Proteomic analysis of PMNs identifies catalase as a novel biomarker of Abdominal Aortic Aneurysm (AAA).

Potential implication of oxidative stress in AAA progression.

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ABSTRACT

BACKGROUND: 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>5cm and 4 small AAA 3-5cm) and 4 controls were analyzed by 2D-DIGE. Among differentially spots, several proteins involved in redox balance were identified by mass spectrometry (e.g. cyclophilin, thioredoxin reductase, catalase). Diminished catalase expression and activity was observed in PMNs from AAA patients compared to controls. In contrast, PMNs from AAA patients display higher H_2O_2 and MPO levels than PMNs from controls. Moreover, a significant decrease in catalase mRNA levels was observed in PMNs after PMA incubation. Catalase plasma levels were also decreased in large (n=47) and small (n=56) AAA patients compared to controls (n=34). We observed catalase expression in AAA thrombus and thrombus-conditioned medium, associated to PMN infiltration. Furthermore, increased H_2O_2 levels were observed in AAA thrombus-conditioned medium compared to the media layer.

CONCLUSIONS: Diminished catalase levels in circulating PMNs and plasma are observed in AAA patients, supporting an important role of oxidative stress in AAA evolution.

KEYWORDS: Abdominal aortic aneurysm; biomarkers; differential proteomics; catalase; thrombus

Abdominal aortic aneurysm (AAAs) 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 2-3% of all deaths (1). Since AAAs are usually asymptomatic, the present clinical challenge is early diagnosis, and to decipher the biological mechanisms responsible for the progressive dilatation and final rupture, in order 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 experimental (4,5) and human (6,7) 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 remodelling, 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 to identify novel diagnostic and prognostic targets in AAA disease. Furthermore, identification of biomarkers could also afford novel pathogenic pathways and may thus

open possibilities for pharmacological inhibition of growth, providing tools for monitoring this inhibition (13). Until now, most studies have focused on the role of individual proteins related to different PMNs function/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 (14). However, no studies have addressed the comparison of PMN proteome in vascular diseases. In the present study, we have performed a comparative 2D-DIGE 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 undergoing infrarenal AAA repair (n=4, large AAA, AAA diameter >5cm) or who visit the vascular surgery department for follow-up assessment (n=4, small AAA, AAA diameter 3-5cm). The control group consisted of 4 volunteers (AAA diameter<3cm) without significant differences in age, sex, risk factors and medications with the patient groups. 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 µM phorbol 12-myristate 13-acetate (PMA, Sigma aldrich) or vehicle during 30 min (for hydrogen peroxide, H_2O_2) or during 4h [for myeloperoxidase (MPO) and catalase/manganese superoxide dismutase (MnSOD) mRNA analysis] at 37ºC.

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

(Table 1 online). We excluded patients with multiple synchronic aneurysms (thoracic, femoral, popliteal), and AAA with a location other than infrarenal. We also excluded patients with active inflammatory or acute infectious processes, surgical procedures or major trauma in the previous 60 days, and chronic antinflammatory or immunosupressive medication. 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 non-dilated (<30 mm, confirmed with abdominal ultrasound) infrarenal aortas. The study was approved by the Scientific Ethical Committee of our Institutions, and an informed consent from the patients and the controls for their inclusion in the study was obtained.

AAA tissue and tissue-conditioned medium

Ten AAA tissue samples were collected during surgical repair and dissected into intraluminal thrombus and medial layer. AAA samples were obtained from patients undergoing surgery, enrolled in the RESAA protocol (REflet Sanguin de l'évolutivité des Anévrysmes de l'Aorte abdominale, CCPPRB Paris-Cochin n° 2095,n° 1930 and n° 1931) (15). All patients gave their written informed consent, and the protocol was approved by a French ethic committee (CCPPRB, Cochin Hospital).AAA thrombus and aortic walls were cut into small pieces (5 mm^2) and separately incubated in RPMI 1640 medium containing antibiotics and an antimycotic (Gibco) for 24 hours at 37°C (6ml/g of wet tissue). The conditioned medium (supernatant containing proteins released by the tissue sample) was obtained after centrifugation as 3,000g 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 hypo-osmotic lysis of residual erythrocytes. Neutrophils were resuspended in lysis buffer (7M

urea, 2M thiourea, 4% CHAPS and 30mM Tris-HCl, pH8.5),and proteins were precipitated to remove salts and other interfering componentsby using the 2D Clean Up Kit (GE Healthcare), and resulting proteins were resuspended in lysis buffer.The protein concentration was measured using the RC/DC Protein Assay (Bio-Rad Laboratories). PMNs purity was assessed by flow cytometry to ensure that they were not contaminated by others cell types. The analysis of CD16 from PMNs showed a strong fluorescence signal (>90-95%) in all samples **(Figure 1 online).**

DIGE protein labeling

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 1μL of 10mM lysine on ice in the dark for 10min.

2DE and image acquisition

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).

DIGE data analysis

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.

Protein identification by MALDI mass spectrometry

Differentially expressed spots were selected from silver-stained gels for gel excision, automated digestion and analysis in an Ultraflex MALDI TOF/TOF mass spectrometer (Bruker Daltonik) to obtain the corresponding MALDI-MS and MALDI-MS/MS spectra. These MS and MS/MS combined data were used to search a non-redundant protein database (NCBInr; ~ 107 entries; National Centre for Biotechnology

Information, Bethesda, US) using the Mascot software (Matrix Science). Detailed information included in the Methods section of the **online-only Data Supplement.**

Western blot

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 resolved 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.

