Impaired high-density lipoprotein anti-oxidant capacity in human abdominal aortic aneurysm

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Aims
Abdominal aortic aneurysm (AAA) is a particular form of atherothrombotic disease characterized by the dilation of the aortic wall and the presence of an intraluminal thrombus (ILT). The objective of the present study was to evaluate the pro-oxidant properties of the ILT and to characterize the anti-oxidant capacity of high-density lipoproteins (HDLs).

Methods and results
Our results show that ILT, adventitia, and plasma from AAA patients contained high concentrations of lipid and protein oxidation products. Mediators produced within or released by the thrombus and the adventitia were shown to induce reactive oxygen species (ROS) production by cultured aortic smooth muscle cells (AoSMCs) and to trigger the onset of apoptosis (an increase in mitochondrial membrane potential). Iron chelation limited these effects. Both concentration and functionality of HDLs were altered in AAA patients. Plasma levels of Apo A-I were lower, and small HDL subclasses were decreased in AAA patients. Circulating HDLs from AAA patients displayed an impaired capacity to inhibit copper-induced low-density lipoprotein oxidation and AoSMC ROS production. Western blot analyses of HDLs demonstrated that myeloperoxidase is associated with HDL particles in AAA patients.

Conclusion
ILT and adventitia are a source of pro-oxidant products, in particular haemoglobin, which may impact on the wall stability/rupture in AAA. In addition, HDLs from AAA patients exhibit an impaired anti-oxidant activity. In this context, restoring HDL functionality may represent a new therapeutic option in AAA.

Keywords
Abdominal aortic aneurysm • Oxidative stress • Haemoglobin • High-density lipoproteins

1. Introduction
Abdominal aortic aneurysms (AAAs) may be considered as a particular form of atherothrombosis characterized by high levels of proteolytic activity,1,2 leading to dilation and eventually to rupture of the aortic wall. Oxidative stress, corresponding to an imbalance between reactive oxygen species (ROS) and antioxidant mechanisms, has been shown to play a contributory role in chronic diseases, including cardiovascular disease, and continuous production of ROS can cause progressive cell and tissue damage.3 The notion that oxidative stress may be involved in AAA pathogenesis is well accepted but poorly documented. Clinical investigations have shown that antioxidants are reduced in tissues and plasma of AAA patients.4,5 In parallel, it has been shown that the AAA vascular wall produces high concentrations of superoxide anion via the activation of NADPH oxidase and that this process is reflected by an increase in circulating oxidative stress markers.6 In addition, haemoglobin may represent an important source of oxidative stress that is not yet well documented. Furthermore, increased intracellular ROS production and oxidation products are major contributors to the induction of smooth muscle cell (SMC) apoptosis.7,8 The presence of an intraluminal thrombus (ILT) appears to be a critical condition for the induction of arterial wall degradation involving SMC apoptosis in the underlying media and elastolysis.9,10 Fontaine et al.11,12 have shown that the ILT is the main source of proteolytic enzymes which inhibit thrombus colonization by aortic smooth muscle cells (AoSMCs), suggesting that proteases, and in particular elastase may be one of the triggers of AoSMC...
apoptosis but the potential implication of oxidative stress in thrombus-induced SMC apoptosis has not yet been evaluated.

In addition to their reverse cholesterol transport activity, high-density lipoproteins (HDLs) possess other potent biological effects including anti-inflammatory, anti-thrombotic, vasodilatory, and antioxidant properties.13,14 Plasma levels of HDL-cholesterol (HDL-C) are inversely correlated with atherothrombosis risk and are significantly lower in AAA patients.15,16 In addition, Golledge et al.17 observed that HDL-C is lower in AAA patients and its concentration was inversely associated with a reduced risk of AAA, whereas triglyceride and LDL levels were not associated with the presence of an AAA. However, to our knowledge, the functionality of HDLs in AAA has not been investigated. In the present study, we evaluated the oxidative stress in the ILT and in the underlying AAA vascular wall. We hypothesized that the production of ROS may participate in AoSMC rarefaction, and that the anti-oxidant properties of HDLs may be blunted in AAA patients.

