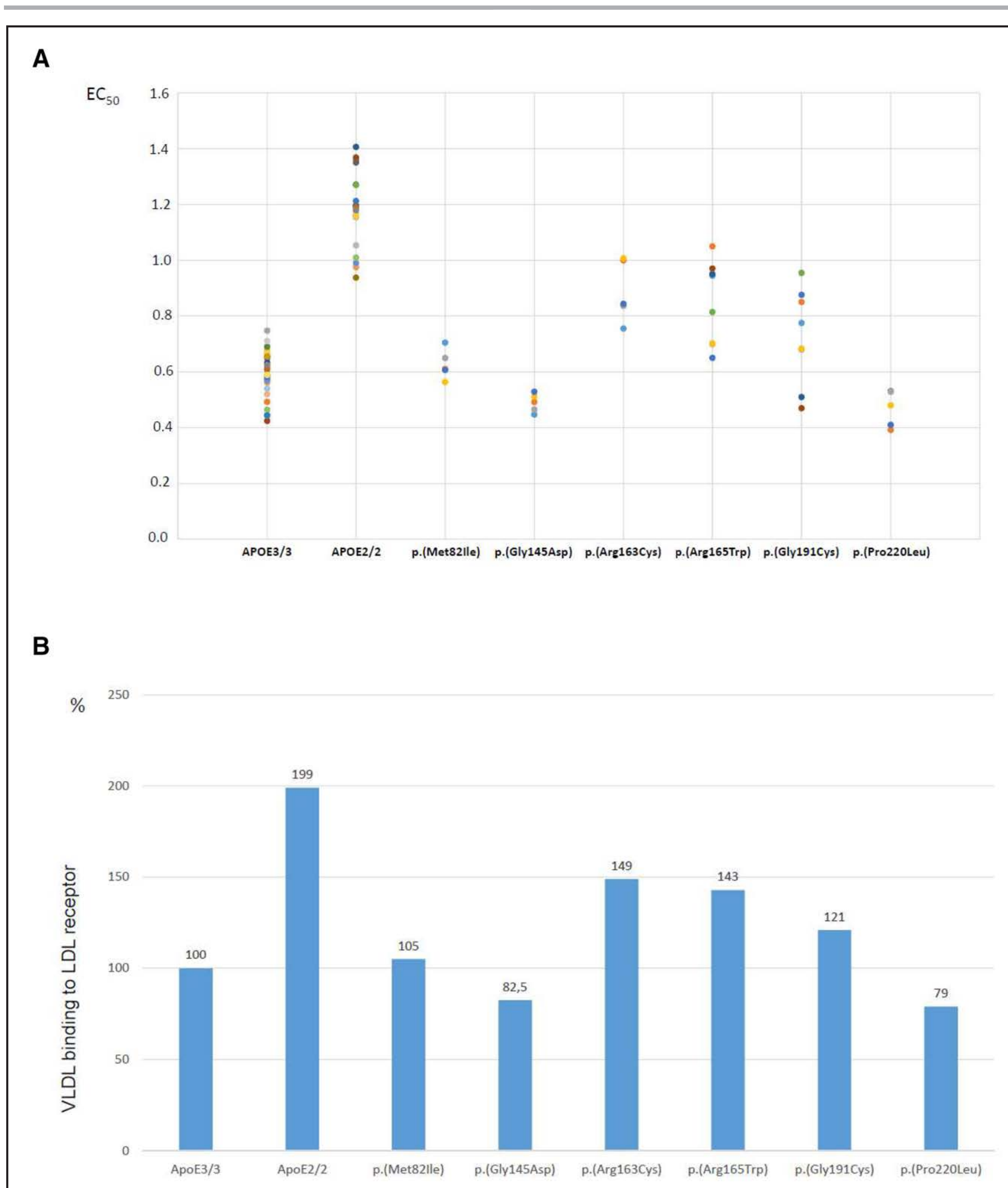

Figure 2. Half-maximal effective concentration (EC_{50}) for VLDL (very low-density lipoprotein) binding to LDL (low-density lipoprotein) receptor for those *APOE* variants (A).
Standardized values to VLDL binding from *APOE3/3* donor subjects (**B**). Footnote Figure 2. Cholesterol from VLDL isolated by ultracentrifugation from APOE3/3 subjects (n=3) and from subjects with *APOE* variants was used for VLDL standardization.

subjects heterozygous for DBL-associated mutations is more benign than the homozygous APOE2/2-associated phenotype. Taking into account that subjects with rare variants in *APOE* are heterozygous, it seems to indicate that these variants have a greater pathogenic effect than heterozygosity for APOE2. The non-HDLc/apoB plus TG/apoB ratio proposed for the diagnosis of DBL seems to be a good screening method for the

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study of pathogenic variants in *APOE* in the absence of *APOE2/2* and genotyping may need to go further than just the APOE2/3/4 variants.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Tables S1–S4

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