Antibodies against HDL Components in Ischaemic Stroke and Coronary Artery Disease

Joana R. Batuca[1] Marta C. Amaral[1,2] Catarina Favas[2] Gonçalo C. Justino[3] Ana L. Papoila[4] Paul R. J. Ames[1] José Delgado Alves[1,2]

[1] CEDOC, NOVA Medical School/Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, Lisboa, Portugal
[2] Department of Medicine IV, Immune-mediated Systemic Diseases Unit, Fernando Fonseca Hospital, Amadora, Portugal
[3] Centro de Química Estrutural, Instituto Superior Técnico, Universidade de Lisboa, Lisboa, Portugal
[4] CEAUL, NOVA Medical School/Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, Lisboa, Portugal

Address for correspondence Joana R. Batuca, CEDOC, PhD, NOVA Medical School/Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, Campo dos Mártires da Pátria, 130, Lisboa 1169-056, Portugal (e-mail: joana.r.batuca@nms.unl.pt).

Thromb Haemost

Abstract

Quantitative and qualitative defects of high-density lipoprotein (HDL) are important in atherogenesis. In this study, we investigated whether antibodies against HDL components had additional value to conventional cardiovascular risk factors for the diagnosis of ischaemic stroke (IS) and coronary artery disease (CAD). Cross-sectional study was conducted on 53 patients with IS, 51 with CAD and 55 healthy controls, and in vitro studies to validate findings of the clinical study. We determined serum immunoglobulin G (IgG) antibodies against HDL (aHDL), apolipoproteins (aApoA-I, aApoA-II and aApoC-I) and paraoxonase-1 (aPON1) as well as PON1 activity (PON1a), total antioxidant capacity and biomarkers of endothelial activation (serum nitric oxide metabolites, 3-nitrotyrosine, VCAM-1 and ICAM-1); in vitro assays tested the capacity of IgG aHDL purified from high titer patients to inhibit PON1a and to reverse protective effect of HDL on endothelial cells. IgG aHDL, aApoA-I and aPON1 were higher in IS and CAD than controls ($p < 0.001$), predicted negatively PON1a and positively VCAM-1 and ICAM-1. By adding IgG aHDL and aApoA-I to a traditional cardiovascular risk factors model for IS and by adding IgG aHDL in a similar model for CAD, we obtained better discrimination of IS and CAD from healthy controls. IgG aHDL purified from IS and CAD inhibited PON1a by 38% ($p < 0.01$) and abrogated the protective effect of HDL on VCAM-1 expression by 126% compared with non-specific human IgG ($p < 0.001$). IgG against HDL components interfere with the antioxidant and anti-inflammatory properties of HDL and may represent novel biomarkers for vascular disease that need to be investigated in prospective studies.

Keywords

- high-density lipoprotein
- auto-antibodies
- coronary artery disease
- ischaemic stroke
- HDL dysfunction

Introduction

Epidemiologic studies show an inverse relationship between high-density lipoprotein (HDL) cholesterol and cardiovascular diseases (CVDs),[1] but despite improved diagnostic techniques, the atherosclerosis-related clinical burden is still raising.[2] Due to its antioxidant, anti-inflammatory, anti-apoptotic and anti-thrombotic properties, HDL represents a major anti-atherogenic factor beyond its reverse cholesterol transport effect[3] as it prevents atheroma formation and

received
October 12, 2017
accepted after revision
March 21, 2018

DOI https://doi.org/10.1055/s-0038-1645857.
ISSN 0340-6245.

stabilizes plaques, preventing rupture and thrombosis.[4] HDL has a very complex protein and lipid structure and its plasma concentration does not necessarily correlate to these protective effects, leading to the concept of HDL dysfunction.[5] Despite increasing plasma HDL, cholesteryl ester transfer protein inhibitors[6,7] and niacin[8,9] failed to achieve their expected clinical outcomes.

We previously identified immunoglobulin G (IgG) antibodies against HDL (aHDL), more specifically anti-apolipoprotein A-I antibodies (aApoA-I) in patients with autoimmune diseases, in association with decreased paraoxonase-1 activity (PON1a), enhanced endothelial activation, heightened disease activity and CV damage.[10–13] Others have recently described IgG anti-PON1 (aPON1) antibodies in patients with rheumatoid arthritis,[14] systemic lupus erythematosus (SLE)[15] and also in patients with primary antiphospholipid syndrome.[16]

Anti-ApoA-I antibodies have been identified also in non-autoimmune patients with atherosclerosis-associated clinical events[17–19]: in particular, IgG aApoA-I antibodies are associated with atherosclerotic plaque vulnerability in patients with acute coronary syndrome,[20] behaving as independent CV prognostic marker of major CV events after myocardial infarction[21] and predicting worse post-stroke outcomes.[18] Likewise, we found aApoA-I antibodies in type 2 diabetes,[22] particularly in patients with diabetes complicated with CVD.[23] Furthermore, we recently reinforced the clinical relevance of ApoA-I antibodies in a phase II, randomized, placebo-controlled, trial with niacin showing that the raise in HDL-C achieved by niacin was hampered by an increase in aApoA-I antibodies, suggesting a possible explanation why the HDL-C-boosting treatments fail to reduce CVD risk.[24]

Herein, we investigated whether antibodies against the HDL complex added diagnostic value to conventional CV risk factors and evaluated their biologic effects on the antioxidant and anti-inflammatory properties of HDL.

Materials and Methods

Study Participants

One hundred and twelve consecutive patients (56 ischaemic stroke [IS] and 56 coronary artery disease [CAD]) with atherosclerosis-associated clinical events recruited at the Internal Medicine outpatients clinic of Fernando Fonseca Hospital in Portugal were invited to participate in this cross-sectional case–control study. Inclusion criteria were: at least one vascular event confirmed by computed tomography (CT) scan (IS) or coronary angiography (CAD) in the past 5 years, without evidence of active systemic inflammation (normal erythrocyte sedimentation rate [ESR] and high-sensitivity C-reactive protein [hsCR*p*] < 1.0 mg/dL) and routine (six-monthly) follow-up appointments.

Exclusion criteria were: infection, cancer, renal or hepatic dysfunction (serum creatinine < 1.2 mg/dL and aspartate transaminase/alanine transaminase [ALT/AST] $< 2\times$ reference value), decompensated cardiac insufficiency and any thrombotic event in the previous 3 months. Of the initial cohort, 5 patients did not have confirmed CAD and 3 patients had a cerebral haematoma rather than IS and were excluded. Therefore, the study enrolled 51 patients with CAD and 53 with IS.

Fifty-five healthy subjects without known CVD or cerebrovascular disease and not taking any medication were recruited during the same time via various advertising means including internal email or direct invitations to staff and patients' relatives. Demographic data of patients and controls and medical history, including current medication data, are summarized in ►**Table 1**.

All subjects signed consent forms approved by the ethics committees of the hospital and the study was performed according to the revised Declaration of Helsinki. Blood samples were collected after a 12-hour fast and were centrifuged at $3{,}000 \times g$ at 4°C for 10 minutes to obtain serum, which was kept at –80°C until assayed.

Measurement of Antibodies against HDL and its Components

Titers of IgG aHDL, aApo A-I, aApo A-II, aApo C-I, aPON1 and immunoglobulin M (IgM) aHDL antibodies were assessed by in-house enzyme-linked immunosorbent assays (ELISAs). IgG aHDL and IgG aApoA-I antibodies were measured as previously described[11–13,24]; ELISA testings for the others antibodies were also adapted from these studies. Briefly, the antigen (HDL or other HDL component) solution was prepared at the appropriate concentration (20 µg/mL human HDL, 10 µg/mL human ApoA-I or ApoA-II, 2 µg/mL human ApoC-I [Sigma-Aldrich, Portugal] and 1 µg/mL PON1 [Abnova, Tebu-bio, Portugal], respectively) in 70% ethanol or bicarbonate (BIC) buffer (pH 9.8) for PON1, and coated to a half 96-well micro-titer plates for a period of 2 hours at 37°C. The plates were then blocked with the addition of 100 µL/well at 1% bovine serum albumin (BSA)—10 mM phosphate buffer saline (PBS) (pH 7.4) for 1 hour at 37°C. The unbound blocking agent was then removed and the plates were washed 4× with PBS. Next, standard, positive and negative controls and samples were diluted (1:100 for IgG and IgM aHDL, aApoA-II, aApoAC-I; 1:200 for IgG aApoA-I and 1:300 for IgG aPON1) in blocking buffer and added in duplicate to each half of the plate for 1 hour at 37°C. The unbound antibodies were removed by repeating the washing step. Alkaline phosphatase conjugated anti-human IgG (1:1,000 in the blocking agent) or anti-human IgM (1:15,000 in the blocking agent) was added for 1 hour. Plates were then washed 2× with PBS and 2× with BIC buffer. P-nitrophenyl phosphate diluted in BIC buffer was added and incubated at 37°C for colour development, and the absorbance read by a Biotrak II plate reader (Amersham Biosciences) at 405 nm after 1 hour.

