Proteomic Analysis of Polymorphonuclear Neutrophils Identifies Catalase as a Novel Biomarker of Abdominal Aortic Aneurysm: Potential Implication of Oxidative Stress in Abdominal Aortic Aneurysm Progression

Priscila Ramos-Mozo, Julio Madrigal-Matute, Roxana Martinez-Pinna, Luis Miguel Blanco-Colio, Juan Antonio Lopez, Emilio Camafeita, Olivier Meilhac, Jean-Baptiste Michel, Cesar Aparicio, Melina Vega de Ceniga, Jesus Egido, Jose Luis Martin-Ventura

Objective—Polymorphonuclear neutrophils (PMNs) play a main role in abdominal aortic aneurysm (AAA) progression. We have analyzed circulating PMNs isolated from AAA patients and controls by a proteomic approach to identify proteins potentially involved in AAA pathogenesis.

Methods and Results—PMNs from 8 AAA patients (4 large AAA >5 cm and 4 small AAA 3–5 cm) and 4 controls were analyzed by 2D differential in-gel electrophoresis. Among differentially expressed spots, several proteins involved in redox balance were identified by mass spectrometry (eg, cyclophilin, thioredoxin reductase, catalase). Diminished catalase expression and activity were observed in PMNs from AAA patients compared with controls. In contrast, PMNs from AAA patients displayed higher H2O2 and myeloperoxidase levels than PMNs from controls. Moreover, a significant decrease in catalase mRNA levels was observed in PMNs after phorbol 12-myristate 13-acetate incubation. Catalase plasma levels were also decreased in large (n=47) and small (n=56) AAA patients compared with controls (n=34). We observed catalase expression in AAA thrombus and thrombus-conditioned medium, associated with PMN infiltration. Furthermore, increased H2O2 levels were observed in AAA thrombus-conditioned medium compared with the media layer.

Conclusion—Diminished catalase levels in circulating PMNs and plasma are observed in AAA patients, supporting an important role of oxidative stress in AAA evolution. (Arterioscler Thromb Vasc Biol. 2011;31:3011-3019.)

Key Words: aneurysms ■ antioxidants ■ leukocytes

Abdominal aortic aneurysm (AAA) is an important health problem in elderly. In cross-sectional studies, the prevalence varies from 3% to 8%.1 In elderly men, AAAs may cause as much as 2% to 3% of all deaths.1 Because AAAs are usually asymptomatic, the present clinical challenges are early diagnosis and deciphering the biological mechanisms responsible for the progressive dilatation and final rupture to develop new diagnostic and therapeutic approaches.

Although polymorphonuclear neutrophils (PMNs) represent the major class of leukocytes, they have received little attention in atherothrombosis.2,3 However, recent evidence is revealing a previously unappreciated role of PMN in experimental4,5 and human6–7 AAAs. PMNs can contribute to main mechanisms of AAA evolution, namely intraluminal thrombus (ILT) formation, oxidative stress, proteolytic degradation of the aortic media, and adventitial inflammation.8 AAAs are characterized by the presence of a mural ILT-containing platelets, red blood cells (RBCs), and PMNs, particularly abundant within the luminal layer of human thrombus.6–8 AAAs are also characterized by destructive connective tissue remodeling, including depletion of aortic elastin and fragmentation of medial elastic fibers.9 Finally, inflammatory cells (macrophages and neutrophils) are also evident within the adventitia of human AAAs.10 Interestingly, PMNs depletion is able to inhibit experimental AAA formation.11 More recently, short-term preoperative doxycycline therapy improved the proteolytic balance in human AAA, presumably via an effect on aortic wall neutrophil content.12

These data highlight the potential interest of analyzing the PMNs proteome with the aim of identifying novel diagnostic and prognostic targets in AAA disease. Furthermore, identification of biomarkers could also afford novel pathogenic...
plasma sample collection was registered (Supplemental Table I, mRNA analysis) at 37°C. [MPO] and catalase/manganese superoxide dismutase [MnSOD] activities. Expression proteomic studies offer the possibility of finding out proteins that could be dysregulated in PMNs under pathological conditions. Previous studies analyzing expression proteome of PMNs have been recently thoroughly reviewed. However, no studies have addressed the comparison of PMN proteome in vascular diseases. In the present study, we have performed a comparative 2D differential in-gel electrophoresis protein expression analysis of circulating PMNs isolated from AAA patients and controls.

