APOA1 oxidation is associated to dysfunctional high-density lipoproteins in human abdominal aortic aneurysm

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ABSTRACT

Background: High-density lipoproteins (HDL) are a complex mixture of lipids and proteins with vasculoprotective properties. However, HDL components could suffer post-translational modifications (PTMs) under pathological conditions, leading to dysfunctional HDL. We studied whether HDL are modified in abdominal aortic aneurysm (AAA) and the effect on HDL functionality.

Methods: HDL were isolated by ultracentrifugation from AAA tissue (HDL-T) and from plasma of healthy volunteers and then incubated with AAA tissue-conditioned medium (HDL-AAA CM). PTMs from these particles were characterized using Comet-PTM. The ability of HDL-AAA CM for promoting cholesterol efflux was determined ex vivo and in vivo by using J774A.1 [3H]cholesterol-labeled mouse macrophages and after injecting [3H]cholesterol-labeled mouse macrophages and HDL into the peritoneal cavity of wild-type C57BL/6 mice, respectively. Trp50 and Trp108 oxidized forms of APOA1 in HDL incubated with conditioned-medium of activated neutrophils and in plasma of AAA patients and controls were measured by targeted parallel reaction monitoring.

Findings: Oxidation was the most prevalent PTM in apolipoproteins, particularly in APOA1. Trp50 and Trp108 in APOA1 were the residues most clearly affected by oxidation in HDL-T and in HDL-AAA CM, when compared to their controls. In addition, cholesterol efflux was decreased in macrophages incubated with HDL-AAA CM in vitro and a decreased macrophage-to-serum reverse cholesterol transport was also observed in mice injected with HDL-AAA CM. Finally, both oxidized Trp50 and Trp108 forms of APOA1 were increased in HDL incubated with conditioned-medium of activated neutrophils and in plasma of AAA patients in relation to controls.

Interpretation: Oxidative modifications of HDL present in AAA tissue and plasma were closely associated with the loss of vasculoprotective properties of HDL in AAA.

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1. Introduction

Abdominal aortic aneurysm (AAA) is a permanent focal dilation of the abdominal aorta [1]. AAA is characterized by the presence of an Intra Luminal Thrombus (ILT), mainly composed by red blood cells (RBCs), neutrophils and platelets and an aortic wall with few vascular smooth muscle cells (VSMC) in the remaining media, along with immune cell infiltrates, fibroblasts and neovessels in the adventitia [2]. Main mechanisms of AAA include proteolysis, oxidative stress and...
A protective role of these drugs in preventing AAA growth and rupture [11–13]. Regardless of the potential contribution of lipids on AAA progression, it is well accepted that low levels of high-density lipoprotein (HDL) cholesterol are associated to both AAA presence and evolution [14,15]. Moreover, raising HDL inhibits AAA formation and progression in experimental models [16,17]. These data support the notion that increasing HDL levels could have a beneficial effect to prevent AAA development. However, it is nowadays being accepted that the quality of HDL is the relevant factor for its vasculoprotective activities [18]. Cardiovascular protective functions of HDL include reverse cholesterol transport (RCT), as well as antioxidant, anti-inflammatory, antiapoptotic and antithrombotic effects. Regarding AAA, we previously demonstrated that HDL particle carries lower alpha-1 antitrypsin and higher myeloperoxidase (MPO) proteins, leading to HDL with decreased antiapoptotic and antioxidant properties [19,20]. Moreover, systemic paraoxonase-1 activity, a surrogate marker of HDL (antioxidant) functionality, is decreased in AAA patients [21]. More recently, we have shown that AAA patients display impaired HDL cholesterol efflux capacity, the first step of RCT [22]. All these data suggest that HDL could be dysfunctional due to impaired concentration or function of specific molecular components of the particle in AAA. In this respect, APOA1, the main protein constituent of HDL, could be oxidatively modified in atherothrombosis, leading to dysfunctional HDL [23,24]. As we previously observed that human AAA tissue (ILT and wall) was characterized by increased oxidation capacity [20] and by high APOA1 immunostaining [15] we thus hypothesized that human AAA tissue could be a privileged site for searching APOA1/HDL oxidative modifications.

