


# ApoL1 levels in high density lipoprotein and cardiovascular event presentation in patients with familial hypercholesterolemia<sup>S</sup>

Judit Cubedo,<sup>\*,†</sup> Teresa Padró,<sup>\*,†</sup> Rodrigo Alonso,<sup>§</sup> Pedro Mata,<sup>\*\*</sup> and Lina Badimon<sup>1,\*,†,††</sup>

Cardiovascular Research Center (CSIC-ICCC),\* Barcelona, Spain; Biomedical Research Institute Sant Pau (IIB-Sant Pau),<sup>†</sup> Barcelona, Spain; Jimenez Diaz Foundation,<sup>§</sup> Madrid, Spain; Fundación Hipercolesterolemia Familiar,<sup>\*\*</sup> Madrid, Spain; and Cardiovascular Research Chair, Autonomous University of Barcelona (UAB),<sup>††</sup> Barcelona, Spain

**Abstract** HDL composition rather than HDL-cholesterol (HDL-C) levels seems to be a key determinant of HDL-induced atheroprotection. Heterozygous familial hypercholesterolemia (FH) patients, with lifelong exposure to high LDL levels, show a high prevalence of premature coronary artery disease. We hypothesized that HDL of FH patients might have a modified protein composition and investigated the proteomic signature of HDL obtained from FH patients and their unaffected relatives. HDLs were characterized by 2D electrophoresis/MS in 10 families from the SAFE-HEART cohort (3 individuals/family: 2 with genetic FH diagnosis and 1 non-FH relative) clinically characterized and treated as per guidelines. FH patients had lower apoA-I levels and a differential HDL distribution profile of apoL1 and apoA-IV. ELISA validation revealed decreased apoL1 serum levels in FH patients. ApoL1 levels were able to predict presentation of an ischemic cardiac event, and apoL1/HDL-C ratio was associated with the survival rate after the event. FH patients who died because of a fatal cardiac event had lower apoL1 and LCAT content in HDL3 an average of 3.5 years before the event than those who survived. Changes in HDL protein composition could affect patients' prognosis.  The proteomic profile of apoL1 is modified in HDLs of high cardiovascular risk patients, and apoL1 plasma levels are significantly lower in serum and in HDL3 of patients that will suffer an adverse cardiac event within 3 years.—Cubedo, J., T. Padró, R. Alonso, P. Mata, and L. Badimon. ApoL1 levels in high density lipoprotein and cardiovascular event presentation in patients with familial hypercholesterolemia. *J. Lipid Res.* 2016. 57: 1059–1073.

**Supplementary key words** apolipoprotein L1 • proteomics • prognosis • mortality • biomarkers

Familial hypercholesterolemia (FH) is one of the most prevalent genetic disorders associated with premature coronary artery disease (CAD) (1). This disorder appears by mutations in the gene that encodes the LDL receptor (LDLR) and it is characterized by a lifelong exposure to high plasma LDL-cholesterol levels (LDL-C). FH patients show an important decrease in their life expectancy because of an increase in CAD frequency, the principal causes of mortality being sudden death and myocardial infarction (2). Clinical expression of CAD in FH patients varies considerably across cohorts and individual patients treated to guidelines (3, 4), suggesting the contribution of additional factors to the atherosclerotic burden in these patients. Indeed, a recent study has reported lipoprotein (a) [Lp(a)] levels as one of those additional factors that influence CVD in FH patients (5).

HDLs have proved to be atheroprotective in several epidemiological, clinical, and experimental studies (6–13). Indeed, HDLs are considered key players in the progression of CAD because of their multiple antiatherogenic effects (14) in addition to their role in reverse cholesterol transport. Increasing evidence suggests that HDL atheroprotective effects lie in HDL composition, highlighting the importance of HDL quality rather than HDL quantity in CVD (14–17). Moreover, the existence of several HDL subpopulations with differential contribution to these HDL-related antiatherogenic properties has underscored the relevance of HDL composition in HDL functionality (15, 18).

A recent study has demonstrated that FH is associated with quantitative and qualitative modifications of HDL

This work was supported by Spanish Ministry of Economy and Competitiveness of Science Grants SAF2013-42962-R (L.B.) and CNIC-082008 (L.B. and P.M.); FEDER “Una manera de hacer Europa” and Institute of Health Carlos III Grant RIC RD12/0042/0027 (L.B.); TERCEL (Red de Terapia Celular) Grant RD12/0019/0026 (L.B.); FIS (Fondo Investigación Sanitaria) Grant PI13/02850 (T.P.), and FIC-Fundación Jesús Serra, Barcelona, Spain.

Manuscript received 23 June 2015 and in revised form 31 March 2016.

Published, JLR Papers in Press, April 25, 2016  
DOI 10.1194/jlr.P061598

Abbreviations: AUC, area under ROC curve; CAD, coronary artery disease; CI, confidence interval; FH, familial hypercholesterolemia; HDL-C, HDL cholesterol; HR, hazard ratio; LDL-C, LDL cholesterol; LDLR, LDL receptor; Lp(a), lipoprotein (a); PON1, paraoxonase-1; ROC, receiver operating characteristic; 2DE, two-dimensional gel electrophoresis.

<sup>1</sup>To whom correspondence should be addressed.

e-mail: lbadimon@csic-iccc.org

<sup>S</sup> The online version of this article (available at <http://www.jlr.org>) contains a supplement.

particles leading to alterations in their functions in the reverse-cholesterol transport pathway (19). Furthermore, we have previously described a differential distribution of the acute phase response protein transthyretin in HDL of FH patients with prior clinical manifestation of CAD (20). However, until now, little has been known about the protein composition of HDL in FH patients and if changes in their proteome distribution might be related to their high risk for premature CAD.

One of the most unexplored protein components of HDL is apoL1. Specifically, apoL1 is found in HDL3 sub-species (21), together with paraoxonase-1 (PON1) and apoF (also known as lipid transfer inhibitor protein) (22). The apoL1 gene belongs to a family of six genes encoding for the six different existing apoL forms (L1–L6) that are found on chromosome 22 and are only present in humans and a few primate species (23). It is a widely expressed apolipoprotein, particularly in the vasculature (24, 25). ApoL1 contains characteristic protein domains that imply important intracellular physiological roles possibly involving ion transport and apoptosis (26). As occurs with other apolipoproteins (27, 28), apoL1 is secreted as a proform, and it is proteolytically activated giving as a result a mature apoL1 form with a lower molecular mass (21). Moreover, a potential role in inflammatory or innate immunity-linked mechanisms has also been proposed (29). However, little is known about its exact function. Until now, apoL1 gene variants have been associated with increased incidence and progression of chronic kidney disease (30), and with renal allograft failure (31) in African Americans, having been proposed as key factors in the future of renal diagnosis (32). Furthermore, it has been shown that these specific apoL1 variants, containing missense residues or presenting deletions of amino acids in the C-terminal domain, contribute to atherosclerotic cardiovascular risk, as a genetic determinant to cardiovascular risk in individuals of African ancestry (29). Nevertheless, how these genetic variations affect apoL1 function is not known. ApoL1 levels have been shown to be positively associated with TG levels and hyperglycemia in patients with low HDL-cholesterol (HDL-C) levels and CAD, not being affected by lipid-altering therapies (33). However, the potential role of this HDL-related apolipoprotein in cardiovascular progression and outcome is yet unknown (34).

In this study, by using differential proteomic profiling approaches, we have investigated the HDL proteome in a population of FH patients and their non-FH relatives, and we have found significant changes in the apoL1 content in HDL, pointing for the first time to apoL1 as a potential causal factor in premature CVD presentation in hypercholesterolemic patients.

## MATERIALS AND METHODS

### Study population characteristics

The present study comprised three phases: *I*) the discovery phase, in which a differential proteomic approach was used as previously described (20, 35–38) to identify differences in the

HDL proteomic signature in 10 families from the Spanish Familial Hypercholesterolaemia Cohort Study (SAFEHEART) (39, 40) with three individuals per family [two members with genetic and clinical diagnosis of heterozygous FH (N = 19) and a control relative without the FH mutation (non-FH; N = 11) matched for age, gender, and demographics (N = 30; **Table 1**)]; *II*) the validation phase, where differential selected proteins were validated by ELISA in the discovery cohort (N = 30) and in a validation cohort of 73 FH patients (with nonrelative relationship between them or with those included in the discovery cohort) who suffered an ischemic event, including acute myocardial infarction [both fatal (exitus patients; N = 32) and nonfatal (no exitus patients; N = 41)] and unstable angina, an average of  $3.5 \pm 0.3$  years after inclusion in the study (FH-CV; **Table 2** shows differences between no CV and CV patients, and **Table 3** shows differences within FH-CV patients between no exitus and exitus patients); and *III*) the characterization phase, where the distribution profile of differential proteins was specifically analyzed in isolated HDL2 and HDL3 subfractions in a subset of randomly selected FH-CV patients from the validation phase (supplementary Table 1) who suffered an ischemic event, both fatal (exitus; N = 5) and nonfatal (no exitus; N = 5) after blood sampling and inclusion in the study. Patients included in the validation and characterization phases were also recruited from the SAFEHEART cohort. The study workflow is shown in **Fig. 1**.

The genetic diagnosis of FH was made using a DNA-microarray (Progenika) and capillary sequencing by multiplex PCR conditions and sequence reactions (41, 42). Negative samples for the DNA array or sequencing were also analyzed for large deletions or insertions using an adapted quantitative fluorescent multiplex PCR methodology (43). As previously reported (44), those mutations entailing an early stop codon and those entailing a shift reading frame that did not produce protein were grouped as receptor-null mutations, whereas receptor-defective mutations were those entailing an amino acid change, mutations with a splicing change, and other mutations inducing the production of a mutated protein. All patients were followed up for 10 years. All the samples were collected following very strict protocols and stored in the same conditions and for the same time period.

It was determined that ischemic event presentation was present if one of the following criteria was documented: *1*) myocardial infarction proved by classic criteria or *2*) classic symptoms of angina pectoris and one positive result on an ischemic test or >70% stenosis on coronary angiography, as previously reported (5).

Sociodemographic data were obtained from all subjects. All samples were from patients of the cohort that had been prescribed lipid-lowering treatment according to guidelines. The Ethics Committee of the Jimenez Diaz Foundation approved the project, and the studies were conducted according to the principles of the Helsinki Declaration. All participants gave written informed consent to take part in the study.