Methods

AAA Patients

The blood samples used for proteomic analysis were obtained from 8 AAA patients before they underwent infrarenal AAA repair (n=4 large AAA, AAA diameter >5 cm) or patients who visited the vascular surgery department for follow-up assessment (n=4 small AAA, AAA diameter 3–5 cm). The control group consisted of 4 volunteers (AAA diameter <3 cm) without significant differences from the patient groups in age, sex, risk factors, or medications. Furthermore, 12 additional patients (6 large AAA and 6 small AAA) and 6 additional controls were used for further validation of the proteomic results. Finally, for further functional studies, PMNs were isolated from 10 additional controls and 10 patients (4 large AAA and 6 small AAA) and incubated with 50 μmol/L phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) or vehicle for 30 minutes (for hydrogen peroxide [H2O2]) or for 4 hours (for myeloperoxidase [MPO] and catalase/manganese superoxide dismutase [MnSOD] mRNA analysis) at 37°C.

In addition, 103 consecutive patients with an asymptomatic infrarenal AAA were recruited, and the AAA size at the time of plasma sample collection was registered (Supplemental Table I, available online at http://atvb.ahajournals.org). Similarly, 34 controls were recruited from a screening program, which is currently being performed among the population in the area under our care. They were randomly selected from the screened individuals with nondilated (<30 mm, confirmed with abdominal ultrasound) infrarenal aortas. The study was approved by the scientific ethics committees of our institutions, and informed consent was obtained from the patients and the controls for their inclusion in the study.

AAA Tissue and Tissue-Conditioned Medium

Ten AAA tissue samples were collected during surgical repair and dissected into ILT and medial layer. AAA samples were obtained from patients undergoing surgery, enrolled in the RESAA protocol (Réflet Sanguin de l’évolution des Anévrismes de l’Aorte abdominale, CCPPRB Paris-Cochin Nos. 2095, 1930, and 1931). All patients gave their written informed consent, and the protocol was approved by a French ethics committee (CCPPRB, Cochin Hospital). AAA thrombus and aortic walls were cut into small pieces (5 mm²) and separately incubated in RPMI 1640 medium containing antibiotics and an antymycotic (Gibco) for 24 hours at 37°C (6 mL/g of wet tissue). The conditioned medium (supernatant containing proteins released by the tissue sample) was obtained after centrifugation at 3000g for 10 minutes at 20°C.

Human Neutrophils

Neutrophils were obtained from heparinized venous blood by centrifugation in Ficoll-Paque (GE Healthcare) followed by 6% dextran sedimentation of the pellet and hypoosmotic lysis of residual erythrocytes. Neutrophils were resuspended in lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, and 30 mmol/L Tris-HCl, pH 8.5), and proteins were precipitated to remove salts and other interfering components by using the 2D Clean Up Kit (GE Healthcare). The resulting proteins were resuspended in lysis buffer. The protein concentration was measured using the RC/DC Protein Assay (Bio-Rad).

PMNs purity was assessed by flow cytometry to ensure that they were not contaminated by other cell types. The analysis of CD16
from PMNs showed a strong fluorescence signal (>90% to 95%) in all samples (Supplemental Figure I).

**Differential In-Gel Electrophoresis Experiment and Data Analysis**
The 6 paired samples were labeled with CyDye Fluor minimal dyes (GE Healthcare) according to the manufacturer’s instructions, and isoelectric focusing and second dimension were performed as described in the supplemental material.

The images were analyzed using the DeCyder software, version 7.0 (GE Healthcare), for spot detection and quantification, intergel matching, and statistics. DeCyder calculates the average abundance of each spot among the 6 gels under study. Statistical significance was assessed for each change in abundance using the Student t test ANOVA. We considered spots present in all of the 18 images (3 images per gel) with statistical significance at the 95% confidence level for standardized average spot volume ratios greater than 1.5.

**Protein Identification by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry**
Differentially expressed spots were selected from silver-stained gels for gel excision, automated digestion, and analysis in an Ultraflex matrix-assisted laser desorption ionization (MALDI) tandem time of flight mass spectrometer (Bruker Daltonik) to obtain the corresponding MALDI–mass spectrometry (MS) and MALDI-MS/MS spectra. These MS and MS/MS combined data were used to search a nonredundant protein database (NCBI; ≈107 entries; National Center for Biotechnology Information, Bethesda, MD) using the Mascot software (Matrix Science). Detailed information is given in the Supplemental Methods.

**Western Blot**
Cell extracts from PMNs were sonicated and resuspended in lysis buffer, and protein concentration was quantified by Bradford reagent (Bio-Rad). Equal amounts of PMN proteins (20 μg) or an equal volume (10 μL) of AAA tissue–conditioned medium (previously normalized to tissue weight: 6 mL of RPMI per 1 g of wet tissue) were used as described in the supplemental material.

