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Malondialdehyde-modified HDL particles elicit a specific IgG response in abdominal aortic aneurysm

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ABSTRACT

High Density Lipoprotein (HDL) plays a protective role in abdominal aortic aneurysm (AAA); however, recent findings suggest that oxidative modifications could lead to dysfunctional HDL in AAA. This study aimed at testing the effect of oxidized HDL on aortic lesions and humoral immune responses in a mouse model of AAA induced by elastase, and evaluating whether antibodies against modified HDL can be found in AAA patients.

HDL particles were oxidized with malondialdehyde (HDL-MDA) and the changes were studied by biochemical and proteomics approaches. Experimental AAA was induced in mice by elastase perfusion and then mice were treated with HDL-MDA, HDL or vehicle for 14 days. Aortic lesions were studied by histomorphometric analysis. Levels of anti-HDL-MDA IgG antibodies were measured by an in-house immunoassay in the mouse model, in human tissue-supernatants and in plasma samples from the VIVA cohort.

HDL oxidation with MDA was confirmed by enhanced susceptibility to diene formation. Proteomics demonstrated the presence of MDA adducts on Lysine residues of HDL proteins, mainly ApoA-I. MDA-modification of HDL abrogated the protective effect of HDL on cultured endothelial cells as well as on AAA dilation in mice. Exposure to HDL-MDA elicited an anti-HDL-MDA IgG response in mice. Anti-HDL-MDA were also detected in tissue-conditioned media from AAA patients, mainly in intraluminal thrombus. Higher plasma levels of anti-HDL-MDA IgG antibodies were found in AAA patients compared to controls. Anti-HDL-MDA levels were associated with smoking and were independent predictors of overall mortality in AAA patients. Overall, MDA-oxidized HDL trigger a specific humoral immune response in mice. Besides, antibodies against HDL-MDA can be detected in tissue and plasma of AAA patients, suggesting its potential use as surrogate stable biomarkers of oxidative stress in AAA.

1. Introduction

Abdominal aortic aneurysm (AAA) is a vascular disease hallmarked by a permanent and chronic dilation of the abdominal aorta [1,2]. Although AAA progression is mostly asymptomatic, AAA rupture is fatal in most of the cases and accounts for 150,000 to 200,000 deaths per year around the globe [3]. Thoracic endovascular aortic repair (TEVAR) and open surgical repair are generally accepted as efficient and safe surgical methods, but they are only applicable to the late stage of the disease. Therefore, in the absence of specific therapeutic approaches, especially

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for the early phase, a better characterization of pathogenic mechanisms underlying AAA progression, as well as potential biomarkers, is imperative to improve clinical management and patient stratification.

Risk factors correlated to AAA onset and progression include hypertension, smoking, and obesity, among others [1]. Recent genetic analysis suggests that lipids play a role in AAA etiology [3–6]. Several epidemiological studies have shown a connection between decreased High-Density Lipoprotein Cholesterol (HDLc) levels and AAA progression [7–9]. These results were supported by experimental animal models reporting that raising HDLc levels can cause abrogation of AAA initiation and progression [10,11]. However, these findings were confronted with clinical trials reporting no clinical benefit of HDLc-raising agents on cardiovascular (CV) patients [12–14]. These findings seem to suggest that the association between HDLc and cardiovascular diseases is more complex than initially considered. On the one hand, HDLc levels may not be as relevant on themselves as previously conceived, and functional properties of HDL particles should be included into the equation. In this respect, the quality of HDL is the relevant factor for its vasculoprotective activities [15]. Cardiovascular protective functions of HDL include reverse cholesterol transport (RCT), as well as antioxidant, anti-inflammatory, anti-apoptotic and antithrombotic effects. Regarding AAA, we previously demonstrated that systemic paraoxonase-1 activity, a surrogate marker of HDL (antioxidant) functionality, is decreased in AAA patients [16]. Moreover, we have shown that AAA patients display impaired HDL cholesterol efflux capacity, the first step of RCT [17]. We have recently demonstrated that HDLs are sensitive to oxidation in the AAA milieu, mainly in the intraluminal thrombus (ILT), which is rich in neutrophils and red blood cells. Moreover, those HDL-oxidized forms in plasma were independently linked to AAA presence [18]. Importantly, oxidized HDL was associated with impaired HDL functionality [18].

Previous studies from our group have reported the presence of antibodies against HDL particles in AAA patients, which were associated with HDLc levels and clinical features [19], hence proposing a role for antibodies against lipoproteins in AAA. Similar results were observed in other conditions [20–22], thus suggesting that antibodies against lipoproteins may help to understand the crosstalk among lipids, inflammation and clinical outcomes. Taking into account that oxidation can lead to modifications in proteins and lipids, it is conceivable that antibodies directed against HDL oxidized forms could be also detected in AAA. In fact, antibodies against malondialdehyde (MDA)-modified LDL have been reported [23] but whether oxidized HDL may represent an antigenic target remains unknown. Therefore, the present study is aimed at (i) testing the effect of HDL-MDA on aortic lesions and immune responses in the elastase-induced AAA mouse model and (ii) assessing whether anti-HDL-MDA antibodies can be detected in tissue and plasma samples from AAA patients.

2. Material and methods

2.1. HDL isolation and oxidation with MDA

HDL was isolated from EDTA plasma samples of healthy volunteers by ultracentrifugation as described [24]. Plasma density was adjusted to $\rho = 1.063$ g/ml with KBr and the sample was overlaid with KBr saline solution (ρ 1.063 g/ml). Samples were ultracentrifuged at 53,000 rpm for 18 h at 4 °C in a TLA-100 rotor (Beckman). After centrifugation, the upper, apolipoprotein-B-containing lipoproteins, 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 63,000 rpm for 24 h at 4 °C. After this step, HDLs (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. ApoA-I concentration in HDL was determined by an immunoturbidimetric assay, using a commercial kit adapted to a COBAS 501c autoanalyzer (Roche Diagnostics).

MDA (0.5 M) was obtained from malonaldehyde-bis-dimethylacetal (Sigma) by acid hydrolysis [25]: 88 µl of malonaldehyde-bis-dimethylacetal were incubated with 12 µl 4 N HCl and 400 µl distilled water at 37 °C for 10 min pH was adjusted to 7.4 adding 1 N NaOH and measuring with a universal pH indicator. Distilled water was added to reach a final volume of 1 ml. MDA-HDL was prepared by incubating HDL with 0.5 M MDA at 37 °C during 3 h at a constant ratio of 100 µl/mg of HDL. Unbound MDA was eliminated using PD-10 desalting columns (GE Healthcare). MDA-HDL was concentrated with a centrifugal filter of 30 KDa (Centricon Merck Millipore). Concentration of MDA-HDL was measured using the measurement of ApoAI as reported above.

HDL susceptibility to copper-induced lipid oxidation was determined by monitoring the formation of conjugated dienes at 234 nm at 37 °C in a BioTek Synergy HT spectrophotometer (BioTek, Agilent). Oxidation was started by adding 2.5 µmol/L $CuSO_4$ in wells each containing HDL (100 µg/ml of apoA1), and the maximum slope during the propagation phase of the kinetics was calculated.