2. Methods

2.1 Plasma and tissues samples

Plasma samples of AAA patients (n = 20) and healthy volunteers (n = 20) were obtained from the AMETHYST (Aneurysm Metalloproteinases and Hypertension) study (Supplementary material online, Methods and Table S1). AAA tissues (n = 20) were obtained from patients undergoing surgery and enrolled in the RESAA protocol (Référent Sanguin de l’évolutivité des Anévrismes de l’Aorte abdominale, Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, CCPBR Paris-Cochn, approval no 2095).18 Control aortas (n = 10) were sampled from deceased organ donors with the authorization of the French Biomedicine Agency (PFS 09-007). These control aortic samples were macroscopically normal, devoid of early atheromatous lesions.

2.2 Conditioned medium and AoSMC culture

Briefly, the ILT (n = 20) was dissected into three parts: luminal (at the interface with the circulating blood), intermediate, and abluminal layers. The media of AAA (n = 20) and control aortas (n = 20) was separated from the adventitia and each layer was cut into small pieces (5 mm × 3). These various tissue samples were then separately incubated (24 h at 37°C) in a standardized volume (6 mL/g of wet tissue) of RPMI 1640 medium (life technologies, France). The media layer was aliquoted and frozen at −80°C until use.

2.3 Oxidative stress markers

The concentration of advanced oxidation protein products (AOPP) was evaluated as previously described by spectrophotometry.19 A chloramine-T solution, which in the presence of potassium iodide absorbs at 340 nm, was used as a calibrator. Samples (conditioned medium and plasma) were diluted at 1:10 (final volume 200 µL) in phosphate buffer saline. Then 20 µL of acetic acid was added to samples and to the chloramine-T solution just before reading at 340 nm. Results were expressed as µM of chloramine-T equivalents. The concentration of thiobarbituric acid-reactive substances (TBARS) was determined in the conditioned medium and in the plasma of controls and AAA patients using the Yagi method.20 Oxidation of DNA was determined by measuring the 8-hydroxydeoxyguanosine (8-OH-dG) concentration in plasma using an ELISA kit (Cayman, France).

2.4 Determination of intracellular ROS production

Intracellular ROS [including peroxyl (ROO.), hydroxyl radicals (OH.), and peroxynitrite anion (ONOO−)] production by AoSMCs was determined using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescin diacetate, acetyl ester (CM-H2DCFDA, life technologies, France). See Supplementary material online.

2.5 Mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm) was determined in AoSMCs using the JC-1 mitochondrial potential assay kit (Cayman, France), as detailed in Supplementary material online.

2.6 Lipoprotein profile and plasma Apo A-I concentration

Plasma Apo A-I levels (control n = 10, AAA n = 10) were determined using the Human Apo A-I ELISA kit from Mabtech (Sweden). HDL profile was evaluated using Lipoprint® technology (Eurobio, France). In brief, 25 µL of plasma (control n = 10, AAA n = 10) was mixed with a lipophilic dye, which allows visualization and quantification of lipoprotein particles after non-denaturing and non-reducing electrophoresis. The gels were then scanned and analysed using the Lipoware software. The resulting specific HDL profile allowed determination of the percentage of large, intermediate, and small HDLs.

2.7 Determination of the capacity of HDLs to inhibit LDL oxidation

Low density lipoproteins (LDLs) (100 µg/mL) oxidation was induced by adding 5 µM of CuSO4. Oxidation was monitored every minute for 2 h by measuring the increase in absorbance at 234 nm due to conjugated diene formation. Oxidation curves were divided into three different phases: the lagtime, the propagation phase, and the decomposition phase (Supplementary material online, Figure S2).

2.8 Western blot analyses of HDLs

The presence of myeloperoxidase (MPO), in control (n = 8) and AAA (n = 8) HDL particles, was determined by western-blot using a polyclonal rabbit anti-MPO (Abcam, France). Apo A-I (polyclonal rabbit anti-Apo A-I, Abcam, France) was used for normalization. See details in Supplementary material online.