**Catalase Activity**
Similar amounts of PMN extracts were analyzed for catalase activity using a commercial enzymatic assay (K773, Biovision) following the manufacturer’s instructions. Catalase units were defined as the amount of enzyme that decomposes 1 μmol H₂O₂ per minute at pH 4.5 at 25°C.

**ELISA**
We quantified concentrations of catalase and MPO with commercial kits (E92418HU, USCN Life Science and Hycult) following the manufacturers’ instructions.

**2,7-Dichlorofluorescein Diacetate**
Intracellular H₂O₂ levels in PMNs were measured with 2,7-dichlorofluorescein diacetate (Sigma-Aldrich) as described previously. Briefly, 1×10⁶ fresh PMNs were loaded with 5 μmol/L 2,7-dichlorofluorescein diacetate in Hanks’ buffered salt solution at 37°C for 30 minutes and then washed twice. Fluorescence was evaluated with a microplate reader (GENios Tecan) at 535 nm with an excitatory wavelength of 485 nm for 30 minutes. The reactive oxygen species (ROS) production was expressed as relative fluorescence units per second.

**RNA Extraction and Real-Time Quantitative-Polymerase Chain Reaction**
Total RNA was isolated from cells using Trizol Reagent (Invitrogen). One μg of RNA was used to perform the reverse transcribed with High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time polymerase chain reactions were performed on ABI Prism 7500 sequence detection polymerase chain reaction system (Applied Biosystems) according to the manufacturer’s protocol using the delta delta Ct method as described. Primers and conditions are described in the supplemental material.

**Immunohistochemistry**
AAA thrombus samples were fixed in 3.7% paraformaldehyde and embedded in paraffin. Immunohistochemistry was performed on 5-μm sections using Catalase (1:100 Abcam) overnight at 4°C as primary antibody, The peroxidase LSAB + system–horseradish peroxidase kit (Dako) followed by the Histogreen peroxidase substrate (AbCys SA) was used for detection. Sections were then counterstained with Nuclear Fast Red for 5 minutes at room temperature before being mounted using Eukitt medium. Control irrelevant rabbit immunoglobulins (Dako) were applied at the same concentrations as primary antibodies to assess nonspecific staining.

**Hydrogen Peroxide Quantification**
Quantitative determination of extracellular hydrogen peroxide in ILT conditioned media was performed with a commercial colorimetric kit (907-015, Assay Design), following the manufacturer’s instructions.

**Statistical Analysis**
Results from Western blot, catalase activity, 2,7-dichlorofluorescein diacetate, MPO levels in PMNs, quantitative polymerase chain reaction, and H₂O₂ extracellular levels are expressed as mean±SEM and were analyzed by the Mann-Whitney nonparametric test (small and large AAA and control groups) or Wilcoxon paired test (between thrombus and media supernatants of the same samples). Results from the ELISA are expressed as median (interquartile ranges) and were analyzed by the ANOVA test. A probability value <0.05 was considered statistically significant.

**Results**

**Proteomic Analysis of Circulating PMNs**
Proteins from neutrophils isolated from different groups of AAA patients (large AAA [AAA diameter >5 cm] and small AAA [AAA diameter 3–5 cm]) and control subjects (AAA diameter <3 cm) were compared by 2D differential in-gel electrophoresis (Figure 1). DeCyder software provided us a list of differentially expressed spots. After silver staining of the gels, only those visible were excised and trypsin digested. Forty-one of them were finally identified by MALDI MS (Supplemental Table II). Proteins known to be expressed by PMN have been identified (eg, lactoferrin, lipocalin 2). In addition, we found proteins involved in different neutrophil functions, such as cytoskeletal proteins (eg, WDR1), inflammatory processes (eg, LT4H) and survival (eg, coronin). Moreover, a high number of proteins involved in redox balance (eg, cyclophilin, catalase, and thioredoxin reductase) were observed.

**Decreased Catalase Levels in Circulating PMNs From AAA Patients**
Among the differentially expressed proteins identified by MALDI MS, we have focused on the antioxidant protein catalase because of the importance of oxidative stress in AAA progression. The results derived from proteomic data were validated in a second, independent group of patients and controls by Western blot, confirming the decreased catalase expression in PMNs from patients with large and small AAA relative to control subjects (10.4±0.8 and 10.7±1.2 versus
Similar results were obtained for thioredoxin reductase (10.8 $\pm$ 1.5 and 10.6 $\pm$ 1.2 versus 14.8 $\pm$ 1.7 arbitrary units, $P<0.05$, not shown). In addition, catalase activity was also assessed in PMNs from patients and controls. As shown in Figure 2B, catalase activity was decreased in PMNs from patients with large and small AAA compared with controls (338 $\pm$ 27 and 312 $\pm$ 32 versus 376 $\pm$ 36 mU/mL, $P<0.05$). Catalase activity and expression showed a positive correlation ($r=0.4$, $P<0.05$, Figure 2C).