In the present paper, we used state of the art proteomics approaches very recently developed [25] to characterize the post-translational modifications (PTMs) on HDL and the changes suffered by the particles in the context of AAA from a completely hypothesis-free perspective. We analyzed PTM alterations in HDL isolated from tissue of AAA patients. We then studied whether these alterations could be reproduced in plasma-isolated HDL incubated with AAA tissue-conditioned medium (HDL-AAA CM) and whether they affected HDL functionality. The ability of HDL-AAA CM for promoting macrophage cholesterol efflux was determined ex vivo and in vivo by using mouse macrophages and after injecting them along with HDL into the peritoneal cavity of mice. Finally, we explored the potential role of these modifications as circulating biomarkers of AAA.

2. Material and methods

2.1. Data statement

Datasets have been deposited in PeptideAtlas (dataset identifier PASS01347).

2.2. Tissue and tissue-conditioned medium

Tissue samples (ILT and wall) were collected during surgical repair from AAA patients and kept at −80°C until further processed. Ethical committee advice and patient informed consent were obtained (RESAA and AMETHYST studies, CPP Paris–Cochin n° 2095, 1930 and 1931, Inserm Institutional Review Board, IRB0000388). Healthy abdominal aortas were sampled from deceased organ donors with the authorization of the French Biomedicine Agency (PBS 09–007). Tissue-conditioned medium was obtained as described [26].

2.3. HDL isolation

Tissue [100 mg of AAA intraluminal thrombus (HDL-ILT), AAA pathological wall (HDL-PW) and healthy aortic wall (HDL-HW)] was homogenized and care was taken throughout homogenization to maintain a low temperature by keeping the homogenization vessel submerged within liquid N₂. After that, lysates were resuspended in buffer
(BR20x, FIP, CIP, PMSF, 1 mM EDTA, Tris-HCl pH 8.5). Samples were vortexed every 15 min for a total of 4 times and kept on ice. The supernatant was obtained after centrifugation at 12,000 rpm for 15 min. Then, lipoproteins were isolated from these tissue supernatants or from EDTA plasma samples of healthy volunteers by ultracentrifugation as described [27]. Plasma density was adjusted to \( \rho = 1.063 \) g/mL with KBr and the sample was overlaid with KBr saline solution (\( \rho = 1.063 \) g/mL). Samples were ultracentrifuged at 53000 rpm for 18 h at 4 °C in a TLA-100 rotor (Beckman). After centrifugation, the upper, apolipoprotein-B-containing fraction was recovered and stored. The bottom fraction, containing whole HDL, was adjusted to 1.25 g/mL with KBr and overlaid with KBr saline solution (\( \rho = 1.22 \) g/mL). The HDL-containing fraction was then ultracentrifuged at 63000 rpm for 24 h at 4 °C. After this step, HDL, in the top layer, were recovered as a single band and were extensively rinsed with saline and concentrated using a centrifugal concentrating device (cutoff 10 kDa). All HDL samples were desalted by centrifugation and 3 washes with saline. In some cases, HDL isolated from plasma were incubated with AAA ILT-conditioned medium (HDL-ILT CM) or with PBS (HDL-control) for 4 h (1:1 proportion), and then reisolated by ultracentrifugation. The protein composition of these particles is shown in Supp. Figs. 1 and 2. Finally, we isolated neutrophils from blood of healthy volunteers and stimulated 5x10^6 cells with phorbol 12-myristate 13-acetate (PMA, 20 nM) for 4 h (1:1 proportion), and then reisolated by ultracentrifugation. Patients were considered as diabetics if they were under treatment (supervised diet, hypoglycemic oral medication, and insulin) or a basal glycemia >120 mg/dL and/or glycosylated hemoglobin >6.5%. We defined hypertension as SBP >140 mmHg and/or DBP >90 mmHg measured during the examination (after the participant had been sitting for at least 30 min) or if the participant was already taking hypotensive medication. Dyslipidemia was diagnosed as the presence of at least 1 of the following characteristics: total cholesterol >5.2 mmol/L; LDL-cholesterol >3.4 mmol/L, or triglycerides >2.3 mmol/L; or the participant was already taking statins or fibrates medication. Smoking was defined as current smokers or nonsmokers (including ex-smokers, those who stopped smoking at least 6 months before the inclusion in the study), with a smoker defined by the history of smoking (>10 cigarettes per day >1 year). Controls with no AAA (n = 28) were recruited from a screening program for AAA between 65-year-old men. The absence of AAA was confirmed with a physical examination and an ultrasound scan (aortic size <3 cm).