Catalase activity

Similar amounts of PMN extracts were analyzed for catalase activity using a commercial enzymatic assay (#K773, Biovision), following manufacturer´s instructions. Catalase units were defined as the amount of enzyme that decomposes 1µmol H_2O_2/min at pH 4.5 at 25ºC.

ELISA

We quantified concentrations of catalase and MPO with commercial kits (E92418HU, USCN LIFE SCIENCE and Hycult), following manufacturer´s instructions.

DCF

Intracellular H_2O_2 levels in PMNs were measured with 2,7-dichlorofluorescein diacetate (DCF, Sigma Aldrich) as described previously (16). Briefly, $1x10^6$ fresh PMNs were loaded with 5uM of DCF in Hanks Buffered salt solution (HBSS) at 37 °C for 30 min and then washed twice. Fluorescence was evaluated with a microplate reader (GENios Tecan) at 535 nm with an excitatory wavelength of 485 nm during 30 min. The ROS production was expressed as relative fluorescence units per second (RFU/s).

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 PCR reactions were performed on ABI Prism 7500 sequence detection PCR system (Applied Biosystems) according to manufacturer's protocol using the DeltaDelta Ct method as described (17). 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′- TTAATCCATTCGATCTCACC -3′, and antisense: 5′- GGCGGTGAGTGTCAGGATAG -3′; MnSOD (NM_000636.2, predicted size 54 bp) sense: 5′- CACTCGTGGCTGTGGTGGCT -3′, and antisense: 5′- GCTGATGCCGCCGATCTGCT -3′; 18S (NR_003286.2, predicted size 125 bp) sense: 5′- CCGTCGTAGTTCCGACCATAA -3′, and antisense 5′- CAGCTTTGCAACCATACTCCC -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 as described (17).

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 4ºC as primary antibody. Peroxidase LSAB + system-HRP kit (Dako) followed by Histogreen peroxidase substrate (AbCys SA) was used for detection. Sections were then counterstained with Nuclear Fast Red for 5 minutes at room temperature before mounting 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 manufacturer´s instructions.

Statistical analysis

Results from western blot, catalase activity, DCF, MPO levels in PMNs, qPCR and H_2O_2 extracellular levels are expressed as mean ± SEM and were analyzed by Mann–Whitney non-parametric test (small and large AAA and control groups) or Wilcoxon paired test (between thrombus and media supernatants of the same samples). Results from ELISA are expressed as median (interquartile ranges, IQR) and were analyzed by the ANOVA test. A p 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 >5cm) and small AAA (AAA diameter=3-5cm)] and control subjects (AAA diameter<3cm) were compared by 2D-DIGE (**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 mass spectrometry (**Table 1**). As Table 1 reports, proteins known to be expressed by PMN has been identified (e.g. lactoferrin, lipocalin 2). In addition, we found proteins involved in different neutrophil functions, such as cytoskeletal proteins (e.g. WDR1), inflammatory processes (e.g. LT4H) and survival (e.g. coronin). Moreover, a high number of proteins involved in redox balance (e.g. cyclophilin, catalase and thioredoxin reductase) were observed.

Decreased catalase levels in circulating PMNs from AAA patients

Among the differentially 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 vs 15.7±2 a.u, $p<0.05$, **Figure 2A**). Similar results were obtained for thioredoxin reductase (10.8±1.5 and 10.6±1.2 vs 14.8±1.7 a.u. $p<0.05$, 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 to controls (338±27 and 312±32 vs 376±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_20_2 and MPO levels from a third additional group of PMNs isolated of 10 AAA patients [AAA>5cm (n=4) and AAA<5cm (n=6)] and controls (n=10). PMNs isolated from both large and small AAA patients display higher H_20_2 intracellular levels compared to PMNs from controls

(**Figure 3A**). In addition, PMNs isolated from both large and small AAA patients release higher MPO concentrations compared to PMNs from controls (**Figure 3B**). In the other hand, baseline catalase mRNA levels were decreased in AAA patients compared to controls (**Figure 3C**), in agreement with the results obtained at the protein level (**Figure 2B**). Similar results were obtained for MnSOD mRNA (**Figure 3D**).

To address whether decreased catalase expression could be associated to the prooxidant conditions that occur in neutrophils during respiratory burst, neutrophils were incubated with PMA, known to induce respiratory burst in PMNs. After PMA incubation, increased H_20_2 and MPO levels were shown in PMNs from controls, reaching levels similar to those of PMNs from patients (**Figure 3A,B**). Interestingly, we have found a significant decrease in catalase mRNA levels after PMA incubation and a similar trend was observed for MnSOD (**Figure 3C,D**).

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) vs 145 (90-208) vs 159 (132-211) U/mL, median (IQR), $p<0.05$ for controls vs small AAA and $p<0.001$ for controls vs large AAA] (**Figure 4A**). In the other hand, MPO plasma levels were increased in patients with large and small AAA as compared to controls [65 (46-118) vs 65 (54-92) vs 41 (31-51) ng/ml, $p<0.001$ for both, large and small AAA vs controls, not shown). Finally, since 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

Since 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 red blood cells, 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 to that of the media layer (15.9±2.7 vs 7.2±1.6 a.u. $p<0.05$, **Figure 5C**). Finally, hydrogen peroxide (H_20_2) levels were also increased in the ILT compared to the media layer (10.4±2.2 vs 5.2±0.7 μM, $p<0.05$, **Figure 5D**).