All assays were validated by the inclusion of internal quality control samples of known activity and the results were calculated after subtraction of the background in the uncoated half of the plate. IgG aApoA-I antibodies were expressed as µg/mL determined by the standard curve present in each plate. This curve was prepared with six standards of commercial Apo A-I antibody (MIA 1404, Thermo Scientific) in a concentration range of 0.001 to 0.04 µg/mL. For all the other antibodies (IgG

Table 1 Demographic characteristics and medical history of the healthy controls and patients with atherosclerosis-associated clinical events

Characteristics[a]	Healthy controls ($n = 55$)	IS ($n = 53$)	p-Value[b]	CAD ($n = 51$)	p-Value[c]
Age, y	57.50 (54.00–79.25)	75.00 (64.00–79.50)	< 0.001	82.00 (77.00–86.00)	< 0.001
Male sex, *n*	27(49.10)	32 (60.40)	0.253	22 (43.10)	0.564
BMI, kg/m^2	22.45 (21.88–24.78)	24.22 (22.12–25.55)	0.373	25.22 (23.44–26.12)	0.490
Systolic BP, mm Hg	128.00 (121.10–141.50)	147.08 (120.30–164.50)	0.318	119.50 (103.80–139.30)	0.171
Diastolic BP, mm Hg	76.40 (61.00–82.00)	79.00 (68.50–92.50)	0.548	62.00 (56.50–83.50)	0.341
Risk factors, *n* (%)					
Hypertension	0	34 (64.15)	< 0.001	36 (70.59)	< 0.001
Type 2 diabetes	0	11 (20.75)	< 0.001	14 (27.46)	< 0.001
Dyslipidaemia	0	11 (20.75)	< 0.001	11 (21.57)	< 0.001
Smoking	0	7 (13.21)	0.006	9 (17.65)	< 0.001
Medication, *n* (%)					
ACE inhibitor	0	7 (13.21)	0.006	12 (23.53)	< 0.001
ARB	0	2 (3.77)	0.238	2 (3.92)	0.229
β-blocker	0	5 (9.43)	0.026	7 (13.73)	0.005
Calcium-channel blocker	0	4 (7.55)	0.055	7 (13.73)	0.005
Diuretics	0	9 (16.98)	0.001	14 (27.46)	< 0.001
Anti-platelet agents	0	12 (22.64)	< 0.001	13 (25.90)	< 0.001
Statin	0	5 (9.43)	0.026	9 (17.65)	<0.001

Abbreviations: ACE, angiotensin-converting enzyme; ANOVA, analysis of variance; ARB, angiotensin receptor blocker; BMI, body mass index; BP, blood pressure; CAD, coronary artery disease; IS, ischaemic stroke.

[a]All continuous variables are reported as median with interquartile range (IQR) and categorical variables were presented as frequencies (*n*) and percentages (%).

[b]Comparison between healthy control and ischaemic stroke (IS) group. *p*-Value obtained by one-way ANOVA applied to log-transformed data or Fisher's exact test as appropriate.

[c]Comparison between the healthy control and coronary artery disease (CAD) group. *p*-Value obtained by one-way ANOVA applied to log-transformed data or Fisher's exact test as appropriate.

aHDL, aApoA-II, aApoC-I, aPON1 and IgM aHDL), the results were expressed as a percentage of the different positive control corresponding to 100% for each antibody assay. Inter-/intra-plate coefficients of variation were less than 10%. The cut-off for the upper normal titers of all antibodies quantified in the study was set at the mean ± 3 standard deviation (SD) of the control group.

Quantification of the Lipid Profile

The plasma lipid profile (total cholesterol, HDL-C, LDL-C and triglycerides) was determined by standard colorimetric enzymatic techniques (commercial kits by Randox, Irlandox, Portugal).

Quantification of Paraoxonase 1 Activity (PON1a) and Total Antioxidant Capacity

Serum PON1a was assessed by quantification of nitrophenol formation by spectrophotometry as previously described.[11–13] Total antioxidant capacity (TAC) was assessed using an ABEL-41M2 antioxidant test kit with Pholasin (Knight Scientific), according to the manufacturer's instructions. An Anthos Zenyth 1100/3100 multimode detector was used and the results were expressed in vitamin E analogue (VEA) equivalent units (μM) derived from a set of standards run at the same time.

Quantification of Nitric Oxide Metabolites and 3-Nitrotyrosine (3-NT)

Serum nitric oxide metabolites nitrite (NO_2^-) and nitrate (NO_3^-) were measured using a modified Griess reaction.[25] Serum were diluted in ice-cold ethanol (1:3, v/v), vortex for 1 minute and incubated on ice for 30 minutes after being centrifuged at 12,000 × *g* for 20 minutes at 4°C and the supernatants were collected for NO_2^- and NO_3^- assessments. The assay was performed in a standard flat-bottomed 96-well plate for simultaneous measurement of nitrite and nitrate concentration. Note that 50 μL of standard or diluted sample were added in duplicate, and PBS was used as blank. In half plate, 6.3 U/L of nitrate reductase (Sigma-Aldrich) and 550 μmol/L of nicotinamide adenine dinucleotide phosphate (NADPH) (Sigma-Aldrich) were added for the reduction of

nitrate to nitrite. After 2 hours, the plate was incubated at room temperature, and Griess reaction was initiated by adding equal volumes of 2% sulphanilamide (Sigma-Aldrich) in 5% H_3PO_4 and 0.2% N-(1-naphthyl)-ethylenediamine dihydrochloride (Sigma-Aldrich) in water, mixed just prior to use. After 10 minutes of incubation at room temperature, the absorbance was read at 540 nm and the levels were expressed as µM. Serum levels of 3-NT were determined by enzyme immunoassay using commercial kits (Oxis Research, Foster City, California, United States), according to the manufacturer's instructions.

Measurement of Soluble Endothelial Cell Adhesion Molecules

Soluble intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) levels were determined by ELISA using commercial kits (R&D Systems, Citomed, Portugal), according to the manufacturer's instructions.

Isolation of the aHDL Antibodies by Immunoaffinity Chromatography

Anti-HDL antibodies from patient's serum were isolated by immunoaffinity chromatography, and human HDL was covalently coupled to N-hydroxysuccinimide (NHS)-activated HiTrap affinity column of 1 mL (GE Healthcare) according to the manufacturer's instructions. Briefly, HDL (10 mg) was dissolved in coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). The column was embedded in isopropanol to preserve the stability of the activated medium, and immediately prior to the coupling of the ligand, the column was flushed with six column volumes of ice-cold 1 mM HCl using a 1 mL syringe at a flow rate of 1 mL/min (½ drop/s). Once completed, the ligand solution was added onto the column that was sealed and left to stand for 30 to 60 minutes at room temperature. Then, to deactivate any excess active groups that did not couple to the ligand and to wash out the non-specifically bound HDL, the column was washed with six column volumes of 0.5 M ethanolamine, 0.5 M NaCl (pH 8.3) (buffer A) followed by six column volumes of 0.1 M acetate, 0.5 M NaCl (pH 4) (buffer B) then again with the same volume of buffer A, after which the column was sealed and allowed to stand for 30 minutes at room temperature. Then, the column was re-washed with six volumes of buffer B, followed by buffer A, and then by buffer B again at the end of which the column was washed with 0.01 M NaH_2PO_4, 0.0027 M KCl and 0.137 mM NaCl (pH 7.4). After this step, the column was ready for use. If not used immediately, it was stored by applying a storage buffer consisting of 0.05 M Na_2HPO_4, 0.1% NaN3 (pH 7).

Before applying the sample, the column was equilibrated with 10 column volumes of binding buffer (0.02 M Tris, pH 7.5). Serum samples from patients with CAD and IS were pooled after being selected according to the highest immunoreactives previously observed, diluted in equal volume of binding buffer and filtered through Millex GV 0.22 µm. Then, the diluted serum was applied to the column and run through at least six times at room temperature. The unbound antibodies were removed by washing the column with 10 column volumes of 0.02 M Tris (pH 7.5) and further by 10 volumes of 0.02 M Tris and 0.5 mM NaCl (pH 7.5). Antibodies retained by the immunosorbent were eluted with 0.1 M glycine pH 2.5 into tubes containing 70 µL neutralization buffer (Tris 1 M, pH 8.8). After elution of antibodies at low pH, the column should be washed with 10 volumes of 0.02 M Tris pH 8.8 and then with 10 volumes of 0.02 M Tris pH 7.5 to re-equilibrate the column. Absorbance (280 nm) of the collected fractions (1 mL) was measured, and the peak fractions were dialyzed against PBS and tested for the presence of aHDL antibodies by ELISA as described above. The fractions positive for antibodies were pooled, concentrated by evaporation under nitrogen and stored at –4°C until their biologic activity was tested.

Inhibition of Paraoxonase 1 Activity (PON1a) by the Antibodies Isolated from Patient's Serum

Dose-dependent inhibition assays of PON1a were performed by incubating human HDL (100 µg/mL) with PBS (basal) or the aHDL antibodies isolated from patients. As control, we used irrelevant human IgG (Sigma-Aldrich) at the same concentration of the fractions tested. After 1 hour of incubation, PON1a was assessed as described above. All experiments were run in triplicate and results were expressed as a percentage of the effect on PON1a in basal conditions.