2.2. Proteomics

Samples (200 µg of total proteins of MDA-HDL or vehicle-treated-HDL) were digested overnight at 37 °C in FASP filters with trypsin (Promega, Madison, WI, USA) at an 40:1 protein:trypsin (w/w) ratio in 50 mM ammonium bicarbonate, pH 8.8, according to manufacter's instructions. 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. The TMT10-plex experiment was composed by 4 biological replicates coming from MDA-treated HDL, 4 from vehicle-treated HDL, and two channels reserved for internal standard (I.S.) samples. The I.S. was created by pooling the four samples from the vehicle-treated-HDL and was used as reference to express relative quantification values. Peptides were fractionated using the high pH reversed-phase peptide fractionation kit (Thermo Fisher Scientific) according to manufacter's instructions. Peptide fractions were analyzed using an Easy nano-flow HPLC system (Thermo Fisher Scientific, Bremen, Germany) coupled via a nanoelectrospray ion source (Thermo Fisher Scientific) to an Orbitrap Fusion 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). Peptides were loaded in buffer A (0.1% formic acid (v/v)) and eluted with a 240-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-speed acquisition method. MS spectra were acquired in the Orbitrap analyzer with a mass range of 400–1500 m/z and 120,000 resolution. HCD fragmentation was performed at 35 normalized collision energy and MS/MS spectra were analyzed at 30,000 resolution in the Orbitrap. Data analysis was performed with Xcalibur 2.2 (Thermo Scientific).

Peptide identification from MS/MS spectra was performed by open search on Comet-PTM [26] against a human database, build by concatenating the human reference proteome database (UniProtKB/Swiss-Prot July 2014, 40555 sequences), with the inverted database constructed from the same target database using DecoyPYrat [27]. Search parameters were: full trypsin digestion with 2 missed cleavages, fixed modifications Cys carbamidomethylation (57.021464 Da) and TMT labeling at N-terminal end and Lys (229.162932 Da). Precursor mass tolerance was set to 500 Da and fragment mass tolerance at 0.03 Da; precursor charge range was set to 2–4; and 3 was the maximum fragment charge. Only y- and b-ions were used for scoring. The FDR (<1%) of PTM identification was controlled at the global, local and peak levels by SHIFTS [26]. Quantitative information was extracted

from MS/MS spectra of TMT-labeled peptides using Proteome Discoverer 2.1 (Thermo Scientific). Modified peptides and protein quantification was performed using the Generic Integration Algorithm [28,29] on the basis of the WSPP model [30] with some modifications [26]. Significant abundance changes of modified peptides across the different samples were detected by Student's t-test. An in-house developed script was used for the semisupervised annotation of peptide modifications as described [26]. For simplicity, artifactual modifications introduced during sample preparation were not considered in further analysis. Met mono-oxidation, or Asn, Gln and alkylated Cys deamidation, were not considered as post-translationally modifications (PTMs). Proteomics data have been deposited at the Proteome Xchange [31] Consortium via the PRIDE partner repository with the dataset identifier PXD025322.

2.3. In vitro studies

Human aortic endothelial cells (HAECs) were isolated from entire arterial segments from aortae of patients undergoing cardiac surgery as described [32]. HAECs were incubated in DMEM/F-12 medium with 0.1% Collagenase I (Gibco, Thermo Fisher Scientific, UK) overnight. After incubation, the reaction was stopped and the ECs were scraped off the vessel walls, centrifuged at 500 g for 5 min at room temperature (RT) and resuspended in DMEM/F-12 complete medium (DMEM/F-12 + 100 U/ml penicillin +100 μg/ml streptavidin +2 mM L-glutamine + 2.5 μg/ml Amphotericin B + 100 μg/ml heparin +30 μg/ml ECGF + 20% FBS). After 2–5 days of expansion, the HAECs were detached from the culture plates using 0.025% trypsin-EDTA and isolated through positive selection using 2.5 g/ml mouse anti-human CD31 antibody (BD Biosciences-EU) followed by $2{\cdot}10^6$ beads/ml magnetic beads coated with anti-mouse IgG (Invitrogen, Thermo Fisher Scientific, UK). Both incubation steps were made for 30 min at 4 °C. The cell suspensions were then passed through a column placed in a magnet, ensuring the isolation of HAECs. The isolated cells were resuspended in complete medium and cultured on 0.5% gelatin-coated plates. Once in confluence, primary cell cultures were FBS 0.5% starved for 24 h before the experiments were performed. All experiments were performed during passages 3–7. Cells were preincubated with HDL and HDL-MDA (0.1 mg/ml for both) for 24 h and then stimulated with TNF-alfa (50 ng/ml) for 4 additional h. After that, cells were pelleted and resuspended for RNA analysis.

The study was approved by the Research Ethics Committee at Hospital Universitario Fundacion Jimenez Diaz (approval number PIC076-18_FJD). Informed consent was obtained from all patients.

2.4. Real time PCR

Lysates from HAECs were resuspended in TRIzol, and total RNA was purified. Duplicate samples were quantified by determining absorbance at 260 nm, and Real-Time PCR was performed as described [33]. The expression of target genes was normalized to housekeeping transcript (GADPH). The TaqMan probes (Hs00174575 m1 CCL5, Hs00234140 m1 CCL2, IL1b Hs01555410_m and Hs02786624_g1 GAPDH) were purchased from Applied Biosystems and optimized according to the manufacturer's protocol.

2.5. Animal model

Wild type (WT) control mice (C57BL6/J) were purchased from The Jackson Laboratory (Bar Harbor). All animals were housed in isolation rooms in the animal facility of our institute. Water and chow-fed diet was available ad libitum. The Ethics Review Board of our institute approved all animal procedures, and the project was authorized by the IIS-FJD-Universidad Autónoma de Madrid (CEI 59-1036-A061) and by the Spanish Authority governing animal experimentation, the Comunidad Autónoma de Madrid (registered approval letter 10/008932.9/15). All animal procedures were performed in accordance with the guidelines of Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Twelve-week-old mice were anesthetized by 2% isofluorane inhalational anaesthesia and a horizontal laparotomy was performed. Using a surgical stereomicroscope, the abdominal aorta was separated from the level of the left renal vein to the bifurcation and temporarily ligated between the renal and iliac arteries. An aortotomy was created with a 30-gauge needle and the aorta was exsanguinated. A PE-26 polyethylene tube was introduced through the aortotomy, and the aorta was infused for 5 min at 100 mmHg with type I porcine pancreatic elastase (specific activity 6 U/mg protein; E1250; Sigma Chemical). The aortotomy was then repaired, the ligation was eliminated, and the restoration of blood flow visually confirmed. Incisions were closed and the mice housed under standard conditions. Experimental groups were: elastase-infused WT mice treated i.v. with saline (WT, n = 9); elastase-infused WT mice treated i.v. with HDL (HDL, 400 μg every day, n = 8) and elastase-infused WT mice treated i.v. with MDA-HDL (MDA-HDL, 400 μg every day, n = 9). On day 14 post-surgery, all mice were anesthetized with a mixture of ketamine/xylazine (100 mg/kg and 10 mg/kg body weight, respectively) and euthanized by cervical dislocation. Lipid and lipoprotein levels were measured in plasma using commercial kits adapted to a COBAS 501c autoanalyzer (Roche Diagnostics).