DISCUSSION

Polymorphonuclear neutrophils (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 (11,12). Since 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 to matrix metalloproteinase (MMP)-9 (18). Cyclophilin, could participate in different mechanisms involved in vascular remodelling by promoting inflammation and smooth muscle cell (SMC) proliferation (19). Moreover, cyclophilin, was shown to enhance vascular oxidative stress and the development of angiotensin II-induced aortic aneurysms (20). 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 pro-oxidant molecules. Among the identified proteins by proteomic analysis, we showed decreased intracellular expression of antioxidant proteins such as catalase and thioredoxin reductase in circulating PMNs from AAA patients compared to controls, while cyclophilin is enhanced. Furthermore, we analyzed catalase activity in circulating PMNs, showing a decreased catalase activity of AAA patients compared to controls. In contrast, we observed that PMNs isolated from AAA patients display higher H_2O_2 levels and release higher MPO concentrations compared to PMNs from controls, paralleled by a decrease in both catalase and MnSOD mRNA expression. Moreover, to test if these antioxidant systems could be modified under the conditions of increased oxidative stress associated to respiratory burst, neutrophils were incubated with PMA, known to induce respiratory burst in PMNs. Incubation of PMNs from controls with PMA increased both H_20_2 and MPO levels, reaching levels similar to those of PMNs from patients, probably associated to 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 towards increased oxidative

stress (increased oxidant species such as H_2O_2 and MPO/decreased antioxidant species such as catalase and MnSOD) is observed in PMNs from AAA patients.

At the systemic level, previous studies have analyzed the circulating levels of different pro-oxidant molecules in AAA patients (21). Among them, blood levels of malondialdehyde were significantly increased in AAA patients (22). Likewise, we have measured MPO in plasma of AAA patients, a well-recognized oxidative stress biomarker of different cardiovascular pathologies (23,24), showing that MPO plasma levels are increased not only in large AAA (7), but also in small AAA. In the other hand, catalase plasma levels were significantly decreased in small and large AAA patients relative to control subjects. However, it should be taken into account that patients and controls exhibited differences in risk factors as well as treatments that could influence oxidative stress. In contrast, small and large AAA patients showed similar risk factors and medications, suggesting that the differences in catalase plasma levels could be associated to 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 non-abdominal segments, showing an increased oxidative stress and associated derived-products in AAA segments (25). In the other hand, MnSOD activity in human diseased aorta was about 65% of controls. Furthermore, ruptured AAA tissue also had low SOD activity and protein (26). However, AAA formation is associated with early increases in SOD expression in an experimental model (27). In contrast, the beneficial effect of flow loading limiting experimental AAA formation was associated to increased antioxidant gene (hemoxigenase-1) expression in the aorta (28). In the present study, immunohistochemical analysis showed catalase in human ILT, associated with PMNs. However, other non-nucleated cells likely to be 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 (29) 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 signalling 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 (e.g. MPO), the trapping of RBCs within the thrombus may lead to hemolysis and subsequent release of hemoglobin, heme and finally, pro-oxydant iron. Among ROS, H_2O_2 is non-radical uncharged oxidant, which is chemically more stable then other ROS and can permeate through the vascular wall. In addition, H_2O_2 can accumulate extracellularly in the tissue and survive long enough to induce numerous paracrine functions (30). H_2O_2 itself is not very reactive; however, the danger of H_2O_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 since they can be formed, among other mechanisms, either by MPO-catalyzed or by Fe^{2+}-catalyzed conversion of H_2O_2 (30). On the whole, the imbalance between oxidant species and antioxidant systems in AAA patients, both at the systemic and the tissue level, further support 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_2O_2 activates endothelial cells to increase PMN adhesion and catalase is able to prevent leukocyte accumulation (33). Moreover, overexpression of catalase suppresses oxLDL-induced aortic SMC death (34) and inhibits SMC 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 to its functions outside cells (36). Interestingly, diminished experimental AAA formation by tamoxifen treatment has been associated to 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 SMCs 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.

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 pro-oxidant factors and for the development of approaches that enhance production or activity of antioxidant enzymes.

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DISCLOSURES

None

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Figure legends

FIG. 1. Differential in-gel electrophoresis analysis

PMN protein extracts of 8 AAA patients and 4 controls were labeled with the corresponding CyDye reagents, mixed, resolved on 6 independent DIGE gels, imaged (red, Cy3; green, Cy5; blue, Cy2) and analyzed with DeCyder software. A) A representative gel image from the DIGE experiment is shown. Proteins were resolved in the 3-11(non-linear) pH range on the first dimension and on 12% polyacrylamide gels on the second dimension. B) Spots showing statistically significant regulation between the two conditions were excised from silver stained gels and identified by MALDI mass spectrometry. Identified spots are numbered as in Table 1.

FIG. 2. Decreased catalase expression and activity in PMNs from AAA patients.

A) Representative western blot of catalase and GAPDH in PMN extracts from AAA patients (aaa=AAA<5cm and AAA=AAA>5cm) and controls (C). Quantification of catalase expression levels normalized by GAPDH levels in PMNs from AAA patients and controls (n=10, densitometric arbitrary units, AU, *$p<0.05$). B) Catalase activity in PMN extracts (n=10, mU/ml, *$p<0.05$ vs controls). C) Correlation between catalase expression and activity in PMN extracts ($r=0.4$, $p<0.05$).