In Vitro Effect of aHDL Antibodies on Cytokine-Induced Expression of VCAM-1 Levels in Human Umbilical Vein Endothelial Cells (HUVECs)

Confluent monolayers of HUVECs seeded in 24-well tissue culture plates were incubated for 16 hours at 37°C in 5% CO_2, in EGM Media (Lonza) supplemented with 10% fetal bovine serum (FBS) (basal), human HDL (1.0 mg/mL) alone or with aHDL antibodies (50 µg/mL) isolated from patient's serum, or a non-specific human IgG (50 µg/mL) or commercial human ApoA-I monoclonal antibody (MIA 1404) (30 µg/mL). After being washed, cells were incubated for a further 4 hours in the basal or stimulated state with 10 ng/mL of tumour necrosis factor-α (TNF-α). After washing with PBS, cells were gently trypsinized (0.5% trypsin, ethylenediaminetetraacetic acid [EDTA]). Trypsin was neutralized with cold PBS and cells were centrifuged for 5 minutes at 1,200 × *g*. Cell pellets were suspended and incubated with Fc block (BD Biosciences) for 10 minutes at 4°C following the stained with fluorescein isothiocyanate (FITC)-conjugated anti-human VCAM-1 (R&D Systems) for 30 minutes on 4°C. After incubation, cells were washed once with cold PBS and re-suspended. Expression of VCAM-1 was measured as mean fluorescence intensity (MFI) in a FACS Canto II cytometer. Ten thousand cells were analysed per sample by using the Cell Quest Software. Data were expressed as the percentage of basal VCAM-1 expression positive cells. All experiments were done in triplicate.

Statistical Analysis

An exploratory analysis was performed for all variables. Categorical data were expressed as frequencies (*n*) and

percentages (%), and continuous variables as median and interquartile range (25th–75th percentile). For quantitative variables, the comparison between healthy controls, IS and CAD groups was performed by one-way analysis of variance (ANOVA) and Dunnett's test for multiple comparisons was applied to log-transformed data (due to the existence of outliers and skewed distributions). To study the association between categorical variables, non-parametric Fisher's exact test was used. The relationship between HDL levels and the titers of IgG aHDL, IgG aApoA-I and aPON1antibodies was evaluated by Spearman's correlation coefficient. Positive antibody titer cut-off was defined as mean plus 3 SDs of healthy subjects, and was used to dichotomize (positive vs. negative) IgG aHDL, aApoA-I and aPON1 antibodies. Chi-square test was used to compare the prevalence of positive antibodies titers between both patients' groups and controls. Linear regression models were applied to study the association between the resulting binary independent variables and PON1a, VCAM-1, ICAM-1 and NO_2^- levels as dependent variables. Normality assumption of the residuals was verified using the Kolmogorov–Smirnov goodness-of-fit test with Lilliefors correction and logarithmic transformation of PON1a, VCAM-1, ICAM-1 and NO_2^- levels was performed for the violation of the normality assumption.

Logistic regression models were fitted to the data to evaluate the incremental value to the performance of a traditional CV risk factors model (reference model) after adding antibodies against HDL complex to the model. Results are presented as odds ratio (OR) and corresponding 95% confidence intervals (95% CIs). The calibration of the models was analysed by Hosmer–Lemeshow goodness-of-fit test and their discriminative ability was measured by the area under the receiver operating characteristic (ROC) curve. A value of 0.50 is obtained when a model discriminates no better than chance, and a value of 1.0 means perfect discrimination. The Hosmer–Lemeshow test compares observed and expected frequencies of patients and healthy subjects based on the values of the estimated probabilities obtained by the logistic regression model. In this test, a high p-value indicates that the model is performing well, i.e., there is not a large discrepancy between observed and expected frequencies. A significance level of $\alpha = 0.05$ was considered. All data were analysed using STATA 13.0. (StataCorp. 2013, Stata Statistical Software: Release 13; StataCorp LP, College Station, Texas, United States) and the Statistical Package for the Social Sciences for Windows 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows; IBM Corp., Armonk, New York, United States).

Results

Comparative Clinical Features of Participants

Demographic characteristics, clinical features and medication use of all participants of this study are shown in ►**Table 1**. IS/CAD participants were older than controls ($p < 0.001$) and were more likely to have hypertension (64%/71%), type 2 diabetes (21%/27%) and dyslipidaemia (21%/22%) than controls.

Comparative Lipid Profile of Participants

HDL was lower in both patient groups than healthy controls ($p < 0.001$). Total cholesterol was similar across groups, whereas LDL was higher in patients with IS ($p = 0.001$) and triglycerides higher in both disease groups ($p < 0.001$ and $p = 0.03$, for IS and CAD, respectively) (►**Table 2**).

Comparative HDL Auto-Antibody Profile and other Laboratory Variables

Mean IgG aHDL, aApoA-I and aPON1 titers were higher in both patient groups compared with healthy controls (►**Fig. 1 A–C**; $p < 0.001$). IgG aHDL titer was positive in 32% (95% CI, 20–46%) of IS patients (17/53) and in 33% (95% CI, 21–48%) of CAD patients (17/51); IgG aApoA-I was positive in 42% (22/53, 95% CI, 28–56%) and 33% (17/51, 95% CI, 21–48%) of IS and CAD patients, respectively; IgG aPON1 was positive in 9% (5/53, 95% CI, 3–21%) and 23% (12/51, 95% CI, 13–38%) of IS and CAD patients, respectively. The prevalence of positive antibody titers between both patient groups and controls was significantly different ($p < 0.001$ for IgG aHDL, aApoA-I, and $p = 0.001$ for aPON1). No differences were observed for the remaining antibodies tested.

IgG aHDL directly correlated with the IgG aApoA-I titers ($p < 0.001$ in CAD and IS) and IgG aPON1 titers ($p < 0.001$), but only in patients with CAD (not shown).

In CAD, four patients presented simultaneous positivity for aHDL, aApoA-I and aPON1, whereas in IS there were only three. Ten patients with CAD were double positive (4 aHDL/aApoA-I and 5 aHDL/aPON1), while in patients with IS 10 were double positive although with a different distribution (8 aHDL/aApoA-I, 1 aHDL/aPON1 and 1 aApoA-I/aPON1). Seventeen patients with CAD were positive to one auto-antibody (4 aHDL, 9 aApoA-I and 3 aPON1), whereas 15 patients with IS were single positive (5 aHDL, 10 aApoA-I).

PON1a was lower in both patient groups compared with healthy controls ($p < 0.001$), although TAC was not different (►**Table 2**). VCAM-1 and ICAM-1 levels were increased in both patient groups compared with healthy controls (VCAM-1: $p < 0.001$ for both; ICAM-1: $p = 0.005$ and $p < 0.001$, for IS and CAD, respectively) (►**Table 2**). NO_2^- was decreased in patients with CAD compared with controls ($p = 0.028$), but no significant differences were noted for NO_3^- and 3-NT levels.

Neither co-morbidities, such hypertension, diabetes mellitus and dyslipidaemia, nor treatment modalities affected mean differences of antibody titers in the patients with CAD and IS. Despite some negative trends, no correlation was found between IgG aHDL, IgG aApoA-I, IgG aPON1 and HDL levels (not shown).

Predictive Value of HDL Auto-Antibody Profile towards PON1 Activity, VCAM-1 and ICAM-1

Several multiple linear regression models analysed the association of IgG aHDL, aApoA-I or aPON1 with PON1a, VCAM-1, ICAM-1 and NO_2^-. For each biomarker, a univariable analysis was performed considering age, sex, co-morbidities, total cholesterol, HDL, LDL, triglycerides and each binary variable resulting from the positive antibody titers cut-off (176.84%

Table 2 Biologic variables of the healthy controls and patients with atherosclerosis-associated clinical events

