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Original Article

Lipoprotien(a) concentration, genetic variants, apo(a) isoform size, and cellular cholesterol efflux in patients with elevated Lp(a) and coronary heart disease submitted or not to lipoprotein apheresis: An Italian case-control multicenter study on Lp(a)

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KEYWORDS:
Lipoprotein apheresis;
HyperLp(a);
Cholesterol loading capacity;
Genetics;

BACKGROUND: Coronary artery disease (CAD) risk is greater with higher plasma lipoprotein(a) [Lp(a)] concentrations or smaller apoisoform size and putatively with increased cellular cholesterol loading capacity (CLC). The relationship between Lp(a) and CLC is not known. Information on Lp(a) polymorphisms in Italian patients is lacking.

OBJECTIVE: The objective of this study was to determine relationships between Lp(a) and CLC, the impact of lipoprotein apheresis (LA), and describe the genetic profile of Lp(a)

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Particle size;
Genetic polymorphisms

METHODS: We conducted a multicenter, observational study in Italian patients with hyperLp(a) and premature CAD with (n = 18)/without (n = 16) LA in which blood samples were analyzed for Lp(a) parameter and CLS. Genetic profiling of LPA was conducted in patient receiving LA.

RESULTS: Mean macrophage CLC of the pre-LA serum was significantly higher than that of normolipidemic controls (19.7 ± 0.9 μg/mg vs 16.01 ± 0.98 μg/mg of protein, respectively). After LA, serum macrophage CLC was markedly lower relative to preapheresis (16.1 ± 0.8 μg/mg protein; $P = .003$) and comparable with CLC of the normolipidemic serum. LA did not significantly affect average apo(a) isoform size distribution. No anthropometric or lipid parameters studied were related to serum CLC, but there was a relationship between CLC and the Lp(a) plasma concentration ($P = .035$). DNA analysis revealed a range of common genetic variants. Two rare, new variants were identified: LPA exon 21, c.3268C>G, p.Pro1090Arg, and rs41259144 p.Arg990Gln, c.2969G>A

CONCLUSIONS: LA reduces serum Lp(a) and also reduces macrophage CLC. Novel genetic variants of the LPA gene were identified, and geographic variations were noted. The complexity of these polymorphisms means that genetic assessment is not a predictor of CAD risk in hyperLp(a).

Introduction

Several studies, including meta-analyses, have reported that risk of coronary artery disease (CAD) is increased as plasma lipoprotein(a) (Lp(a)) concentration increases.[1–3] Various studies have been conducted on the association between small apo(a) isoform size and increased CAD risk,[4] showing that not only is small size associated with high Lp(a) concentration[5] but also that the size predicts CAD with greater strength and independence than Lp(a) concentration.[6] In patients with high levels of Lp(a) and progressive CAD, lipoprotein apheresis (LA) is at present the only effective therapeutic option.[3]

Cholesterol loading capacity (CLC) is a cellular parameter used for the evaluation of the proatherogenic capacity of the serum to load cells with cholesterol. CLC informs on both the free cholesterol and multigenic lipoprotein influx[7] and on the efflux capacity of lipoprotein in circulating macrophages.[8] Increases in CLC contribute to the development of foamy infiltrate and is therefore considered to be a proatherogenic cellular index. CLC appears to be increased in cardiovascular disease and is associated with increased cardiovascular risk.[9,10]

The gene coding for Lp(a) is the LPA gene localized on chromosome 6q27 and is characterized by high sequence homology (78%–100%) to human plasminogen in the untranslated and coding regions.[11] A characteristic feature of apo(a) is the presence of loop-like structures called kringles. Kringle domains are triple-loop structures stabilized by 3 internal disulfide bonds. The kringle IV (KIV) domain in Lp(a) results in ten different types of KIV domains, all unique in their amino acid composition. Among the KIV encoding domains, KIV-1 and KIV-3 to KIV-10 are present only as single copies. The kringle IV type 2 (KIV-2) domain is further expanded resulting in a multiallelic, intragenic copy number variation (from 1 to 40 repeat sequences) known as the KIV-2 copy number variation polymorphism.[11] This polymorphism is the most important predictor of variations in Lp(a) concentration in the range 40%–70%.[12]

Considering single-nucleotide LPA polymorphisms, 2 genetic variants—rs3798220 (a nonsynonymous single-nucleotide polymorphisms [SNPs] in the protease domain) and rs10455872 (an intronic SNP)—have been found to be associated with particularly high levels of Lp(a), and both are associated with an elevated cardiovascular risk.[13] Stepwise regression identified 7 SNPs that each had a significant association with Lp(a) levels and together explained 40% of the total variation. Genome-wide studies and subsequent meta-analysis confirmed the strongest association between CAD and rs10455872 and rs3798220 polymorphisms.[14,15]

Lp(a) levels are increased in familial hypercholesterolemia (FH)[16] and in familial defective apolipoprotein B-100.[17] Only recently, 2 additional genes were found to be implicated into Lp(a) metabolism: *APOE* and *PCSK9*. The isoform *APOE2* is associated with 15% lower Lp(a) concentrations, whereas the Lp(a)-increasing effect of *APOE4* is controversial and vanishes after apo(a) isoform adjustment.[15] In line with the effects of proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, the *PCSK9* LOF mutation R46L (rs11591147) decreases Lp(a) levels.[18]

In the present study, we evaluated the relationship between Lp(a) and CLC, as an index of cellular proatherogenicity of the serum, by determining Lp(a) concentration and apo(a) size in patients with a high level of serum Lp(a) and CAD. We also examined how LA influences the concentration and the size of Lp(a) particles and the serum CLC levels in these patients. Genetic sequencing for LPA polymorphisms was conducted on a case series of patients with elevated Lp(a). The number of cases studied is relatively small. However, it is to be considered that complex and in-depth laboratory evaluations were carried out.