Redox Balance of PMNs From AAA Patients and Controls

To address the prooxidant status of neutrophils, we analyzed H$_2$O$_2$ and MPO levels from a third additional group of PMNs isolated of 10 AAA patients (AAA $>5$ cm [$n=4$] and AAA $<5$ cm [$n=6$]) and controls ($n=10$). PMNs isolated from both large and small AAA patients displayed higher H$_2$O$_2$ intracellular levels compared with PMNs from controls (Figure 3A). In addition, PMNs isolated from both large and small AAA patients released higher MPO concentrations compared with PMNs from controls (Figure 3B). In the other hand, baseline catalase mRNA levels were decreased in AAA patients compared with controls (Figure 3C, in agreement with the results obtained at the protein level (Figure 2A). Similar results were obtained for MnSOD mRNA (Figure 3D).

To address whether decreased catalase expression could be associated with the prooxidant conditions that occur in neutrophils during respiratory burst, neutrophils were incubated with PMA, which is known to induce respiratory burst in PMNs. After PMA incubation, increased H$_2$O$_2$ and MPO levels were shown in PMNs from controls, reaching levels similar to those of PMNs from patients (Figure 3A and 3B). Interestingly, we found a significant decrease in catalase mRNA levels after PMA incubation, and a similar trend was observed for MnSOD (Figure 3C and 3D).

Decreased Catalase Plasma Levels in AAA Patients

To address whether the redox imbalance observed in circulating PMNs could also occur in plasma of AAA patients, MPO and catalase were assessed in AAA patients and controls. Similar to the results obtained in circulating PMNs, catalase plasma levels were significantly decreased in large and small AAA patients relative to control subjects (111 [63–175] versus 145 [90–208] versus 159 [132–211] U/mL, median [interquartile range], $P<0.05$ for controls versus small AAA and $P<0.001$ for controls versus large AAA) (Figure 4A). In the other hand, MPO plasma levels were increased in patients with large and small AAA as compared with controls (65 [46–118] versus 65 [54–92] versus 41 [31–51] ng/mL, $P<0.001$ for both large and small AAA versus controls; data not shown). Finally, because aortic diameter is a surrogate marker of the growth rate, we studied the correlation between catalase plasma levels and aortic diameter. Interestingly, a significant negative correlation between catalase plasma levels and aortic diameter was found ($r=-0.4$, $P<0.001$, Figure 4B).

Catalase in AAA Thrombus and Thrombus-Conditioned Media

Because the luminal layer of ILT of human AAA is enriched in PMNs, we evaluated the levels of catalase in both ILT and ILT-conditioned media. As shown in Figure 5A, the luminal part of the thrombus showed an important staining for catalase, associated with poly-lobed nuclei cells, likely to be...
neutrophils; however, other catalase-positive cells from non-neutrophil origin are observed in AAA tissue (possibly RBCs). Both cellular and diffuse staining was observed, which suggests the presence of catalase in the extracellular compartment. In this respect, catalase levels were increased in the conditioned media of ILT of AAA compared with that of the media layer (15.9 ± 2.7 versus 7.2 ± 1.6 arbitrary units, \( P < 0.05 \), Figure 5C). Finally, hydrogen peroxide (H\(_2\)O\(_2\)) levels were also increased in the ILT compared with the media layer (10.4 ± 2.2 versus 5.2 ± 0.7 \( \mu \)mol/L, \( P < 0.05 \), Figure 5D).

**Discussion**

PMNs represent the major class of leukocytes. PMNs contribute to main pathological mechanisms of human AAA, such as proteolysis, oxidative stress, and adventitial immune-inflammatory processes.\(^6\)–\(^8\) The key role of PMNs in the pathogenesis of AAA is supported by recent studies in animal models of AAA.\(^4\),\(^5\) Furthermore, therapies modifying PMN content in both human and experimental models of AAA have shown a protective effect on AAA development.\(^1\)\(^1\),\(^1\)\(^2\) Because PMNs are key cells in AAA pathophysiology, we have comparatively analyzed circulating PMNs from AAA patients and controls to unveil proteins differentially expressed in pathological conditions, which could provide information about mechanisms involved in AAA evolution. Among identified proteins previously related to AAA, increased lipocalin 2 and cyclophilin have been observed in PMNs of AAA patients in our study. Lipocalin-2 was previously localized in the luminal part of AAA thrombus, associated with matrix metalloproteinase-9.\(^1\)\(^8\) Cyclophilin could participate in different mechanisms involved in vascular remodeling by promoting inflammation and smooth muscle cell proliferation.\(^1\)\(^9\) Moreover, cyclophilin was shown to enhance vascular oxidative stress and the development of angiotensin II-induced aortic aneurysms.\(^2\)\(^0\) These results reinforce the interest and feasibility of analyzing circulating PMNs by proteomic approaches to unveil biomarkers of AAA pathogenesis.