### Blood collection, biochemical analysis, and sample preparation

Venous blood was collected, with informed consent, both in EDTA-containing tubes (1 g/l) and in tubes to obtain serum after a 12 h overnight fast. Plasma and serum were then isolated by centrifugation (2,500 g for 10 min at 4°C). All analyses, except the one for TGs, were performed at the end of the study on aliquoted samples stored at –80°C, in order to minimize assay variability. Plasma TGs and cholesterol concentrations were measured using standard enzymatic methods (45, 46). HDL-C was measured using phosphotungstic acid/MgCl<sub>2</sub>, after precipitation of apoB-containing lipoproteins (47). Quality controls were applied to every measurement using commercial kits (Precinorm, Precilip, Boehringer-Mannheim). LDL-C levels were calculated using the

TABLE 1. Background description and biochemical parameters of FH patients and non-FH relatives included in the discovery phase

	FH Patients	Non-FH Relatives	<i>P</i> <sup>a</sup>
	(N = 19)	(N = 11)	
Age	57 ± 3	50 ± 3	0.144
Females/males	6/13	6/5	0.432
Risk factors (%)			
Tobacco consumption	53	45	>0.999
Hypertension	37	18	0.419
Diabetes	0	0	>0.999
BMI (kg/m <sup>2</sup> )	28 ± 1.3	29 ± 1.1	0.644
Years of statin treatment	9.6 ± 1.6	5.6 ± 3.4	0.281
Total cholesterol (mg/dl)	235 ± 16	230 ± 14	0.851
LDL-C (mg/dl)	164 ± 14	145 ± 10	0.367
HDL-C (mg/dl)	50 ± 3	58 ± 4	0.085
TG (mg/dl)	103 ± 11	141 ± 42	0.289
Non-HDL-C (mg/dl)	184 ± 16	172 ± 14	0.588
ApoB (mg/dl)	116 ± 9	106 ± 8	0.447
CRP (mg/l)	4.3 ± 1.3	2.4 ± 0.6	0.282
Glc (mg/dl)	89 ± 2	94 ± 3	0.172
Xanthomas (%)	16	0	0.279
Corneal arcus (%)	53	9	0.023
FH mutation (%)			
Null	63	0	0.0006
Defective	37	0	0.029
Indeterminate	0	0	>0.999
Treatment (%)			
Statins	100	45	0.0008
ASA	37	0	0.029
Clopi	5	0	>0.999
Beta-blockers	5	0	>0.999
Ca <sup>2+</sup> antagonists	11	0	0.520
ACEI	21	0	0.268
A2RA	11	0	0.520
OAD	0	0	>0.999

ACEI, angiotensin-converting enzyme inhibitors; ASA, acetylsalicylic acid; A2RA, angiotensin-2-receptor antagonists; Clopi, clopidogrel; CRP, C-reactive protein; Glc, glucose; OAD, oral antidiabetic drugs. Data are expressed as mean ± SEM.

<sup>a</sup>Student's *t*-test for continuous variables and Chi-square test or Fisher's exact test, when any of the expected values was <5, for categorical variables.

Friedewald formula (48). ApoA-I and apoB were determined by turbidimetry (49). High-sensitivity C-reactive protein was measured by immunoturbidimetry in a DDPP-800 autoanalyzer (Roche/Hitachi, Roche Diagnostics GmbH).

For proteomic studies, HDL samples were prepared as previously described (20, 35–37). Briefly, human HDL, HDL2, and HDL3 were obtained by ultracentrifugation in KBr gradient of EDTA plasma (density gradients for total HDL, 1.063–1.210 g/ml; HDL2, 1.063–1.125 g/ml; and HDL3, 1.125–1.210 g/ml), and the protein fraction was obtained by precipitation with pure ice-cold acetone (protocol that enables the delipidation of HDL samples) and solubilized in a urea/thiourea buffer (7 M urea, 2 M thiourea, 2% CHAPS).

Protein concentration was measured with 2D-Quant kit (GE Healthcare). All processed samples were stored at –80°C until used.

### Proteomic analysis

In the discovery phase of the study, the total HDL fraction was analyzed in FH patients (N = 19) and their non-FH relatives (N = 11) to identify the differential HDL proteome associated with FH. In the second phase and in order to find out the relevance of the detected changes in the outcome of FH patients, the differential proteomic profile associated with FH was specifically analyzed in HDL2 and HDL3 subfractions in a subset of patients who suffered an ischemic event, both fatal (exitus; N = 5) and nonfatal (no exitus; N = 5) after blood sampling and inclusion in the study.

**2DE.** For analytical and preparative gels, respectively, a protein load of 100 µg and 300 µg protein of the urea/thiourea HDL, HDL2, and HDL3 extracts were applied to 17 cm dry strips (pH 4–7 linear range, BioRad). Second dimension was resolved in 12% SDS-PAGE gels. Gels were developed by fluorescent staining (Flamingo, BioRad). For each independent experiment, two-dimensional gel electrophoresis (2DE) analyses for protein extracts from each group of patients were processed in parallel to guarantee a maximum of comparability. Each 2DE run was at least repeated twice to ensure the reproducibility. In 2DE analyses, the proteomic profile of the analyzed groups was compared by using the PD-Quest 8.0 software (BioRad) that specifically analyzes the differences in protein patterns by using a single master that includes all the gels of each independent experiment (samples from all the groups included in the experiment). In this analysis, each spot in the gel is assigned a relative value that corresponds to the single spot volume compared with the volume of all spots in this gel in order to avoid potential differences due to technical variability, as previously described (20, 35–38). Afterwards, this value is subjected to background extraction and the final intensity value is then normalized by the local regression model (LOESS) method of the software.

**MS analysis.** Proteins were identified after in-gel tryptic digestion and extraction of peptides from the gel pieces, as previously described (20, 35–38) by MALDI-TOF using an AutoFlex III Smartbeam MALDI-TOF/TOF (Bruker Daltonics). Samples were

TABLE 2. Background description and biochemical parameters of FH patients included in the validation phase with and without an ischemic event after blood sampling and inclusion in the study

	FH Patients		<i>P</i> <sup>a</sup>
	No CV	CV	
	(N = 19)	(N = 73)	
Age	57 ± 3	57 ± 2	0.887
Females/males	6/13	25/48	0.827
Risk factors (%)			
Tobacco consumption	53	62	0.475
Hypertension	37	37	0.991
Diabetes	0	16	0.04
BMI (kg/m <sup>2</sup> )	28 ± 1.3	29 ± 0.6	0.844
Years of statin treatment	9.6 ± 1.6	10.3 ± 0.9	0.735
Total cholesterol (mg/dl)	235 ± 16	278 ± 10	0.042
LDL-C (mg/dl)	164 ± 14	207 ± 9	0.027
HDL-C (mg/dl)	50 ± 3	43 ± 1	0.017
TG (mg/dl)	103 ± 11	132 ± 9	0.119
Non-HDL-C (mg/dl)	184 ± 16	235 ± 10	0.02
ApoB (mg/dl)	116 ± 9	139 ± 6	0.07
CRP (mg/l)	4.3 ± 1.3	3.3 ± 0.4	0.285
Glc (mg/dl)	89 ± 2	98 ± 3	0.111
Xanthomas (%)	16	26	0.351
Corneal arcus (%)	53	47	0.638
FH mutation (%)			
Null	63	48	0.237
Defective	37	48	0.387
Indeterminate	0	4	0.369
Treatment (%)			
Statins	100	100	>0.999
ASA	37	38	0.904
Clopi	5	12	0.378
Beta-blockers	5	22	0.096
Ca <sup>2+</sup> antagonists	11	10	0.903
ACEI	21	18	0.746
A2RA	11	18	0.444
OAD	0	8	0.196

Data are expressed as mean ± SEM.

<sup>a</sup>Student's *t*-test for continuous variables and Chi-square test or Fisher's exact test, when any of the expected values was <5, for categorical variables.

applied to Prespotted AnchorChip plates (Bruker Daltonics) surrounding the calibrants provided on the plates. Spectra were acquired with flexControl on reflector mode, (mass range *m/z* 850–4,000; reflector 1, 21.06 kV; reflector 2, 9.77 kV; ion source 1 voltage, 19 kV; ion source 2, 16.5kV; detection gain, 2.37×) with an average of 3,500 added shots at a frequency of 200 Hz. Each sample was processed with flexAnalysis (version 3.0, Bruker Daltonics) considering a signal-to-noise ratio >3, applying statistical calibration and eliminating background peaks. For identification, peaks between *m/z* 850 and 1000 were not considered as in general only matrix peaks are visible on this mass range. After processing, spectra were sent to the interface BioTools (version 3.2, Bruker Daltonics), and MASCOT search on Swiss-Prot 57.15 database was done [taxonomy, *Homo sapiens*; mass tolerance, 50 to 100; up to two trypsin miss cleavages; global modification: carbamidomethyl (C); variable modification: oxidation (M)]. Identification was carried out by peptide mass fingerprinting (pmf) where a mascot score >56 and at least five matched peptides was accepted, as previously reported (20, 35). Identified proteins were then confirmed by peptide fragmentation working on the reflector mode (MS/MS).

### Quantification of apoL1 and apoA-IV serum levels

Systemic total apoL1 and apoA-IV concentrations were determined in serum by quantitative sandwich ELISAs (MyBioSource and Millipore, respectively) in the population used in the discovery phase (non-FH, N = 11; and FH, N = 19). The differences found were then further validated in the validation cohort [73

FH patients who suffered an ischemic event after blood sampling and inclusion in the study (FH-CV)].

Specifically, the antibody of the ELISA kit for apoL1 detection uses a combination of monoclonal/polyclonal antibodies and recognizes as epitope the amino acid sequence of apoL1 from the residue 196 to 357 (amino acids present in both the precursor and the mature form of the protein). The apoA-IV ELISA kit uses two polyclonal antibodies. However, the epitope recognized by this kit is undisclosed by the providers.

The detection limits of the assays were 0.884 ng/ml for apoL1 and 0.02 µg/ml for apoA-IV. The intra-assay variability reported by the providers was of 10% for apoL1 and 4.6% for apoA-IV, and the interassay variability was of 12% for apoL1 and 12.3% for apoA-IV. Each sample was run in duplicate within the same assay and was repeated at least twice in two independent assays to ensure reproducibility.

### Western blot analysis

Total HDL, HDL2, and HDL3 protein profiles were validated by Western blot. Protein extracts were resolved by 1D gel electrophoresis under reducing conditions and electrotransferred to polyvinylidene difluoride membranes in semidry conditions (Semidry transfer system, BioRad). Protein detection was performed using monoclonal antibodies against apoA-IV (1:5,000 dilution, ab108200 Abcam), apoL1 (1:5,000 dilution, ab108315 Abcam), LCAT (1:10,000 dilution, ab51060 Abcam), and PON1 (1:1,000, H00005444-M01 Abnova), in combination with the Dye Double Western Blot kit (Invitrogen). Band fluorescence was determined



TABLE 3. Background description and biochemical parameters of FH patients included in the validation phase who suffered an ischemic event after blood sampling and inclusion in the study

	FH-CV Patients		<i>P</i> <sup>a</sup>
	No Exitus	Exitus	
	(N = 41)	(N = 32)	
Age	53 ± 2	62 ± 3	0.016
Females/males	12/29	13/19	0.310
Risk factors (%)			
Tobacco consumption	63	59	0.725
Hypertension	29	47	0.122
Diabetes	12	22	0.268
BMI (kg/m <sup>2</sup> )	29 ± 0.7	29 ± 0.9	0.919
Years of statin treatment	11.5 ± 1.2	8.8 ± 1.4	0.143
Total cholesterol (mg/dl)	229 ± 14	265 ± 13	0.240
LDL-C (mg/dl)	218 ± 13	194 ± 12	0.170
HDL-C (mg/dl)	42 ± 2	45 ± 2	0.176
TG (mg/dl)	130 ± 11	134 ± 13	0.831
Non-HDL-C (mg/dl)	247 ± 14	220 ± 13	0.183
ApoB (mg/dl)	142 ± 9	135 ± 7	0.557
CRP (mg/l)	3.6 ± 0.6	2.8 ± 0.3	0.293
Glc (mg/dl)	96 ± 4	99 ± 6	0.755
Xanthomas (%)	27	34	0.860
Corneal arcus (%)	25	63	0.016
FH mutation (%)			
Null	49	47	0.872
Defective	49	47	0.872
Indeterminate	2	6	0.416
Treatment (%)			
Statins	100	100	>0.999
ASA	41	34	0.537
Clopi	10	16	0.449
Beta-blockers	20	25	0.574
Ca <sup>2+</sup> antagonists	5	16	0.199
ACEI	17	19	0.853
A2RA	20	16	0.667
OAD	7	9	0.751

Data are expressed as mean ± SEM.