FIG. 3. Oxidative status of PMNs from AAA patients and controls.

PMNs were incubated with vehicle (basal, black) or with 50 µM PMA (white) during 30 min for A) intracellular H_20_2 quantification by DCF or during 4h for B) MPO determination by ELISA (conditioned media) and mRNA expression of C) catalase and D) MnSOD analysis by Q-PCR [controls (n=10), aaa=AAA<5cm (n=6) and AAA=AAA>5cm (n=4), *$p<0.05$ vs basal, †$p<0.05$ vs controls].

FIG. 4. Plasma catalase levels in AAA patients.

A) Catalase levels are significantly increased in plasma from controls (n=34) compared to patients with small (n=56) and large (n=47) AAA (*p< 0.05 small AAA vs large AAA, †p<0.001 control vs large AAA). B) Correlation between catalase levels and aortic size (Pearson rho=-0.4, p<0.001, n=137 subjects).

FIG. 5. Catalase expression in AAA thrombus and thrombus-conditioned medium.

A)Immunodetection of catalase in AAA thrombus and B) negative control (10X). Inset (20X). Positivity is shown in green, nuclei are in red.

C) Western blot of anti-catalase in conditioned medium from ILT and media layer (M)(n=10, *p<0.001). D) Hydrogen peroxide quantification in conditioned media of ILT and healthy media (n=10, p<0.05).

TABLE 1. *Proteins altered in PMNs from AAA patients and control subjects found by 2D-DIGE/MS*

	DeCyder †							Mascot #						
Spot *	C vs aaa		C vs AAA		aaa vs AAA		Protein description §	Acc. code §	Score	Expect.	Ions score	MW(KDa)/ pI**	Matched ††	Unmatch. ††	Cov.††
	Av.Ratio	p-value	Av.Ratio	p-value	Av.Ratio	p-value									
1	--	--	-1.2	8.2E-02	-1.35	3.1E-02	Lactoferrin	gi|28948741	108	1.60E-04	NA	77.93/8.47	8	4	12
2	1.68	4.2E-02	3.6	6.3E-02	--	--	Guanine nucleotide-binding protein G(i)	gi|23398623	214	4.00E-15	97	39.10/5.27	6	0	14
3	1.52	4.5E-02	--	--	--	--	Actin. cytoplasmic 1	gi|15277503	172	1.40E-12	93	40.54/5.55	4	0	16
4	-1.34	7.4E-02	1.58	4.7E-02	2.12	4.3E-02	Actin. cytoplasmic 2	gi|178045	162	1.40E-11	102	26.15/5.65	3	0	20
5	--	--	1.41	6.3E-02	--	--	glyoxalase domain containing 4	gi|217330598	191	8.00E-13	NA	33.55/5.4	11	3	41
6	1.86	5.4E-02	2.6	6.7E-02	--	--	Actin. cytoplasmic 1	gi|15277503	535	7.10E-49	346	40.54/5.55	11	3	35
7	1.88	3.4E-02	2.98	2.1E-02	--	--	Actin. cytoplasmic 1	gi|15277503	139	2.80E-09	NA	40.54/5.55	7	0	25
8	--	--	--	--	-1.83	4.2E-02	Copine-3	gi|4503015	172	6.30E-11	68	60.95/5.6	6	0	12
9	--	--	-128	1.1E-02	--	--	ubiquitin-activating enzyme E1	gi|23510338	126	2.50E-06	NA	118.86/5.49	9	1	10
10	--	--	1.26	3.3E-02	--	--	alpha-actinin 1	gi|54304187	79	2.60E-03	54	10.19/5.41	1	0	13
11	1.48	0.03	1.66	6.7E-02	--	--	Major vault protein	gi|1097308	203	5.00E-14	58	100.14/5.34	9	0	15