The ethical committee on human research at IIS, Fundacion Jimenez Diaz, Autonoma University (Madrid, Spain) approved the study, which was performed in accord with the principles outlined in the Declaration of Helsinki, and all participants gave written informed consent.

2.5. Proteomics

2.5.1. Sample preparation

Samples (200 μg of total proteins of HDL) were digested overnight at 37 °C in FASP filters with trypsin (Promega, Madison, WI, USA) at an enzyme/protein ratio of 1:50. The resulting peptides were desalted on C18 Oasis cartridges (Waters Corporation, Milford, MA, USA) using 50% acetonitrile (ACN) (v/v) in 0.1% trifluoroacetic acid (v/v) as eluent, and vacuum dried. The peptides were then subjected to isobaric TMT labelling following manufacturer’s instructions. HDL samples isolated from tissue (healthy wall, AAA pathological wall and ILT) were analyzed in three biological replicates. An internal control was constructed by pooling the three samples from the healthy wall and was used as reference to express relative quantification values. HDL isolated from plasma of healthy volunteers were incubated with ILT-conditioned media or PBS. An internal control was constructed by pooling HDL incubated with PBS and was used as reference to express relative quantification values.

2.5.2. LC-MS analysis

Peptide samples were analyzed using an Easy nano-flow HPLC system (Thermo Fisher Scientific, Bremen, Germany) coupled via a nanoelectrospray ion source (Thermo Fisher Scientific) to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). C18-based reverse phase separation was used with a 2-cm trap column and a 50-cm analytical column (EASY column, Thermo Fisher Scientific). For the study of PTMs, peptides were loaded in buffer A (0.1% formic acid (v/v)) and eluted with a 180-min linear gradient of buffer B (80% acetonitrile, 0.1% formic acid (v/v)) at 200 nL/min. Mass spectra were acquired in a data-dependent manner, with an automatic switch between MS and MS/MS using a top 15 method. MS spectra were acquired in the Orbitrap analyzer with a mass range of 400–1500 m/z and 60,000 resolution. HCD fragmentation was performed at 27 normalized collision energy and MS/MS spectra were analyzed at 60,000 resolution in the Orbitrap. For the parallel reaction monitoring assays, peptides were loaded in buffer A and eluted with a 90 min linear gradient of buffer B at 200 nL/min. MS spectra were acquired in the Orbitrap analyzer with a mass range of 400–1500 m/z and 15,000 resolution followed by data-independent MS/MS spectra of selected precursor ions. HCD fragmentation was performed at 27 of normalized collision energy and MS/MS spectra were analyzed at 60,000 resolution in the Orbitrap. Data analysis was performed with Xcalibur 2.2 (Thermo Scientific).

2.5.3. Database search

For protein identification, searches were performed with Comet release 2016.01 using 5 ppm precursor ion tolerance, Met oxidation as a dynamic modification, trypsin digestion with 1 missed cleavage (unless otherwise specified) and fixed Cys carbamidomethylation (57.021464 Da) [29,30]. TMT labeling at N-terminal end and Lys was also considered as a fixed modification (229.162932 Da). Fragment ion tolerance was 0.02 bin, 0 mass offset. Precursor tolerance type and isotope error were set to 1. Precursor charge range was 2–4, maximum precursor charge 5 and maximum fragment charge 3. Only y- and b-ions were used for scoring. Peptide identification from MS/MS data was performed using the probability ratio method [31]. False discovery rate (FDR) of peptide identifications were calculated using the refined method [32]; 1% FDR was used as the default criterion for peptide identification.

2.5.4. Comet-PTM

For PTM analysis, open searches using a precursor ion tolerance of 500 Da were performed using Comet-PTM, as described [25]. Comet-
PTM is a novel open search algorithm that calculates the difference between theoretical and experimental precursor mass (ΔMass), and adds up this mass iteratively to each one of the amino acid masses in the peptide sequence, calculating a Xcorr score in each one of the possible modified forms of the peptide. The selected candidate is the modified peptide form that produces the highest Xcorr. Comet-PTM produces the same score than Comet with a preselected modification, with the difference that Comet-PTM identifies all possible modifications within + − 500 Da of precursor ion [25]. The accuracy of Comet-PTM to assign the modification to the correct site has been estimated to be between 80 and 85% using both high-throughput data and synthetic peptides [25].