<sup>a</sup>Student's *t*-test for continuous variables and Chi-square test or Fisher's exact test, when any of the expected values was <5, for categorical variables.

with Typhoon 9400 (GE Healthcare), and band quantification was performed using ImageQuant TL v7.01. software (GE Healthcare). Protein load was normalized using total protein fluorescent signal, as previously published (20, 35–37).

The intraindividual variability of Western blot loading detected by relative fluorescence was of 3.24%. The fluorescent signal showed a highly significant linear correlation with protein abundance ( $R = 0.991$ ,  $P = 0.001$ ).

### Statistical analysis

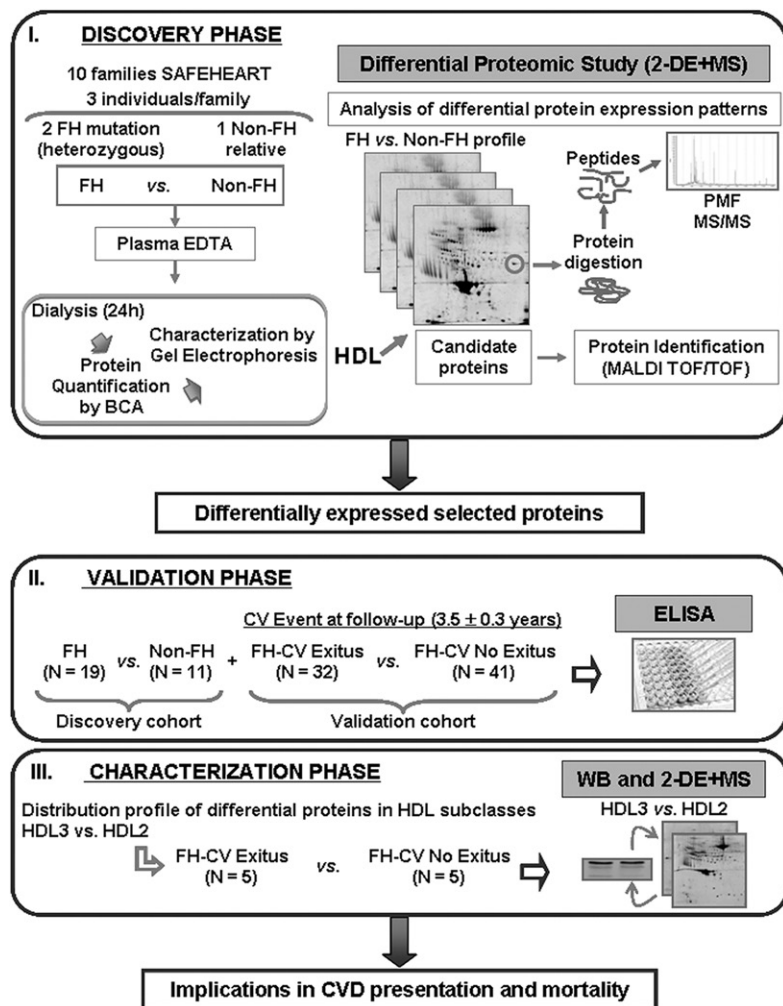
Data are expressed as mean and standard error except when indicated. N indicates the number of subjects tested. Statistical analyses were performed with Stat View 5.0.1 software. A test for normality was performed using the Kolmogorov-Smirnov test. Student's *t*-test was used for comparison between groups. The ANOVA test was used in analyses where the effect of variables acting as covariates was added to the model, and in multiple comparisons analyses using Fisher's protected least significant difference as post hoc test. Correlations between variables were determined by single and multiple regression models. Chi-square test ( $\chi^2$ ) or Fisher's exact test, when any of the expected values was <5, was used for categorical variables. Receiver operating characteristic (ROC) curves (to assess the discriminating power of selected variables), and Kaplan-Meier and Cox regression survival analyses (to analyze the impact of different variables in the survival rate of FH patients after the presentation of an ischemic event) were performed with IBM SPSS Statistics v19.0. A *P* value ≤ 0.05 was considered significant. Differences between areas under the ROC curves (AUCs) were analyzed as previously described (50).

Minimal required sample size for validation analyses was calculated and validated using the JavaScript-based method for simple power/sample size calculation when two independent groups are compared (provided at <http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html>) as previously described (51). Based on the mean value of FH patients and non-FH relatives and the standard deviation of the studied population, our sample size gave a study power of >0.75 (type I error = 0.05, two-sided test).

## RESULTS

### Differential HDL proteomic profile in FH patients

Within the selected conditions (pI range, 4–7; molecular mass range, 120–10 kDa), the proteomic analysis of isolated HDL revealed 19 main nonredundant proteins being predominantly apolipoproteins and lipoprotein metabolism enzymes, acute phase reactants, and complement components (Fig. 2A and supplementary Table 2). Among those, 15 proteins were represented by clusters of various spots. The main HDL protein, apoA-I, depicted three clusters with different molecular masses (35, 28, and 17 kDa), the 28 kDa form being the most abundant ( $99.7 \pm 0.13\%$ ). ApoA-IV was represented by two clusters, one of 45 kDa and another of 26 kDa, representing  $80.5 \pm 3.2\%$  and  $19.5 \pm 3.2\%$  of the total apoA-IV intensity, respectively. FH patients



**Fig. 1.** Study workflow. The present study comprised three phases: (I) the discovery phase, in which a differential proteomic approach was used as previously described to identify differences in the HDL proteomic signature in 10 families from the SAFEHEART with three individuals per family [two members with genetic and clinical diagnosis of heterozygous FH (N = 19) and a control relative without the FH-mutation (non-FH; N = 11) matched for age, gender and demographics (N = 30; Table 1)]; (II) the validation phase, where differential selected proteins were validated by ELISA in the discovery cohort (N = 30) and in a validation cohort of 73 FH patients (with nonrelative relationship between them or with those included in the discovery cohort) who suffered an ischemic event, including acute myocardial infarction [both fatal (exitus patients; N = 32) and nonfatal (no exitus patients; N = 41)] and unstable angina, an average of  $3.5 \pm 0.3$  years after inclusion in the study (FH-CV; Table 2 shows differences between no CV and CV patients, and Table 3 shows differences within FH-CV patients between no exitus and exitus patients); and (III) the characterization phase, where the distribution profile of differential proteins was specifically analyzed in isolated HDL2 and HDL3 subfractions in a subset of randomly selected FH-CV patients from the validation phase who suffered an ischemic event, both fatal (exitus; N = 5) and nonfatal (no exitus; N = 5) after blood sampling and inclusion in the study (supplementary Table 1). Patients included in the validation and characterization phases were also recruited from the SAFEHEART cohort.

showed a significant decrease in the apoA-IV cluster of 26 kDa ( $P = 0.013$ ; Fig. 2B) when compared with their non-FH relatives.

ApoL1 also showed two clusters with different molecular masses, one of  $\sim 45$  kDa and the other of 39 kDa, representing  $77.6 \pm 3.2\%$  and  $22.5 \pm 3.2\%$  of total apoL1, respectively. The distribution of apoL1 clusters showed a significant change in FH patients who depicted a 15.7% decrease of the 45 kDa form ( $P = 0.015$ ) and the subsequent increase of the 39 kDa form ( $P = 0.015$ ) when compared with their non-FH relatives (Fig. 2C).

The presence of the different apoA-IV and apoL1 clusters was validated by Western blot analysis (Fig. 2D).

#### Validation of apoL1 and apoA-IV changes in serum samples in FH patients

In order to validate whether the observed changes in the proteomic profile of apoL1 and apoA-IV in HDL led to changes in total levels of these apolipoproteins, serum concentrations of apoL1 and apoA-IV were determined by commercially available ELISAs in the same group of patients used in the discovery proteomic analysis (non-FH, N = 11; and FH, N = 19). Additionally, a group of FH patients that suffered an ischemic cardiac event 3.5 years after their inclusion in the study (N = 73; Tables 2 and 3) was also

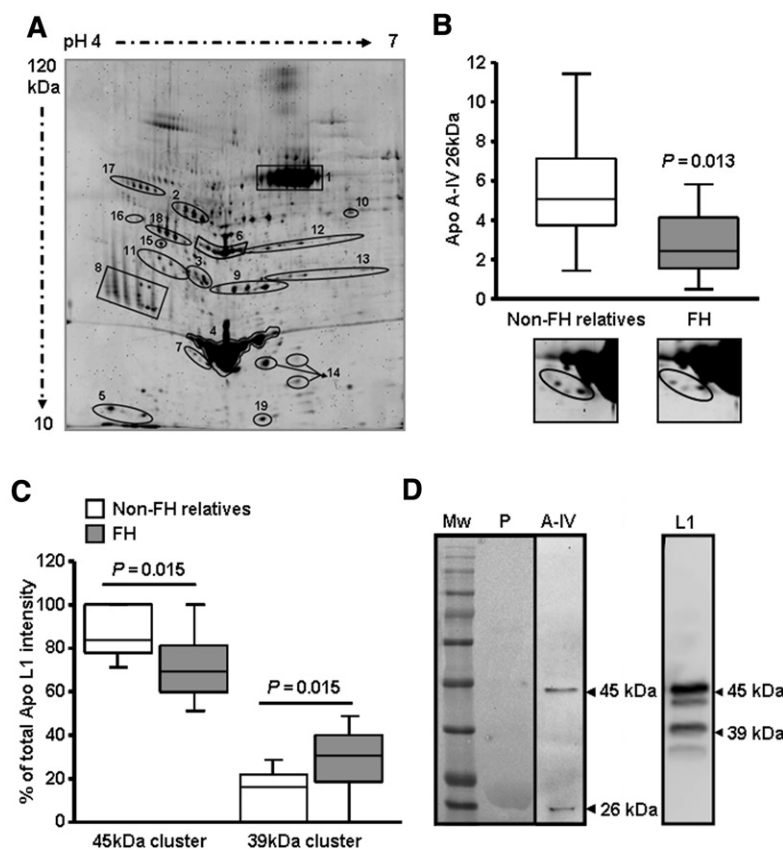
analyzed to find out the potential implications of the observed changes in patients' outcome.

The intra-assay variability of apoL1 ELISA was of  $3.5 \pm 0.3\%$ , and the interassay variability assessed in 16 samples measured in three independent assays was of  $6.2 \pm 0.9\%$ .

ApoA-IV levels assessment was performed in a single assay with  $5.4 \pm 0.6\%$  of intra-assay variability.

ApoL1 serum levels were normally distributed in the analyzed population ( $P = 0.179$  Kolmogorov-Smirnov test). Moreover, apoL1 levels were not significantly associated with age, gender, BMI, presence of diabetes, tobacco smoking or hypertension, or LDL-C, HDL-C, Glc, or Lp(a) levels (supplementary Table 3).