Oxidative stress is the result of an imbalance between antioxidant and prooxidant molecules. Among the proteins identified by proteomic analysis, we showed decreased intracellular expression of antioxidant proteins, such as catalase and thioredoxin reductase, in circulating PMNs from AAA patients compared with controls, whereas cyclophilin was enhanced. Furthermore, we analyzed catalase activity in circulating PMNs, showing a decreased catalase activity of AAA patients compared with controls.

In contrast, we observed that PMNs isolated from AAA patients displayed higher H\(_2\)O\(_2\) levels and released higher...
MPO concentrations compared with PMNs from controls, paralleled by a decrease in both catalase and MnSOD mRNA expression. Moreover, to test whether these antioxidant systems could be modified under the conditions of increased oxidative stress associated with respiratory burst, neutrophils were incubated with PMA, known to induce respiratory burst in PMNs. Incubation of PMNs from controls with PMA increased both $H_2O_2$ and MPO levels, reaching levels similar to those of PMNs from patients, probably associated with the saturation of the prooxidant capacity of these cells. Interestingly, after PMA incubation, a significant decrease in catalase mRNA levels were observed, and a similar trend was obtained for MnSOD, which could suggest a global decrease in antioxidant enzymes in PMNs under respiratory burst conditions. On the whole, our data suggest that a redox imbalance toward increased oxidative stress (increased oxidant species such as $H_2O_2$ and MPO/decreased antioxidant species such as catalase and MnSOD) was observed in PMNs from AAA patients.

At the systemic level, previous studies have analyzed the circulating levels of different prooxidant molecules in AAA patients. Among them, blood levels of malondialdehyde were significantly increased in AAA patients. Likewise, we have measured MPO in plasma of AAA patients, a well-recognized oxidative stress biomarker of different cardiovascular pathologies, showing that MPO plasma levels were increased not only in large AAA, but also in small AAA. In the other hand, catalase plasma levels could be associated with the progression of the disease. At the tissue level, superoxide anions, as well as lipid peroxidation products have been assessed in human AAA arterial wall as compared with adjacent nonabdominal segments, showing an increased oxidative stress and associated derived-products in AAA segments.

On the other hand, MnSOD activity in human diseased aorta was $\approx 65\%$ of controls. Furthermore, ruptured AAA tissue also had low SOD activity and protein. However, AAA formation is associated with early increases in SOD expression in an experimental model. In contrast, the beneficial effect of flow loading limiting experimental AAA formation was associated with increased antioxidant gene (hemoxigenase-1) expression in the aorta. In the present study, immunohistochemical analysis showed catalase in human ILT, associated with PMNs. However, other nonnucleated cells, likely RBCs, exhibited a strong immunostaining for catalase. Interestingly, diffuse extracellular staining of catalase was observed in ILT tissue by immunohistochemistry, suggesting its potential release to the extracellular medium. Accordingly, we have shown increased extracellular catalase levels in ILT-conditioned medium, which could be a response to counteract the formation of ROS from extracellular $H_2O_2$ observed in ILT. In agreement, other authors have shown the presence of catalase in the extracellular medium, and they suggest that catalase secretion could be a response to avoid neutrophil-induced oxidative damage at a local level or to regulate the function of ROS as extracellular signaling molecules. However, high catalase levels in ILT could also be due to cell lysis of both PMNs and RBCs. In this respect, the ILT of AAA is characterized by the presence of several blood cells (among them, PMNs and RBCs) and apoptotic cells, and all of them could contribute to increased oxidative stress. In addition to the release of PMN intracellular content (eg, MPO), the...
trapping of RBCs within the thrombus may lead to hemolysis and subsequent release of hemoglobin, heme, and, finally, prooxidant iron. Among ROS, H$_2$O$_2$ is a nonradical, uncharged oxidant that is chemically more stable than other ROS and that can permeate through the vascular wall. In addition, H$_2$O$_2$ can accumulate extracellularly in the tissue and survive long enough to induce numerous paracrine functions. $^{30}$ H$_2$O$_2$ itself is not very reactive; however, the danger of H$_2$O$_2$ comes from its rapid conversion to hydroxyl radical by interaction with a range of transition metal ions, of which the most important in vivo is probably iron. Thus, ILT is a privilege site for ROS formation because they can be formed, among other mechanisms, either by MPO-catalyzed or by Fe$^{2+}$-catalyzed conversion of H$_2$O$_2$. $^{30}$ On the whole, the imbalance between oxidant species and antioxidant systems in AAA patients, both at the systemic level and the tissue level, further supports the importance of oxidative stress in AAA evolution.