2.6. Histomorphometry and immunohistochemistry

Mouse aortic samples were embedded in paraffin. Serial sections (4 μm) of aortas were cut for histomorphometry. Histomorphometry was performed on Masson trichrome-stained histological sections as previously described [16]. Aortic diameter increase was obtained after normalization to normal aorta diameter before surgery. AAA was defined as aortic diameter expansion 100% of that before perfusion [34]. Elastin fragmentation were graded from grade 1 (intact well-organized elastin laminae) to grade 4, severe elastin fragmentation or loss. For immunohistometry, primary Ab was CD68 (Ab53444; Abcam) and donkey anti-rat HRP (Jackson) was used as secondary antibody. HRP was then added and sections were stained with DAB substrate-chromogen (DAKO) and counterstained with haematoxylin. Computer-assisted morphometric analysis was performed with the Image-Pro Plus software (version 1.0 for Windows) as described [16]. The threshold setting for area measurement was equal for all images. Samples from each animal were examined in a blinded manner. Results of CD68 were expressed as % positive area versus total area.

2.7. Tissue-conditioned media

Tissue-conditioned media were obtained as previously reported [35]. Briefly, tissue samples from diseased (AAA) aortas were obtained during surgical repair and dissected into intraluminal thrombus (AAA-ILT) and arterial wall (AAA-wall). Healthy aortas were collected from brain-deceased organ donors and included abdominal and thoracic aortas. Tissue sections were cut into small pieces and incubated in RPMI 1640 medium (at 6 ml/g of wet tissue) for 24 h at 37 °C. Then, cultures were centrifuged (3000 g, 10 min, 20 °C) and supernatants containing proteins released from the tissue samples (tissue-conditioned media) were collected and frozen at −80 °C until analyzed. Procedures were approved by the local Institutional Review Board (INSERM Institutional Review Board: RESAA and AMETHYST studies, CPP Paris-Cochin n° 2095, 1930 and 1931; French Biomedicine Agency: PFS 09-007, BBMRI network, BB-0033-00029) and patient informed consent was obtained.

2.8. AAA patients and controls

Plasma samples were obtained from individuals participating in the VIVA study (ClinicalTrials.gov NCT00662480), a population-based image-screening trial for AAA in men aged between 64 and 74 years old from Denmark.

The study recruitment protocol has been reported in detail elsewhere

[36,37]. In brief, 50,156 male adults were randomized between October 2008 and October 2010 to receive either an invitation for vascular screening or participate as control subjects. The study protocol was performed in compliance with the Declaration of Helsinki and received approval from the Institutional Review Board from the Mid Region of Denmark (M20080028). All participants gave written informed consent prior enrolment.

Study participants underwent an ankle-brachial index (ABI) measurement and an ultrasound scan of the abdominal aorta. The ABI was calculated as the mean of the two-recorded ankle arterial blood pressures divided by the brachial systolic blood pressure. The presence of peripheral arterial disease (PAD) was defined by an ABI <0.90 or >1.4. Questionnaires regarding lifestyle parameters, medical pre-existing conditions and smoking status were administered by trained nurses. Study participants were followed-up for up to 5 years and information about death was obtained from the Civil Registration System. No patients experiencing AAA rupture were registered in our cohort.

A final cohort consisting on 427 AAA patients and 139 HC was analyzed (Table 1). Blood samples were obtained by venipuncture. Plasma samples were immediately processed and stored at −80 °C until analyses.

2.9. Quantification of IgG anti-HDL-MDA and anti-HDL antibodies

IgG anti-HDL antibodies were measured as previously reported [20]. IgG anti-HDL-MDA antibodies were measured by means of an in-house immunoassay. ELISA plates (Maxisorp, Nunc) were coated with either 20 μg/ml of HDL-MDA (test half) or HDL-cholesterol (unfractioned HDL isolated from human plasma, Sigma) in 70% ethanol (control half) overnight at 4 °C. Plates were then blocked with blocking solution (PBS containing 1% BSA (Sigma)) for 1 h at room temperature and washed with PBS. Then, 1:50-diluted plasma samples and standard curves from patients' pooled sera (diluted 1:16 to 1:512) (in PBS containing 0.1% BSA) were incubated in both plate halves for 2 h at room temperature. Plates were then washed twice with TBS and alkaline phosphatase-conjugated anti-human IgG or anti-mouse IgG (both 1:1000) (Immunostep, Spain) was added for 1 h. Lastly, p-nitrophenylphosphate (Sigma) in diethanolamine buffer (pH 9.8) was added and absorbance at 405 nm was recorded. For each measurement, the absorbance in the control half was substracted to that of the test half. For plasma samples, IgG anti-HDL-MDA Arbitrary Units (AU) were calculated according to the standard curves. Intra- and inter-assay variations for our assay were 6.5% and 11.2%, respectively. Standard curves using pooled sera were used for anti-HDL-MDA measurements in plasma from patients. Due to the lack of a standard material, internal controls were used instead for anti-HDL-MDA measurements in mouse plasma and tissue-conditioned supernatants. For mouse plasma samples, plasma was diluted 1:50 and the raw absorbances were analyzed. For tissue-conditioned media, samples were assayed undiluted and the raw absorbances were analyzed.

Total IgG plasma levels were quantified by conventional ELISA techniques and AU values obtained from the anti-HDL-MDA and anti-HDL ELISAs were corrected using total IgG levels (anti-HDL-MDA/IgG and anti-HDL/IgG ratios).

Table 1
Demographical and clinical parameters of the study participants (VIVA study). Variables were summarized as mean ± SD or n(%). Differences between groups were assessed by χ^2 or unpaired t tests for categorical or continuous variables, respectively.

	AAA (n = 427)	Healthy controls (n = 139)	p-value
Age	69.5 ± 2.8	69.2 ± 2.9	0.321
Sex, n male	427 (100)	139 (100)	–
BMI, kg/cm2	27.3 ± 3.6	24.7 ± 3.3	0.003
ABI	0.8 ± 0.2	1.1 ± 0.1	0.004
Aortic size, mm	39.5 ± 11.8	18.2 ± 2.8	<0.001
AAA repair, n(%)	171 (40.0)	0 (0.0)	<0.001
Death, n(%)	95 (22.2)	16 (139)	0.006
CV risk factors, n(%)			
Current smoking	182 (42.6)	24 (17.3)	<0.001
Hypertension	230 (53.7)	62 (44.9)	0.070
Diabetes	49 (11.5)	23 (16.7)	0.115
History of CV events, n(%)			
Former CVD	90 (20.8)	19 (13.7)	0.062
Former PAD	6 (1.4)	1 (0.7)	0.525
Former angina	43 (10.1)	10 (7.2)	0.312
Former AMI	24 (5.6)	3 (2.2)	0.096
Former stroke	17 [4]	2 (1.4)	0.148
Treatments, n(%)			
Use of aspirin	192 (45.0)	36 (25.9)	<0.001
Use of ACE inhibitors	116 (27.2)	30 (21.6)	0.184
Use of b-blockers	123 (28.8)	33 (23.7)	0.250
Use of calcium antagonist	106 (24.8)	24 (17.3)	0.067
Use of statins	219 (51.3)	48 (34.5)	0.001
Use of glucocorticoids	37 (8.7)	10 (7.2)	0.574
Use of bronchodilator	45 (10.6)	12 (8.6)	0.500

2.10. Statistical analyses

Variables were checked for normal distribution using graphical methods and the Shapiro–Wilk test. Residuals were assessed in histograms and p-p plots. Continuous variables were summarized as median (interquartile range) or mean ± standard deviation (SD) depending on the distribution of the data. Categorical variables were expressed as n (%).