FH patients showed a significant decrease in total apoL1 serum levels when compared with non-FH relatives ( $P = 0.002$ ; Fig. 3A). However, no differences were detected between FH patients and non-FH relatives in total apoA-IV serum levels (FH,  $730.8 \pm 37.1$  vs. non-FH,  $733.0 \pm 47.4$   $\mu\text{g/ml}$ ;  $P = 0.974$ ) highlighting that the observed changes in the 2DE analysis only referred to a specific form of apoA-IV but not to total apoA-IV levels. FH patients also showed a significant decrease in total apoA-I serum levels when compared with non-FH relatives ( $P = 0.012$ ; Fig. 3B).



**Fig. 2.** Identified HDL proteomic changes in FH patients. A: Representative 2DE image of the HDL profile from a non-FH subject showing the main protein clusters identified in the proteomic analysis. Mainly identified proteins were albumin (1),  $\alpha$ -1-antitrypsin (2), apoA-I 35 kDa cluster (3), apoA-I 28 kDa cluster (4), apoA-I 17 kDa cluster (5), apoA-IV 45 kDa cluster (6), apoA-IV 26 kDa cluster (7), apoD (8), apoE (9), apoH (10), apoJ (11), apoL1 45 kDa cluster (12), apoL1 39 kDa cluster (13), apoM (14), complement component 3 (15), complement component 4a (16), LCAT (17), PON1 (18), and transthyretin (TTR; 19). B: Box plot showing changes in the intensity of the 26 kDa apoA-IV cluster detected by 2DE analysis. C: Box plot showing changes in apoL1 distribution detected by 2DE analysis in FH patients ( $N = 19$ ) when compared with non-FH relatives ( $N = 11$ ; Student's  $t$ -test). D: Representative image of the detection of apoA-IV and apoL1 forms in Western blot analysis. Mw, molecular weight markers; P, Ponceau staining.

Interestingly, within the FH population, patients who had an ischemic cardiac event an average of 3.5 years after inclusion (within a follow-up of 10 years) showed significantly decreased apoL1 serum levels before the event when compared with those FH patients who did not have any event ( $P = 0.004$ ; Fig. 3C). On the contrary, those patients did not show significant differences in total apoA-I serum levels ( $P = 0.867$ ; Fig. 3D). These results suggest that reduced apoL1 levels are associated with lesion complication and event presentation (Fig. 3E), whereas reduced apoA-I levels are associated with FH but are independent of event presentation (Fig. 3F).

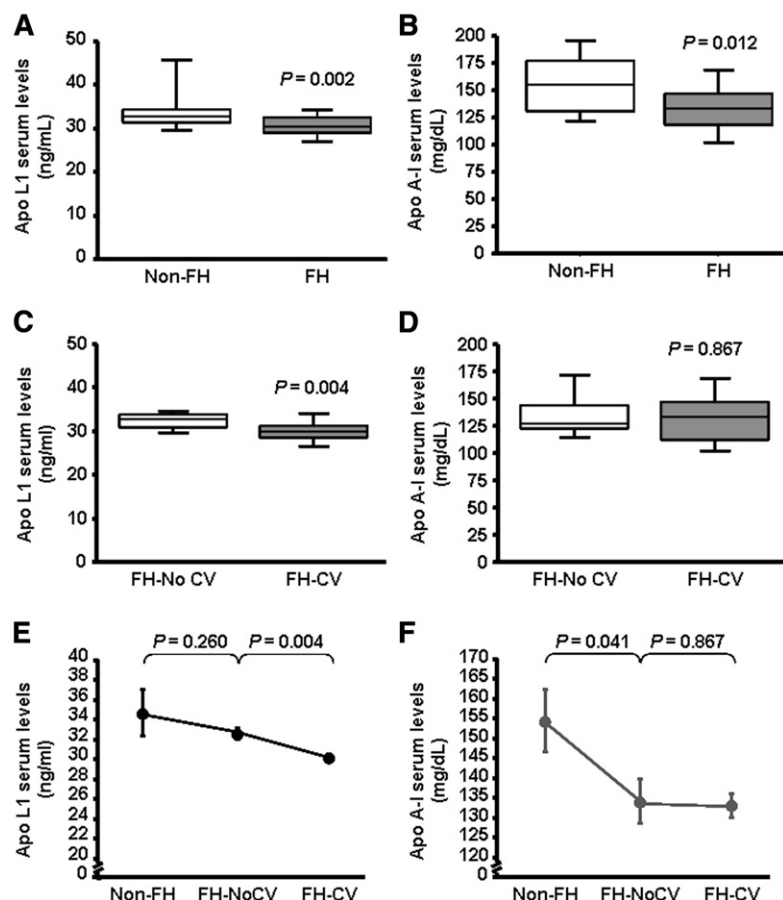
C-statistics analysis revealed that apoL1 serum levels measured by ELISA had a predictive value for the presentation of an ischemic cardiac event in FH patients with an AUC of 0.759 [95% confidence interval (CI), 0.634–0.884;  $P = 0.001$ ] and a cutoff value of 31 ng/ml with 77% of sensitivity and 75% specificity (Fig. 4A). The only significant difference found between FH patients above and below the 31 ng/ml apoL1 cutoff value was in the frequency of null and defective LDLR mutation (supplementary Table 4). However, no significant differences were found in apoL1 levels between patients with null and defective mutation (supplementary Fig. 1).

The predictive power, in the C-statistics analysis, of a conventional cardiovascular risk factor model [CVRF1 (including age; LDL-C, HDL-C, and Glc levels; BMI; and systolic blood pressure); AUC, 0.823; 95% CI, 0.708–0.939;  $P < 0.0001$ ] was increased when apoL1 levels were included in the model (AUC, 0.901; 95% CI, 0.820–0.982;  $P <$

0.0001; Fig. 4B). When the same analysis was performed including gender, LDLR mutation, smoking, and Lp(a) levels in the model (CVRF2; AUC, 0.927; 95% CI, 0.854–0.999;  $P < 0.0001$ ), apoL1 levels still showed an additive predictive power achieving the maximal predictive value (AUC, 0.948; 95% CI, 0.892–1.000;  $P < 0.0001$ ; Fig. 4C). The inclusion of apoL1 improved the predictive value of both models, CVRF1 and CVRF2, although the observed differences in the AUC did not reach significance ( $P = 0.326$  and  $P = 0.687$  for models CVRF1 and CVRF2, respectively). The addition of apoL1 in the CVRF2 model afforded the maximal sensitivity (supplementary Table 5).

### HDL protein composition changes and survival analysis

Cox proportional hazards analysis (stepwise selection of variables) was performed to assess the potential impact of age, lipid-related variables (including total cholesterol, LDL-C, HDL-C, TG, apoA-I, apoB, and apoL1 levels), and corneal arcus in the survival rate in FH patients treated to guidelines after suffering an ischemic cardiac event 3.5 years later ( $N = 73$ ; Table 3). The presence/absence of corneal arcus was included in the model as it has been previously described that this typical symptom of FH is associated with a higher CVD risk (52), and our discovery proteomic analysis revealed that FH patients with corneal arcus showed significantly lower total apoA-IV, apoL1, and PON1 contents, as well as a decreasing trend in LCAT levels (supplementary Table 6), when compared with those without. There were no significant differences in the duration of statin treatment between patients with corneal



**Fig. 3.** Serum levels of differential proteins. Box plots showing apoL1 measured by ELISA and apoA-I serum levels determined by turbidimetry in FH patients (N = 92) and non-FH subjects (N = 11; Student's *t*-test; A and B, respectively), and in FH patients who suffered an ischemic cardiac event 3.5 years after blood collection and inclusion (FH-CV; N = 73) compared with those who did not have an event (FH-no CV; N = 19; Student's *t*-test; C and D, respectively). Plots showing changes in apoL1 (E) and apoA-I (F) in the three analyzed groups (non-FH, FH-no CV, and FH-CV).

arcus and those without this typical sign of FH ( $P = 0.324$ ). As shown in **Fig. 5**, age appeared as the strongest determinant of mortality ( $P = 0.003$ ; HR, 1.039; 95% CI, 1.013–1.065). Interestingly, apoL1 was the second variable associated with the survival rate in FH patients ( $P = 0.022$ ; HR, 0.836; 95% CI, 0.717–0.974) affording a 16% reduction in the risk of mortality due to a fatal cardiac event in FH patients. ApoA-I was the third variable associated with the survival rate in FH patients ( $P = 0.044$ ; HR, 0.983; 95% CI, 0.966–0.999), but only affording a 2% reduction in the risk of mortality because of a fatal cardiac event in FH patients. None of the other analyzed variables had a significant impact on the survival rate. When smoking, BMI, and Glc and Lp(a) levels were included in the analysis, only age appeared as a determinant of mortality ( $P = 0.04$ ; HR, 1.042; 95% CI, 1.001–1.084). ApoL1, although not reaching significance, was the second variable more closely associated with the survival rate in FH patients ( $P = 0.066$ ; HR, 0.338; 95% CI, 0.169–0.676) after suffering an ischemic event.

Changes in apoL1 were normalized to HDL-C levels in order to investigate their prognostic power after the presentation of an ischemic event. This analysis showed that those patients who died because of a fatal ischemic event 3.5 years after their inclusion in the study showed a significantly lower apoL1/HDL-C ratio than those who survived ( $P = 0.027$ ; **Fig. 6A**). When the same analysis was performed with apoA-I levels, no differences were found in apoA-I/HDL-C ratio ( $P = 0.428$ ; **Fig. 6B**).

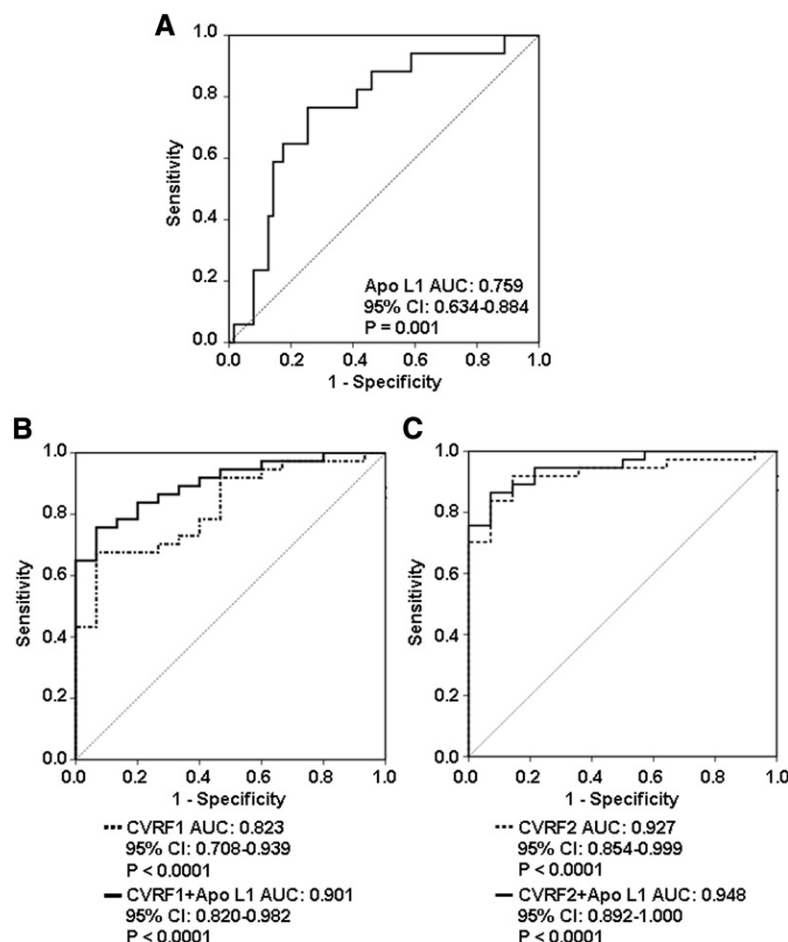
Kaplan-Meier survival analysis revealed that a cutoff value of apoL1/HDL-C ratio  $<0.7$  (median value of FH-CV patients) was associated with a significant decrease in the survival rate after an acute ischemic event ( $P = 0.038$  log rank; **Fig. 6C**) pointing to a potential impact of apoL1 content as an indicator of HDL protective properties. In line with these results, a cutoff value of apoL1 levels  $<30$  ng/ml (median value of FH-CV patients) was associated with a significant decrease in the survival rate after an acute ischemic event ( $P = 0.05$  log rank; **Fig. 6D**). On the contrary, no differences were observed in the survival rate of FH patients after suffering an acute ischemic event between patients carrying the null LDLR mutation and those carrying the defective mutation ( $P = 0.68$  log rank; **Fig. 6E**). As expected, older patients ( $>57$  years old; median value of the FH-CV patients) showed a significantly lower survival rate than younger patients ( $<57$  years old;  $P = 0.039$  log rank; **Fig. 6F**).