12	1.52	1.4E-02	--	--	--	--	ARP3 actin-related protein 3 homolog B	gi|5031573	149	1.30E-08	NA	47.80/5.61	9	4	23
13	--	--	-1.69	0.03	-1.48	9.2E-02	Nucleoside diphosphate kinase A	gi|35068	136	5.70E-09	62	20.74/7.07	4	2	26
14	-1.31	7.8E-02	-2.14	5.6E-03	-1.63	2.3E-02	gelsolin	gi|55960301	135	7.10E-09	54	29.11/7.71	4	0	13
15	1.92	3.6E-02	2.41	7.1E-02	--	--	Leukotriene A4 Hydrolase	gi|51247429	231	8.00E-17	49	69.65/5.73	11	1	25
16	--	--	1.42	3.3E-02	1.46	0.02	Thioredoxin reductase 1. cytoplasmic	gi|3820535	101	1.80E-05	NA	55.32/6.36	7	4	16
17	1.43	3.1E-02	1.93	9.9E-02	--	--	Coronin-1A	gi|5902134	257	4.50E-21	119	51.38/6.25	9	4	18
18	1.54	4.3E-02	--	--	--	--	Actin-related protein 3B	gi|5031571	233	1.10E-18	115	45.02/6.3	8	6	29
19	--	--	--	--	1.28	3.9E-02	Proteasome subunit alpha type-7	gi|4506179	265	7.10E-22	137	29.82/6.15	7	2	36
20	-2.29	2.3E-02	--	--	NS		phosphoglycerate mutase 1	gi|114326546	141	8.00E-08	NA	28.93/6.67	7	4	25
21	--	--	-1.76	2.8E-02	-1.51	1.6E-02	phosphoglycerate mutase 1	gi|4505753	162	1.40E-11	NA	28.90/6.67	10	1	51
22	--	--	--	--	2.12	3.3E-02	phosphoglycerate mutase 1	gi|114326546	141	8.00E-08	NA	28.93/6.67	7	1	38
23	--	--	-1.43	8.4E-02	--	--	Rho GDP-dissociation inhibitor 1	gi|4757768	263	1.10E-21	163	23.25/5.02	6	2	25
24	1.58	0.03	--	--	--	--	transaldolase	gi|5803187	104	4.00E-04	NA	37.69/6.36	7	2	24
25	--	--	1.61	0.03	1.62	8.4E-02	adenosine kinase	gi|32484975	107	2.00E-04	NA	40.92/6.24	6	0	12
26	--	--	--	--	1.38	4.3E-02	Coronin-1A	gi|5902134	206	2.50E-14	51	51.68/6.25	12	7	20
27	--	--	3.48	3.80E-03	--	--	catalase	gi|4557014	440	1.00E-37	175	59.95/6.9	17	5	42
28	1.62	5.6E-02	2.27	9.5E-02	--	--	esterase D/formylglutathione hydrolase	gi|33413400	256	2.50E-19	123	31.96/6.54	8	5	30
29	--	--	1.69	3.4E-02	--	--	phosphoglycerate mutase 1	gi|4505753	400	2.30E-35	189	28.90/6.67	12	7	56
30	--	--	-1.86	2.8E-02	--	--	adenylyl cyclase-associated protein	gi|5453595	146	5.70E-10	69	51.93/8.07	5	0	10
31	--	--	1.19	9.1E-03	1.36	5.7E-02	Ras-related protein Rab-7a	gi|34147513	214	9.00E-17	87	23.76/6.4	6	0	40
32	--	--	2.04	2.9E-03	2.14	6.3E-02	adenylyl cyclase-associated protein	gi|5453595	193	1.10E-14	96	51.93/8.07	7	3	21
33	--	--	2.69	8.4E-03	3.01	5.02E-03	DNA-(apurinic or apyrimidinic site) lyase	gi|18375501	172	1.40E-12	52	35.93/8.33	7	2	30
34	--	--	3.46	3.1E-02	5.47	3.2E-02	gelsolin	gi|38044288	380	2.30E-33	284	80.88/5.58	8	7	11
35	--	--	3.39	2.4E-02	4.37	0.02	gelsolin	gi|38044288	176	5.70E-13	104	80.88/5.58	5	1	6
36	2.12	5.8E-02	3.1	2.9E-02	--	--	carbonic anhydrase I	gi|4502517	124	9.00E-08	NA	28.91/6.59	6	1	38
37	--	--	-1.73	2.8E-02	-1.39	2.3E-02	Human Seminal Lactoferrin	gi|28948741	1220	1.00E-115	647	77.93/8.47	38	2	61