Differences across groups were assessed by Student's t tests, Mann-Whitney *U* test, ANOVA or Kruskal-Wallis tests, as appropriate. Dunn correction for multiple comparisons tests was performed when more than 2 groups were analyzed. Levels of IgG anti-HDL-MDA were log-transformed in order to being entered in parametric and multivariate tests. Independent associations between AAA prevalence and IgG anti-HDL-MDA levels were assessed by logistic regression analyses with adjustments for confounders. Selection of confounders was performed for each model by identifying those variables associated with the outcome, dependent variable with a p-value below 0.1 in univariate analyses as previously described [38,39].

A p-value<0.050 was considered as statistically significant. Data were analyzed by SPSS 23.0 for Windows (IBM).

3. Results

3.1. Effect of HDL-MDA on aortic lesions and immune responses in the elastase-induced AAA mouse model

As we recently showed that oxidation of HDL leads to an impaired HDL activity [18], we tested the functional impact of the oxidative modification of HDL by MDA on AAA. We firstly characterized the effect of MDA treatment on diene formation in HDL. MDA-oxidized HDL particles exhibited a higher diene formation compared to their naïve counterparts (HDL-MDA: 8.99 ± 1.08 vs HDL: 5.85 ± 0.91) (Fig. 1A). We next applied a novel mass spectrometry-based technology for the unbiased characterization and quantification of PTMs [26], which we have applied to biomedical research [40], including characterization of HDL on the context of AAA [18]. We observed that the majority of PTMs induced by MDA treatment were MDA adducts on Lys residues (Fig. 1B). Out of the 22 ApoA1 Lys sites, 18 were detected, and from those, 15 were found to be modified by MDA (Fig. 1C). MDA also produced kynurenin oxidation on Trp residues and other additional PTMs, although in much lower abundance.

We then analyzed the effect of MDA-treated HDL particles in vitro (human aortic endothelial cells, HAEC) and in vivo (elastase-induced AAA lesions in mice). In HAEC, HDL prevented TNF-induced IL-1b, CCL-2 and CCL-5 mRNA expression, while this protective anti-inflammatory

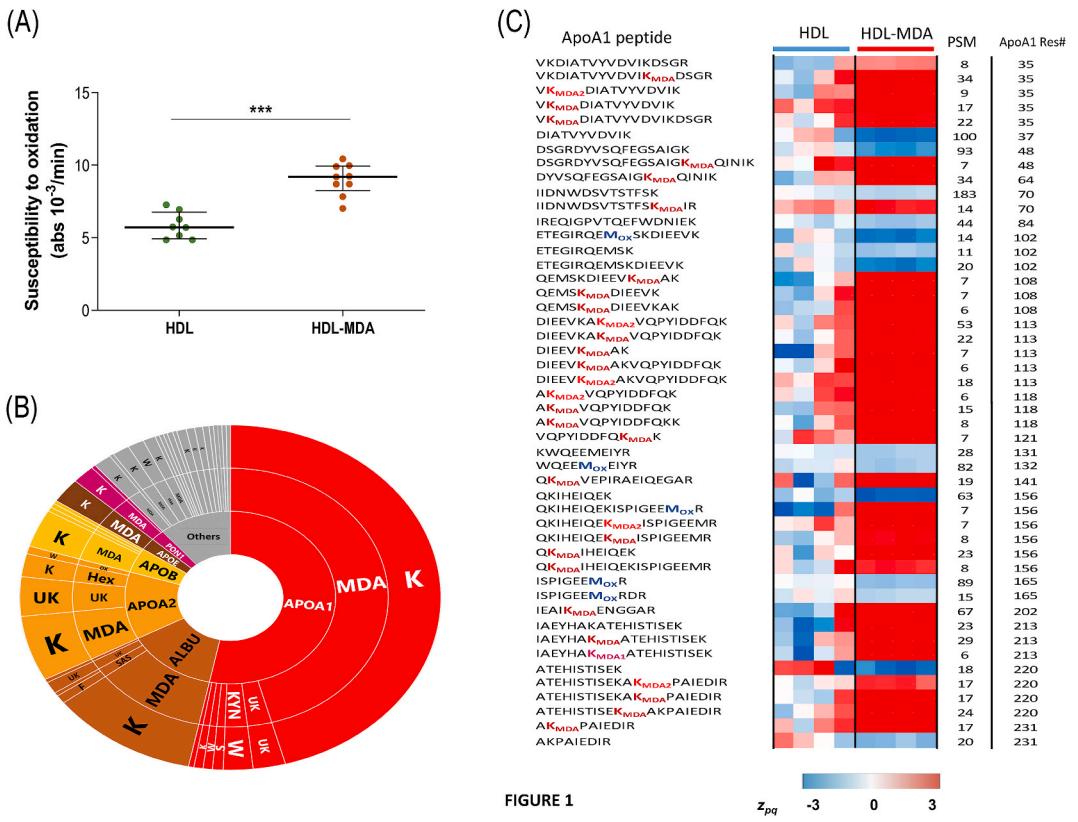

Fig. 1. MDA-induced modifications in human HDLs. (A) Susceptibility to oxidation of HDL particles (with or without MDA) was induced by adding 2.5 μM $CuSO_4$ and conjugated dienes formation monitored at 234 nm. Data are expressed as the absorbance increase per minute during the phase of maximal slope. Bars indicate 25th, median and 75th percentiles. Differences between HDL-MDA (orange, n = 9) and native HDL (green, n = 8) were assessed by T tests. ***$p < 0.001$. (B) An absolute abundance-proportional graph of the post-translationally modified peptides (PTMs) (with more than 2 quantified spectra) significantly increased in MDA-treated HDLs as compared to the vehicle-treated ones, is shown [n = 4, *t*-test, $p < 0.050$]. The adscription of the type of modification and the modified residue to HDL proteins is depicted. The abundance of PTMs was assessed by spectral counting. (C) Heat-maps showing changes in ApoA1 peptide abundance produced by MDA treatment (in units of standard deviation, z_{pq}), whose magnitude is shaded according to the color scale at the bottom. For simplicity, the figure only includes non-modified or MDA-modified peptide forms quantified with more than 5 spectra. The number of scans used to quantify each peptide (PSM) as well as the ApoA1 (P02647-1) residue numbering of the N-terminal residue of each peptide sequence is shown. Note that a minor proportion of peptides containing MDA-modified Lys (MDA) are also detected in other forms where they have an additional TMT labeling moiety (MDA1) or are unlabeled (MDA2) in other amino groups. NM: non-modified; MDA: malondialdehyde; UK: unknown; KYN: Kynurenine; HEX: hexose; SAS: single amino acid substitution; Ox and diox: addition of one or two oxygens, respectively.

effect was lost when HAEC were treated with HDL-MDA (Fig. 2a). In addition, treatment with HDL decreased aortic dilation induced by elastase in mice, in agreement with previous studies in other AAA models [10,11]. In contrast, MDA-oxidized HDL did not prevent elastase-induced AAA dilation (HDL-MDA: 228.2 ± 29.98% vs HDL: 201.3 ± 29.04% and control: 255.5 ± 38.58%) (Fig. 2B and C). Neither treatment (HDL or HDL-MDA) lead to significant changes in plasma lipid and lipoprotein levels, elastin or macrophage content (Supplementary Fig. 1).