#### Differential proteomic signature of HDL subclasses in FH patients: implication of outcomes

To further prove the implication of apoL1 changes in the HDL protective profile and in the survival rate after an acute ischemic event, the differential distribution of the identified proteins in the two most important HDL subclasses (HDL3 and HDL2) was analyzed by 2DE (**Fig. 7A**).

ApoL1, LCAT, and PON1 were predominantly transported by HDL3. Western blot validation in both non-FH and FH patients confirmed that apoL1, LCAT, and PON1





**Fig. 4.** ApoL1 serum levels and ischemic cardiac event presentation. ROC curve showing the predictive value for the presentation of an ischemic cardiac event of total apoL1 serum levels (A); improvement of the predictive value of two cardiovascular risk factor models when apoL1 serum levels are included in the model: CVRF1 (B) (including age; LDL-C, HDL-C, and Glc levels; BMI; and systolic blood pressure) and CVRF2 (C) [including age; gender; LDL-C, HDL-C, and Glc levels; BMI; systolic blood pressure; type of LDLR mutation; smoking; and Lp(a) levels].

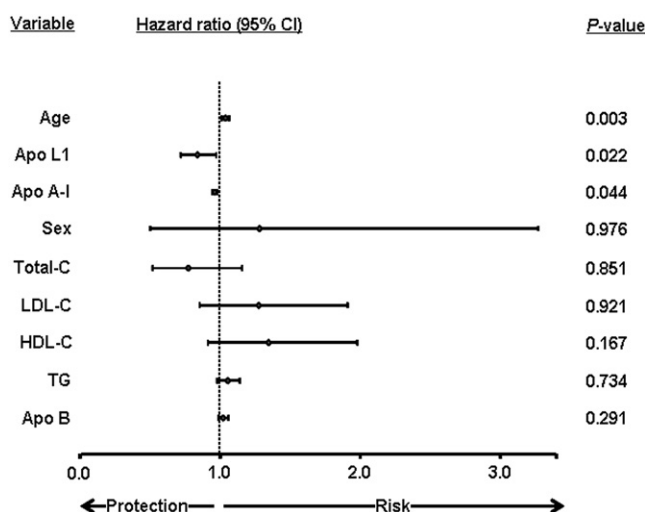
levels were significantly higher in HDL3 than in HDL2 (Fig. 7B). On the contrary, no differences were detected in apoA-IV and apoA-I levels (data not shown).

The proteomic profile of HDL3 and HDL2 of patients that died (exitus; N = 5) and patients that survived after the cardiac event (no exitus; N = 5) was compared (Fig. 8A;

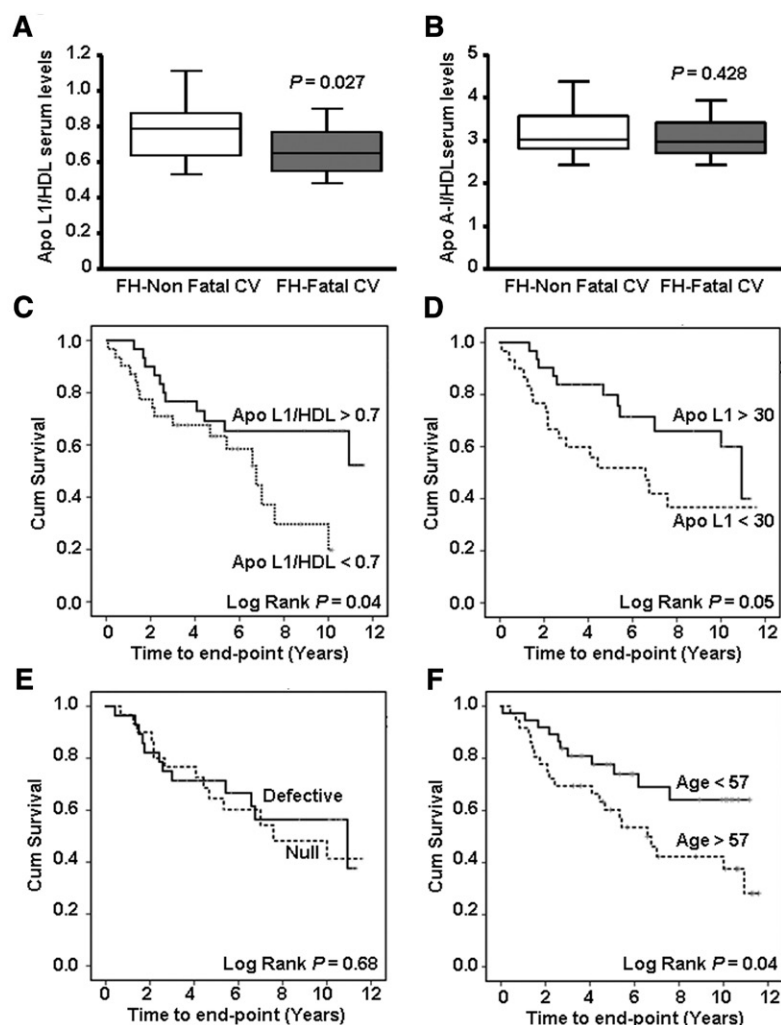
supplementary Table 1). The total intensity of apoL1 showed a significant decrease in the HDL3 subfraction in exitus patients when compared with no exitus patients ( $P = 0.003$ ; Fig. 8B). Similarly, total LCAT intensity was significantly decreased in HDL3 of exitus patients ( $P = 0.009$ ; Fig. 8C). There were no significant changes in PON1 intensity between exitus and no exitus patients (data not shown). All the observed changes were specifically related to the HDL3 subfraction, the most atheroprotective, but not to the HDL2 subclass. Despite the small sample size, C-statistics analysis revealed that apoL1 content in HDL3 showed the highest predictive power for the presentation of a fatal cardiac event in FH patients with an AUC of 1.000 (95% CI, 1.000–1.000;  $P = 0.009$ ) and a 100% sensitivity and specificity. On the contrary, LCAT content in HDL3 did not show a significant predictive value for the presentation of a fatal cardiac event in FH patients (supplementary Table 7).

## DISCUSSION

The use of statins has markedly decreased coronary and total mortality and morbidity in FH patients (53); however, there is a need for decreasing their still high risk for premature CAD. Previous studies have reported changes in HDL-C and apoA-I levels in patients with hypercholesterolemia and high risk of CVD, reflecting perturbed lipid



**Fig. 5.** Impact of differential proteins in survival. Cox regression analysis showing the hazard ratios (HRs) and 95% CI of the association of age, sex, and lipid-related variables, with the survival rate in FH patients (N = 73).



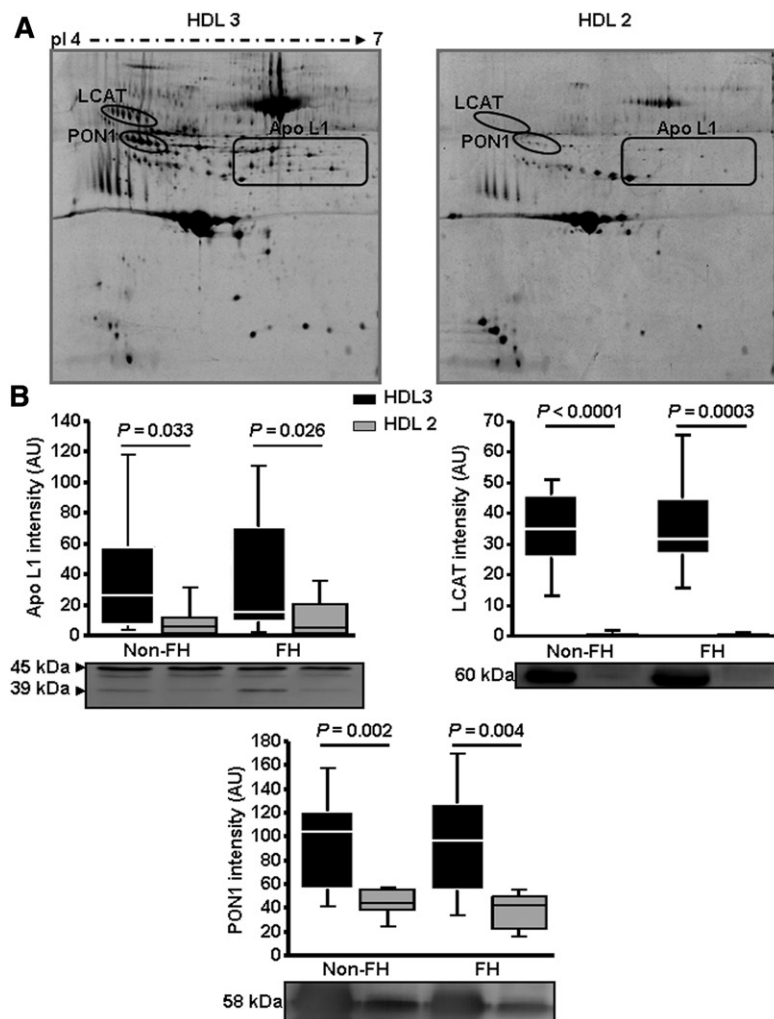
**Fig. 6.** Impact of differential proteins in prognosis of FH patients after suffering an ischemic event. Box plots showing the ratios between apoL1 and HDL-C levels (apoL1/HDL-C) (A) and apoA-I and HDL-C levels (apoA-I/HDL-C) (B) in FH patients who died because of cardiac event at follow-up (FH-fatal CV; N = 32) compared with those who did not die (FH-nonfatal CV; N = 41; Student's *t*-test). C: Kaplan-Meier curves showing the significant differences in the survival rate of FH patients after an ischemic cardiac event in relation to the apoL1/HDL-C ratio (log rank analysis  $P = 0.038$ ) (C) and the levels of apoL1 (log rank analysis  $P = 0.05$ ) (D). E: Kaplan-Meier curves showing the lack of differences observed in the survival rate of FH patients after suffering an acute ischemic event between patients carrying the null LDLR mutation when compared with those carrying a defective mutation (log rank analysis  $P = 0.68$ ). F: Kaplan-Meier curves showing the effect of age in the survival rate of FH patients after suffering an acute ischemic event (log rank analysis  $P = 0.039$ ).

metabolism with compositional anomalies in HDL particles (54, 55). This differential composition of HDL micelles in FH patients was attributed to changes in phospholipid and TG levels (56). Until now, HDL protein composition in FH patients had not been analyzed. In this study, by applying differential proteomic profiling technologies we have demonstrated for the first time significant changes in the HDL proteomic profile of FH patients with potential implications in the presentation of adverse coronary events.