2.5.5. SHIFTS

SHIFTS (Systematic Hypothesis-free Identification of modifications with controlled FDR based on ultra-Tolerant database Search) was used to identify peaks in the ΔMass distribution, assign PSMs to peaks and calculate the FDR for peptide identification. Further details about SHIFTS are described in [26].

2.5.6. Annotation of modifications

An in-house developed script was used for the semisupervised annotation of peptide modifications as described [25]. Briefly, the script matches ΔMass values against the Unimod database, considering the amino acid modification positions revealed by Comet-PTM as well as the preceding and following residues. For simplicity, artificial modifications introduced during sample preparation and modifications that could not be annotated in Unimod were not considered in further analysis. Met mono-oxidation (+16 Da) is not considered as a PTM.

2.5.7. Peptide quantification and statistical analysis

The quantitative information from TMT reporter intensities was integrated from the spectrum level to the peptide level and then to the protein level on the basis of the WSPP model using the Generic Integration Algorithm (GIA), as described [25,33,34]. The algorithm was modified to incorporate the quantitative values of modified peptides as part of the automated workflow [25]. Hence, quantitative peptide values are referred to the weighted averages of the non-modified peptides from the same protein and therefore are not affected by protein abundance changes [25]. This statistical model describes accurately the error distribution of abundance changes of both non-modified and modified peptides in null hypothesis experiments [25]. Significant abundance changes of modified peptides across the different samples were detected by Student's t-test.

2.6. Immunochemistry

Samples of ILT and wall obtained from AAA and healthy arterial wall were embedded in paraffin, and 4 μm cross-sections were cut. Immunochemistry was performed with antibodies against APOA1 (ab7613, abcam) and the lipid peroxidation marker MDA (ab6463, abcam), as described [35].

2.7. Lipoprotein oxidation

We measured basal HDL conjugated dienes at 234 nm by using a 96-microwell plate for UV detection. HDL was also oxidized with 2.5 μM CuSO4 and the oxidation kinetics was followed by continuous monitoring of conjugated diene formation at 37 °C for 1 h. The lag phase was calculated from the intersection point between the maximal slope of the curve and initial absorbance.

2.8. Cholesterol efflux analyses

Cholesterol efflux capacity to HDL-control or HDL-ILT CM (50 μg/ml APOA1) was determined by using [J774A.1] [3H]-cholesterol-labeled macrophages after loaded with acetylated (ac)-LDL as previously reported [36]. Ten to 12 weeks-old female wild-type C57BL/6 mice received intraperitoneal injections of HDL-control or HDL-ILT CM (100 μg APOA1) together with [3H]-cholesterol-labeled J774 macrophages. Mice were randomized into two groups and the HDL injections were performed blinded by the technician. Cell viability of the macrophage preparations ranged from 85 to 90% and each mouse received an i.p. dose of [3H]-cholesterol-labeled mouse macrophages (0.92 × 10^5 cells/mouse, 0.99 × 10^5 cpm/mouse). Serum [3H]-cholesterol was determined after 3 h of the macrophage injection as described [36]. All mice were kept in a temperature-controlled environment (20 °C) with a 12-h light/dark cycle and maintained on a regular chow diet (Safe, Scientific Animal Food & Engineering; A04C, 3% fat, 2900 kcal/kg). Food and water were provided ad libitum. All animal procedures were conducted in accordance with published regulations and reviewed and approved by the Institutional Animal Care Committee of the Institut de Recerca of the Hospital de la Santa Creu i Sant Pau (ref. 7281).

3. Results

3.1. Characterization of PTMs of HDL-like particles recovered from AAA tissue highlights APOA1 oxidation

To analyze whether HDL are modified in the context of AAA, we decided to recover HDL-like particles from homogenized AAA tissue [ILT (HDL-ILT) and pathological wall (HDL-PW)] and from healthy aortic wall (HDL-HW) by ultracentrifugation.