Then, we aimed to evaluate whether MDA modifications on HDL elicited a specific immune response in mice. To this end, the presence of anti-HDL-MDA IgG was tested in plasma samples obtained from animals treated with HDL-MDA, HDL or vehicle (control). Our results revealed that animals treated with HDL-MDA mounted an anti-HDL-MDA specific response (HDL-MDA: 0.74 ± 0.25 vs controls: 0.55 ± 0.17). This response was absent when HDL was administered (HDL: 0.27 ± 0.10) (Fig. 3A), which in turn prompted a specific anti-HDL response (HDL: 0.56 ± 0.21 vs controls: 0.07 ± 0.05 and HDL-MDA: 0.18 ± 0.11) (Fig. 3B). Furthermore, adjustment by total IgG levels allowed us to confirm that this effect was not attributed to differences in the production of total IgG levels (Fig. 3C).

All these results suggest that oxidative modification of HDL with MDA, mostly related to MDA adducts on Lys residues, impaired the protective effects of HDL on aortic dilation in an elastase-induced AAA model. Moreover, exposure to HDL-MDA particles triggered a specific anti-HDL-MDA response in mice, which was different to that of HDL-prompted.

3.2. IgG Anti-HDL-MDA levels in tissue-conditioned media and plasma of AAA patients

Next, we assessed whether IgG anti-HDL-MDA antibodies could be found in human samples obtained from patients at the local (tissue) and systemic (plasma) level. First, the levels of IgG anti-HDL-MDA were analyzed in tissue-conditioned media samples from AAA patients and controls (Supplementary Table 1). Interestingly, the levels of IgG anti-HDL-MDA antibodies were found to be elevated in ILT samples compared to those obtained from the AAA wall or control aortic tissues (AAA-ILT: 0.28 ± 0.22 vs AAA-wall: 0.13 ± 0.27 and HC: 0.06 ± 0.07) (Fig. 4A). Of note, differences were noted between anti-HDL-MDA and anti-HDL, showing increased anti-HDL levels in the AAA wall (AAA-ILT: 0.08 ± 0.10 vs AAA-wall: 0.15 ± 0.13 and HC: 0.05 ± 0.07) (Fig. 4B), in line with previous findings [19]. Again, a potential effect of differences in total IgG production was ruled out (Fig. 4C). IgG anti-HDL-MDA levels were not correlated with age in any of the groups (AAA-ILT: r = 0.018, p = 0.919, AAA-wall: r = 0.129, p = 0.454, and controls: r = 0.036, p =

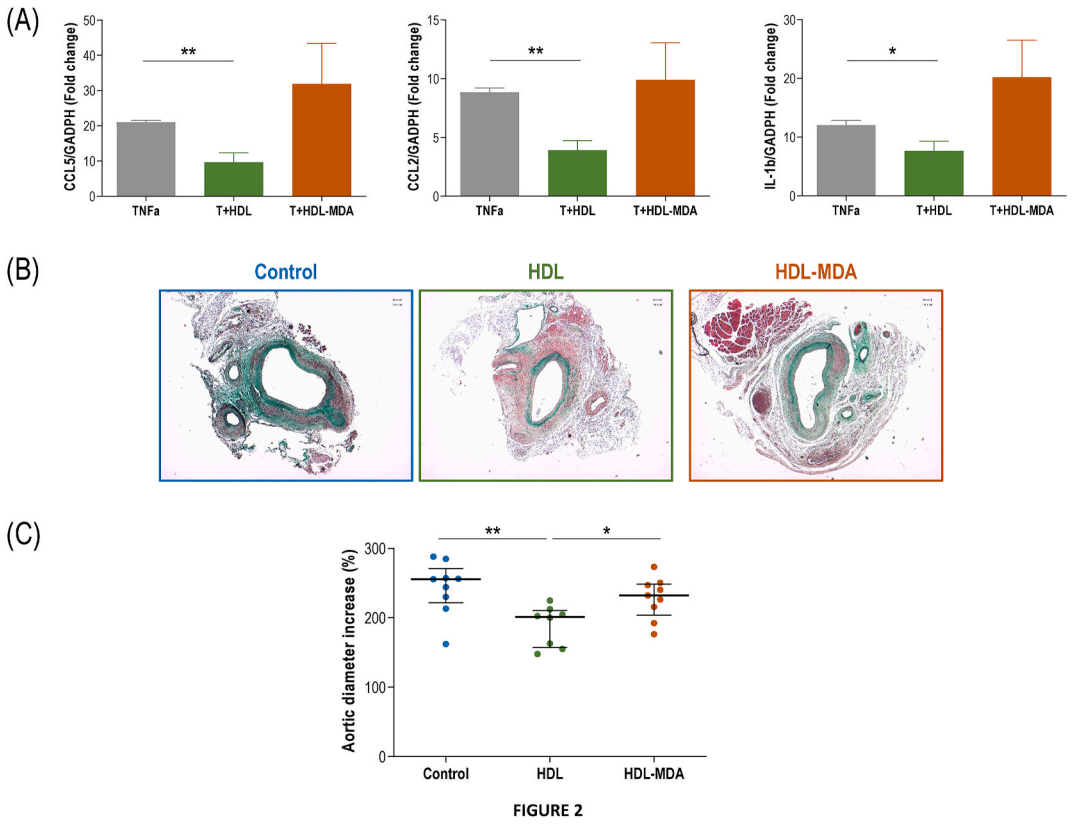

Fig. 2. Effect of HDL-MDA treatment in vitro and in vivo. The effect of HDL-MDA in vitro on endothelial cells stimulated with TNFa was evaluated by measuring the gene expression of IL-1b, CCL2 and CCL5 (A). Bars indicate mean and standard error of the means. Results are expressed as fold change vs basal. Differences were assessed by T-test. The effect of HDL-MDA treatment in an elastase-induced AAA mouse model (B, representative images) was evaluated by measuring aortic diameter (C). Bars indicate 25th, median and 75th percentiles. Differences among mice treated with HDL-MDA (orange), HDL (green) or vehicle (control) (blue) were assessed by one-way ANOVA and Dunn tests for multiple comparisons. The p-values from multiple comparisons are indicated in the graphs as follows: *p < 0.050, **p < 0.010. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

0.779). A similar picture was observed for IgG anti-HDL levels (all p > 0.050). Furthermore, these antibodies were not associated with sex in any of the study groups (all p≫0.050). No differences between origins (abdominal vs thoracic) were observed for anti-HDL-MDA (p = 0.241) or anti-HDL (p = 0.321) levels in control samples. Excluding samples from thoracic donors did not change these findings (data not shown).