Specifically, patients with genetic diagnosis of FH showed a significant decrease in the 26 kDa cluster of apoA-IV when compared with their non-FH relatives. ApoA-IV is a glycoprotein produced by epithelial cells in the small intestine (57), and besides its known implication in reverse cholesterol transport, apoA-IV has also been shown to enhance the formation of small HDL particles by activating LCAT (58, 59). Previous studies have suggested a protective effect of apoA-IV against atherosclerosis. Indeed, human apoA-IV overexpression in apoE-knockout mice has been shown to confer considerable protection against atherosclerosis development (60). Moreover, an inverse association between plasma apoA-IV levels and CAD has also been shown (61). Our proteomic study demonstrates the existence of several HDL-associated apoA-IV forms that we have validated by Western blot analysis, and that FH

patients show an important decrease in the apoA-IV low molecular mass cluster. Interestingly, this specific apoA-IV 26 kDa cluster is positively correlated with apoA-I levels ( $R = 0.458$ ,  $P = 0.013$ ), pointing to its protective role in CVD. Therefore, this decrease in apoA-IV could induce changes in the HDL properties as apoA-IV has been shown to decrease the secretion of proinflammatory cytokines (62). Despite the observed differences in the HDL proteome, in the apoA-IV 26 kDa cluster, we did not detect significant changes when apoA-IV total levels were measured by a commercial ELISA. This is probably because the polyclonal antibody of the kit is recognizing both apoA-IV forms (45 and 26 kDa) and thus the observed differences in the 26 kDa form in the 2DE analysis are masked by the lack of differences in the form of 45 kDa, which is more abundant.

Here we have demonstrated for the first time that there is a significant change in the apoL1 proteomic distribution profile in HDL of FH patients. ApoL1 is a plasma apolipoprotein that belongs to a family of programmed cell death genes whose proteins can initiate host apoptosis (63) or autophagic cell death (64). Specifically, we have identified apoL1 as two clusters with different molecular masses and found that the 45 kDa cluster shows a significant decrease in FH patients when compared with their

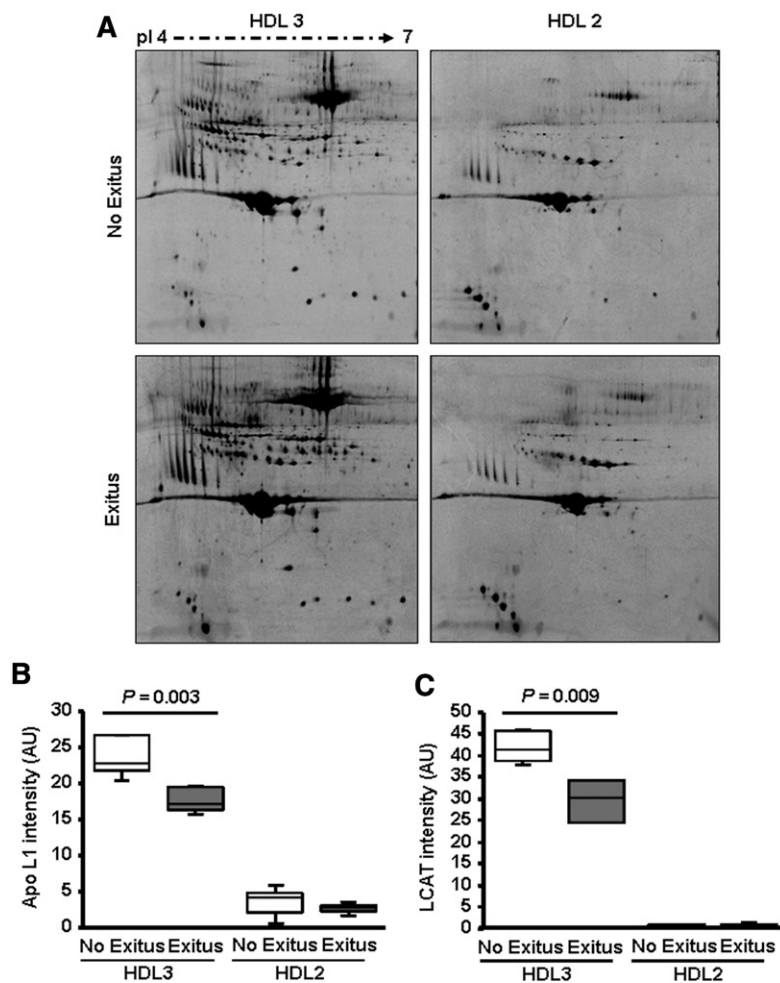


**Fig. 7.** Differential protein distribution in HDL sub-classes. A: Representative images of the HDL3 and HDL2 proteomes in FH patients obtained by 2DE in a pI range between 4 and 7 (N = 10). ApoL1, LCAT, and PON1 clusters are marked. B: Box plots showing the increased intensity of apoL1, LCAT, and PON1 levels, measured by Western blot, in the HDL3 subfraction (Student's *t*-test). Protein load was normalized using total protein fluorescent signal.

control relatives, while the 39 kDa cluster is significantly increased in FH patients. Interestingly, this low molecular mass apoL1 cluster agrees with the previously described apoL1 truncated form that has been suggested as the proteolytically activated form of apoL1 (21), as happens with other apolipoproteins (27, 28). Previous studies have reported changes in apoL levels in the presence of risk factors such as hyperlipidemia and hyperglycemia highlighting the implication of apoL in lipid metabolism (65). However, its potential implication in CVD progression and clinical event presentation has not yet been elucidated. Interestingly, the ELISA analysis (using a commercial method recognizing the amino acid sequence from residue 196 to 357) has revealed a significant decrease in apoL1 serum levels in FH patients. This decrease in apoL1 circulating levels is accompanied by a decrease in apoA-I levels pointing to a coordinated change in HDL-related apolipoproteins that might have an impact on HDL functionality. Indeed, we have found significantly decreased apoL1 levels in FH patients previous to the presentation of an acute ischemic event, showing a predictive value of the levels of this apolipoprotein in prognosis. Importantly, apoL1 improved the predictive value for the presentation of acute ischemic events in FH patients over conventional

risk factors and other relevant parameters such as the type of LDLR mutation, highlighting that role of apoL1 is independent of the effect of other markers. Furthermore, apoL1 levels, together with age and apoA-I levels, are significantly associated with the survival rate of FH patients underscoring a potential protective role of this apolipoprotein. Specifically, apoL1 levels normalized by HDL-C levels appear as a potential determinant of patients' outcome after the presentation of an ischemic cardiac event. All these results point to a potential shift in HDL properties associated with the decrease in apoL1 content that might directly impact prognosis. Similarly, our proteomic study has revealed that FH patients with corneal arcus, a clinical symptom of FH, showed lower levels of apoA-IV, apoL1, PON1, and LCAT suggesting an association between lower levels of those proteins and a worse prognosis in FH individuals, as the presence of corneal arcus has been associated with a higher risk of CVD (52).

In order to find out the potential implication of the observed changes in the HDL proteome for clinical event presentation, the proteomes of the HDL3 and HDL2 fractions were analyzed in a subgroup of FH patients that were to suffer an ischemic cardiac event within 3.5 years. The specific analysis of HDL3 and HDL2 subproteomes revealed



**Fig. 8.** Differential protein distribution in HDL subclasses and prognosis. **A:** Representative 2DE images of the HDL3 and HDL2 proteomes in FH patients who suffered a fatal (exitus;  $N = 5$ ) and nonfatal (no exitus;  $N = 5$ ) ischemic cardiac event 3.5 years after blood collection and inclusion in the study. Box plots showing apoL1 (**B**) and LCAT (**C**) distribution in HDL 3 and HDL 2 subfractions in FH patients who suffered a fatal and nonfatal cardiac event (Student's  $t$ -test).

significant differences in their proteomic profile (as expected), with the most relevant changes detected in apoL1, LCAT, and PON1 contents, highlighting their relevance in the differential atheroprotective properties of HDL3. In fact, several previous studies have shown that small dense HDL subspecies (i.e., HDL3 subclass), besides their role in reverse cholesterol transport, show additional properties, such as antioxidant, anti-inflammatory, cytoprotective, anticoagulant, and anti-infectious properties, not exhibited by large light HDL (18). This increased value of HDL3 in atheroprotection has been recently supported by several epidemiological studies that have shown an inverse association of HDL3 and not HDL2 with disease progression and clinical event presentation (66, 67). Interestingly, both enzymes, LCAT and PON1, have been shown to inhibit LDL oxidation in the absence of HDL and to enhance the antioxidant activity of HDL (68). Moreover, the coordinated distribution of apoL1 with LCAT and PON1 raises the possibility of a potential protective role of apoL1. Interestingly, the proteomic analysis of HDL3 and HDL2 subclasses has revealed that patients who died because of an adverse cardiac event showed lower apoL1 and LCAT contents in HDL3 than those FH individuals that survived after having a cardiac event. In fact, it has been previously shown that antioxidant treatment induces an

increase in apoL1 in plasma samples of CAD patients with low HDL (33) suggesting a potential implication of apoL1 in the differential antioxidant abilities of HDL subclasses. Indeed, the abundance of apoL1 in HDL3 particles strongly correlates with the capacity of HDL to attenuate LDL oxidation (22). In addition, the apoL1 trypanosomic activity has been described to be responsible, at least in part, for the anti-infectious properties of HDL3 that are not present in HDL2 (18). Furthermore, a possible role for LCAT in the generation of apoL-containing HDL particles has been suggested (65). Therefore, the observed changes in the HDL3 subfraction could lead to the presence of highly modified HDL3 micelles contributing to the previously described lower atheroprotective properties of HDL3 in FH patients (56). The presence of these modified HDL3 micelles may explain, at least in part, the high prevalence of adverse CAD in FH patients (69). In line with our results, it has been recently shown that low HDL3-C, but not HDL2-C levels, are associated with an increased risk for long-term clinical events in secondary prevention highlighting the differential impact of distinct HDL subclasses in prognosis (66). Moreover, these changes in the HDL3 proteomic profile could underscore the already reported differential susceptibility across FH patients to develop major ischemic events (69).