Subject and methods

Subjects and study design

This was a multicenter, observational study in patients with hyperLp(a) (isolated and combined forms) and premature CAD. The study included 34 patients (mean age

59.5 ± 8.9 years, men 70%) collected from 3 Italian hospitals (Roma 24 of 34, Verona 9 of 34, Pistoia 1 of 34). All patients were on maximally tolerated lipid-lowering therapy: 14 patients were treated with statins, 16 with statins plus ezetimibe, one patient with alirocumab, and one patient with lomitapide. Eighteen patients were chronically treated with LA (LA group), whereas 16 patients with hyperLp(a) and CAD never submitted to LA and were used as the control group.

Ethics

MightyMedic.org projects are conducted in accordance with the Declaration of Helsinki and International Conference on Harmonization Good Clinical Practice guidelines. Where required, approval has been obtained from the institutional review board at each participating center. Written informed consent is received from all enrolled patients.

Lipoprotein apheresis

Selective LA procedures were conducted weekly or every 2 weeks (Q2W) using one of the 3 most common techniques: heparin-induced LDL precipitation apheresis (HELP, Plasmat Futura; B. Braun, Melsungen, Germany; 11 of 18 patients), dextran sulfate absorption from plasma (Liposorber-LA systems; Kaneka, Osaka, Japan; 4 of 18 patients), or direct adsorption of lipoproteins (Fresenius Medical Care DALI 1000/1250; 3 of 18 patients).

Blood sampling was conducted before and immediately after one LA session to evaluate plasma lipids, apo(a) concentration, and average apo(a) isoform size, and to determine CLC. Total cholesterol, HDL cholesterol, and triglycerides were measured by standard enzymatic techniques. Lp(a) levels were determined by nephelometry. LDL cholesterol (LDL-C) was calculated as per the Friedewald formula.

Apo(a) isoform size estimation

Chemicals

Ammonium bicarbonate (ABC), dithiothreitol, iodoacetamide, sodium deoxycholate (DOC), and formic acid were supplied by Sigma-Aldrich Srl (Milan, Italy). Mass Spectrometry Grade Trypsin Gold (code number: V5280) was supplied by Promega Srl (Promega, Milan, Italy). Proteotypic peptide LFLEPTQADIALLK used for determining apo(a) concentration and GTYSTTVTGR used for identification of KIV-2 repeating sequence, together with the corresponding $^{13}C^{15}$ N-labeled standards, were obtained from New England Peptides (Gardner, MA)—purity of these was >95% according to high-performance liquid chromatography (HPLC) analysis by the manufacturer. Recombinant apo(a) isoform calibrators for apo(a) size attribution containing 10, 14, and 18 kringles, respectively, were provided by Prof. Eduardo Angles-Caño.

Tryptic digestion of human serum samples

For the digestion of human serum samples, the protocol published by Lassman et al[19] was used with minor modifications. Briefly, 4 μL of plasma of each patient was diluted with 132 μL of 50 mM ABC, pH 8.0, 10 μL of a 10% w/v DOC solution, and 50 μL of a 100 nM solution of labeled internal standards in ABC, to reach a final volume of 200 μL. The samples were then subjected to a standard reduction/alkylation/digestion protocol using 2 μL of a 500 mM solution of dithiothreitol for 30 min at 60°C, followed by 2 μL of a 1 M solution of iodoacetamide for 1 h at room temperature in the dark. Trypsin digestion occurred overnight using 3 μg of trypsin per sample, as already described. Ten microliter of a 20% v/v formic acid solution was added to stop digestion and to precipitate the DOC. The samples were centrifuged (15 min, 3000 g, 4°C), and 10 μL of the acid supernatant was directly injected into the HPLC-MS/MS system for analysis.

Tryptic digestion of recombinant apo(a) isoforms

The 3 recombinant apo(a) isoforms used as calibrators were supplied as stock solutions in culture medium (complete RPMI medium + 10% fetal calf serum) harvested from a CELLine bioreactor. They were received in dry ice, split into aliquots, and stored at −70°C until use. Their concentration had been previously measured using IED assay rocket electrophoresis and was equal to 0.948 mg/mL for the 10 kringles apo(a), 0.339 mg/mL for the 14 kringles apo(a), and 0.675 mg/mL for the 18 kringles apo(a). The stock solutions of recombinant apo(a) were serially diluted and digested overnight with trypsin in the presence of 4 μL of human serum, which, having been previously digested following the same protocol described previously, had no detectable levels of the corresponding proteotypic peptides.