Characteristics, median (IQR)	Healthy controls ($n = 55$)	IS ($n = 53$)	p-Value[a]	CAD ($n = 51$)	p-Value[b]
Cholesterol (mg/dL)	148.50 (114.00–192.75)	160.00 (107.75–200.00)	0.258	137.50 (104.00–159.00)	0.989
HDL (mg/dL)	49.71 (38.75–67.28)	38.5 (26.50–46.50)	< 0.001	32.00 (26.50–44.25)	< 0.001
LDL (mg/dL)	79.00 (45.85–105.96)	104.00 (73.50–138.50)	0.001	93.50 (67.75–108.50)	0.099
Triglycerides (mg/dL)	85.00 (63.00–149.50)	116.00 (96.00–149.50)	< 0.001	99.50 (72.50–140.80)	0.030
IgG aHDL (% p. control)	87.82 (68.23–105.80)	150.22 (104.11–220.20)	< 0.001	149.26 (111.40–205.60)	< 0.001
IgM aHDL (% p. control)	34.49 (19.42–63.69)	23.99 (9.66–55.72)	0.223	22.86 (17.23–47.18)	0.497
IgG aApoA-I (μg/mL)	0.09 (0.07–0.14)	0.22 (0.14–0.36)	< 0.001	0.21 (0.14–0.38)	< 0.001
IgG aApoA-II (% p. control)	116.70 (63.02–182.80)	121.50 (95.71–195.20)	0.436	171.50 (83.62–222.00)	0.209
IgG aApoC-I (% p. control)	242.50 (157.00–362.50)	242.90 (116.40–394.80)	0.891	250.70 (105.80–541.70)	0.872
IgG aPON1 (% p. control)	70.59 (39.83–144.12)	121.95 (82.99–201.40)	< 0.001	155.93 (70.98–305.90)	< 0.001
PON1a (U/L)	310.41 (236.62–333.05)	203.95 (146.29–272.74)	< 0.001	166.60 (119.50–278.70)	< 0.001
TAC (VEA-equivalent units, μM)	7.66 (6.06–9.05)	8.06 (6.71–9.42)	0.716	8.38 (6.55–10.79)	0.188
VCAM-1 (ng/mL)	606.14 (501.95–710.75)	1100.18 (683.55–1411.19)	< 0.001	1360.15 (1016.00–2146.00)	< 0.001
ICAM-1 (ng/mL)	297.94 (251.88–334.05)	333.18 (288.43–404.99)	0.005	404.36 (348.20–595.70)	< 0.001
NO_2^- (μM)	12.06 (6.63–19.78)	8.38 (5.86–12.04)	0.076	7.19 (5.11–11.50)	0.028
NO_3^- (μM)	23.26 (11.55–30.95)	16.19 (7.56–29.49)	0.153	27.89 (13.92–51.45)	0.192
3-NT (nM)	17.42 (14.91–21.21)	17.49 (15.98–21.45)	0.296	17.48 (15.84–19.62)	0.083

Abbreviations: 3-NT, 3-nitrotyrosine; aApoA-I, anti-apolipoprotein A-I antibodies; aApoA-II, anti-apolipoprotein A-II antibodies; aApoC-I, anti-apolipoprotein C-I antibodies; aHDL, anti-HDL antibodies; ANOVA, analysis of variance; aPON1, anti-paraoxonase 1 antibodies; CAD, coronary artery disease; HDL, high-density lipoprotein; ICAM-1, intercellular adhesion molecule 1; IgG, immunoglobulin G; IgM, immunoglobulin M; IQR, interquartile range; IS, ischaemic stroke; LDL, low-density lipoprotein; NO_2, nitrite; NO_3, nitrate; PON1a, paraoxonase 1 activity; TAC, total antioxidant capacity; VCAM-1, vascular cell adhesion molecule 1; VEA, vitamin E analogue.

Note: SI conversions: To convert total LDL and HDL and total cholesterol to mmol/L, multiply by 0.0259; and to convert triglycerides to mmol/L, multiply by 0.0113.

[a]Comparison between the healthy control and ischaemic stroke (IS) group.

[b]Comparison between the healthy control and coronary artery disease (CAD) group. p-Value obtained by one-way ANOVA applied to log-transformed data.

for IgG aHDL, 0.28μg/mL for aApoA-I and 311.7% for aPON1 antibodies): variables with p-value of < 0.25 were considered for the multivariable study.

First, three multivariable regression models considered the log of PON1a as dependent variable and age, co-morbidities, HDL, LDL and dichotomized antibody titers (positive vs. negative) separately, as independent variables. IgG aHDL, aApoA-I or aPON1 higher than the cut-off of positivity negatively predicted PON1a corresponding to a decrease of 17.63% ($\hat{\beta} = -0.194$, $p = 0.020$), 31.82% ($\hat{\beta} = -0.383$, $p < 0.001$) and 25.77% ($\hat{\beta} = -0.298$, $p = 0.007$), respectively, when compared with lower antibody titers (►**Table 3**).

Second, three multivariable regression models considered the log of VCAM-1 as dependent variable and age, HDL, LDL and dichotomized antibody titers separately as independent variables. IgG aHDL, aApoA-I or aPON1 higher than the cut-off of positivity independently predicted VCAM-1 by 42.19% ($\hat{\beta} = 0.352$, $p < 0.001$), 44.05% ($\hat{\beta} = 0.365$, $p < 0.001$) and 51.89% ($\hat{\beta} = 0.418$, $p = 0.001$), respectively, when compared with lower antibodies titers (►**Table 3**).

Lastly, three multivariable regression models considered the log of ICAM-1 as dependent variable and age, sex, co-morbidities, HDL, LDL, triglycerides and dichotomized antibody titers separately as independent variables. Results

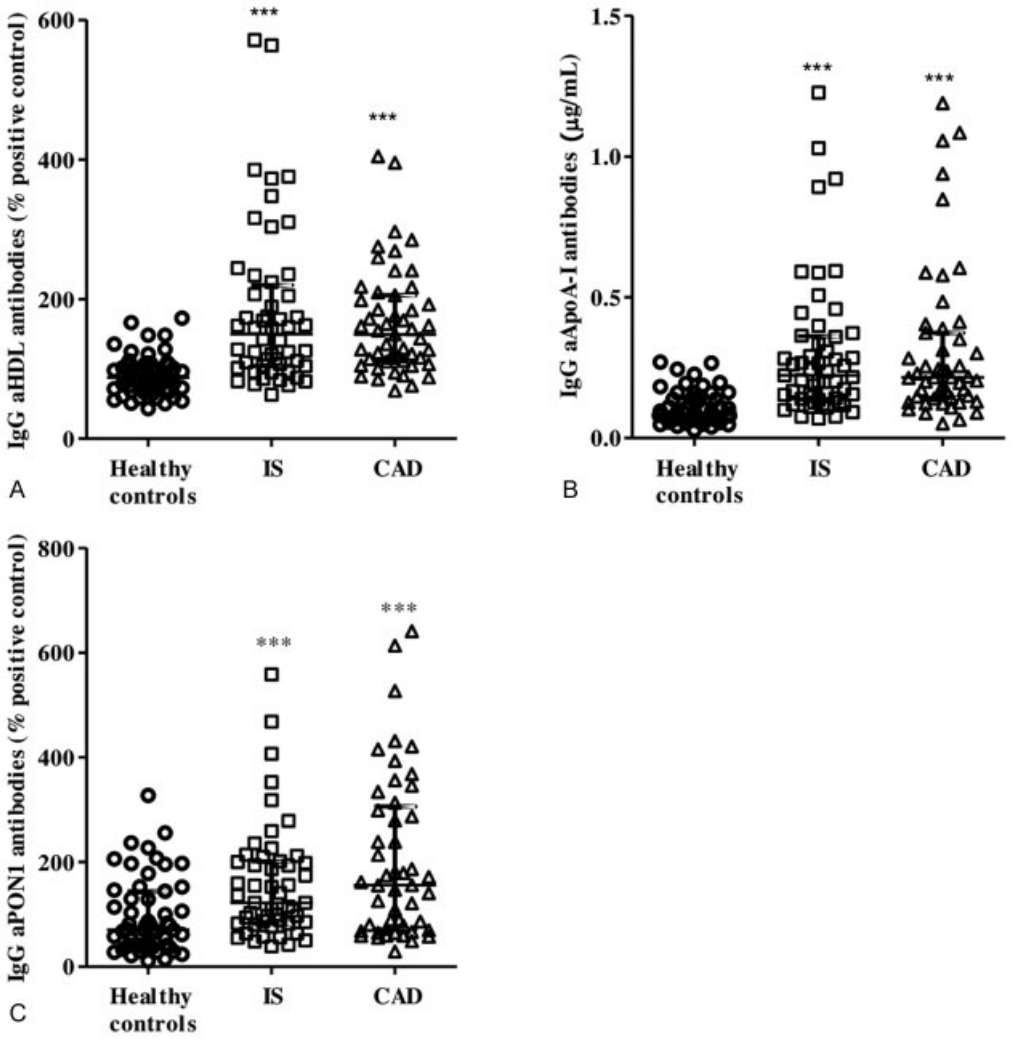

Fig. 1 Levels of antibodies against high-density lipoprotein (aHDL) complex. Immunoglobulin G (IgG) aHDL (**A**), anti-apolipoprotein A-I (aApoA-I) (**B**) and paraoxonase-1 activity (aPON1) (**C**) antibodies in healthy controls and patients with atherosclerosis-associated clinical events: ischaemic stroke (IS) and coronary artery disease (CAD). Bars show the median with interquartile range. ***p-Value of < 0.001 for both IS and CAD patients when compared with healthy controls.

showed that only IgG aApoA-I and aPON1 higher than the cut-off of positivity independently predicted higher ICAM-1 levels of 14.68% ($\hat{\beta} = 0.137$, $p = 0.035$) and 35.39% ($\hat{\beta} = 0.303$, $p < 0.001$), respectively, when compared with lower antibody titers (►**Table 3**). No association was observed between NO_2^- levels and the antibody titers.