2.9 Statistical analysis

Results are expressed as box plots in which the boxes represent the 25th and 75th percentiles, or as means ± SD. Differences between groups were assessed by the Mann–Whitney non-parametric test. Statistical significance was accepted when P < 0.05. Statistical analyses were performed using Prism 5 (GraphPad software, USA). Owing to non-normal distribution, non-parametric ANOVA analyses were performed on plasma APOP, TBARS, and 8-OH-dG values after sex and smoking adjustment (SAS 9.3, SAS Institute, Inc., USA).

3. Results

3.1 Histological characterization of the AAA wall shows an intense peroxidase activity

All three tunica of the AAA wall (ILT, media, and adventitia) displayed peroxidase activity, chiefly associated with the presence of red blood cells (RBCs) (Figure 1A). The most luminal layer of the ILT, at the interface with circulating blood, was characterized by the presence of freshly (intact) RBCs and cells with polylobed nuclei (Figure 1B, black arrowheads). Patchy areas of RBC agglutination, associated with a fibrin network, were observed in the intermediary layer. The ability of RBCs to generate peroxidase activity was visualized by the precipitation of 3′,3″-Diaminobenzidine (DAB) (brown precipitate). The media layer...
Figure 1 DAB staining for peroxidase activity in AAA samples. (A) Topography of the different layers constituting the AAA wall thrombus, media, and adventitia. (B, C, and D) (B) higher magnifications of selected areas of the luminal part of the thrombus, (C) the residual media covered by a necrotic core containing cholesterol crystals (intima, black arrowheads) and the underlying proximal adventitia containing ATLOs (surrounded with a discontinuous black line), and (D) the highly vascularized adipose tissue. The lower panel shows higher magnifications with details of the thrombus (back arrowheads show cells with polylobed nuclei), the DAB-positive media/intima, ATLOs, and the inflamed adipose tissue. (E) Haemosiderin (black arrowheads) was visualized by Perl’s blue staining and (F) the presence of ceroids (white arrowheads) in the intraluminal thrombus was detected by autofluorescence at 330 nm, nuclei were labelled with DAPI. ATLOs, adventitial tertiary lymphoid organs; RBCs, red blood cells; VSMCs, vascular smooth muscle cells.
and residual cholesterol crystal area also displayed peroxidase activity detected with reaction with DAB (Figure 1C). The underlying adventitia, rich in lymphocytes (CD20+ staining data not shown) that constitute ATLOs (adventitial tertiary lymphoid organs) was also positive locally for peroxidase (Figure 1C), as well as the more remote adipose tissue particularly vascularized (rich in RBCs, Figure 1D). Haemosiderin (Figure 1E), visualized by Perl's reaction, was mainly associated with phagocytic activity in the media/intima interfacing with ILT. Ceroid (Figure 1F) were present within the luminal layer of the ILT and colocalized with toluene-resistant lipids.

3.2 Oxidative stress markers are increased in AAA
Our results show that the release of TBARS, a marker of lipid oxidation, was significantly enhanced in AAA arterial wall, especially in the adventitia, when compared with control aortic samples (Figure 2A). The luminal layer was the major site of lipid peroxidation within the ILT (P < 0.0001 vs. abluminal layer). Similarly, the release of AOPP, a marker of protein oxidation, was significantly increased in AAA-conditioned medium when compared with control (P < 0.0001, Figure 2B) in particular in the adventitia of AAA (P < 0.0001 vs. AAA media). In the ILT, the main source of AOPP was the luminal layer (P < 0.0001 vs. intermediate and abluminal layers).

In plasma, TBARS (lipid marker), AOPP (protein marker), and 8-OH-dG (DNA marker) levels were significantly higher (Supplementary material online, Table SII) in AAA patients when compared with controls. Furthermore, the statistical differences persisted between control and AAA groups after adjustment for sex and smoking status (TBARS: P = 0.0006, AOPP: P = 0.047; 8-OH-dG: 0.0002).