High-performance liquid chromatography coupled to mass spectrometry (HPLC-MS/MS) analytical method

The digested samples were analyzed on a Thermo Accela UHPLC system coupled to a Thermo TSQ Quantum Access Max triple quadrupole mass spectrometer (Thermo, Milan, Italy) equipped with a heated electrospray ionization (H-ESI) ion source. The separation occurred by gradient Q13 elution; eluent A: acetonitrile + 0.1% formic acid; eluent B: water + 0.1% formic acid. Time: t = 0 minute: 2%A; 98%B; t = 5 minute: 2%A; 98%B; t = 20 minute: 30% A; 70%B; t = 22 minute: 95%A; 5%B; t = 23 minute: 95%A; 5%B; and t = 24 minute: 2%A; 98%B followed by 4-minute re-equilibration time. Total run time: 28 minute. The HPLC column was a Phenomenex Synergi Fusion C_{18} (150 × 2.1 mm, 3 μm particle size) with a flow rate of 350 μL/min. Injected volume was 10 μL. The mass spectrometer acquired data in electrospray positive ionization

(ESI$^+$) and multiple reaction monitoring mode. ESI of peptides of interest was optimized by flow injection analysis of a 10 μM solution in 50% water containing 0.1% HCOOH: 50% MeOH. The following parent–product ion transitions were applied: GTYSTTVTGR $[M+2H]^{2+}$ m/z = 521.8→721.4; 634.5; 136.0 (tube lens (TL) = 74V; collision energy (CE) = 19; 20; 27 eV); internal standard GTYSTTVTGR[$^{13}C_6{}^{15}N_4$] $[M+2H]^{2+}$ m/z = 526.8 →731.4; 543.2; 135.9 (TL = 75 V; CE = 19; 20; 28 eV); LFLEPTQADIALLK $[M+2H]^{2+}$ m/z = 786.5→1070.0; 260.8; 232.9 (TL = 101 V; CE = 27; 31; 38 eV); internal standard LFLEPTQADIALLK[13C615N2] $[M+2H]^{2+}$ m/z = 790.5→1078.0; 260.8; 232.8 (TL = 101 V; CE = 26; 30; 36 eV). HPLC-MS/MS data were acquired and processed by Thermo Xcalibur software (version 1.3). GraphPad Prism (GraphPad Software Inc, version 6.01) was used for data analysis and graphs.

Cholesterol loading capacity

Measurement of intracellular cholesterol was conducted via enzymatic determination on cell extracts as already described.[9] Human macrophages derived from THP-1 were used as a model to evaluate the accumulation of intracellular cholesterol induced by exposure to patient sera.

THP-1 monocytes were plated in 24-well plates in RPMI at 10% of fetal calf serum in a number of 500,000 cells per well and treated with PMA at a concentration 100 ng/mL to induce macrophage differentiation and substrate adhesion. After 72 hours, after a wash with PBS, the incubation of the cells began with 5% of patient's sera and the control sera for 8 hours; the negative control consists of cells incubated with only the medium. The cells were then lysed to dose the cholesterol and the protein content.

The macrophages were lysed with a 24-hour incubation at room temperature in 250 μL of 1% cholic acid solution and DNA-ase 50 U/mL. Each well was filled with 62.5 μL of a reaction buffer containing 0.5% triton-X-100, potassium phosphate 0.5 M (pH = 7.4), and 1% sodium cast, left to incubate for 30 min. The lysis of the cell monolayers was completed with repeated aspirations and extrusions through a 1 mL thin needle syringe. Aliquots (300 μL) were transferred from each well to plastic tubes then sonicated for 30 minutes. The samples were incubated at 60°C in a thermostatic bath to inactivate any enzymes interfering with the cholesterol quantification reaction. The standard cholesterol scale was prepared in duplicate in 96-well plates by adding 0, 2, 5, 10, 20, and 30 μL of 0.1 μg/μL cholesterol to the wells and resulting in a final volume of 50 μL with the reaction buffer contained in the Amplex Red Cholesterol Assay Kit (Molecular Probes, Life Technologies). A 50-μL aliquot of each cell extract was then added to each well. At the end, 50 μL of Amplex Red reagent was added to each sample and standards. A fluorimeter reading was taken with excitation wavelength at 535 nm and emission at 585 nm. The results obtained were expressed in relation to the protein content of each extract. The protein content in each extract was measured by the bicinchoninic acid method. 96-well plates were used to plate-out the standard protein scale (0, 3.125, 6, 12, 5, 25, 50, and 100 μg of albumin). The scale was prepared in duplicate using a 4 mg/mL albumin stock and taking 1: 2 serial dilutions starting from 100 μg up to 3.125 μg. Standards were made up to the volume with the sample lysis buffer. All samples (25 μL; standard scale and cell extracts) were mixed with 200 μL of a mixture of A and B solutions from the kit in a 50:1 ratio, with an incubation of 20 minutes at room temperature. Solution A consisted of 20 g of sodium carbonate, 4 g of soda, and 0.2 g of sodium tartrate (or 0.237 g of sodium hydrochloride tartrate) in 1 L of deionized water; solution B is instead composed of 5 g of copper sulfate in 1 L of deionized water. The developed color was measured at the spectrophotometer with a 550 nm filter. Protein concentration was calculated based on the standard albumin curve, and the total protein content per well was taken against the dilution volume.

Statistical analysis

The results were expressed as mean ± SEM. Differences in 7 variables associated with the lipid profile before and after LA were evaluated by means of a paired *t*-test. Because the analysis concerned multiple comparisons, the Bonferroni correction was applied, which sets the statistical cutoff for each comparison to α/n set at 0.007.