Incremental Value of Adding the HDL Auto-Antibody Profile to a Lipid Atherosclerotic Disease Risk Prediction Model

By using univariable logistic regression analysis, we explored which CV risk factors (age, sex, co-morbidities, total cholesterol, HDL, LDL, triglycerides) were related to HDL antibodies: those with a p-value of < 0.25 were considered for the multivariable study.

In IS, age, HDL and LDL levels remained the same in the multivariable model (IS reference model). By adding separately each antibody titer in IS patients (IgG aHDL, aApo A-I or aPON1 antibodies), a 10% increment of IgG aHDL, 0.04 ug/mL of aApoA-I or 10% of aPON1 raised the odds of having IS ($\widehat{OR} = 1.47$, 95% CI ,1.20–1.81, $p < 0.001$; $\widehat{OR} = 1.35$, 95% CI, 1.16–1.58, $p < 0.001$; $\widehat{OR} = 1.11$, 95% CI, 1.03–1.19, $p = 0.005$, respectively) (►**Table 4**).

In CAD, age and HDL levels remained the same in the multivariable model (CAD reference model). By adding separately each antibody titers, a 10% increment of IgG aHDL, 0.04 ug/mL of aApoA-I or 10% of aPON1 raised the odds of having CAD ($\widehat{OR} = 1.61$, 95% CI, 1.21–2.14, $p = 0.001$; $\widehat{OR} = 1.16$, 95% CI, 1.02–1.32, $p = 0.028$; $\widehat{OR} = 1.11$, 95% CI, 1.02–1.20, $p = 0.015$, respectively) (►**Table 4**).

With regards to calibration, all models had a good performance (Hosmer-Lemeshow test p-value = 0.106, 0.885, 0.998 and 0.871 respectively, for reference model, reference model plus aHDL, plus aApoA-I or plus aPON1 antibodies) in patients with IS as in patients with CAD (Hosmer-Lemeshow test p-value =0.978, 0.758, 0.999 and 0.740, respectively for the same models) (►**Table 5**).

With regards to the discriminative ability of the models, we obtained acceptable to outstanding AUC values[26] for the reference model, reference model plus IgG aHDL, reference

Table 3 Multivariable linear regression models to study the association between PON1 activity, VCAM-1, ICAM-1 and NO_2^- levels and IgG aHDL, aApo A-I and aPON1 antibodies

Variables[a]	Log PON1a		Log VCAM-1		Log ICAM-1		Log NO_{2-}	
	$\hat{\beta}$ (95% CI)	*p*-Value	$\hat{\beta}$ (95% CI)	*p*-Value	$\hat{\beta}$ (95% CI)	*p*-Value	$\hat{\beta}$ (95% CI)	*p*-Value
IgG aHDL								
Age	−0.011 (−0.016 to 0.005)	< 0.001	0.019 (0.013–0.024	< 0.001	0.007 (0.003–0.011)	< 0.001	−0.015 (−0.024 to 0.007)	0.001
LDL	0.002 (0.001–0.004)	0.011	−0.002 (−0.004 to 0.000)	0.04	–	–	−0.004 (−0.007 to 0.001)	0.013
Triglyceride	–	–	–	–	–	–	0.003 (0.001–0.005)	0.008
HDL	0.004 (0.000–0.0008)	0.038	−0.007 (−0.011 to 0.002)	0.002	−0.005 (−0.007 to 0.002)	0.001	–	–
IgG aHDL > cut-off +	−0.194 (−0.357 to 0.031)	0.020	0.352 (0.175–0.529)	< 0.001	0.076 (−0.043 to 0.195)	0.210	−0.203 (−0.493 to 0.087)	0.169
IgG aApoA-I								
Age	−0.010 (−0.015 to 0.005)	< 0.001	0.017 (0.012–0.023)	< 0.001	0.007 (0.003–0.011)	< 0.001	−0.015 (−0.024 to 0.007)	0.001
LDL	–	–	–	–	–	–	−0.004 (−0.007 to 0.001)	0.013
Triglyceride	–	–	–	–	–	–	0.003 (0.001–0.005)	0.008
HDL	–	–	−0.005 (−0.010 to 0.001)	0.008	−0.003 (−0.006 to 0.001)	0.018	–	–
IgG aApoA-I > cut-off +	−0.383 (−0.545 to 0.222)	< 0.001	0.365 (0.182–0.549)	< 0.001	0.137 (0.009–0.265)	0.035	−0.201 (−0.507 to 0.105)	0.195
IgG aPON1								
Age	−0.010 (−0.016 to 0.005)	< 0.001	0.017 (0.011 to 0.023)	< 0.001	0.007 (0.003–0.010)	0.001	−0.015 (−0.024 to 0.007)	0.001
Co-morbidities	−0.175 (−0.326 to 0.024)	0.023	–	–	0.133 (0.033–0.232	0.009	–	–
LDL	0.002 (0.001–0.004)	0.009	–	–	–	–	−0.004 (−0.007 to 0.001)	0.013
Triglyceride	–	–	–	–	–	–	0.003 (0.001–0.005)	0.008
HDL	–	–	−0.006 (−0.010 to 0.002)	0.005	–	–	–	–
IgG aPON1 > cut-off +	−0.298 (−0.513 to 0.083)	0.007	0.418 (0.185–0.651)	0.001	0.303 (0.159–0.447)	< 0.001	−0.336 (−0.703 to 0.030)	0.072

Abbreviations: $\hat{\beta}$, regression coefficient estimate; aApoA-I, anti-apolipoprotein A-I antibodies; aHDL, anti-HDL antibodies; aPON1, anti-paraoxonase 1 antibodies; CI, confidence interval; HDL, high-density lipoprotein; ICAM-1, intercellular adhesion molecule 1; IgG, immunoglobulin G; LDL, low-density lipoprotein; Log, natural logarithm; NO_2^-, nitrite; PON1a, paraoxonase 1 activity; TAC, total antioxidant capacity; VCAM-1, vascular cell adhesion molecule 1.

[a]All variables that were not statistically significant in each multivariable model were excluded, with the exception of the antibodies titers.

model plus IgG aApoA-I or reference model plus IgG aPON1 in IS and CAD (►**Table 5**). Moreover, only the models resulting from adding IgG aHDL or aApoA-I in IS and IgG aHDL in CAD had statistically different AUCs from the reference model ($p = 0.006$, $p < 0.001$ and $p = 0.021$, respectively).

To further quantify the improvement resulting from adding IgG aHDL, aApoA-I or aPON1 to the reference model, we calculated category-free net re-classification improvement (NRI) and integrated discrimination improvement (IDI) measures with corresponding bootstrap of 95% CIs. The NRI quantifies the correctness of upward and downward movement of predicted probabilities resulting from adding the three referred antibodies to the reference model. The IDI measures the size of changes in these probabilities.[27]

For IS, according to NRI events and non-events (45.8%, 95% CI, 16.2–71.4 and 73.1%, 95% CI, 45.6–87.8, respectively) and according to IDI events and non-events (0.115, 95% CI, 0.058–0.230 and 0.114, 95% CI, 0.053–0.211, respectively), adding IgG aHDL to the reference model resulted in an improvement in identifying patients with an increased CV risk (►**Table 5**).

A similar improvement in classifying patients with CAD was observed by adding IgG aHDL antibodies to the reference model according to NRI events and non-events (65.2%, 95% CI, 16.3–85.7 and 73.6%, 95% CI, 25.0–88.9, respectively), and IDI events and non-events (0.101, 95% CI, 0.044–0.190 and 0.086, 95% CI, 0.041–0.163, respectively) (►**Table 5**).

For patients with IS, the change in identifying patients with increased CV risk from adding IgG aApoA-I to the reference model resulted in an improvement of NRI events

Table 4 Multivariable logistic regression models to study the incremental value of adding IgG aHDL, aApo A-I or aPON1 antibodies to traditional cardiovascular risk factors in patients with atherosclerosis-associated clinical events

Variable	Reference model (RM)		RM + IgG aHDL		RM + IgG aApoA-I		RM + IgG aPON1	
	$\widehat{OR}$ = (95% CI)	*p*-Value	$\widehat{OR}$ = (95% CI)	*p*-Value	$\widehat{OR}$ = (95% CI)	*p*-Value	$\widehat{OR}$ = (95% CI)	*p*-Value
IS								
Age[a]	1.04 (1.00–1.08)	0.052	1.08 (1.02–1.14)	0.007	1.05 (1.00–1.11)	0.070	1.04 (1.00–1.08)	0.065
HDL	0.94 (0.91–0.97)	0.001	0.93 (0.89–0.98)	0.010	0.94 (0.90–1.00)	0.041	0.95 (0.91–0.99)	0.007
LDL	1.02 (1.01–1.04	0.001	1.03 (1.01–1.05	0.007	1.04 (1.02–1.07)	0.001	1.03 (1.01–1.04	< 0.001
IgG aHDL[a]			1.47 (1.20–1.81)	< 0.001				
IgG aApoA-I[a]					1.35 (1.16–1.58)	< 0.001		
IgG aPON1[a]							1.11 (1.03–1.19)	0.005
CAD								
Age[a]	1.14 (1.08–1.21)	< 0.001	1.17 (1.08–1.27)	< 0.001	1.13 (1.07–1.20)	< 0.001	1.15 (1.08–1.22)	< 0.001
HDL	0.93 (0.89–0.97)	0.001	0.95 (0.90–1.00)	0.056	0.94 (0.90–0.99)	0.013	0.94 (0.90–0.99)	0.012
IgG aHDL[a]			1.61 (1.21–2.14)	0.001				
IgG aApoA-I[a]					1.16 (1.02–1.32)	0.028		
IgG aPON1[a]							1.11 (1.02–1.20)	0.015

Abbreviations: $\widehat{OR}$, odds ratio estimate; aApoA-I, anti-apolipoprotein A-I antibodies; aHDL, anti-HDL antibodies; aPON1, anti-paraoxonase 1 antibodies; CAD, coronary artery disease; CI, confidence interval; HDL, high-density lipoprotein; IgG, immunoglobulin G; IS, ischaemic stroke; LDL, low-density lipoprotein; RM, reference model.