Antioxidant systems are crucial for tissues to detoxify free radical species and protect organisms against oxidative stress. In a previous study, vitamin E attenuated formation of AAA. $^{31}$ Importantly, animals treated with vitamin E showed a 44% reduction in the combined end point of fatal and nonfatal aortic rupture. More recently, ROS inhibition has been shown to attenuate aneurysm formation. $^{32}$ Among pathological mechanisms potentially modulated by catalase, it was previously shown that ROS/H$_2$O$_2$ activates endothelial cells to increase PMN adhesion, and catalase is able to prevent leukocyte accumulation. $^{33}$ Moreover, overexpression of catalase suppresses oxidized-low-density lipoprotein–induced aortic smooth muscle cell death $^{34}$ and inhibits smooth muscle cell proliferation. $^{35}$ In addition, catalase delivery has been successfully used to reduce lipid peroxidation in mice. $^{36}$ In these studies, the doses used are lower than its endogenous levels, $^{37}$ suggesting that its therapeutic effects could be associated with its functions outside cells. $^{36}$ Interestingly, diminished experimental AAA formation by tamoxifen treatment has been associated with increased catalase expression, which was accompanied by decreased PMN infiltration. $^{38}$ Furthermore, catalase supplementation inhibited experimental aneurysm formation. $^{38}$ Very recently, catalase overexpression in aortic smooth muscle cells prevents pathological mechanical changes underlying AAA formation. $^{39}$ On the whole, these studies, including ours, suggest a potential protective role of catalase in the mechanisms underlying AAA.

**Figure 5.** Catalase expression in abdominal aortic aneurysm (AAA) thrombus and thrombus-conditioned medium. A and B, Immunodetection of catalase in AAA thrombus (A) and negative control (B) (×10; inset, ×20X). Positivity is shown in green, and nuclei are in red. C, Western blot of anticatalase in conditioned medium from intraluminal thrombus (ILT) and medial layer (M) (n=10, *P<0.001). D, Hydrogen peroxide quantification in conditioned media of ILT and healthy media (n=10, P<0.05).
In conclusion, following a proteomic approach to compare circulating PMNs from AAA patients and controls, several proteins have been identified. Among them, we have shown decreased catalase expression and activity in circulating PMNs from AAA patients, paralleled by decreased catalase plasma levels, supporting the main role of oxidative stress in AAA evolution. These results suggest the need for early prevention and treatment of prooxidant factors and for the development of approaches that enhance production or activity of antioxidant enzymes.

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Disclosures
None.

References


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SUPPLEMENT MATERIAL

METHODS

DIGE protein labeling, 2DE and image acquisition and DIGE data analysis

Samples were labeled with CyDye Fluor minimal dyes (GE Healthcare) according to manufacturer’s instructions. Briefly, 50µg of protein extracts were mixed with 400pmol of the N-hydroxysuccinimide esters of Cy3 or Cy5 fluorescent cyanine dyes on ice in the dark for 30 min. An equal amount of each sample included in the experimental sample set was combined to create the internal standard. The internal standard was labeled with Cy2. The labeling reactions were stopped with 1 µL of 10mM lysine on ice in the dark for 10min.

The six-paired samples of Cy3 and Cy5-labeled proteins were mixed with 50µg of Cy2-labeled internal standard. The mixtures were diluted in Rehydration Buffer (7M urea, 2M thiourea, 4% CHAPS, 0.8% IPG Buffer 3-11NL and bromophenol blue) containing 50mM DTT, and resolved on 24cm IPG strips pH3–11 non-linear gradient IPG strips. The samples were applied by cup loading to the previously rehydrated IPG strips with 450µL of the mentioned Rehydration Buffer containing 97mM DeStreak reagent (GE Healthcare). The isoelectric focusing (IEF) was performed using an IPGphor II IEF system (GE Healthcare) until a total of 42kVh following a stepwise voltage increase: 300V for 3h, linear gradient to 1000V in 4h, linear gradient to 8000V in 2h and 8000V until the steady state was reached. IEF strips were then equilibrated in buffer containing 6M urea, 30% glycerol, 2% SDSand 30mM Tris-HCl and trace amounts of bromophenol blue for 15 minutes with addition of 1% DTT. Finally, strips were incubated with the same buffer containing 4% iodoacetamide instead of DTT for 15 additional minutes. Second dimension was performed on 12% polyacrylamide gels at 17W/gel using an Ettan Dalt Six device (GE Healthcare). Gels
were scanned on a Typhoon 9400 (GE Healthcare) and Cy2-, Cy3-, and Cy5-labeled images of each gel were acquired at excitation/emission wavelength values of 488/520, 523/580 and 633/670 nm respectively. Finally, gels were fixed in 12% methanol and 7% acetic acid, and silver stained using a commercial kit (GE Healthcare).