Two nested linear regression models were estimated to check for the effects of a set of variables on the levels of the CLC. The first model involves 2 demographic variables (age and gender), the BMI, the smoking attitude of the patient, the concentrations of LDL, Lp(a), and triglycerides as explanatory variables. Gender and smoking attitude are categorical variables, whose reference categories are men and nonsmokers, respectively. The second model adds the variable concerning the Lp(a) size (Kn). The improvement in model fit between the 2 nested models was tested and assessed by performing an F test of statistical significance on the change in R^2.

On account of the small size of the sample, the regression models described previously were estimated using a limited number of variables. Therefore, total cholesterol was excluded because of collinearity problems with other cholesterol variables such as triglycerides and LDL, whereas HDL was excluded because it had little biological relevance to accumulation of intracellular cholesterol.

Gene sequencing

Next-generation sequencing based on a custom AmpliSeq panel designed for sequencing of human genes related to the lipoprotein metabolism (including *LPA*, *PCSK9*, *APOE*, and *LDLR*) was performed with Ion PGM Sequencer (Fig. 1). The genomic DNA from venous blood

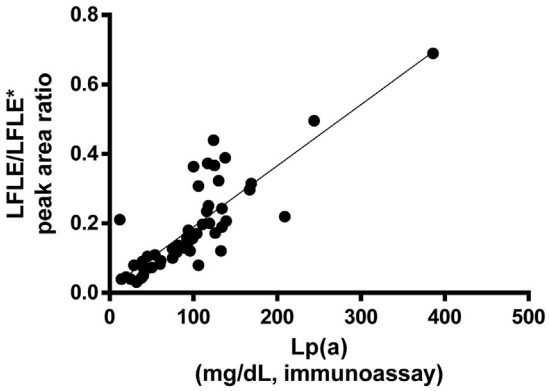

Figure 1 Correlation between MS- and Ab-based apo(a) and Lp(a) serum quantification. Ab, antibody; Lp(a), lipoprotein(a); MS, mass spectrometry.

samples was extracted from 200 μL blood applying the blood and body fluid spin protocol provided in the QIAamp DNA Blood Mini Kit (Qiagen, Milan, Italy). A multiplex PCR amplification strategy for the coding DNA sequences was accomplished online (Ion AmpliSeq Designer; http://www.ampliseq.com) to amplify the target region with 25 base pair exon padding. About 20 ng DNA per sample was used for the target enrichment by a multiplex PCR, and each DNA pool was amplified with the Ion AmpliSeq Library Kit in conjunction with the Ion AmpliSeq "Custom Primer Pool." Protocols were conducted as per the manufacturer's procedures (Life Technologies, Darmstadt, Germany) using the Ion Chef System. Enriched ISPs, which carried many copies of the same DNA fragment, were subjected to sequencing on an Ion 316 Chip to sequence pooled libraries with 12 samples. The 316 chip was chosen to obtain a mean sequencing depth of coverage of 50× which means that, on average, each base had been sequenced 50× when eleven samples were loaded. Sequencing was performed using a sequencing kit (Ion PGM reagents; Life Technologies, Monza (MI), Italy). The raw data (unmapped BAM-files) from the sequencing runs were processed using Torrent Suite software (Version 4.4.2, Life Technologies, Monza (MI), Italy) to generate read alignments, which are filtered by the software into mapped BAM files using the reference genomic sequence (hg19-human genome 19) of target genes. Variant calling was performed with the Torrent Variant Caller Plugin using as key parameters: minimum allele frequency = 0.015, minimum quality = 10, minimum coverage = 20, and minimum coverage on either strand = 3. The annotation of called variants was performed using the Ion Reporter software (Version 4.4; Life Technologies, Monza (MI), Italy) for the VCF files that contained the nucleotide reads.

All new missense variants were analyzed with in silico software PolyPhen and SIFT, predicting the effect of mutation on the function of a protein. These 2 tools predict the effect resulting in an amino acid substitution on the structure and function of a protein.

Results

Subjects

Demographic, disease, and treatment parameters are shown in Table 1. The baseline lipid parameters and CLC are shown in Table 2. Age, BMI, lipid-lowering therapy, carotid artery disease, smoking habit, and baseline lipid profile were similar in the 2 groups, whereas in the LA-group, CAD was more severe and CLC was higher (19.6 ± 3.7 vs 15.7 ± 3.5 μg/mg of protein, $P = .004$) than in the control group. The 18 patients in the LA group were on chronic LA for a median period of 5 years (range 1–27 years).

Comparison of MS-based and Ab-based methods for determination of serum levels of Lp(a)

We used an HPLC-MS/MS method to analyze human serum samples (n = 52) of the MM multicentric study. The area ratio of the LFLE peptide and its labeled internal standard LFLE* were correlated to the Lp(a) levels, expressed in mg/dL unit, which had been measured by conventional antibody-based (Ab-based)

Table 1 Patients' demographics, diagnosed diseases, and pharmacological treatment

Parameter	LLT + LA (N = 18)	LLT (N = 16)	*P**
Sex (F/M)	2/16	8/8	
Age (y)	60 ± 10	59 ± 8	ns
BMI (kg/m^2)	26.0 ± 3.0	24.8 ± 2.0	ns
CAD (#vessels involved) (mono-/bi-/trivasal) N	4/6/6	2/4/2	ns
Carotid (stenosis<69%/CAS or CEA) N	3/3	4/2	ns
Cigarette smoking (previous/never/current) N	11/7/-	5/9/2	ns
Lipid-lowering therapy (statins/statins + ezetimibe/PCSK9i/lomitapide) N	5/10/1/1	9/6/-/-	ns

CAD, coronary artery disease; CAS, carotid angioplasty and stenting; CEA, carotid endarterectomy; LA, lipoprotein apheresis; LLT, lipid-lowering treatment.