[a]Age, IgG aHDL, aApoA-I and aPONI1 antibodies' odds ratios were estimated for each increase of 1 year, 10%, 0.04 ug/mL and 10%, respectively.

and non-events (54.2%, 95% CI, 21.5–77.1 and 80.4%, 95% CI, 54.5–92.9, respectively) and IDI events and non-events (0.148, 95% CI, 0.063–0.245 and 0.134, 95% CI, 0.066–0.230, respectively) (►**Table 5**). However, for patients with CAD adding IgG aApoA-I antibodies to the reference model resulted in a negligible change of NRI events and non-events and IDI events and non-events (►**Table 5**). For both groups of patients, the change in identifying those with an increased CV risk after adding IgG aPON1 to the reference model was also negligible as reflected by the low values of NRI events and non-events and IDI events and non-events (►**Table 5**).

Isolation of IgG aHDL Antibodies by Immunoaffinity Chromatography

►**Supplementary Fig. S1** (available in the online version) shows a typical elution profile of IgG aHDL from patient serum. All the collected fractions were tested for the presence of IgG aHDL (►**Supplementary Fig. S2A**, available in the online version). The recovery of IgG aHDL from the column was greater than 90% and the purified IgG aHDL retained the ability to bind to human HDL (►**Supplementary Fig. S2B**, available in the online version).

IgG aHDL Inhibition of PON1 Activity In Vitro

IgG aHDL purified from patients inhibited PON1a in a dose-dependent fashion from 12 to 38% ($p < 0.01$, ►**Fig. 2A**). The non-specific human IgG used as control did not influence PON1a.

IgG aHDL Antibodies Abolish the Protecting Effect of HDL on Endothelial Activation

Unstimulated HUVECs do not express VCAM-1 but they express it after exposure to an inflammatory stimulus such as TNF-α; indeed, we observed a 250.40 ± 77.79% up-regulation of VCAM-1 expression as compared with baseline. The pro-inflammatory effect of TNF-α was prevented by the pre-incubation of HUVECs with HDL (69.97 ± 42.79% VCAM-1 expression), which led to a marked reduction of 72% in the subsequent expression of VCAM-1 ($p < 0.001$). In contrast, the addition of purified IgG aHDL to the culture system increased VCAM-1 expression to 175.80 ± 27.43% and abrogated the inhibitory effect of HDL on VCAM-1 expression by more than 126% ($p < 0.001$). The incubation of HDL with non-specific human IgG did not abolish the anti-inflammatory effect of HDL. Moreover, the addition of recombinant human IgG aApoA-I prevented the protective effect of HDL on VCAM-1 expression by 112% (VCAM-1 expression, 156.60 ± 32.36%; $p = 0.02$) (►**Fig. 2B**). The presence of IgG aHDL antibodies alone or with TNF-α did not modify the expression of VCAM-1 on HUVECs.

Discussion

The novel and important findings of our study is the detection of IgG aHDL antibodies, in particular, aPON1 antibodies, in non-autoimmune patients and the confirmation of aApoA-I in patients with stable chronic IS and CAD. In addition, this is the first study to simultaneously determine antibodies against HDL (IgG and IgM) in general and against different HDL components (ApoA-I, PON1, ApoA-II, ApoC-I) in particular.

In previous reports, IgG aHDL, aApoA-I and aPON1 were associated with decreased PON1a, endothelial activation, increased disease-related CV damage and activity in patients with autoimmune diseases[10–14,16] and were considered an early biomarker of premature atherosclerosis in SLE[15]; at

Table 5 Improvement from adding IgG aHDL, aApoA-I or aPON1 antibodies to a reference model in patients with atherosclerosis-associated clinical events

Variable	Reference model (RM)	RM + IgG aHDL	RM + IgG aApoA-I	RM + IgG aPON1
IS				
Goodness-of-fit (Hosmer–Lemeshow test)	0.106	0.885	0.998	0.871
AUC	0.79 (0.63–0.85)	0.89 (0.81–0.93)	0.92 (0.85–0.96)	0.83 (0.72–0.89)
p-Value (AUC difference)		= 0.006	< 0.001	= 0.063
cfNRI, % (CI)				
cfNRI events		45.8 (16.2–71.4)	54.2 (21.5–77.1)	4.2 (–14.6 to 40.5)
p-Value		< 0.001	< 0.001	= 0.562
cfNRI non-events		73.1 (45.6–87.8)	80.4 (54.5–92.9)	34.6 (9.0–66.7)
p-Value		< 0.001	< 0.001	= 0.008
IDI, (CI)				
IDI events		0.115 (0.058–0.230)	0.148 (0.063–0.245)	0.047 (0.009–0.117)
IDI non-events		0.114 (0.053–0.211)	0.134 (0.066–0.230)	0.044 (0.008–0.103)
IDI		0.229 (0.117–0.421)	0.282 (0.135–0.462)	0.091 (0.016–0.214)
p-Value		< 0.001	< 0.001	< 0.001
CAD				
Goodness-of-fit (Hosmer–Lemeshow test)	0.978	0.758	0.999	0.740
AUC	0.90 (0.82–0.94)	0.96 (0.90–0.98)	0.92 (0.87–0.97)	0.92 (0.85–0.95)
p-Value (AUC difference)		= 0.021	= 0.160	= 0.310
cfNRI, % (CI)				
cfNRI events		65.2 (16.3–85.7)	17.4 (–11.8 to 62.8)	11.1 (–14.9 to 47.6)
p-Value		< 0.001	= 0.120	= 0.453
cfNRI non-events		73.6 (25.0–88.9)	19.2 (–27.3 to 71.4)	39.6 (–3.9 to 76.1)
p-Value		< 0.001	= 0.087	= 0.070
IDI, (CI)				
IDI events		0.101 (0.044–0.190)	0.030 (0.000–0.087)	0.041 (0.006–0.105)
IDI non-events		0.086 (0.041–0.163)	0.026 (0.003–0.076)	0.030 (0.004–0.080)
IDI		0.187 (0.086–0.346)	0.056 (0.003–0.154)	0.071 (0.011–0.186)
p-Value		< 0.001	= 0.080	= 0.003

Abbreviations: aApoA-I, anti-apolipoprotein A-I antibodies; aHDL, anti-HDL antibodies; aPON1, anti-paraoxonase 1 antibodies; AUC, area under the receiver operating characteristic curve; CAD, coronary artery disease; cfNRI, category-free net reclassification improvement; CI, confidence interval; HDL, high-density lipoprotein; IDI, integrated discrimination improvement; IgG, immunoglobulin G; IS, ischaemic stroke.

variance, IgG aApoA-I predicted a poorer outcome in patients with acute IS,[18] were associated with CVD in the general population[19] being linked to plaque vulnerability[20,28] and bore prognostic relevance in myocardial infarction.[21,29]

This study also measured IgM aHDL antibodies for the first time, although both patient groups presented non-significant lower average levels than controls as opposed to what we observed for IgG aHDL. Additional average levels of IgM aHDL antibodies were much lower than IgG aHDL levels in the three groups, which is in line with the concept that upon chronic (repeated) antigen exposure, the IgM response is usually absent or relatively low as compared with the secondary antibody response. We cannot exclude that IgG antibodies have greater affinities than IgM antibodies and often out-compete them for antigens, making it difficult to reliably measure IgM levels.

Taken together, our data suggest that aHDL represents a new family of auto-antibodies, in a similar fashion to the anti-nuclear antibodies (ANA), which are present in many autoimmune diseases. The strong correlation between IgG aHDL, aApoA-I and aPON1 titers suggests that ApoA-I and PON1 might be the key targets, rather the lack of a positive titer of IgG aApoA-II and IgG aApoC-I across the two study groups rules against ApoA-II and ApoC-I as relevant (auto)-antigens within the HDL complex.