Finally, we aimed to assess whether anti-HDL-MDA antibodies could be detected in plasma samples from AAA patients. Then, levels of IgG anti-HDL-MDA were measured in AAA patients and controls from the VIVA study (Table 1). AAA patients exhibited higher IgG anti-HDL-MDA levels compared to age-matched controls (AAA: 39.53 ± 23.63 vs HC: 28.73 ± 19.01 AU) (Fig. 5A). These differences were not explained by total IgG levels (Fig. 5B). IgG anti-HDL-MDA levels were associated with current smoking habit (40.00 ± 25.50 vs 35.89 ± 21.18 AU, p = 0.040), whereas no associations were observed for other risks factors including hypertension (p = 0.906), diabetes (p = 0.986), history of previous CV events (p = 0.120) nor with treatments (all p > 0.050). Moreover, multivariate regression analysis adjusted for confounders revealed that anti-HDL-MDA antibodies were independent predictors of AAA (Table 2). Lastly, associations between anti-HDL-MDA and clinical outcomes in AAA were analyzed. No differences were found between AAA patients undergoing surgical repair during follow-up and their non-repair counterparts (38.77 ± 22.23 vs 39.43 ± 23.77, p = 0.776). Interestingly, IgG anti-HDL-MDA levels were associated with overall mortality in AAA patients in both univariate and multivariate analyses (Table 3).

In sum, these results confirm that IgG anti-HDL-MDA are enriched in the ILT of AAA samples, thus suggesting an enrichment of oxidized epitopes in this location compared to the vascular wall. AAA patients are hallmarked by elevated IgG anti-HDL-MDA plasma levels, irrespective of traditional risk factors. Anti-HDL-MDA antibodies are independent predictors of overall mortality.

4. Discussion

Despite the vast evidence supporting an association between low HDLc levels and AAA, this notion has been challenged by recent findings, hence pointing to a more complex scenario. Oxidative stress and immune responses are central mechanisms in AAA pathogenesis. However, how lipoproteins interplay with these processes in AAA is poorly characterized. In the present study, we reported that oxidized HDL particles not only were associated with a loss of its protective functions in vivo to counteract AAA progression, but also triggered a specific adaptive immune response. To the best of our knowledge, the findings herein reported demonstrate, for the first time, the involvement of anti-HDL-MDA response in AAA, thereby pointing to a connection among oxidative stress, HDL dysfunction and immune response.

Oxidative stress has been proposed to contribute to AAA, although the exact mediators are not completely elucidated [41]. In addition to causing direct cell injury, DNA and protein damage, free radicals are known to prompt lipid peroxidation [1,2,42]. Certain molecules such as MDA are able to enhance oxidative stress via lipid peroxidation [43]. Previous findings from our group have documented that oxidized HDL was associated with impaired activity [18]. In the present study, we further expand these findings. We first show that in our conditions, MDA induces oxidative modifications of HDL in vitro, predominating the generation of MDA adducts on Lys residues, which were identified in the most abundant HDL protein components, among them ApoA-I. Among

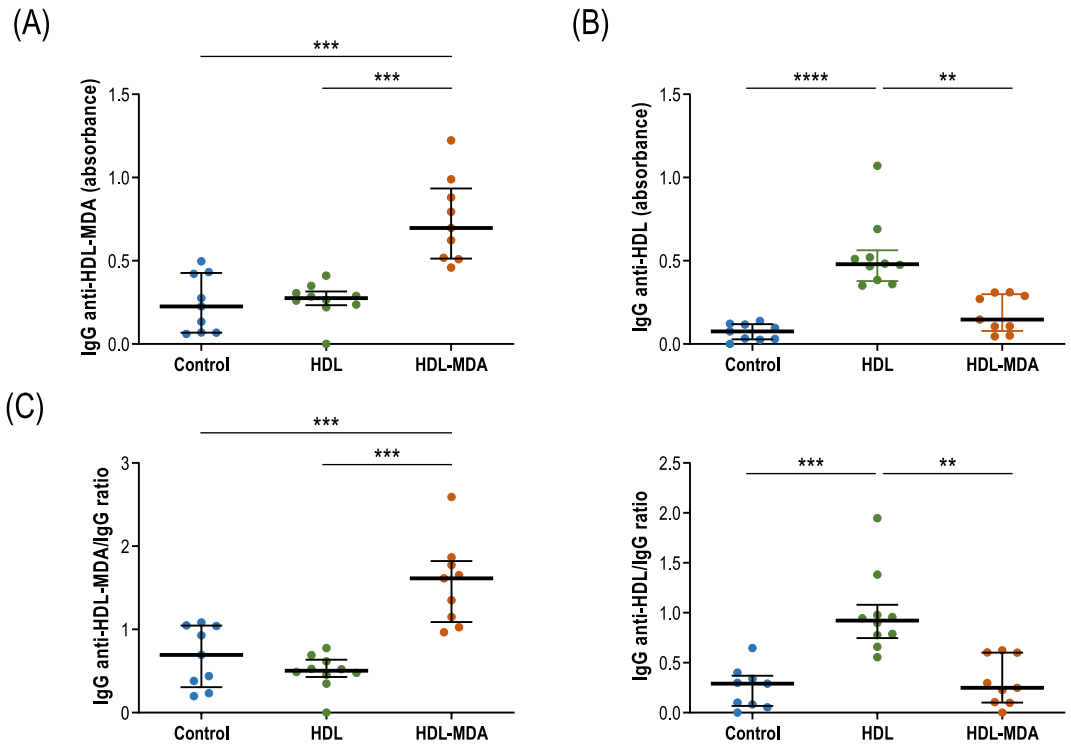

Fig. 3. Anti-HDL-MDA responses in elastased-induced AAA mouse model. IgG anti-HDL-MDA (A) and IgG anti-HDL (B) levels (measured as absorbance) were quantified at day 14 in plasma samples from mice treated with HDL-MDA (orange, n = 9), HDL (green, n = 8) or vehicle (control) (blue, n = 9). (C) Anti-HDL-MDA and anti-HDL levels were corrected for the total IgG levels and ratios were analyzed. Bars indicate 25th, median and 75th percentiles. Differences across groups were assessed by Kruskal-Wallis tests and Dunn tests for multiple comparisons. The p-values from multiple comparisons are indicated in the graphs as follows: **$p < 0.010$, ***$p < 0.001$, ****$p < 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

other functions, ApoA-I contributes to the inhibition of lipid hydroperoxides accumulation in HDL, while lipid hydroperoxides in HDL could impair a variety of HDL's vasculoprotective functions by oxidizing ApoA-I via a reactive aldehyde-mediated reaction [44]. In addition, it was previously shown that modifying ApoA-I by MDA, but not by an array of other reactive carbonyls, blocks cholesterol efflux [45]. We then demonstrate that MDA-induced modifications abrogate the vasculoprotective functions of native HDL in HAEC and in the elastase-induced mouse model of AAA. In vitro, we have shown that HDL prevented the overexpression of inflammatory mediators such as IL-1b, CCL-2 and CCL-5, while this protective effect was lost by HDL-MDA treatment. In vivo, HDL prevented elastase-induced AAA dilation, while HDL-MDA did not. The mechanisms underlying this protective effect was not related to changes in plasma lipid and lipoprotein levels, elastin preservation and/or macrophage infiltration. Thus, further studies are needed to completely understand the beneficial effect of HDL in vivo. Moreover, we have found that HDL-MDA is able to elicit a specific immune humoral response in both mouse and AAA patients. Oxidative stress is known to cause changes in proteins and lipids that can not only generate new epitopes either by prompting oxidation-specific epitopes (OSE) (neo-antigens) [46], but also by inducing conformational changes which can expose additional epitopes [47,48]. Interestingly, smoking has been associated with higher oxidative stress in aneurysmal diseases [49,50] and other conditions [51]. Similarly, smoking has been proposed to induce protein modifications that trigger specific humoral responses [52,53], which in turn participate in disease pathogenesis in other conditions. These lines of evidence are in accordance with our findings, which point to a similar pathogenic circuit in the framework of AAA, antibodies against HDL-MDA being a missing link among oxidative stress, HDL dysfunction and immune responses.