Table 2 Baseline lipids, lipoproteins, and cholesterol loading capacity

Parameter, mean ± SD	LLT + LA (N = 18)	LLT (N = 16)	P*
Total cholesterol (mg/dL)	171 ± 39	180 ± 39	ns
LDL cholesterol (mg/dL)	97 ± 34	106 ± 34	ns
HDL cholesterol (mg/dL)	50 ± 10	50 ± 8	ns
Triglycerides (mg/dL)	123 ± 56	121 ± 34	ns
Lp(a) mg/dL; median	117	115	ns
[range i.q.]	[94.3-135.0]	[99.2-137.7]	
CLC ug/mg of protein	19.7 ± 3.7	16.1 ± 3.57	.009

CLC, cholesterol loading capacity; LLT, lipid-lowering treatment; LA, lipoprotein apheresis; Lp(a), lipoprotein(a).

immunoturbidimetric methods.[20] A Spearman nonparametric correlation rho of 0.8315 was obtained, suggesting a positive monotonic correlation among the 2 variables ($P < .0001$) (Fig. 1).

Average size of apo(a)

The tryptic digestion of 3 recombinantly expressed apo(a) isoforms containing a different KIV-2 copy number (K10; K14; and K18) was used to relate the GTYS/LFLE peptide area ratios to the average size of apo(a) in unknown patient samples (Materials and Methods). As it can be seen from data in Supplementary Table 1, the GTYS/LFLE peptide area ratio remained constant as the concentration of each trypsin-digested recombinant apo(a) increased but proportionally increased among the different recombinant apo(a)s as a function of the KIV-2 number copies. Thus, the equation GYTS/LFLE ratio = 0.569 (±0.002) KIV-2 - 4.702 (±0.010); r^2 = 0.999; s = 0.10; F = 132,300; n = 3, was used to calculate the average KIV-2 copy number (ie, average apo(a) size) in the undetermined patient samples. Then, the derived apo(a) average size was related to in vitro CLC and was used to evaluate the effect of LA on apo(a) isoform size distribution.

Effect of lipoprotein apheresis on plasma lipids

Data on circulating lipid levels before and after LA are shown in Table 3. As expected, levels of TC, TG, LDL-C, and Lp(a) markedly reduced immediately after LA [−51,64% (P = .00001), −52,03% (P = .00001), −69,67% (P = .00001), and −65,43% (P = .00001), respectively]. HDL cholesterol was also reduced by LA [−15,69% (P = .0003)]. The HPLC-MS/MS method was also used to evaluate the efficiency of the LA treatment in decreasing the plasma concentration of Lp(a), by evaluating the percentage decrease in the LFLE/LFLE* peptide area ratios in preapheresis and postapheresis samples (Fig. 2). The mean percentage decrease was 66.2 ± 11.7% and not significantly different from values obtained by the Ab-based method (65.0 ± 11.7%). Only for n = 4 patients, the difference between the percentage decrease provided by the 2 methods was >15%.

Patient serum macrophage CLC before and after lipoprotein apheresis

Serum macrophage CLC was evaluated using patient sera obtained before and after LA to deliver cholesterol to

Table 3 Paired *t*-tests for a set of variables before and after lipoprotein apheresis

Lipid parameter	Before lipoprotein apheresis	After lipoprotein apheresis	t	P-value
TC (mg/dL)	171.2 ± 9.2	82.8 ± 4.7	**11.452**	**<.001**
TG (mg/dL)	123.4 ± 13.1	59.2 ± 9.8	**8.116**	**<.001**
LDL-C (mg/dL)	97.6 ± 7.9	29.6 ± 3.0	**9.478**	**<.001**
HDL-C (mg/dL)	49.7 ± 2.3	41.9 ± 2.0	**3.677**	**<.001**
Lp(a) (mg/dL)	131.6 ± 17.9	45.5 ± 6.3	**6.639**	**<.001**
CLC (mg/dL)	19.7 ± 0.9	16.1 ± 0.8	**3.139**	**.003**
apo(a) size (average KIV-2 copy number)	19.8 ± 0.7	18.1 ± 0.5	2.646	.009

CLC, cholesterol loading capacity; TC, total cholesterol; TG, triglyceride; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; KIV-2, kringle IV type 2; Lp(a), lipoprotein (a).

Values are expressed as mean ± SEM (n = 18). Variables in bold are statistically significant at $P < .007$, after Bonferroni's correction for 7 comparisons.