## Study limitations

As a limitation of this study, we may consider the low sample size. However, this is a proteomic proof of concept study that looks for specific characteristics of protein processing associated with the HDL proteome of FH patients and not yet a biomarker qualification analysis. In addition, the population used is highly homogeneous, and the observed differences are highly significant despite being a small population. Furthermore, patients were followed up for 10 years giving the possibility to analyze the potential impact of the observed changes on the prognosis of the patients. Moreover, the apoL1 observed changes in the proteomic study have been validated by ELISA giving robustness to the obtained results. Another element that could limit the present study is the use of ultracentrifugation methods for HDL isolation that may have removed the more weakly associated HDL proteins; however, as samples of different groups were processed at the same time and under the same conditions, the loss of weakly associated proteins was proportional in all the groups ensuring reliable results. Besides, the aim of the present study was not to perform an in-depth analysis of the entire HDL proteome, a study that has been previously performed in several high-throughput studies (70), but rather to analyze for the first time the main changes in the HDL proteome associated with the presence of hypercholesterolemia that could afford additional cardiovascular risk to those patients independently of the type of LDLR mutation. In this context, despite that the use of a high-throughput LC/MS methodology would have added complementary information, our results show for the first time a potential implication of this yet largely unknown apolipoprotein, apoL1, in the setting of FH.

Finally, the lack of functional analysis of HDL could be considered a limitation of the present study. However, changes in HDL functionality have already been reported in FH patients (54, 55). On the contrary, the novelty of our study is that we have analyzed the potential association between HDL proteomic changes and cardiovascular event presentation and mortality in FH patients.

## CONCLUSIONS

In summary, by applying differential proteomic profiling approaches we have described for the first time a coordinated change in the profile of the HDL proteome of FH patients together with a decrease in total serum levels of apoL1. Our results underscore the importance of apoL1 as a predictor of CVD presentation and mortality in FH patients independently of the type of LDLR mutation. Furthermore, we have found a differential contribution of HDL3 to the total pool of apoL1 together with significant changes in a relevant enzyme involved in lipid metabolism, LCAT. Those changes could induce modifications in HDL atheroprotective properties, not only leading to a higher cardiovascular risk but also contributing to poorer prognosis after an event presentation.

These results warrant validation in a larger population of patients with cardiovascular risk.

The authors thank María Dolores Fernández and Ona Catot for technical assistance. The authors are indebted to the Spanish Familial Hypercholesterolemia Foundation for the recruitment of participants and to patients for their valuable contribution.

## REFERENCES

1. Goldstein, J. L., H. H. Hobbs, and M. S. Brown. 2001. The metabolic and molecular basis of inherited disease. *In* Familial hypercholesterolemia. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 2863–2913.
2. Wierzbicki, A. S., S. E. Humphries, R. Minhas; Guideline Development Group. 2008. Familial hypercholesterolaemia: summary of NICE guidance. *Br. Med. J.* **337**: a1095.
3. Jansen, A. C., E. S. van Aalst-Cohen, M. W. Tanck, M. D. Trip, P. J. Lansberg, A. H. Liem, H. W. van Lennep, E. J. Sijbrands, and J. J. Kastelein. 2004. The contribution of classical risk factors to cardiovascular disease in familial hypercholesterolaemia: data in 2400 patients. *J. Intern. Med.* **256**: 482–490.
4. Besseling, J., I. Kindt, M. Hof, J. J. Kastelein, B. A. Hutten, and G. K. Hovingh. 2014. Severe heterozygous familial hypercholesterolemia and risk for cardiovascular disease: a study of a cohort of 14,000 mutation carriers. *Atherosclerosis*. **233**: 219–223.
5. Alonso, R., E. Andres, N. Mata, F. Fuentes-Jimenez, L. Badimon, J. Lopez-Miranda, T. Padro, O. Muniz, J. L. Diaz-Diaz, M. Mauri, et al. 2014. Lipoprotein(a) levels in familial hypercholesterolemia: an important predictor of cardiovascular disease independent of the type of LDL receptor mutation. *J. Am. Coll. Cardiol.* **63**: 1982–1989.
6. Wilson, P. W., R. D. Abbott, and W. P. Castelli. 1988. High density lipoprotein cholesterol and mortality. The Framingham Heart Study. *Arteriosclerosis*. **8**: 737–741.
7. Badimon, J. J., L. Badimon, A. Galvez, R. Dische, and V. Fuster. 1989. High density lipoprotein plasma fractions inhibit aortic fatty streaks in cholesterol-fed rabbits. *Lab. Invest.* **60**: 455–461.
8. Badimon, J. J., L. Badimon, and V. Fuster. 1990. Regression of atherosclerotic lesions by high density lipoprotein plasma fraction in the cholesterol-fed rabbit. *J. Clin. Invest.* **85**: 1234–1241.
9. Ibanez, B., C. Giannarelli, G. Cimmino, C. G. Santos-Gallego, M. Alique, A. Pinero, G. Vilahur, V. Fuster, L. Badimon, and J. J. Badimon. 2012. Recombinant HDL(Milano) exerts greater anti-inflammatory and plaque stabilizing properties than HDL(wild-type). *Atherosclerosis*. **220**: 72–77.
10. Badimón, J. J., C. G. Santos-Gallego, and L. Badimón. 2010. Importance of HDL cholesterol in atherothrombosis: how did we get here? Where are we going? [Article in Spanish] *Rev. Esp. Cardiol.* **63** (Suppl. 2): 20–35.
11. Assmann, G., H. Schulte, A. von Eckardstein, and Y. Huang. 1996. High-density lipoprotein cholesterol as a predictor of coronary heart disease risk. The PROCAM experience and pathophysiological implications for reverse cholesterol transport. *Atherosclerosis*. **124** (Suppl.): S11–S20.
12. Goldbourt, U., S. Yaari, and J. H. Medalie. 1997. Isolated low HDL cholesterol as a risk factor for coronary heart disease mortality. A 21-year follow-up of 8000 men. *Arterioscler. Thromb. Vasc. Biol.* **17**: 107–113.
13. Olsson, A. G., G. G. Schwartz, M. Szarek, W. J. Sasiela, M. D. Ezekowitz, P. Ganz, M. F. Oliver, D. Waters, and A. Zeiher. 2005. High-density lipoprotein, but not low-density lipoprotein cholesterol levels influence short-term prognosis after acute coronary syndrome: results from the MIRACL trial. *Eur. Heart J.* **26**: 890–896.
14. Badimon, L., and G. Vilahur. 2012. LDL-cholesterol versus HDL-cholesterol in the atherosclerotic plaque: inflammatory resolution versus thrombotic chaos. *Ann. N. Y. Acad. Sci.* **1254**: 18–32.
15. Lüscher, T. F., U. Landmesser, A. von Eckardstein, and A. M. Fogelman. 2014. High-density lipoprotein: vascular protective effects, dysfunction, and potential as therapeutic target. *Circ. Res.* **114**: 171–182.
16. Riwayto, M., L. Rohrer, A. von Eckardstein, and U. Landmesser. 2015. Dysfunctional HDL: from structure-function-relationships to biomarkers. *Handb. Exp. Pharmacol.* **224**: 337–366.
17. Landmesser, U., A. von Eckardstein, J. Kastelein, J. Deanfield, and T. F. Luscher. 2012. Increasing high-density lipoprotein cholesterol by cholesteryl ester transfer protein-inhibition: a rocky road and