3.3 AAA-conditioned medium induced aortic SMC ROS production
The incubation of AoSMCs with medium conditioned by the adventitial layer of AAA samples induced an increase in ROS production relative to that of healthy aorta and unstimulated cells (Figure 3A; P < 0.0001). ILT-conditioned medium also induced an enhanced ROS production by AoSMCs. This effect was highly significant when SMCs were incubated with the luminal and abluminal layer-conditioned medium. In a second set of experiments (Figure 3B), we have tested the hypothesis that haemoglobin-associated iron contained in the luminal layer may be a major effector of ROS production and we observed that pre-treatment of luminal and adventitial conditioned medium with desferrioxamine significantly reduced the ROS production.

3.4 Mediators released by the luminal layer induced aortic SMC apoptosis
A potential pro-apoptotic effect of the conditioned medium (obtained from the different layers of AAA samples) on AoSMCs was evaluated by measuring the mitochondrial membrane potential (∆Ψm, change in JC-1 fluorescence). As shown in Figure 4A, the medium conditioned by the AAA arterial wall (media and adventitia) induced a significant increase (P < 0.0001) in the ∆Ψm intensity (ratio of fluorescent intensity J-aggregate fluorescence to monomers) relative to untreated cells or cells incubated with control aortic wall-conditioned medium. ILT-conditioned medium induced SMC apoptosis in contrast to intermediate and abluminal conditioned medium. Desferrioxamine significantly reduced this pro-apoptotic effect (Figure 4B).

3.5 HDLs from AAA patients: decreased levels, size, and anti-oxidant capacity
HDL levels are inversely correlated with the development of cardiovascular diseases and reported to be low in AAA patients. Their functionality is associated with anti-proteases, anti-apoptotic, and anti-oxidant capacity that could be modulated under pathological conditions. We tested whether HDL levels, size, and functionality were altered in AAA.

3.5.1 Apo A-I concentration and HDL size
Our results confirmed that the plasma apo A-I concentration of AAA patients is significantly lower than that of healthy subjects (Supplementary material online, Figure S4A). HDL particle size was suggested to be related with their function and anti-atherogenic properties. We evaluated HDL size by non-reducing/non-denaturating electrophoresis (Lipoprint system). Lipoprint analyses suggested that the HDL profile of AAA patients was modified vs. that of controls (Supplementary material online, Figure S4B). The percentage of small HDL particles was significantly lower in AAA than in control subjects, whereas the percentage of intermediate and large HDLs did not differ between the two groups.

3.5.2 Effect on LDL oxidability
In a preliminary experiment, we showed that HDLs (1–10 μg/mL) prevented copper-induced LDL oxidation in a dose-dependent manner, reaching statistical significance at 8 μg/mL (data not shown). HDLs from healthy volunteers protected LDLs against oxidation (Figure 5A). This protective effect was significantly reduced in HDLs from AAA subjects (decreased T1/2 and lag time and increased Vmax).

We then tested whether the effectors released by the luminal thrombus and the adventitia layer may alter the antioxidant capacity of HDLs. For this purpose, HDLs from healthy volunteers were incubated with luminal or adventitial-conditioned medium and then re-isolated by ultracentrifugation. Our results (Figure 5B) show that these HDLs exhibited a decreased protective effect on copper-induced LDL oxidation relative to that of native HDLs (significant increase in T1/2 and decrease in Vmax). Furthermore, our results suggest that haemoglobin-associated iron could be, at least in part, responsible for the inhibition of the protective effect of HDLs since desferrioxamine restored in part the antioxidant effect of HDLs.

3.5.3 Effect on ROS production
We then tested the capacity of HDLs to inhibit ROS production by AoSMCs; our results show that control HDLs (100 μg/mL) significantly decreased ROS production induced by LT and adventitial conditioned medium. It is noteworthy that HDLs isolated from AAA patients were significantly less protective than HDLs isolated from controls (Figure 6A).

3.5.4 Myeloperoxidase is associated with HDLs from AAA patients
Plasma MPO levels are increased in AAA patients. In addition, MPO-modified HDLs are dysfunctional. We tested whether HDLs from AAA patients carried more MPO than those from control subjects. Western blot analyses showed that more MPO was associated with modified HDLs are dysfunctional. We tested whether HDLs from AAA patients than with those from control individuals (Figure 6B).
4. Discussion

Our study shows that the oxidative activity of AAA is mainly linked to haemoglobin-associated iron (or haeme) release by the ILT and contained in the adventitia. ILT and adventitial conditioned medium triggers ROS production by AoSMCs that could induce apoptosis and subsequent dilation of the aortic wall. We have also demonstrated that HDLs isolated from AAA patients displayed a modified subfraction profile and that their ability to inhibit oxidative stress was impaired.