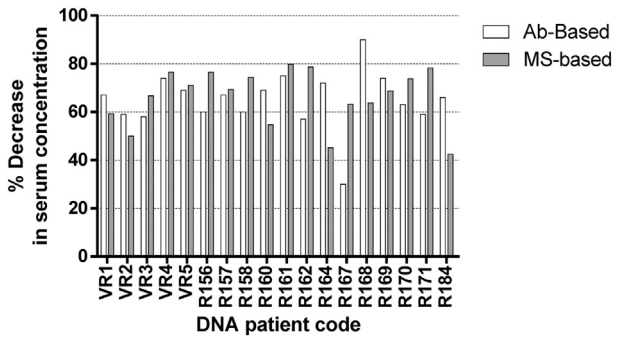

Figure 2 Comparison of Ab- and MS-based methods in evaluating the effect of LA on Lp(a) serum concentrations. Ab, antibody; Lp(a), lipoprotein(a); LA, lipoprotein apheresis; MS, mass spectrometry.

human macrophages THP-1. THP-1 initial cholesterol content before the exposure with serum was 13.90 ± 0.54 μg/mg of protein. The macrophage CLC of the pre-LA serum was significantly higher than that of the normolipidemic serum added as a control (mean ± SD 19.7 ± 0.9 μg/mg of protein and 16.01 ± 0.98 μg/mg of protein, respectively). After LA, serum macrophage CLC was markedly lower relative to preapheresis sera (mean ± SD 16.1 ± 0.8 μg/mg protein; $P = .003$) and comparable with CLC of the normolipidemic serum (Table 2). The LA did not significantly affect average apo(a) isoform size distribution, evaluated by means of the GTYS/LFLE peptide area ratios and back-calculated KIV-2 copy number (Fig. 3).

Serum CLC and Lp(a) concentration and size

Preliminary univariate analyses of the relationship between CLC and Lp(a) (Fig. 4) have shown a poor positive association with concentration (rho = 0.094, $P = .596$) and a moderate/high negative association with size (rho = −0.637, $P < .001$).

Once controlled for various potential confounding factors in a multiple linear regression (Table 4), in model 1, Lp(a) was significantly related to serum CLC ($P = .035$), whereas all the other anthropometric and lipid parameters studied were not. However, the overall model fit is not statistically significant (F = 2.1, $P = .088$), which means that the linear model does not describe adequately the experimental data. In model 2, the inclusion of Lp(a) size (KIV-2 copy number) allows highlighting either its significant inverse relationship with CLC (β = −0.707, $P = .001$) or the fading of Lp(a) statistical significance ($P = .412$). Moreover, in this model, the overall fit is statistically significant (F = 4.3, $P = .003$), which ensures more statistical robustness to the results. The statistical relevance of the improvement in the model fit between the 2 models is also supported by the F test on the change in R^2 (Table 5). Such parameter increases, in fact, by 0.321 (from 0.252 in model 1 to 0.573 in model 2), which results in a highly statistically significant improvement ($P = .001$). The partial R^2 of Lp(a) size in model 2 is 0.429, which means that Lp(a) size explains about 43% of the total variability of CLC, once controlled for the other variables in the model.

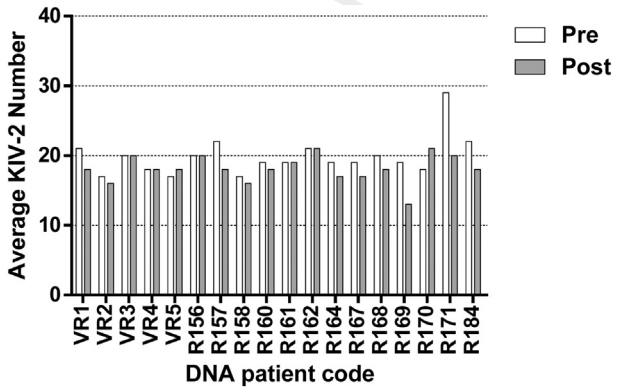

Figure 3 Effect of LA on apo(a) isoform distribution, assessed by changes in the GTYS/LFLE peptide area ratio. KIV-2, kringle IV type 2; LA, lipoprotein apheresis.

DNA sequencing

In our study of 18 cases in LA therapy and 16 controls with increased levels of Lp(a), we identified 5 of the 16 genetic variants described in PROCARDIS study: rs3798220, rs3798221, rs10755578, rs7765781, and rs7765803 (Fig. 6). In particular, 2 cases and one control Q17 were the heterozygous carrier of rs3798220 variant (allelic frequency: C: 0.04, PROCARDIS allele frequency: C: 0.02). In our patients, we did not find the Lp(a)-raising rs10455872 variant; the rs3798221 variant was identified in 2 cases and one control; the rs10755578 intronic variant was identified in 7 cases and 7 controls (heterozygous carriers). The rs7765781 intronic variant was identified in 5 cases and one control (heterozygous carrier). rs7765803 p.Leu1858Val is a missense, nonpathogenic variant on exon 26; in our study, we identified this variant in 4 cases and 3 controls (heterozygous carriers)—one control was homozygous for this rare variant. We did not observe differences between Lp(a) levels or cardiovascular disease severity in 13 patients without PROCARDIS variants in comparison with 21 patients with one or more of these variants.

In our study, we described one or more of the following benign or silent variants in 26 patients: rs3124784 (p.Arg2016Cys), rs41267809 (p.Leu1961Pro), rs41264848, and rs1801693 (p.Met1679Thr). We did not observe any differences in Lp(a) levels between carriers of more than 4 alleles in comparison with carriers of less than 4 alleles.