High-throughput analysis of PTMs using Comet-PTM revealed that one third of peptides from lipid metabolism proteins had modifications that could be assigned to known PTMs, while two-thirds of the peptides were non-modified (Fig. 1A). Oxidation accounted for the majority of modifications in HDL-like particles (Fig. 1A and Supp. Table 1). The majority of PTMs were found in APOA1 (Fig. 1B). Of note, the proportion of modified peptides in APOA1 was higher than in the rest of proteins (Fig. 1C). Oxidative modifications in APOA1 affected mainly Trp residues, while Tyr, Pro, His, Asp, Met and Lys were also affected, although to a lesser extent (Fig. 1D). Quantitative analysis taking as reference the peptides from the same protein that were not modified revealed that the pattern of PTM changes suffered by APOA1 peptides was...
remotely similar in HDL-ILT and HDL-PW in comparison with those of HDL-HW (Fig. 1E). A generalized increase in the abundance of peptides bearing oxidized Trp forms in HDL-ILT and HDL-PW in comparison with HDL-HW was observed (Fig. 1D). The peptides contained mono-, di- and tri-oxidations and also Trp in the form of kynurenine (Fig. 1E). Peptide forms containing the four Trp residues of APOA1 were detected by mass spectrometry; from these, oxidation was found in three of them, corresponding to Trp50, Trp72 and Trp108. This trend was not detected in peptides bearing oxidized forms in other aminoacids (Fig. 1E). Interestingly, we found by immunohistochemistry that APOA1 in ILT and wall of AAA colocalizes with malonaldehyde (MDA), a marker of lipid peroxidation, which was not present in healthy aortic wall (Fig. 1F).

3.2. Incubation of plasmatic HDL with AAA tissue-conditioned medium produces APOA1 oxidation and impacts HDL functionality

Our previous results indicated that HDL-like particles retained in AAA tissue are affected by specific oxidative modifications. We hypothesized that oxidizing agents present in the AAA tissue caused this oxidative damage on HDL and could affect HDL functionality.

In an attempt to reproduce the modifications taking place in the AAA tissue environment, we performed an ex vivo approach preparing HDL isolated from plasma of healthy volunteers and incubating them with tissue-conditioned medium (CM) during 4 h; then, HDL were reisolated by ultracentrifugation and subjected to proteomics analysis following the same approach as above. However, as the limited amount of healthy wall (and then, healthy-CM) to be used in the functional in vivo studies made this task unfeasible, we decided to use HDL incubated with PBS (HDL-control). We firstly confirmed that healthy wall-CM did not produce oxidative HDL modifications when compared to HDL-control (Supp. Fig. 3A). Analysis of HDL incubated with ILT-conditioned medium (HDL-ILT CM) showed that the basal proportions of PTMs were similar to those of HDL-like particles from ILT, being oxidative modifications the most abundant and APOA1 the protein mostly affected by PTM (Fig. 2 A-C and Supp. Table 1). Quantitative analysis confirmed the generalized increase of peptides bearing oxidized Trp forms in the HDL-ILT CM samples in comparison to those from HDL-control (Fig. 2D). In this case, oxidation affected to Trp50 and Trp108 (Fig. 2E). Finally, to analyze if incubation of HDL with ILT CM also produces oxidation at the lipid level, we evaluated the basal HDL conjugated dienes. HDL-ILT CM had significantly increased basal levels of conjugated dienes when compared with those of HDL-control (Fig. 2F). Moreover, we also evaluated the potential of HDL-ILT CM to be further oxidized in an experimental system, in which isolated HDL were exposed to the prooxidant Cu²⁺. Under these conditions, HDL-ILT CM exhibited a significantly shorter lag phase when compared with HDL-control, indicating that extensive oxidation was promoted earlier in the HDL-ILT CM (Fig. 2G).

Then, we used the HDL-ILT CM particles to interrogate the functional consequences that oxidative modifications may produce on HDL. We firstly confirmed that incubation of HDL with healthy wall-CM did not affect the ability of macrophages to act as acceptors of cholesterol ex vivo when compared with HDL-control (Supp. Fig. 3B). In contrast, when HDL-ILT CM particles were added to macrophages, we found that their ability to promote cholesterol efflux was blunted compared with HDL-control (Fig. 3A). Moreover, to study the in vivo effect of HDL-ILT CM, [3H]cholesterol-labeled J774A.1 macrophages were injected together with HDL into the peritoneal cavity of mice. Three hours after injection of the macrophages, [3H]cholesterol in plasma was reduced in the HDL-ILT CM injected group as compared to HDL-control (Fig. 3B).