The role of oxidative stress in AAA progression has already been suggested. ROS-generating systems and decreased antioxidant systems have been reported in AAA subjects. In animal models, the use of free-radical scavengers limited the formation and progression of AAA. In contrast to most animal models, human AAAs are characterized by the presence of biologically active ILT, rich in pro-oxidant haemoglobin derived from erythrocytes and where neutrophil accumulation may represent a source of proteases and ROS. However, the pro-oxidant effect of the ILT is poorly documented. The ILT in AAA...
is composed of fibrin and agglutinated RBC. Tissue RBCs release haemoglobin and heme–iron component, with considerable oxidative power. DAB precipitates in the presence of haemoglobin-dependent peroxidase activity, and the accumulation of free iron (haemosiderin) is revealed by Perl’s reaction.

The ILT in AAA, as in other forms of atheroma, is also characterized by the formation of autolysosomal ceroids. Ceroids are highly cytotoxic insoluble polymers of oxidized cholesterol and proteins that can be formed extracellularly, and that colocalize with iron deposits within the tissue. The presence of haemoglobin has been recently reported in ceroids by the combination of the Raman and fluorescence spectral microscopy. Ceroid antibodies are present in the serum of patients with AAA aortitis, suggesting that outwardly convected ceroids could serve as neo-antigens for promoting the adaptive immune response in the adventitia.

Here, we demonstrate that mediators released by the ILT and the adventitia induced intracellular oxidative stress in AoSMCs and increased mitochondrial membrane potential, a step initiating apoptosis. Moreover, we observed that the mediators accumulated at the luminal pole of the thrombus are the most effective for inducing ROS generation in AoSMCs. Hydraulic conductance occurring across the AAA wall could convey soluble luminal mediators to the AoSMCs. In particular, the luminal layer contains large amounts of haemoglobin that can easily diffuse towards the residual aortic media. In the present study, we have shown that pre-treatment of ILT and adventitial conditioned medium with desferrioxamine (an iron chelator that protect protein against oxidative damages and inhibit neuronal death induced by haemoglobin) decreased intracellular ROS production by AoSMCs and limited mitochondrial membrane changes. These changes are a marker of apoptosis. Haeme-containing proteins are able to induce intracellular oxidative stress but may also participate in the oxidation of lipids and thus produce toxic products of lipid peroxidation such as 4-hydroxy-trans-2-nonenal (4HNE) and malondialdehyde (MDA).

In our study, we show that TBARS (chiefly aldehydes including 4HNE and MDA) were found in greater amounts in the medium conditioned by the luminal layer of the thrombus, which is particularly rich in haemoglobin compared with the other layers. Simvastatin was recently reported to decrease levels of 4HNE in human AAA wall via a NFKB-dependent pathway. The use of statins in AAA patients is recommended, although their beneficial effects are controversial. In particular, statins have a clear impact on LDL levels but their effect on HDL concentrations and/or functionality is not well established. Several studies report that HDL and Apo A-I levels are decreased in AAA patients and that their functionality might be blunted. Moreover, experimental data on an AAA mouse model have shown that the elevation of HDL concentration by fenofibrate treatment or by the injection of recombinant HDLs limited AAA growth.

Using non-denaturing electrophoresis (Lipoprint system), we show here that the HDL profiles of AAA patients were modified. Small HDL particle levels were decreased while large and intermediate subfractions were unchanged. Different studies report that large HDLs are decreased and small HDLs increased in patients with cardiovascular diseases, suggesting that large HDLs could be more protective than small HDLs. However, it has been shown that small HDL3 particles possess anti-atherogenic properties, in particular antioxidant activity. In our study, we observed that the antioxidant capacity of AAA HDLs was blunted, showing: (i) decreased inhibition of LDL oxidation in vitro and (ii) reduced inhibition of ROS production by AoSMCs.