PON1 is the enzyme that accounts for most of the anti-oxidant activity of HDL against LDL and a reduced activity favours CVD[30] and cerebrovascular disease[31]; in line with

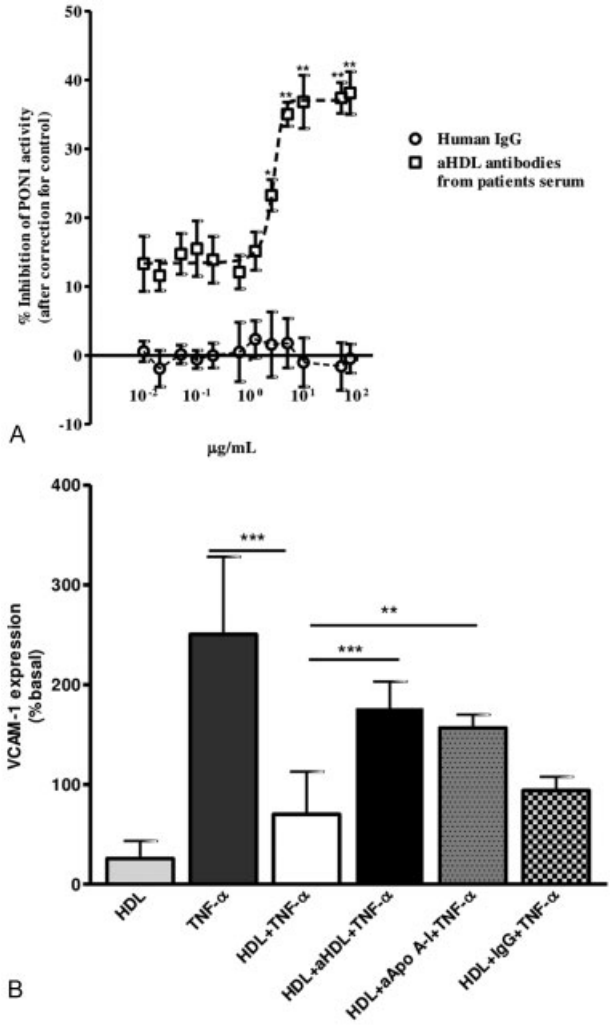

Fig. 2 Biologic activity of the anti-high-density lipoprotein (aHDL) antibodies isolated from serum patients with atherosclerosis-associated clinical events. (**A**) In vitro inhibition of paraoxonase-1 (PON1) activity by aHDL antibodies isolated from serum patients with atherosclerosis-associated clinical events (ischaemic stroke [IS] and coronary artery disease [CAD]). Human immunoglobulin G (IgG) was used as control and 0% effect is the PON1 activity at basal conditions (HDL, 100 µg/mL). Differences between means were evaluated using Bonferroni's multiple comparison test. $^{**}p$-Value of < 0.01 with reference to IgG at the same concentration. (**B**) Effect of aHDL antibodies isolated from patients serum on vascular cell adhesion molecules 1 (VCAM-1) expression in human umbilical vein endothelial cells (HUVECs) exposed to tumour necrosis factor-alpha (TNF-α). Confluent cultures HUVECs were incubated in basal condition (0% effect), human HDL (1.0 mg/mL), TNF-α (10 ng/mL), HDL + TNF-α alone or with aHDL antibodies (50 µg/mL) isolated from serum patients with atherosclerosis-associated clinical events or a non-specific human IgG (50µg/mL) or commercial human anti-apolipoprotein A-I (ApoA-I) monoclonal antibody (30 µg/mL). Bars show the means ± standard deviation (SD). Differences between means were evaluated using a Kruskal–Wallis test. $^{***}p$-Value of < 0.001 and $^{**}p = 0.002$ with reference to HDL + TNF-α.

these studies, our CAD and IS patients showed a 50 and 35% decrease of PON1a, respectively. Our regression model demonstrates that the greater than the cut-off of positivity of IgG aHDL, aApoA-I and aPON1 were significant predictors of the impaired PON1a with respective decrements of 17.63, 31.82 and 25.77%. Furthermore, our in vitro experiments showed that the IgG aHDL isolated from patients inhibited PON1a in a dose-dependent fashion. This may represent a further mechanism by which IgG aApoA-I and aPON1 may contribute to the pro-oxidative state which is characteristic of atherosclerosis.[32]

VCAM-1 and ICAM-1 mediate the recruitment of circulating leukocytes to the vessel wall and are important factors in the early inflammatory and immune activation phases of atherogenesis. Opinions vary when it comes to the clinical relevance of plasma adhesion molecules; in healthy individuals, increased ICAM-1 is a significant predictor of future CAD events, whereas VCAM-1 is a significant predictor of future coronary events only in a sub-group of patients with pre-existing CAD.[33–36] In patients with IS, a plasma concentration of VCAM-1 is related to an increased risk of recurrent stoke.[37] Indeed, both molecules were increased in our patient groups: our regression models show that IgG aHDL, aApoA-I or aPON1 titers greater than the cut-off of positivity were significant predictors of VCAM-1 increase, whereas only IgG aApoA-I and aPON1 antibodies titers higher than the cut-off of positivity, independently predicted ICAM-1 increase.

Our validation experiments in vitro showed that IgG aHDL suppressed the HDL inhibition of VCAM-1 expression induced by TNF-α in HUVECs and therefore monocyte adhesion to endothelial cells, which preludes their transmigration within the intima of the arterial vessels.[38,39]

Given these direct effects of IgG aHDL, we investigated whether this family of antibodies were better predictors of IS or CAD than the traditional CV risk factors. The incremental value of adding separately each antibody titer to a traditional CV risk factors model was shown by the increased ORs of having IS or CAD, obtained for aHDL, aApoA-I and aPON1.

The addition of each antibody to the model results in a significant change in the goodness of fit and in the AUCs between the reference and the new models including the antibodies. However, a good discriminative ability of the models to predict between patients with IS or CAD and healthy subjects was only observed with the addition of aHDL and aApoA-I in patients with IS and the addition of aHDL in patients with CAD. These additions yielded a correct re-classification which further improved the NRI and IDI. This was not observed with the addition of aApoA-I antibodies for patients with CAD or the addition of aPON1 antibodies in both groups of patients. In other words, the addition of IgG aHDL and aApoA-I to the model provides a more accurate diagnosis of IS and the addition of IgG aHDL antibodies contributes to a more accurate diagnosis of CAD.

Our study has some limitations: (1) poor accrual of participants leading to the age difference between patients and controls that could partially confound relations, although our statistical models accounted for this; (2) the cross-sectional design that does not inform on causality supported; however, this was accounted for by our in vitro data; and (3) in the analyses regarding the incremental prognostic value of the antibodies, the reference model was adjusted for the traditional CV risk factor; however, we could not use the CVD risk scoring system.

The presence of either IgM or IgG antibodies towards HDL components may allow the formation of IgM/IgG aHDL/HDL complex and their removal: this may partly explain why many patients have low levels of HDL and why others remain at risk for clinical events despite having normal HDL levels. On that note, these antibodies may explain the recent failure of drugs meant to increase HDL to improve clinical outcomes.[24]

In clinical practice and in clinical trials, quantification of total HDL is still a test of choice because of ease of determination, reproducibility and affordability: unfortunately, the measurement of HDL does not inform on its functionality and tests assessing the latter do not currently exist, but measurement of IgG against HDL components may provide indirect information.

In conclusion, IgG antibodies against HDL components are a new family of auto-antibodies that interfere with some anti-atherogenic functions of HDL contributing another piece to the mosaic where atherosclerosis is depicted as an autoimmune disease. Prospective studies are required to assess the prognostic value of anti-HDL antibodies and eventually re-define the treatment strategies having HDL as their target.

What is known about this topic?

- IgG aHDL, aApoA-I and aPON1 antibodies are present in autoimmune diseases in association with decreased PON1 activity, endothelial activation, disease activity and cardiovascular damage.
- IgG aApoA-I antibodies relate to top atherosclerotic plaque vulnerability with acute coronary syndrome, have negative prognostic value in myocardial infarction and predict worse post-stroke outcomes.
- IgG aApoA-I antibodies have been shown to hamper the protective effect of increase in HDL, suggesting an explanation for the recent failure of drugs meant to increase HDL to improve clinical outcomes.

What does this paper add?

- IgG aHDL, aApoA-I and aPON1 antibodies represent a new family of auto-antibodies present in IS and CAD.
- IgG aHDL and aApoA-I antibodies provide greater diagnostic accuracy for CAD and IS.
- IgG aHDL, aApoA-I and aPON1 antibodies interfere with some anti-atherogenic functions of HDL.
- Testing for this new auto-antibody family might be an indirect way of assessing HDL functionality.

Funding
This work was funded by a grant from AstraZeneca Foundation Portugal, by www.fondazioneaps.org, Italy and by Portuguese Foundation for Science and Technology (FCT) via the post-doctoral SFRH/BPD/112411/2015 and iNOVA4Health - UID/Multi/04462/2013, and co-funded by FEDER under the PT2020 Partnership Agreement.

Conflict of Interest
None.

Acknowledgement
The authors are grateful to Dr. Michel Kranendonk for his collaboration in measuring TAC.