The images were analyzed using the DeCyder version 7.0 software (GE Healthcare) for spot detection and quantification, inter-gel matching and statistics. DeCyder calculates the average abundance of each spot among the six gels under study. Statistical significance was assessed for each change in abundance using Student’s t-test ANOVA analysis. We considered spots present in all of the 18 images (three images per gel) with statistical significance at 95% confidence level for standardized average spot volume ratios over 1.5.

**In-Gel Trypsin Digestion**

Protein spots from silver-stained gels were manually excised from gels, and transferred to pierced V-bottom 96-well polypropylene microplates (Bruker Daltonik). Samples were digested automatically using a Proteineer DP protein digestion station (Bruker Daltonik). The gel pieces were submitted to reduction with 10 mM DTT (GE Healthcare) in 50mM ammonium bicarbonate (99.5% purity; Sigma Chemical) and alkylation with 55mM iodoacetamide (Sigma Chemical) in 50mM ammonium bicarbonate. Gel pieces were then washed with 50mM ammonium bicarbonate and acetonitrile (gradient grade; Merck) and dried with a nitrogen stream. The samples were digested with trypsin 8ng/µL at 37°C for 8h (sequencing grade; Promega) 50mM ammonium bicarbonate was added to the dry gel pieces. After digestion, the peptides were extracted with 0.5% trifluoroacetic acid (99.5% purity; Sigma Chemical).
MALDI Mass spectrometry
Dried samples were dissolved in 0.2g/l α-cyano-4-hydroxycinnamic acid (Bruker Daltonik) in 50% aqueous acetonitrile and 0.2% trifluoroacetic acid (99.5% purity; Sigma Chemical). This solution was deposited onto a 600µm AnchorChip prestructured MALDI probe (Bruker Daltonik) and allowed to dry at room temperature. Samples were automatically analyzed in an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonik) with an automated analysis loop controlled by the flexControl 2.2 software (Bruker Daltonik). In a first step, MALDI-MS spectra were acquired by averaging 400 individual spectra in the positive ion reflector mode at 50 Hz laser frequency in a mass range from 800 to 3500 Da. Internal calibration of MALDI-MS mass spectra was performed using two trypsin autolysis ions with m/z = 842.510 and m/z = 2211.105. In a second step, precursor ions showing in the MALDI-MS mass spectrum were subject to fragment ion analysis in the tandem (MS/MS) mode to average 1200 spectra. For MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800-3200 m/z region. Automated analysis of mass data was performed using the flexAnalysis 2.2 software (Bruker Daltonik). No smoothing or any further spectral processing was applied. MALDI-MS and MS/MS spectra were manually inspected in detail and reacquired, recalibrated and/or relabelled using the aforementioned programs and homemade software when necessary.

MALDI-MS Database searching
MALDI-MS and MS/MS data were combined through the BioTools 3.0 program (Bruker Daltonik) to search a nonredundant protein database (NCBI nr 20091015, ~10⁷ entries, National Center for Biotechnology Information, Bethesda US), using the Mascot software (Matrix Science, London, UK; http://www.matrixscience.com).
Other relevant search parameters were set as follows: enzyme, trypsin; fixed modifications, carbamidomethyl (C); allow up to 1 missed cleavage; peptide tolerance ±20 ppm; MS/MS tolerance ±0.5 Da. Protein scores greater than 82 were considered significant (p<0.05).