In cardiovascular diseases, LDL oxidation represents a crucial event participating in the major deleterious effects of oxidative stress on the arterial wall: foam cell formation, endothelial, and smooth muscle cell apoptosis. The decreased capacity of AAA HDLs to limit LDL oxidation could be related in part to reduced levels of small HDLs in the plasma of these patients. This is in accordance with previous reports showing that small HDLs inhibit LDL oxidation more effectively than large HDLs. Another mechanism by which HDLs may inhibit oxidation could rely on their capacity to limit ROS production, notably through the inactivation of NADPH oxidase. Here, we observed...
that AAA HDLs had a limited effect relative to HDLs from controls on AoSMC ROS production and apoptosis induced by the mediators released by the luminal and adventitial part of the thrombus.

Different proteomic studies have reported that the HDL cargo is modified under pathological conditions and that protein oxidation generates dysfunctional HDLs. Ortiz-Munoz et al. demonstrated that AAA HDLs contain less alpha-1 antitrypsin, the major circulating elastase inhibitor, that could impact on SMC apoptosis induced by elastase and subsequent AAA growth. Here, we observed that AAA HDLs bind MPO, one of the main neutrophil pro-oxidant enzymes. Indeed, western blot experiments showed that AAA HDLs contain larger amounts of MPO than control HDLs. A similar result had been described earlier for HDLs isolated from atherosclerotic lesions. The implication of neutrophils via the release of proteolytic enzymes and activation of pro-oxidant systems in AAA progression is now well documented. This is consistent with our results showing that the luminal part of the thrombus released AOPPs, chiefly originating from the modification of proteins by MPO. MPO binding to HDLs leads to Apo A-I oxidation by HOCl and renders HDLs dysfunctional. We thus tested the ability of the ILT and adventitial conditioned medium to modify, in vitro, the antioxidant capacity of control HDLs. Such modified HDLs displayed reduced capacity to inhibit LDL oxidation. Modification of HDLs within the thrombus and the adventitia could thus explain, at least in part, why AAA HDLs are dysfunctional.

In conclusion, the results of the present study indicate that mediators released by the ILT, particularly by the luminal layer and the adventitia, have pro-oxidant properties. It is noteworthy that haemoglobin accumulated in these layers play a central role. In addition, we have observed that HDLs from AAA patients displayed a different profile (less small HDL particle than controls) and had a reduced antioxidant function. Taken together, our results and those from Ortiz-Munoz et al. underline the impaired functionality of HDLs in AAA and suggest that treatments raising HDL concentration and/or improving their functionality could represent a new strategy for reducing AAA progression.

**Figure 5** HDLs from AAA patients exhibit a blunted protective effect against LDL oxidation. LDL (100 μg/mL) oxidation was induced by 5 μM of CuSO₄ and the formation of conjugated dienes was monitored at 234 nm for 2 h. (A) Co-incubation of LDLs with 8 μg/mL of HDLs from eight AAA or eight control (CTL) subjects. *P < 0.05, **P < 0.01 (Mann–Whitney test). (B) HDLs were modified by incubation with luminal and adventitial conditioned medium in the presence of desferroxamine (100 μM) or not. After re-isolation by ultracentrifugation, their effect on LDL oxidation was assessed as described above. *P < 0.05 (Wilcoxon matched paired test).
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Figure 6 Inhibition of AoSMC ROS production by HDLs and MPO binding. (A) Aortic SMCs were incubated with ILT-conditioned medium for 2 h ± HDLs (100 μg/mL) isolated from controls (CTL) or AAA patients. After washing, 5 μM of CM-H2DCFDA were added and the fluorescence intensity (λ excitation 485 nm, λ emission 538 nm) was monitored every 2 min for 2 h. (B) MPO (upper band) and Apo A-1 (lower band) were detected by western blot in HDLs (5 μg) isolated from 8 control and 8 AAA plasma samples. Results are expressed as the ratio of MPO intensity relative to Apo A-1 intensity. *P < 0.05 (n = 8; Mann–Whitney analysis).

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