3.3. Increased oxidized Trp50 and Trp108 of APOA1 in HDL incubated with PMN-conditioned medium and in plasma of patients with AAA

Given the increased abundance of oxidized Trp in APOA1 observed in HDL from AAA tissue or in HDL incubated with AAA-tissue conditioned media, we tested potential cellular sources of ROS involved in these oxidative processes. As it has been previously shown that MPO is involved in APOA1 oxidation [23,24] and we previously showed increased MPO in HDL and PMN of AAA patients [19,28], we hypothesized that oxidants (including MPO) released by activated PMNs could mediate the oxidation of APOA1 in AAA. Then, we incubated HDL isolated from plasma of healthy volunteers with the conditioned medium obtained after activation of neutrophils with PMA; after that, HDL were reisolated by ultracentrifugation.

To test specific APOA1 oxidation, we setup a targeted mass spectrometry assay (Fig. 4A and B) and used it to quantify non-modified and oxidized Trp (Trp8, 50, 72 and 108)–containing peptides in HDL incubated with conditioned medium of PMA-activated PMNs. The oxidized form of Trp8 was not detected in this preparation. We observed an increase of oxidation forms of Trp 50 and Trp 108 in HDL-incubated with PMN-conditioned medium (Fig. 4C). Moreover, oxidized Trp72 was also increased (not shown), as previously observed in atherosclerotic plaques [38] and here, in AAA tissue.

Finally, we hypothesized that oxidized Trp in APOA1 found in tissue could also be present in plasma, where they could be used as circulating surrogate markers of HDL dysfunction. To explore this possibility, we used the same targeted mass spectrometry assay described above. The oxidized forms of Trp8 and Trp72 could not be directly detected in plasma; however, we could accurately monitor the oxidized Trp50 and Trp108 peptide forms in control subjects and AAA patients. The proportions of the oxidized forms of both Trp50 and Trp108 were
significantly increased in plasma of AAA patients as compared to controls (Fig. 4C).

Multivariate logistic regression analysis also revealed that the proportions of each one of the two dioxidized Trp forms were significantly associated to the presence of AAA after adjustment by age (Table 2A). ROC analysis revealed that the proportions of the two dioxidized Trp forms of APOA1 were good predictors of the disease (Table 2B). Moreover, a panel with the two dioxidized Trp forms significantly increased the power to predict AAA over age alone (Table 2B).

4. Discussion

In this work we have used state of the art proteomics approaches to characterize the whole landscape of PTM in HDL and the specific alterations under AAA tissue environment. We have used as models HDL-like particles recovered from AAA tissue and HDL isolated from plasma incubated with AAA tissue-conditioned media. To our knowledge, this is the first time that such a comprehensive, quantitative and hypothesis-free analysis of PTM has been performed in HDL particles. It is interesting to note that the majority of modifications found in HDL are of oxidative nature; although HDL are known to suffer oxidative modifications, the proportion of these modifications with respect to that of unmodified peptides and other potential modifications has never been studied before. Our analysis revealed that the major hallmark of AAA is the oxidative modifications on Trp residues in APOA1. We were able to detect all Trp-containing peptides in APOA1, and those affected by oxidation were found in different oxidation states, including not only mono- and di-oxidation, but also in kynurenine form, reflecting gradual stages of oxidative damage. Other residues, like Tyr and Pro, were also affected but to a lesser extent. Pro residues were presumably hydroxylated. A remarkable finding of this study is that the abundance of oxidative modifications in Trp was consistently increased in HDL-like particles obtained from AAA tissue, a trend that was not detected in other residues. This apparently selective effect of AAA-derived oxidants on Trp residues was confirmed when HDL particles isolated from plasma of healthy individuals were incubated with conditioned media obtained from ILT. All these results suggested that Trp residues in APOA1 are the most sensitive to oxidants present in the human AAA environment. These oxidants were also manifested by the presence of lipid oxidation products whose tissue distribution closely colocalized with that of APOA1, suggesting the presence of oxidized lipids and proteins in HDL particles retained in the AAA tissue.