**Western-blots**

Cell extracts from PMNs were sonicated, resuspended in lysis buffer and protein concentration was quantified by Bradford reagent (BioRad). Equal amount of PMN proteins (20µg) or equal volume (10µl) of AAA tissue conditioned-media (previously normalized to tissue weight: 6mL RPMI/1g of wet tissue) was runned on denaturing SDS/12% (w/v) polyacrylamide gels. Proteins were then blotted onto PVDF (Immobilion-P; Millipore) membranes and the blots were blocked with 10% (w/v) non-fat dry milk in TBST (0.01M Tris (pH 7.7), 0.1M NaCl and 0.1% Tween 20). The membranes were incubated with monoclonal antibodies against either Catalase (1:1000 abcam) or GADPH (1:5000 Santa Cruz). After, they were incubated with HRP (horseradish peroxidase)-conjugated anti-(rabbit or mouse IgG) antibodies at a dilution of 1:2500. The proteins were then detected by enhanced chemiluminescence (ECL Western Blotting Detection Reagents, GE Healthcare) and evaluated by densitometry (Quantity One; BioRad Laboratories). Pre-stained protein markers (PageRuler™ Prestained Protein Ladder; Fermentas) were used for molecular mass determinations

**Real-time PCR**

Quantification of Catalase and MnSOD mRNA levels were done by amplification of cDNA using SYBR® Green. The primer sequences were as follows: Catalase (NM_001752.3; predicted size 210 bp) sense: 5’- TTATCCATTCTGATCTCACC -3’, and antisense: 5’- GGCGGTGAGTGTACGGATAG -3’; MnSOD (NM_000636.2,
predicted size 54 bp) sense: 5’- CACTCGTGCTGCTGCTGCTGCT-3’, and antisense:
5’- GCTGATGCGGCCGATCTGCT-3’; 18S (NR_003286.2, predicted size 125 bp)
sense: 5’- CCGTCGTAGTTCCGACCATAA -3’, and antisense 5’-
CAGCTTTGCAAACCATACTCCC -3’. Expression levels are given as ratio to
housekeeping gene 18S and data is expressed as ΔCt. The relative quantification was
done using the comparative CT method and expressed as arbitrary units.
Table I online. Characteristics of the patients included in the study.

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<th></th>
<th>Small aaa patients</th>
<th>Large AAA patients</th>
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<tbody>
<tr>
<td></td>
<td>(n=56)</td>
<td>(n=47)</td>
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<tr>
<td>Age (years)</td>
<td>72±8</td>
<td>71±8</td>
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<td>Sex (Male/Female)</td>
<td>54 (96%) / 2 (4%)</td>
<td>45 (96%) / 2 (4%)</td>
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<td>Active smoking</td>
<td>18/56 (32%)</td>
<td>15/47 (32%)</td>
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<tr>
<td>Hypertension</td>
<td>38/56 (68%)</td>
<td>23/47 (49%)</td>
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<tr>
<td>Diabetes mellitus</td>
<td>14/56 (25%)</td>
<td>8/47 (17%)</td>
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<tr>
<td>Hypercholesterolemia</td>
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<td>29/47 (62%)</td>
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<tr>
<td>Cardiac disease</td>
<td>13/56 (23%)</td>
<td>7/47 (15%)</td>
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<tr>
<td>Chronic Obstructive Pulmonary Disease</td>
<td>10/56 (18%)</td>
<td>9/47 (19%)</td>
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<tr>
<td>Chronic renal failure</td>
<td>0/56 (0%)</td>
<td>4/47 (9%)</td>
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<td>Spot</td>
<td>C vs aaa</td>
<td>Av.Ratio p-value</td>
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**TABLE II online. Proteins altered in PMNs from AAA patients and control subjects found by 2D-DIGE/MS**
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<tr>
<th>Spot</th>
<th>Volume</th>
<th>Protein Name</th>
<th>Accession Number</th>
<th>Mascot Score</th>
<th>Expectation Value</th>
<th>Ions Score</th>
<th>Peptide Count</th>
<th>Coverage</th>
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<td>22.65/9.33</td>
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</table>

* Spot number according to Figure 1
† Average volume ratio and p-values from t-test as quantified by DeCyder software (---: Spots without statistical significance; C, Control; aaa, small AAA; AAA, large AAA; Higher protein expression levels is indicated by positive average ratios)
§ Protein ID and accession number according to NCBI database
# Mascot score, expectation value and ions score (NA: Not applicable, that applies to proteins identified just with their corresponding Protein Mass Fingerprint. Ions score applies to peptide MS/MS fragmentation spectra)
** Theoretical protein molecular weight (MW) and isoelectric point (pI)
†† Number of peptides matching the protein sequence, number of unmatched peptides and percentage of protein sequence coverage (Cov).
Figure I online. Schematic representation of the isolation of human PMNs.

- **Control group**
- **aaa group (<5cm)**
- **AAA group (>5cm)**

EDTA venous blood

Neutrophils were obtained by Ficoll-Paque centrifugation and sedimentation by 6% dextran

Hypo-osmotic lysis of residual erythrocytes

Analysis of CD16 from PMNs by flow cytometry