In one case (age 46 years, Lp(a) levels 138 mg/dL, pCAD), we identified a rare heterozygous *LPA* gene mutation on exon 21, c.3268C>G, p.Pro1090Arg predicted as "pathogenic" at in silico analysis (SIFT = 0.0 PolyPhen = 1.0). One patient (age 68 years, Lp(a) levels 106 mg/dL, in primary prevention) was a carrier of the

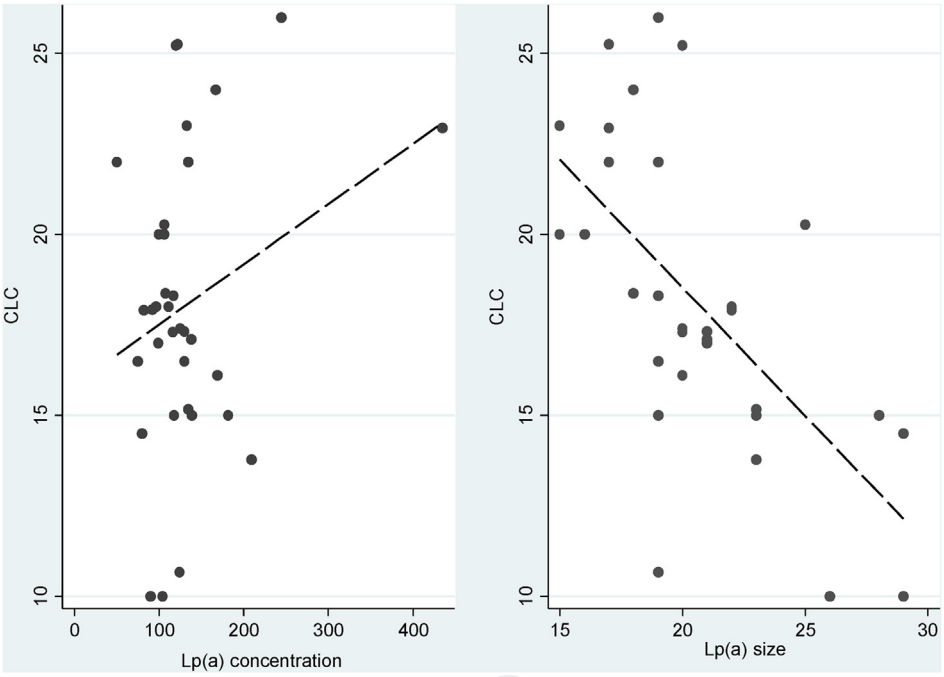

Figure 4 Scatterplot of the relationship between CLC and Lp(a) concentration (on the left) and CLC and Lp(a) size (on the right). CLC, cholesterol loading capacity; Lp(a), lipoprotein(a).

rare variant rs41259144 p.Arg990Gln, c.2969G>A (SIFT = 0 PolyPhen = 0.999). The clinical significance of these 2 new rare variants remains uncertain: in silico programs define "pathogenic" an amino acid substitution likely entails a significant change of the protein structure; however, this alteration can also be advantageous for the species, so the "pathogenic" term may be inappropriate.

Seven patients of our case series are heterozygous FH patients and one of them was homozygous FH patient. Seven patients were carriers of the APOE ε3ε4 genotype, and 4 patients were carriers of the APOE ε3ε2 genotype, but we did not observe significant clinical differences according to the LDLR and APOE genotype. Only one case was a carrier of p.R46L mutation on the PCSK9 gene.

Discussion

This study has demonstrated that patients with elevated Lp(a) and with an increased risk of CAD, who underwent LA, have elevated CLC (Table 2). We also showed that apheresis lowered Lp(a) and CLC and that these changes were statistically concordant. LA did not alter the apo(a) isoform size. LA markedly reduced serum lipid levels,

Table 4 Linear regression of CLC on a set of covariates: nested models

	Model 1			Model 2		
Variables	β	*P*-value	Partial R^2	β	*P*-value	Partial R^2
Age (years)	0.013	.875	0.001	0.007	.907	<0.001
BMI (kg/cm^2)	−0.247	.314	0.029	0.007	.980	<0.001
Sex	−3.060	.176	0.095	−2.435	.263	0.104
Nonsmoker (ref. cat. smoker)	0.304	.869	0.001	0.172	.892	0.001
LDL-C (mg/dL)	−0.017	.555	0.024	−0.013	.561	0.020
Lp(a) (mg/dL)	**0.023**	**.035**	0.106	0.012	.412	0.051
Triglycerides (mg/dL)	0.015	.463	0.030	0.020	.163	0.093
apo(a) size (average KIV-2 copy number)				**−0.707**	**.001**	**0.429**
F (7, 22)	2.1			**4.3**		
P-value	.088			**.003**		
R^2	0.252			0.573		

B, regression coefficient; CLC, cholesterol loading capacity; LDL-C, LDL cholesterol; Lp(a), lipoprotein (a).

Table 5 Improvement in model fit between the 2 nested models: F test on the change in R^2

	Block residual					
Block	F	df	Df	*P*-value	R^2	Change in R^2
1	2.1	7	22	.088	0.252	
2	16.2	1	21	**.001**	**0.573**	**0.321**

and we demonstrated that it was possible to use HPLC-MS/MS to determine apo(a)- and Lp(a)-related parameters, thereby offering the possibility of a simplified, low-volume analysis.