Of note, our study revealed that immune response against oxidized HDL is different and independent from anti-HDL response in the context of AAA. Although both can be detected in serum in AAA patients, our analyses on AAA tissue-conditioned media showed diverging patterns at the local level. Whereas anti-HDL-MDA are increased in the ILT, their anti-HDL counterparts were found to be elevated in the AAA wall. The latter observation is in line with previous findings from our group in a proof-of-concept study [19]. Importantly, the ILT is an oxidative environment, mostly due to the ferrous iron released by trapped erythrocytes and MPO-derived reactive oxygen species (ROS) released by activated neutrophils [54]. In this respect, we previously shown increased levels of oxidized lipids and proteins in the ILT [55]. Specifically, ApoA-I, the main component of HDL, co-localized with MDA in ILT, suggesting the oxidation of HDL in AAA ILT [18]. Moreover, an enhanced proteolytic activity characterizes the ILT [1], which could generate new epitopes. All these notions may explain the higher anti-HDL-MDA levels observed at the ILT compared to the AAA wall, since oxidized epitopes are highly enriched in the latter, thus provoking an enhanced anti-HDL-MDA depot. Elevated anti-HDL responses at the AAA wall may be caused by lipoprotein retention and local immune response in the adventitial tertiary lymphoid organs [56]. Moreover, it must be noted that whereas the anti-HDL-MDA response should be regarded as 'common' immune response towards a new antigen, the anti-HDL response represents an autoimmune phenomenon, whose origin in human patients cannot be established in the present study. All these findings uncover a functional and pathophysiological diversity of humoral responses in AAA.

Taken together, our findings shed new light on the role of adaptive responses in the pathogenesis of AAA. Early studies have demonstrated an overt B-cell infiltration in AAA lesions from patients and animal models [57,58]. In fact, experimental B-cell depletion has been associated with smaller AAA lesions [59], and enhanced IgG deposition have been reported in arterial wall and thrombi in AAA [58], although the relevance of the latter remains unclear. Our findings demonstrate that an important, multifaceted humoral response against modified lipoproteins hallmarks AAA. It is tempting to speculate that these antibodies can further contribute to tissue damage and remodeling once deposited,

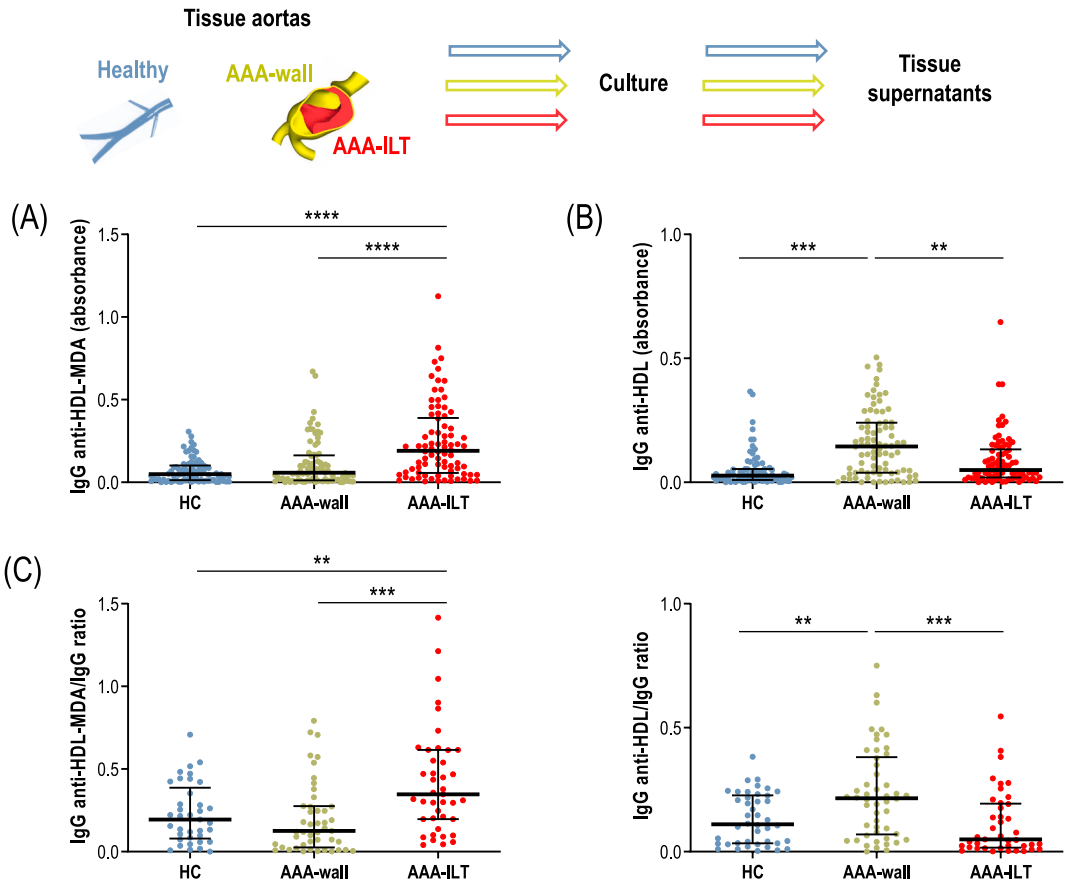

Fig. 4. Anti-HDL-MDA responses in tissue-conditioned media. IgG anti-HDL-MDA (A) and IgG anti-HDL (B) levels (measured as absorbance) were quantified in tissue-conditioned media samples obtained from thrombus (AAA-ILT, n = 82) and media layer (AAA-wall, n = 84) samples from AAA patients as well as from aortas obtained from healthy controls (HC, n = 83). Bars indicate medians, whereas whiskers represent 25th and 75th percentiles. Differences across groups were assessed by Kruskal-Wallis tests and Dunn tests for multiple comparisons. The p-values from multiple comparisons are indicated in the graphs as follows: **p < 0.010, ***p < 0.001, ****p < 0.0001.

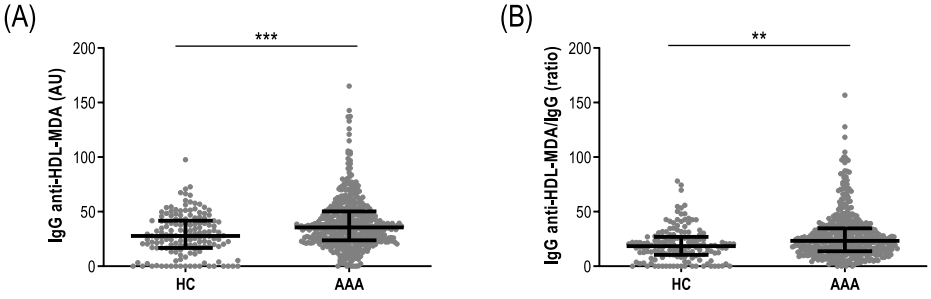

Fig. 5. IgG anti-HDL-MDA antibodies in AAA patients. IgG anti-HDL-MDA plasma levels measured as AU (A) or normalized after total IgG correction (B) in AAA patients (n = 427) and healthy controls (n = 139). Bars indicate 25th, median and 75th percentiles. Differences were assessed by Mann-Whitney U tests. **p < 0.010, ***p < 0.001.

due to antigen recognition or as immune-complexes. The activation of complement fragments, mainly in the ILT of AAA, supports this notion [60]. However, although the findings herein reported are mostly supportive of a detrimental effect of anti-HDL-MDA antibodies, potential protective actions may be also hypothesized. By virtue of their highly specific binding capacity, anti-HDL-MDA antibodies could also act by blocking and scavenging the circulating HDL-MDA particles, hence counteracting the negative effects of the latter. As potential double-edge swords, to what extent detrimental effects outweigh potential beneficial roles in different scenarios must be established. Thus, further research is needed to determine their net effect in AAA progression along disease course.