References

1 Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. Am J Med 1977;62(05): 707–714
2 Baigent C, Keech A, Kearney PM, et al; Cholesterol Treatment Trialists' (CTT) Collaborators. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet 2005;366(9493):1267–1278
3 Besler C, Lüscher TF, Landmesser U. Molecular mechanisms of vascular effects of high-density lipoprotein: alterations in cardiovascular disease. EMBO Mol Med 2012;4(04):251–268
4 Nicholls SJ, Tuzcu EM, Sipahi I, et al. Relationship between atheroma regression and change in lumen size after infusion of apolipoprotein A-I Milano. J Am Coll Cardiol 2006;47(05):992–997
5 Navab M, Hama SY, Hough GP, Subbanagounder G, Reddy ST, Fogelman AM. A cell-free assay for detecting HDL that is dysfunctional in preventing the formation of or inactivating oxidized phospholipids. J Lipid Res 2001;42(08):1308–1317
6 Schwartz GG, Olsson AG, Abt M, et al; dal-OUTCOMES Investigators. Effects of dalcetrapib in patients with a recent acute coronary syndrome. N Engl J Med 2012;367(22):2089–2099
7 Cannon CP, Shah S, Dansky HM, et al; Determining the Efficacy and Tolerability Investigators. Safety of anacetrapib in patients with or at high risk for coronary heart disease. N Engl J Med 2010; 363(25):2406–2415
8 Boden WE, Probstfield JL, Anderson T, et al; AIM-HIGH Investigators. Niacin in patients with low HDL cholesterol levels receiving intensive statin therapy. N Engl J Med 2011;365(24): 2255–2267
9 HPS2-THRIVE Collaborative Group, Landray MJ, Haynes R, et al. Effects of extended-release niacin with laropiprant in high-risk patients. N Engl J Med 2014;371:203–312
10 Delgado Alves J, Kumar S, Isenberg DA. Cross-reactivity between anti-cardiolipin, anti-high-density lipoprotein and anti-apolipoprotein A-I IgG antibodies in patients with systemic lupus erythematosus and primary antiphospholipid syndrome. Rheumatology (Oxford) 2003;42(07):893–899
11 Batuca JR, Ames PR, Isenberg DA, Alves JD. Antibodies toward high-density lipoprotein components inhibit paraoxonase activity in patients with systemic lupus erythematosus. Ann N Y Acad Sci 2007;1108:137–146
12 Batuca JR, Ames PR, Amaral M, Favas C, Isenberg DA, Delgado Alves J. Anti-atherogenic and anti-inflammatory properties of high-density lipoprotein are affected by specific antibodies in systemic lupus erythematosus. Rheumatology (Oxford) 2009;48(01):26–31
13 Ames PR, Matsuura E, Batuca JR, et al. High-density lipoprotein inversely relates to its specific autoantibody favoring oxidation in thrombotic primary antiphospholipid syndrome. Lupus 2010;19 (06):711–716
14 Rodríguez-Carrio J, Alperi-López M, López-Mejías R, et al. Antibodies to paraoxonase 1 are associated with oxidant status and endothelial activation in rheumatoid arthritis. Clin Sci (Lond) 2016;130(21):1889–1899
15 López P, Rodríguez-Carrio J, Martínez-Zapico A, et al. Serum levels of anti-PON1 and anti-HDL antibodies as potential biomarkers of premature atherosclerosis in systemic lupus erythematosus. Thromb Haemost 2017;117(11):2194–2206

16 Oku K, Shibata U, Batuca J, et al. Autoantibodies against high density lipoprotein-associated proteins are related to elevated oxidized low density lipoprotein levels in antiphospholipid syndrome [abstract]. Arthritis Rheumatol 2016;68(Suppl 10):2706-2707
17 Vuilleumier N, Reber G, James R, et al. Presence of autoantibodies to apolipoprotein A-1 in patients with acute coronary syndrome further links autoimmunity to cardiovascular disease. J Autoimmun 2004;23(04):353–360
18 Carbone F, Satta N, Montecucco F, et al. Anti-ApoA-1 IgG serum levels predict worse poststroke outcomes. Eur J Clin Invest 2016; 46(09):805–817
19 Antiochos P, Marques-Vidal P, Virzi J, et al. Association between anti-apolipoprotein A-1 antibodies and cardiovascular disease in the general population. Results from the CoLaus study. Thromb Haemost 2016;116(04):764–771
20 Montecucco F, Vuilleumier N, Pagano S, et al. Anti-apolipoprotein A-1 auto-antibodies are active mediators of atherosclerotic plaque vulnerability. Eur Heart J 2011;32(04):412–421
21 Vuilleumier N, Rossier MF, Pagano S, et al. Anti-apolipoprotein A-1 IgG as an independent cardiovascular prognostic marker affecting basal heart rate in myocardial infarction. Eur Heart J 2010;31(07):815–823
22 Batuca JR, Ferreira I, Favas C, Amaral M, Delgado Alves J. Antibodies towards HDL components in type 2 diabetes patients are associated with modifications in the anti-atherogenic properties of HDL. Diabetologia 2011;54:S277–S278
23 El-Lebedy D, Rasheed E, Kafoury M, Abd-El Haleem D, Awadallah E, Ashmawy I. Anti-apolipoprotein A-1 autoantibodies as risk biomarker for cardiovascular diseases in type 2 diabetes mellitus. J Diabetes Complications 2016;30(04):580–585
24 Batuca JR, Amaral MC, Favas C, et al. Extended-release niacin increases anti-apolipoprotein A-I antibodies that block the antioxidant effect of high-density lipoprotein-cholesterol: the EXPLORE clinical trial. Br J Clin Pharmacol 2017;83(05):1002–1010
25 Giovannoni G, Land JM, Keir G, Thompson EJ, Heales SJ. Adaptation of the nitrate reductase and Griess reaction methods for the measurement of serum nitrate plus nitrite levels. Ann Clin Biochem 1997;34(Pt 2):193–198
26 Hosmer DW Jr, Lemeshow S, Sturdivant RX. Applied Logistic Regression. 3rd ed. New Jersey, United States: John Wiley & Sons, Inc.; 2013
27 Pencina MJ, D'Agostino RB Sr, Steyerberg EW. Extensions of net reclassification improvement calculations to measure usefulness of new biomarkers. Stat Med 2011;30(01):11–21
28 Montecucco F, Braunersreuther V, Burger F, et al. Anti-apoA-1 auto-antibodies increase mouse atherosclerotic plaque vulnerability, myocardial necrosis and mortality triggering TLR2 and TLR4. Thromb Haemost 2015;114(02):410–422
29 Roux-Lombard P, Pagano S, Montecucco F, Satta N, Vuilleumier N. Auto-antibodies as emergent prognostic markers and possible mediators of ischemic cardiovascular diseases. Clin Rev Allergy Immunol 2013;44(01):84–97
30 Abelló D, Sancho E, Camps J, Joven J. Exploring the role of paraoxonases in the pathogenesis of coronary artery disease: a systematic review. Int J Mol Sci 2014;15(11):20997–21010
31 Shenhar-Tsarfaty S, Waiskopf N, Ofek K, et al. Atherosclerosis and arteriosclerosis parameters in stroke patients associate with paraoxonase polymorphism and esterase activities. Eur J Neurol 2013;20(06):891–898
32 Witztum JL, Steinberg D. The oxidative modification hypothesis of atherosclerosis: does it hold for humans? Trends Cardiovasc Med 2001;11(3-4):93–102
33 Ridker PM, Hennekens CH, Roitman-Johnson B, Stampfer MJ, Allen J. Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. Lancet 1998;351(9096):88–92
34 Blankenberg S, Rupprecht HJ, Bickel C, et al. Circulating cell adhesion molecules and death in patients with coronary artery disease. Circulation 2001;104(12):1336–1342
35 Mulvihill NT, Foley JB, Murphy RT, Curtin R, Crean PA, Walsh M. Risk stratification in unstable angina and non-Q wave myocardial infarction using soluble cell adhesion molecules. Heart 2001;85 (06):623–627
36 Postadzhiyan AS, Tzontcheva AV, Kehayov I, Finkov B. Circulating soluble adhesion molecules ICAM-1 and VCAM-1 and their association with clinical outcome, troponin T and C-reactive protein in patients with acute coronary syndromes. Clin Biochem 2008;41 (03):126–133
37 Castillo J, Alvarez-Sabín J, Martínez-Vila E, Montaner J, Sobrino T, Vivancos J; MITICO Study Investigators. Inflammation markers and prediction of post-stroke vascular disease recurrence: the MITICO study. J Neurol 2009;256(02):217–224
38 Cockerill GW, Rye KA, Gamble JR, Vadas MA, Barter PJ. High-density lipoproteins inhibit cytokine-induced expression of endothelial cell adhesion molecules. Arterioscler Thromb Vasc Biol 1995;15(11):1987–1994
39 Baker PW, Rye KA, Gamble JR, Vadas MA, Barter PJ. Ability of reconstituted high density lipoproteins to inhibit cytokine-induced expression of vascular cell adhesion molecule-1 in human umbilical vein endothelial cells. J Lipid Res 1999;40(02): 345–353