38	2.06	4.1E-02	--	--	--	--	phosphoglycerate kinase 1	gi|4505763	178	3.60E-13	84	44.99/8.3	6	1	20
39	--	--	2.69	8.4E-03	3.01	5.03E-03	adenylyl cyclase-associated protein	gi|5453595	170	2.30E-12	119	51.93/8.07	3	1	11
40	-1.27	5.56E-03	--	--	--	--	lipocalin 2	gi|55961101	146	5.70E-10	67	22.95/8.66	4	1	22
41	-1.35	9.9E-02	--	--	--	--	cyclophilin	gi|181250	190	2.30E-14	97	22.65/9.33	5	0	23

* 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: No 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

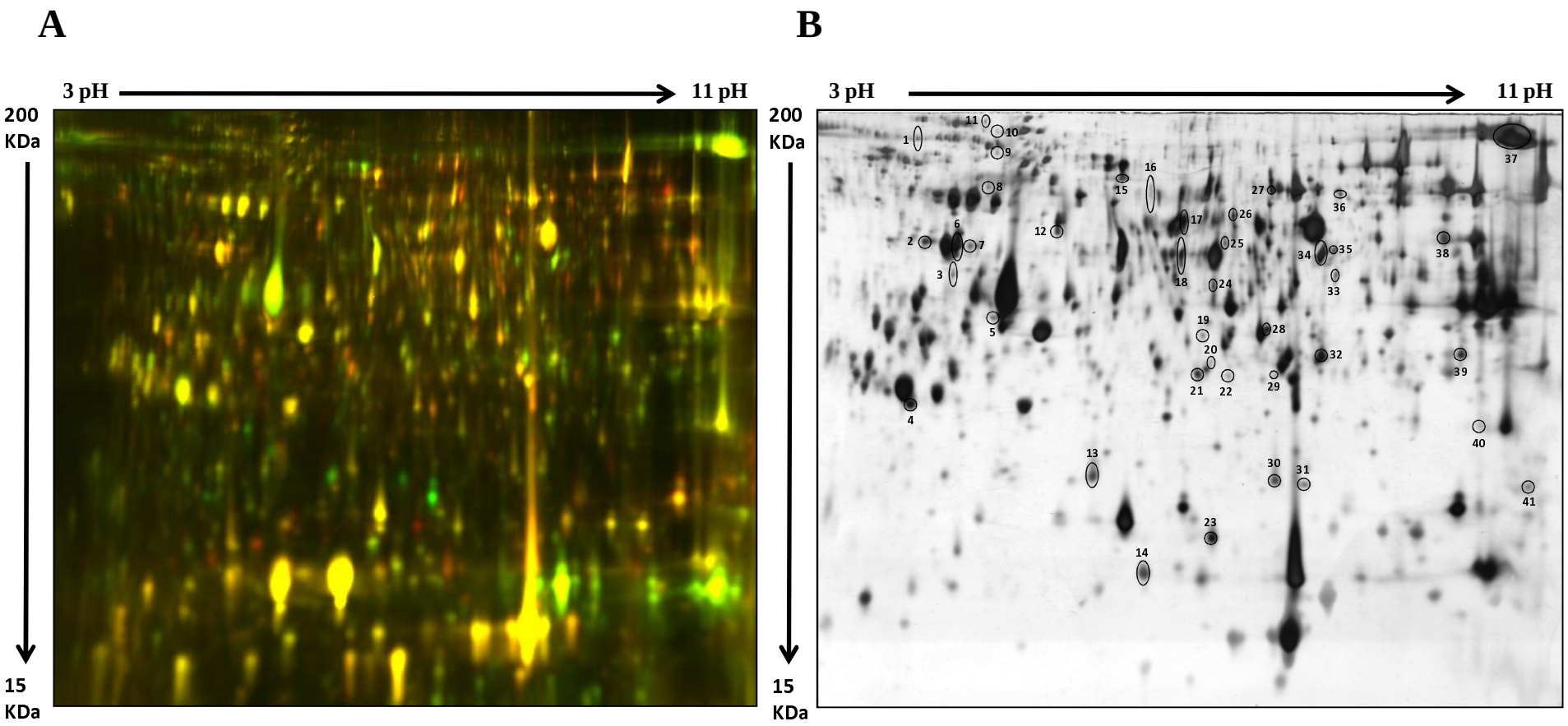


FIGURE 1

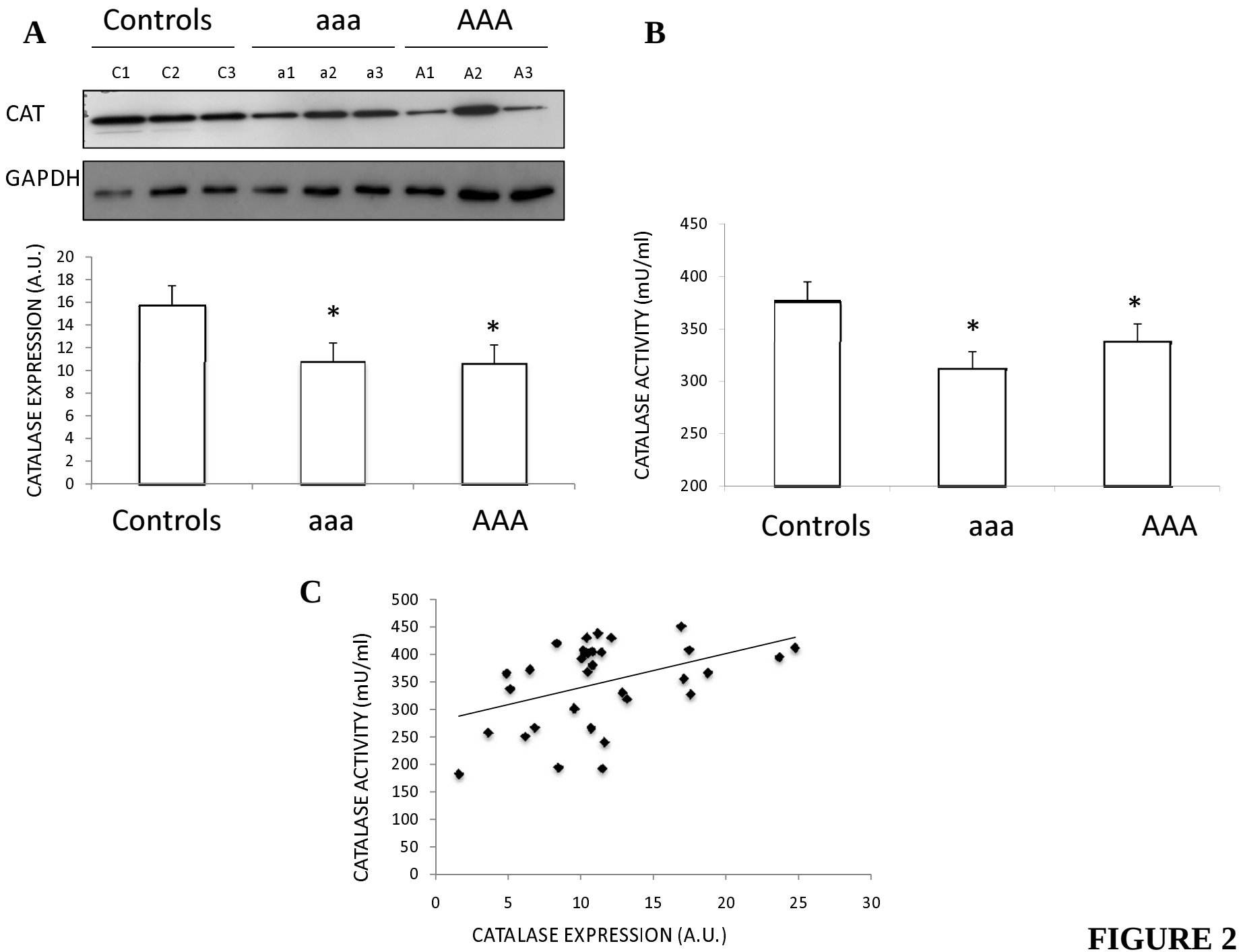


FIGURE 2

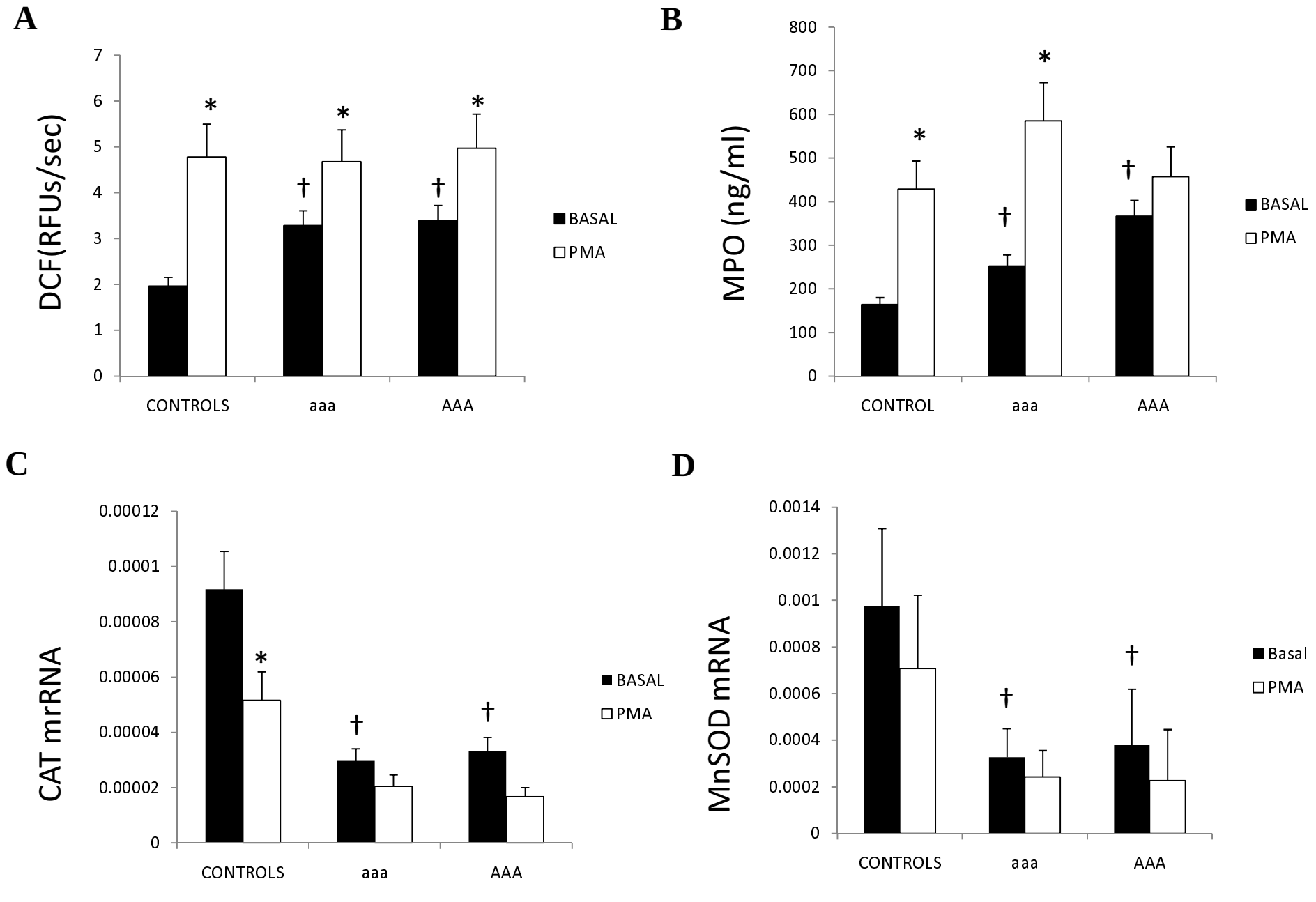
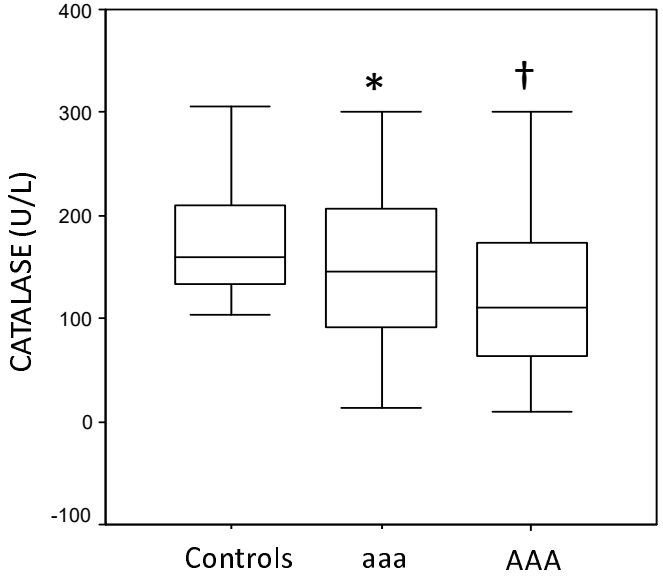