Our experimental approach combining a pre-clinical model of AAA and human samples from AAA patients allowed us to suggest that anti-HDL-MDA emergence is a consequence of previous exposure to HDL-MDA. This antibody response was not generated upon treatment with native HDL particles. Actually, our method allows us to establish that all the anti-HDL-MDA response arise from oxidized epitopes, thus excluding any potential effect of the native HDL particle (and thus, of potential inter-species reactivity). Therefore, anti-HDL-MDA antibodies can be surrogate readouts of HDL-MDA exposure. In fact, MDA plasma levels are increased in AAA patients and are associated with higher risk of AAA rupture [61,62]. However, measuring oxidative stress status is challenging from a technical point of view and difficult to translate into

Table 2
Association between IgG anti-HDL-MDA levels and AAA by univariate and multivariate regression analyses. The association between IgG anti-HDL-MDA levels and AAA presence was analyzed in the cohort presented in Table 1.

	OR	95% CI	p-value
Univariate model			
Anti-HDL-MDA, per unit	1.16	1.006–1.026	0.001
Multivariate model			
Anti-HDL-MDA, per unit	1.015	1.003–1.026	0.012
Current smoking, yes	2.372	1.369–4.110	0.002
Lowest ABI, per unit	0.000	0.000–0.004	<0.001
Former CVD, yes	0.777	0.381–1.581	0.486
Use of aspirin, yes	1.945	1.078–3.510	0.027
Use of statins, yes	1.274	0.716–2.172	0.435

Table 3
Association between IgG anti-HDL-MDA levels and overall mortality by univariate and multivariate regression analyses. The association between IgG anti-HDL-MDA levels and overall mortality was analyzed in AAA patients from the cohort presented in Table 1.

	HR	95% CI	p-value
Univariate model			
Anti-HDL-MDA, per unit	1.008	1.001–1.016	0.023
Multivariate model			
Anti-HDL-MDA, per unit	1.009	1.002–1.017	0.016
Current smoking, yes	1.170	0.770–1.777	0.462
Lowest ABI, per unit	0.237	0.084–0.673	0.007
Former CVD, yes	1.212	0.700–2.097	0.493
Use of aspirin, yes	1.175	0.408–1.094	0.511
Use of statins, yes	0.668	0.408–1.094	0.109

clinical routine. Regarding free MDA, thiobarbituric acid reactive substances (TBARS) assay is the method of choice but with limited specificity. Further methods have been developed in order to improve the specificity of MDA measurement, such as HPLC combined with UV, fluorescence detection or reversed-phase HPLC coupled with fluorescence detection [63]. However, most of these methods lack of broad availability. Conversely, the determination of anti-HDL-MDA levels is a relatively simple and feasible approach, which can indirectly account for the HDL-MDA burden, and thus lipid peroxidation, in AAA patients. The detection of autoantibodies against known lipo-oxidation adducts, namely MDA, malondialdehyde-acetaldehyde (MAA), MDA-LDL, and MAA-LDL, has been used to evaluate their potential as biomarkers of CV diseases [64,65]. In this respect, circulating levels of anti-MAA antibodies were elevated in patients with AAA [66]. More importantly, an IgG anti-HDL-MDA response would imply a certain and sustained exposure to HDL-MDA, which is more likely to have a biological effect, whereas the exact significance of a single assessment with elevated ROS or oxidative stress status may be difficult to evaluate. Capturing the exposure to lipid peroxidation and oxidative stress can be of interest for the clinical setting. In fact, oxidative stress has been reported as a predictor of overall (all-cause) mortality in a number of conditions [67–69], in part due to its widespread involvement in pathology. This is in line with our findings of anti-HDL-MDA as predictors of overall mortality in AAA. Therefore, the quantification of anti-HDL-MDA levels may have potential applications in the decision-taking process in order to guide preventive and/or therapeutic measures to counteract the oxidative stress-induced traits. However, clinical validation and appraisal of their added value are needed. Overall, our findings align with the idea that the association between HDL and AAA is more complex than initially proposed. Then, additional features such as lipoprotein functionality, lipoprotein quality [70] or the presence of (auto)antibodies should be considered as well, hence moving from a conventional lipid levels-centered model to a much more sophisticated approach that accounts for their actual biological relevance.

4.1. Strengths and limitations

This study has important strengths that should be remarked, such as the use of a large, prospective cohort of AAA patients, and the simultaneous assessment in an animal, pre-clinical model. Although a potential effect of heterologous lipoproteins in immunity may be hypothesized, infused, native lipoproteins were observed to maintain a functional activity and our protocol (dosages, time schemes and administration pathway) can be characterized as tolerogenic. Moreover, our experimental procedure allowed to distinguish the signal produced by oxidized epitopes compared to that of the native lipoprotein, hence ruling out a potential confounding effect. The combined analyses of the systemic (plasma) and local (tissue supernatants) from AAA patients further strengthen our results. However, as we do not have paired data in tissue and plasma of the same patients, we could not ascertain in this observational study the potential mechanism(s) behind the observed association of anti-HDL-MDA with AAA presence. Regarding information bias, follow-up data were based upon a nation-wide registry where reporting is mandatory for reimbursement causing a close to 100% follow-up, and validation studies of the registries have shown a high internal validity. Consequently, information bias is unlikely. Finally, we used a systematic approach to identify confounders, but in the end, residual confounding by nature is always a risk in observational studies.

In conclusion, MDA-modified HDL particles elicit a specific humoral response and antibodies directed against HDL-MDA can be detected in AAA patients, both in the systemic and local compartments. Furthermore, anti-HDL-MDA levels were associated with overall mortality in AAA patients. These findings expand the previous knowledge on the contribution of humoral adaptive responses in AAA and point to a connection with oxidized HDL as a potential trigger.

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Author contributions

Conceptualization, JRC, AS, JLMV; methodology, JRC, JSL, AS, JLMV; formal analysis, JSL; resources, JRC, ICP, JSL, EBB, DML, RRM, JCEG, JBM, LMBC, JV, AS, JLMV; writing—original draft preparation, JRC, ICP, AS, JLMV; writing—review and editing, JRC, ICP, JSL, EBB, DML, RRM, JCEG, JBM, LMBC, JV, AS, JLMV; funding acquisition, JCEG, AS, JLMV.

Declaration of competing interest

The authors declared no conflicts of interest and no financial interests or competing interests. Funders have no role in study conception and design, data analysis and interpretation or decision to publish.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2021.08.004.

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