Lp(a) is an LDL-like particle in which the hydrophobic apoB100 is linked via a disulfide bridge to the hydrophilic glycoprotein, apo(a), containing on average 23% carbohydrate content by weight.[21] There is a single apo(a) molecule for Lp(a) particle, but at least 34 apo(a) isoforms have been identified.[22] The most important size determinant for apo(a) isoforms is the number of structural subunits called kringles (molecular weight ~17 kDa) and specifically KIV-2, which can range from 3 to over 40 copies. Commonly used Ab-based diagnostic methods have been standardized to measure Lp(a) levels as mass per unit volume (ie, mg/dL). Nevertheless, mass-based assays do not take into consideration the size polymorphism and heterogeneity of Lp(a) particles among different individuals. Using immunoassays to measure Lp(a) levels in terms of mass rather than molarity can also lead to size-related bias if the used antibodies are directed to epitopes present in the variable portion of Lp(a) particles. Moreover, immunoassays use an assay calibrator of fixed size, which is often not representative of the heterogeneity of the Lp(a) population present. In this context, diagnostic assays based on Lp(a) or apo(a) molarity, such as MS-based assays, could provide a more accurate assessment of Lp(a) concentration such as low Lp(a) concentration for the individuals with large particles and high Lp(a) concentrations for those with small particles.

Our results support the employment of an MS-based approach for apo(a) serum level quantification in future studies. This technique has already been reported for several other serum apolipoproteins and is valid provided the conversion of peptide area ratios to nanomolar concentrations of apo(a) is performed using calibration data in line with the WHO-IFCC–certified reference material of apo(a), SRM-2B.[23,24]

As previously shown by Lassman et al.,[19] the tryptic peptide LFLEPTQADIALLK (LFLE) is unique to apo(a), in human serum proteome, following BLAST search in the UniProtKB/SwissProt database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This peptide is only present in the constant portion of apo(a), so, after tryptic digestion, the area of the LFLE peptide signal is expected to be proportional to the molar concentration of apo(a), independently from the heterogeneity of its molecular weight—its isotope labeled internal standard LFLE*, eluting at the same retention time in the HPLC run, can be used to normalize matrix effects due to coeluting serum components.

Lassmann et al.[19] also provided an interesting insight into the evaluation of apo(a) average size using HPLC-MS/MS. The tryptic peptide GTYSTTVTGR (GTYS) was used as a marker of the KIV-2 copy number. In principle, the bigger is the apo(a) size, the higher is the number of KIV-2 repeats and, relating this after tryptic digestion, the higher the concentration of the corresponding peptide GTYS will be with respect to the concentration of peptide LFLE. Thus, the area ratio of peptide GTYS and its labeled internal standard GTYS* divided by the area ratio of peptide LFLE and LFLE* could function as a useful parameter to describe the mean size of the apo(a) serum population. The main limitation of this approach has been clearly declared by their proponents. Briefly, if apo(a) was expressed in serum as a single isoform, this MS-based approach could be a valid alternative to the gold standard method for apo(a) size measurement (ie, high resolution SDS-PAGE electrophoresis). However, as most individuals express 2 different alleles encoding for at least 2 isoforms of apo(a) of different size, the value retrieved by HPLC-MS/MS after tryptic digestion has to be considered an average size value derived from generally 2 or even more apo(a) populations. Despite this declared limitation, the average size determination by HPLC-MS/MS could be used within the present study as a high-throughput method to assess the size distribution of apo(a) isoforms in the serum of our patient samples.

Marked reductions in levels of LDL-C and Lp(a) after LA in our study were consistent with previous reports.[9] The good correlation between MS- and Ab-based results for determination of Lp(a) levels confirms that the MS-based method would be, after a proper validation, a valid high-throughput assay to check and confirm conventional Ab-based results in hospital settings—particularly as the system only requires very low volumes (ie, 4 μL) of human serum per analysis.

This is a study confirming the significance of CLC as proatherogenic parameter, although it has never been correlated with clinical atherosclerosis, much less validated as a predictor independent of atherogenic lipoprotein parameters such as LDL-C, Lp(a), and apoB. However, despite the need for better validation of the role of CLC assay, it is well known that cholesterol may increase the risk of CAD via direct action on macrophage inducing foam cell formation, leading to the development of atherosclerotic plaque.[25] It has been also reported that the

aforementioned atherogenic evolution may also be related to different degrees[26] and feature/phenotype of in vivo dyslipidemia, such as in our study. Because it has been demonstrated that proatherogenic lipoproteins directly promote the cellular influx of cholesterol,[27,28] we made the choice to focus our attention on CLC parameter in these patients with high Lp(a) levels and CAD, submitted or not to LA. Moreover, the approach directed at investigating CLC, rather than CEC, has been already reported in the literature.[29]

For the first time, our data from hyperLp(a) patients with CAD demonstrate a direct association between Lp(a) size and its proatherogenic capacity, which can be directly measured as CLC. In particular, we found that the greater is the Lp(a) size, the lower is the CLC patient concentration. On the other hand, no significant relationship was highlighted between CLC and Lp(a) serum concentration.

Ours is the first systematic study of nonsize polymorphism of the *LPA* gene in Italian patients in LA therapy. Our data confirmed that the *LPA* gene is extremely polymorphic but some variants, previously described in the literature, are recurrent or absent in relation to the geographical origin. We identified 2 novel variants never described before, but their clinical significance remains uncertain. The sample size of our study is not sufficient to explore the predictive role of each variant on cardiovascular risk. On a case-by-case basis, observing the predictive role of each genetic variant of *LPA* gene vs Lp(a) levels and cardiovascular risk is not reliable because of the complexity of the genetic regulation of Lp(a) levels. In our study, other genes such as *APOE, LDLR,* and *PCSK9* did not influence the clinical phenotype or cardiovascular risk.

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