Previous papers have shown that the oxidative modification of different residues of APOA1 are present in atherosclerotic plaques and are associated to HDL dysfunctionality [23,24]. In this work, by combining immunohistochemical and biochemical approaches in addition to proteomics, we demonstrate for the first time that APOA1/HDL is oxidized within the AAA tissue environment. ILT in human AAA is characterized by the presence of neutrophils and RBC, which are potent sources of oxidative stress through the release of MPO and its potentialization by redox-active Fe^{2+} from free hemoglobin. We previously demonstrated that the levels of MPO are higher in human AAA tissue and tissue-conditioned medium [16]. MPO mainly released by activated neutrophils, catalyzes the reaction between hydrogen peroxide and chloride ions to produce hypochlorous acid as the primary oxidant. Interestingly, MPO is associated to HDL and we showed that HDL from patients with AAA carries higher MPO levels as compared to HDL from controls [19]. Specifically, we have now demonstrated that HDL incubated with conditioned medium of activated neutrophils display oxidative modifications similar to those observed in HDL-like particles from AAA tissue and HDL-ILT CM. Among those oxidative modifications, it has been previously observed that MPO is able to oxidize Trp72 [38], which was confirmed in our *in vitro* study. These data suggest that neutrophil-derivered oxidants may play a relevant role in APOA1/HDL oxidation in AAA tissue. We cannot, however, discard the contribution of other sources of ROS such as red blood cell-derived ferrous iron (highly abundant in AAA) [39] or other enzymatic sources. In these regard, neutrophil extracellular trap-derived enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidases have been shown to oxidize HDL in autoimmune diseases [40].

The best-known HDL’s protective function is their ability to promote cholesterol efflux from cells, including that from macrophages in the arterial wall. In the present study, we show that HDL-ILT CM particles are characterized by decreased cholesterol efflux capacity (CEC). Since Trp residues are key factors regulating the MPO-mediated loss of the cholesterol acceptor activity of APOA1 [41], the oxidative modifications in Trp residues described in this study could partially explain the decreased CEC observed under our experimental conditions, although we cannot discard the contribution of other modifications. To further assess this effect *in vivo*, we used the peritoneal cavity as a surrogate to evaluate the potential impact of HDL-ILT CM on macrophage-to-serum RCT in vivo. Because of the highly turnover of HDL particles in the peritoneal fluid and the highly dynamic nature of the cholesterol efflux assay, we shortened our macrophage-specific RCT assay for directly assessing the local effects of HDL-ILT CM, showing a decreased macrophage-to-serum transport in mice injected with HDL-ILT CM. The importance of effective cholesterol efflux in AAA is due to the fact that cholesterol crystals are present in AAA wall, where they can contribute not only to immune responses but also to SMC death, two main mechanisms underlying AAA progression. Whether therapies aimed at increasing HDL cholesterol efflux could prevent AAA progression deserves further studies. Interestingly, we previously demonstrated that increasing HDL function by using 4DF peptide (which is resistant to APOA1 oxidation) or by overexpressing the major HDL-antioxidant protein (PON-1), was able to decrease experimental AAA formation [15,21].

There is a great interest to find biomarkers of HDL quality and function that can improve risk prediction and target drug treatments. Efforts

### Table 2

Multivariate statistical analysis of Trp50 and Trp108.

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<tr>
<th>A) Plasma levels of dioxidized Trp50 and Trp108 in ApoA1 are independent predictors of AAA</th>
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<tbody>
<tr>
<td>p-Value</td>
</tr>
<tr>
<td>W50diox</td>
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<td>W108diox</td>
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(1) OR for variables expressed in units of standard deviation. Logistic regression was adjusted by age; no other risk factors were found to associate with AAA.