FIGURE 3

A



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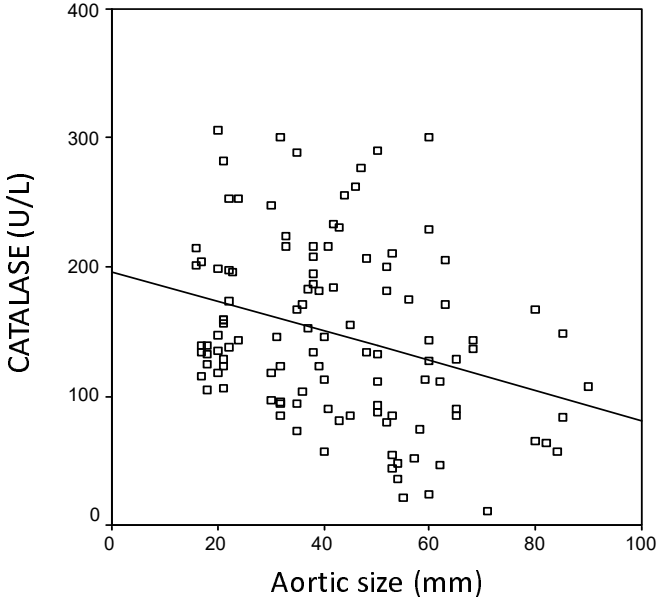

FIGURE 4

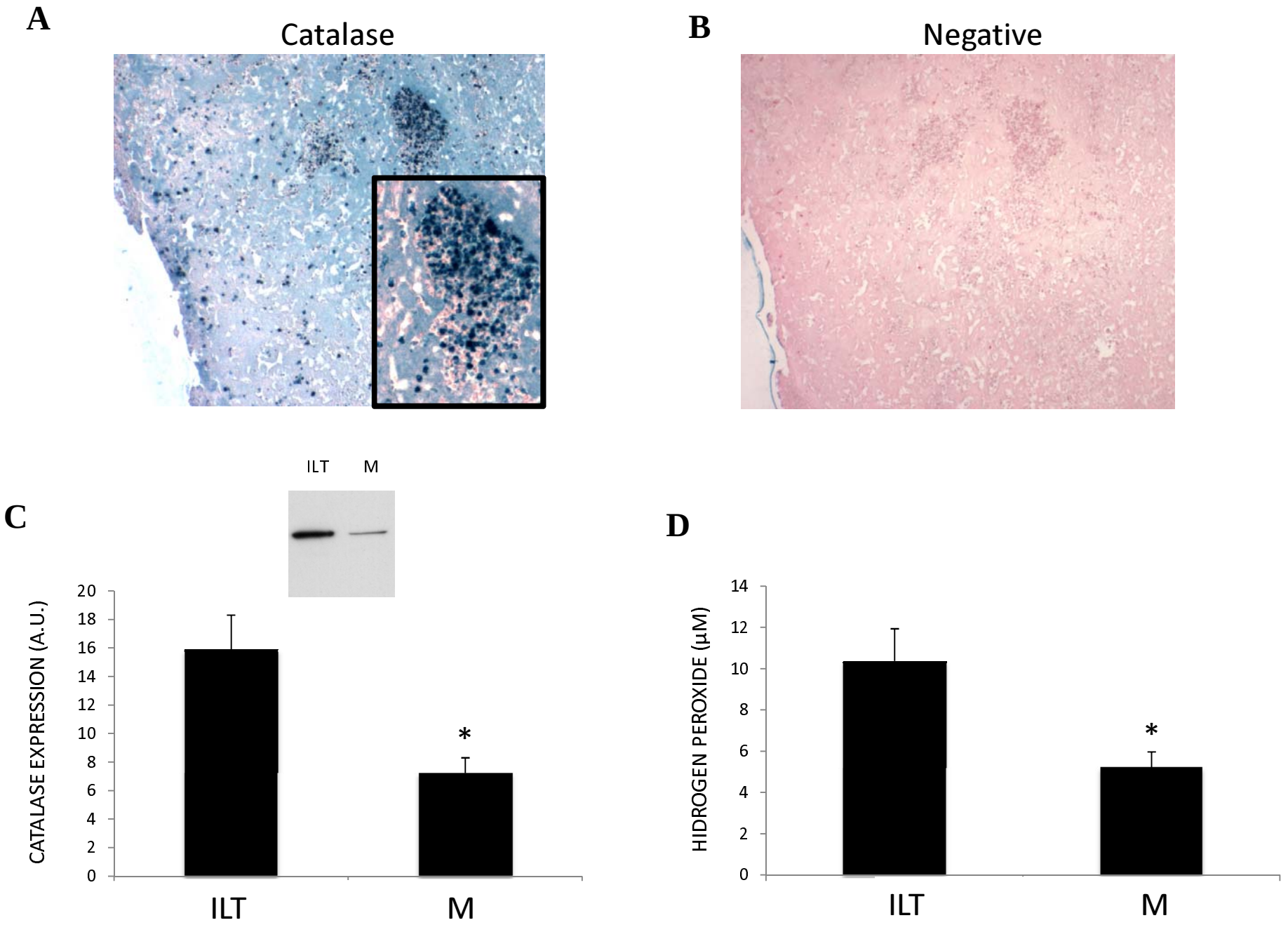

FIGURE 5