- lessons learned? The early demise of the dal-HEART programme. *Eur. Heart J.* **33**: 1712–1715.
18. Camont, L., M. J. Chapman, and A. Kontush. 2011. Biological activities of HDL subpopulations and their relevance to cardiovascular disease. *Trends Mol. Med.* **17**: 594–603.
  19. Bellanger, N., A. Orsoni, Z. Julia, N. Fournier, E. Frisdal, E. Duchene, E. Bruckert, A. Carrie, D. Bonnefont-Rousselot, J. Pirault, et al. 2011. Atheroprotective reverse cholesterol transport pathway is defective in familial hypercholesterolemia. *Arterioscler. Thromb. Vasc. Biol.* **31**: 1675–1681.
  20. Cubedo, J., T. Padro, R. Alonso, J. Cinca, P. Mata, and L. Badimon. 2012. Differential proteomic distribution of TTR (pre-albumin) forms in serum and HDL of patients with high cardiovascular risk. *Atherosclerosis*. **222**: 263–269.
  21. Duchateau, P. N., C. R. Pullinger, R. E. Orellana, S. T. Kunitake, J. Naya-Vigne, P. M. O'Connor, M. J. Malloy, and J. P. Kane. 1997. Apolipoprotein L, a new human high density lipoprotein apolipoprotein expressed by the pancreas. Identification, cloning, characterization, and plasma distribution of apolipoprotein L. *J. Biol. Chem.* **272**: 25576–25582.
  22. Davidson, W. S., R. A. Silva, S. Chantepie, W. R. Lagor, M. J. Chapman, and A. Kontush. 2009. Proteomic analysis of defined HDL subpopulations reveals particle-specific protein clusters: relevance to antioxidative function. *Arterioscler. Thromb. Vasc. Biol.* **29**: 870–876.
  23. Smith, E. E., and H. S. Malik. 2009. The apolipoprotein L family of programmed cell death and immunity genes rapidly evolved in primates at discrete sites of host-pathogen interactions. *Genome Res.* **19**: 850–858.
  24. Duchateau, P. N., C. R. Pullinger, M. H. Cho, C. Eng, and J. P. Kane. 2001. Apolipoprotein L gene family: tissue-specific expression, splicing, promoter regions; discovery of a new gene. *J. Lipid Res.* **42**: 620–630.
  25. Monajemi, H., R. D. Fontijn, H. Pannekoek, and A. J. Horrevoets. 2002. The apolipoprotein L gene cluster has emerged recently in evolution and is expressed in human vascular tissue. *Genomics*. **79**: 539–546.
  26. Vanhellebeke, B., and E. Pays. 2006. The function of apolipoproteins L. *Cell. Mol. Life Sci.* **63**: 1937–1944.
  27. Edelstein, C., J. I. Gordon, K. Toscas, H. F. Sims, A. W. Strauss, and A. M. Scanu. 1983. In vitro conversion of proapoprotein A-I to apoprotein A-I. Partial characterization of an extracellular enzyme activity. *J. Biol. Chem.* **258**: 11430–11433.
  28. Bojanovski, D., R. E. Gregg, G. Ghiselli, E. J. Schaefer, J. A. Light, and H. B. Brewer, Jr. 1985. Human apolipoprotein A-I isoprotein metabolism: proapoA-I conversion to mature apoA-I. *J. Lipid Res.* **26**: 185–193.
  29. Ito, K., A. G. Bick, J. Flannick, D. J. Friedman, G. Genovese, M. G. Parfenov, S. R. Depalma, N. Gupta, S. B. Gabriel, H. A. Taylor, Jr., et al. 2014. Increased burden of cardiovascular disease in carriers of APOI genetic variants. *Circ. Res.* **114**: 845–850.
  30. Limou, S., G. W. Nelson, J. B. Kopp, and C. A. Winkler. 2014. APOI kidney risk alleles: population genetics and disease associations. *Adv. Chronic Kidney Dis.* **21**: 426–433.
  31. Freedman, B. I., B. A. Julian, S. O. Pastan, A. K. Israni, D. Schladt, M. D. Gautreaux, V. Hauptfeld, R. A. Bray, H. M. Gebel, A. D. Kirk, et al. 2015. Apolipoprotein L1 gene variants in deceased organ donors are associated with renal allograft failure. *Am. J. Transplant.* **15**: 1615–1622.
  32. Larsen, C. P., and B. I. Freedman. 2015. Apolipoprotein L1-associated nephropathy and the future of renal diagnostics. *J. Am. Soc. Nephrol.* **26**: 1232–1235.
  33. Albert, T. S., P. N. Duchateau, S. S. Deeb, C. R. Pullinger, M. H. Cho, D. C. Heilbron, M. J. Malloy, J. P. Kane, and B. G. Brown. 2005. Apolipoprotein L-I is positively associated with hyperglycemia and plasma triglycerides in CAD patients with low HDL. *J. Lipid Res.* **46**: 469–474.
  34. Farrall, M. 2014. Cardiovascular twist to the rapidly evolving apolipoprotein L1 story. *Circ. Res.* **114**: 746–747.
  35. Cubedo, J., T. Padro, X. Garcia-Moll, X. Pinto, J. Cinca, and L. Badimon. 2011. Proteomic signature of apolipoprotein J in the early phase of new-onset myocardial infarction. *J. Proteome Res.* **10**: 211–220.
  36. Cubedo, J., T. Padro, and L. Badimon. 2014. Glycoproteome of human apolipoprotein A-I: N- and O-glycosylated forms are increased in patients with acute myocardial infarction. *Transl. Res.* **164**: 209–222.
  37. Cubedo, J., T. Padro, M. Garcia-Arguinzonis, G. Vilahur, I. Minambres, J. M. Pou, J. Ybarra, and L. Badimon. 2015. A novel truncated form of apolipoprotein A-I transported by dense LDL is increased in diabetic patients. *J. Lipid Res.* **56**: 1762–1773.
  38. Cubedo, J., T. Padro, E. Pena, R. Aledo, F. Formiga, A. Ferrer, G. Padros, and L. Badimon. 2015. High levels of antifibrinolytic proteins are found in plasma of older octogenarians with cardiovascular disease and cognitive decline. *J. Am. Coll. Cardiol.* **65**: 2667–2669.
  39. Mata, N., R. Alonso, L. Badimon, T. Padro, F. Fuentes, O. Muniz, F. Perez-Jimenez, J. Lopez-Miranda, J. L. Diaz, J. I. Vidal, et al. 2011. Clinical characteristics and evaluation of LDL-cholesterol treatment of the Spanish Familial Hypercholesterolemia Longitudinal Cohort Study (SAFEHEART). *Lipids Health Dis.* **10**: 94.
  40. Perez de Isla, L., R. Alonso, G. F. Watts, N. Mata, A. Saltijeral Cerezo, O. Muniz, F. Fuentes, J. L. Diaz-Diaz, R. de Andres, D. Zambon, et al. 2016. Attainment of LDL-cholesterol treatment goals in patients with familial hypercholesterolemia: 5-year SAFEHEART registry follow-up. *J. Am. Coll. Cardiol.* **67**: 1278–1285.
  41. Tejedor, D., S. Castillo, P. Mozas, E. Jimenez, M. Lopez, M. T. Tejedor, M. Artieda, R. Alonso, P. Mata, L. Simon, et al. 2005. Reliable low-density DNA array based on allele-specific probes for detection of 118 mutations causing familial hypercholesterolemia. *Clin. Chem.* **51**: 1137–1144.
  42. Tejedor, D., S. Castillo, P. Mozas, E. Jimenez, M. Lopez, M. T. Tejedor, M. Artieda, R. Alonso, P. Mata, L. Simon, et al. 2006. Comparison of DNA array platform vs DNA sequencing as genetic diagnosis tools for familial hypercholesterolemia. *Clin. Chem.* **52**: 1971–1972.
  43. Heath, K. E., I. N. Day, and S. E. Humphries. 2000. Universal primer quantitative fluorescent multiplex (UPQFM) PCR: a method to detect major and minor rearrangements of the low density lipoprotein receptor gene. *J. Med. Genet.* **37**: 272–280.
  44. Alonso, R., N. Mata, S. Castillo, F. Fuentes, P. Saenz, O. Muniz, J. Galiana, R. Figueras, J. L. Diaz, P. Gomez-Enterria, et al. 2008. Cardiovascular disease in familial hypercholesterolemia: influence of low-density lipoprotein receptor mutation type and classic risk factors. *Atherosclerosis*. **200**: 315–321.
  45. Allain, C. C., L. S. Poon, C. S. Chan, W. Richmond, and P. C. Fu. 1974. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20**: 470–475.
  46. Fossati, P., and L. Prencipe. 1982. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin. Chem.* **28**: 2077–2080.
  47. Assmann, G., H. Schriewer, G. Schmitz, and E. O. Hagele. 1983. Quantification of high-density-lipoprotein cholesterol by precipitation with phosphotungstic acid/MgCl<sub>2</sub>. *Clin. Chem.* **29**: 2026–2030.
  48. Friedewald, W. T., R. I. Levy, and D. S. Fredrickson. 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.* **18**: 499–502.
  49. Riepponen, P., J. Marniemi, and T. Rautaoja. 1987. Immunoturbidimetric determination of apolipoproteins A-I and B in serum. *Scand. J. Clin. Lab. Invest.* **47**: 739–744.
  50. Hanley, J. A., and B. J. McNeil. 1983. A method of comparing the areas under receiver operating characteristic curves derived from the same cases. *Radiology*. **148**: 839–843.
  51. Ramaola, I., T. Padro, E. Pena, O. Juan-Babot, J. Cubedo, V. Martin-Yuste, M. Sabate, and L. Badimon. 2015. Changes in thrombus composition and profilin-1 release in acute myocardial infarction. *Eur. Heart J.* **36**: 965–975.
  52. Fernandez, A. B., M. J. Keyes, M. Pencina, R. D'Agostino, C. J. O'Donnell, and P. D. Thompson. 2009. Relation of corneal arcus to cardiovascular disease (from the Framingham Heart Study data set). *Am. J. Cardiol.* **103**: 64–66.
  53. Neil, A., J. Cooper, J. Betteridge, N. Capps, I. McDowell, P. Durrington, M. Seed, and S. E. Humphries. 2008. Reductions in all-cause, cancer, and coronary mortality in statin-treated patients with heterozygous familial hypercholesterolemia: a prospective registry study. *Eur. Heart J.* **29**: 2625–2633.
  54. Kajinami, K., H. Mabuchi, J. Koizumi, and R. Takeda. 1992. Serum apolipoproteins in heterozygous familial hypercholesterolemia. *Clin. Chim. Acta.* **211**: 93–99.
  55. Hogue, J. C., B. Lamarche, D. Gaudet, A. J. Tremblay, J. P. Despres, J. Bergeron, C. Gagne, and P. Couture. 2007. Association of heterozygous familial hypercholesterolemia with smaller HDL particle size. *Atherosclerosis*. **190**: 429–435.

56. Balstad, T. R., K. B. Holven, I. O. Ottestad, K. Otterdal, B. Halvorsen, A. M. Myhre, L. Ose, and M. S. Nenseter. 2005. Altered composition of HDL3 in FH subjects causing a HDL subfraction with less atheroprotective function. *Clin. Chim. Acta.* **359**: 171–178.
57. Green, P. H., R. M. Glickman, J. W. Riley, and E. Quinet. 1980. Human apolipoprotein A-IV. Intestinal origin and distribution in plasma. *J. Clin. Invest.* **65**: 911–919.
58. Steinmetz, A., R. Barbaras, N. Ghalim, V. Clavey, J. C. Fruchart, and G. Ailhaud. 1990. Human apolipoprotein A-IV binds to apolipoprotein A-I/A-II receptor sites and promotes cholesterol efflux from adipose cells. *J. Biol. Chem.* **265**: 7859–7863.
59. Steinmetz, A., and G. Utermann. 1985. Activation of lecithin: cholesterol acyltransferase by human apolipoprotein A-IV. *J. Biol. Chem.* **260**: 2258–2264.
60. Ostos, M. A., M. Conconi, L. Vergnes, N. Baroukh, J. Ribalta, J. Girona, J. M. Caillaud, A. Ochoa, and M. M. Zakin. 2001. Antioxidative and antiatherosclerotic effects of human apolipoprotein A-IV in apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **21**: 1023–1028.
61. Kronenberg, F., M. Stuhlinger, E. Trenkwalder, F. S. Geethanjali, O. Pachinger, A. von Eckardstein, and H. Dieplinger. 2000. Low apolipoprotein A-IV plasma concentrations in men with coronary artery disease. *J. Am. Coll. Cardiol.* **36**: 751–757.
62. Recalde, D., M. A. Ostos, E. Badell, A. L. Garcia-Otin, J. Pidoux, G. Castro, M. M. Zakin, and D. Scott-Algara. 2004. Human apolipoprotein A-IV reduces secretion of proinflammatory cytokines and atherosclerotic effects of a chronic infection mimicked by lipopoly-saccharide. *Arterioscler. Thromb. Vasc. Biol.* **24**: 756–761.
63. Vanhamme, L., F. Paturiaux-Hanocq, P. Poelvoorde, D. P. Nolan, L. Lins, J. Van Den Abbeele, A. Pays, P. Tebabi, H. Van Xong, A. Jacquet, et al. 2003. Apolipoprotein L-I is the trypanosome lytic factor of human serum. *Nature.* **422**: 83–87.
64. Wan, G., S. Zhaorigetu, Z. Liu, R. Kaini, Z. Jiang, and C. A. Hu. 2008. Apolipoprotein L1, a novel Bcl-2 homology domain 3-only lipid-binding protein, induces autophagic cell death. *J. Biol. Chem.* **283**: 21540–21549.
65. Duchateau, P. N., I. Movsesyan, S. Yamashita, N. Sakai, K. Hirano, S. A. Schoenhaus, P. M. O'Connor-Kearns, S. J. Spencer, R. B. Jaffe, R. F. Redberg, et al. 2000. Plasma apolipoprotein L concentrations correlate with plasma triglycerides and cholesterol levels in normolipidemic, hyperlipidemic, and diabetic subjects. *J. Lipid Res.* **41**: 1231–1236.
66. Martin, S. S., A. A. Khokhar, H. T. May, K. R. Kulkarni, M. J. Blaha, P. H. Joshi, P. P. Toth, J. B. Muhlestein, J. L. Anderson, S. Knight, et al.; Lipoprotein Investigators Collaborative. 2015. HDL cholesterol subclasses, myocardial infarction, and mortality in secondary prevention: the Lipoprotein Investigators Collaborative. *Eur. Heart J.* **36**: 22–30.
67. Kim, D. S., A. A. Burt, E. A. Rosenthal, J. E. Ranchalis, J. F. Eintracht, T. S. Hatsukami, C. E. Furlong, S. Marcovina, J. J. Albers, and G. P. Jarvik. 2014. HDL-3 is a superior predictor of carotid artery disease in a case-control cohort of 1725 participants. *J. Am. Heart Assoc.* **3**: e000902.
68. Hine, D., B. Mackness, and M. Mackness. 2012. Coincubation of PON1, APO A1, and LCAT increases the time HDL is able to prevent LDL oxidation. *IUBMB Life.* **64**: 157–161.
69. Neefjes, L. A., G. J. Ten Kate, R. Alexia, K. Nieman, A. J. Galema-Boers, J. G. Langendonk, A. C. Weustink, N. R. Mollet, E. J. Sijbrands, G. P. Krestin, et al. 2011. Accelerated subclinical coronary atherosclerosis in patients with familial hypercholesterolemia. *Atherosclerosis.* **219**: 721–727.
70. Karlsson, H., P. Leanderson, C. Tagesson, and M. Lindahl. 2005. Lipoproteomics II: mapping of proteins in high-density lipoprotein using two-dimensional gel electrophoresis and mass spectrometry. *Proteomics.* **5**: 1431–1445.