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<tr>
<th>B) Plasma levels of dioxidized Trp50 and Trp108 in ApoA1 improve prediction of AAA over risk factors</th>
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<tbody>
<tr>
<td>p-Value</td>
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<tr>
<td>Age, W50diox and W108diox</td>
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1. Area under the curve obtained by ROC analysis.
2. Statistical significance vs reference value (AUC = 0.5).
to develop reproducible, cost-effective, validated assays that measure the potential functions of HDL are recognized as a major challenge in the cardiovascular field. Growing evidence indicates an inverse relationship between HDL CEC and the incidence of CVD [42]. We have recently shown an impairment of macrophage CEC in ApoB depleted plasma ("plasma HDL") from AAA patients [22]. In addition to the analysis of CEC in plasma HDL, other authors have measured oxidized forms of circulating APOA1 in CVD [38,43]. In general, the authors focused on oxidized forms of APOA1 which negatively regulated HDL functions and that were detected in atherosclerotic plaques. In the largest study performed to date, oxidized APOA1 (Trp72) was assessed in plasma using specific antibodies [38]. In this work, we observed that oxidized forms of Trp in APOA1 were increased within AAA tissue environment, and also in HDL incubated with activated neutrophils, and tested whether these forms could serve to monitor a pathophysiologically relevant process occurring locally during AAA. Although we detected peptides containing Trp72 by targeted mass spectrometry, we were unable to detect their oxidized forms in plasma. However, we could readily detect the oxidized forms of Trp50 and Trp108, finding that their relative proportions are increased in plasma of AAA patients when compared to controls. It is also interesting to note that the oxidized forms of Trp50 and Trp108 were independently associated to AAA. Moreover, the model including the oxidized forms of both Trp50 and Trp108 significantly improved the accuracy of AAA prediction over that provided by known risk factors alone. However, for a potential translation to the clinics, these data must be validated in independent cohorts and novel approaches based on antibodies specifically recognizing the modified peptides should be developed. On the whole, our in vitro and in vivo data support the association of these novel oxidized forms of APOA1 with dysfunctional HDL in AAA patients.

4.1. Limitations

One main limitation of our study is the scarce amount of APOA1 in HDL-like particles that can be obtained from AAA tissue, which makes it infeasible to perform functional in vivo studies. This low level is probably also potentially related to APOA1 oxidation, that promotes APOA1 dissociation [38]. To circumvent this problem, we have used HDL isolated from plasma of healthy volunteers incubated ex vivo with ILT-conditioned media, which contains a representative pathophysiological composition of AAA molecules. Moreover, it could have been desirable to compare the effects of ILT-CM with that of healthy-CM instead of HDL incubated with PBS (HDL-control). However, no oxidative or functional differences were observed between HDL incubated with healthy-CM and HDL-control. Moreover, the oxidation of APOA1 in HDL-like particles recovered from AAA tissues preferentially affects the same residues (Trp50 and Trp108) when using the ex vivo model of HDL-ILT CM. Thus, despite the potential limitations of recovering APOA1/HDL from tissue or obtaining healthy-CM to perform functional experiments, the present study showed that the methods used herein to examine HDL in the AAA environment could be relevant for testing the quantity and functional significance of modified HDL in vivo.

On the whole, our results indicate that APOA1 oxidation in AAA patients can contribute to dysfunction of HDL particles in AAA characterized by decreased cholesterol efflux capacity. Moreover, our preliminary data in plasma suggest new potential biomarkers (oxidized Trp50 and Trp108 on APOA1) that could be useful as a surrogate systemic metrics of tissue–HDL functionality.

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Declaration of interest

All authors have reported that they have no relationships relevant to the contents of this paper to disclose.

Author contributions

Diego Martínez-López = HDL isolation, TMT studies, PTM and PRM analysis, revising the work and final approval of the manuscript.

Emilio Camafeita = PRM analysis, revising the work and final approval of the manuscript.

Lidia Cedó = Functional experiments (Eflux and RCT), revising the work and final approval of the manuscript.

Raquel Roldán-Montero = HDL isolation and functional experiments, revising the work and final approval of the manuscript.

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Francisco Blanco-Vaca, Luis Miguel Blanco-Colio = Interpretation of the data, revising the work and final approval of the manuscript.

Jean-Baptiste Michel = Providing human AAA biobank, revising the work and final approval of the manuscript.

Joan Carles Escoila-Gil, Jesús Vázquez, Jose Luis Martín-Ventura = Conception and design of the study, analysis of results and writing the manuscript.

Appendix A. Supplementary data

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References


