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# Label-free proteomic analysis of red blood cell membrane fractions from abdominal aortic aneurysm patients

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**Abbreviations: AAA**, abdominal aortic aneurysm; **Hb**, hemoglobin; **ILT**, intraluminal thrombus; **RBCs**, red blood cells

**Purpose:** To test whether red blood cell (RBC) membrane composition is modified in abdominal aortic aneurysms (AAA) patients.

**Experimental design:** RBC membrane extracts from AAA patients (aortic diameter >3 cm, *n* = 7) and control subjects (*n* = 4) were analyzed by a label-free quantitative MS-based strategy, using spectral count data. Additional validation was performed by western-blot.

**Results:** Data analysis based on spectral count from MS/MS-based experiments provided us a signature of 39 proteins differentially expressed in RBC membranes between AAA and controls (changes equal/over 1.515-fold; *p*-values equal/lower 0.05). MS data revealed altered levels of structural membrane proteins (e.g. spectrins and ankyrin), components of the degradation machinery (proteasome subunits), and oxidative stress related proteins (e.g. catalase and peroxiredoxin-2) among others. Decreased catalase and peroxiredoxin-2 expression in RBC membrane of AAA patients compared to controls were further validated by Western blot, confirming the proteomic results.

**Conclusions and clinical relevance:** RBCs membrane protein composition is altered in AAA patients, which could be involved in the pathological role of RBCs in aortic tissue and become potential targets to prevent AAA progression.

**Clinical Relevance**

Abdominal aortic aneurysm (AAA) is a chronic disease characterized by the presence of a chronic intraluminal thrombus, medial degradation, and an adventitial response. Red blood cells (RBCs) in intraluminal thrombus could contribute to oxidative stress, while RBC in adventitia may induce the recruitment of phagocytes. Since both pathological mechanisms could be favored by alterations in the membrane protein composition of RBCs, we addressed a proteomic analysis of RBCs membranes from AAA patients and controls by a label-free quantitative MS-based strategy. MS data revealed altered levels of structural proteins, components of the protein degradation machinery, and antioxidant proteins. In this respect, previous studies on RBC composition of chronic thrombus in AAA but also acute thrombus in patients with atherothrombosis (myocardial infarction/acute coronary syndromes), have suggested that oxidative stress could be linked to the clinical consequences and therapeutic options in these diseases. Understanding the complex cellular

and molecular mechanisms underlying redox balance associated to RBCs could help to define novel potential therapeutic targets to decrease AAA risk.

## Introduction

Abdominal aortic aneurysm (AAA) occurs in up to 9% of adults older than 65 years of age, causing about 1–2% of male deaths in Western countries. AAA is a chronic disease characterized by the presence of a chronic red blood cell RBC-rich (where RBC is red blood cell) intraluminal thrombus (ILT), medial degradation, and an adventitial response [1]. Main mechanisms involved in AAA development and progression are proteolysis and oxidative stress, associated to the presence of neutrophils and RBC in ILT, and phagocytosis and immune response of the arterial wall. The trapping of RBCs within the ILT of AAA may lead to hemolysis and subsequent release of hemoglobin (Hb), heme, and finally, iron, which generates ROS via the Fenton reaction. Hydrogen peroxide ($H_2O_2$) produced by autoxidation of Hb is a predominant ROS in RBCs [2]. In addition, RBCs and iron deposits associated to phagocytes are observed in the aneurismal wall, potentially favoring the recruitment of monocytes through adventitial neovessels [1]. Moreover, this local RBC retention may impede iron recycling and erythropoiesis leading to anemia, which is a predictor of long-term mortality in patients with AAA [3].

Since mature RBCs do not contain RNA or DNA, proteomics has been previously used to understand the role of RBCs in different diseases [4]. Moreover, as human RBCs have a 120 days lifespan, alterations on RBC proteins could afford interesting information on the long-term status of the body in chronic diseases [4].A main difficulty to analyze RBCs proteome is the large dynamic range for cellular protein concentration, mainly the high concentration of cytosolic Hb (97%). One alternative is analyzing the membrane fraction, which his not devoid of some technical difficulties (e.g.proteins from other compartments) [4]. The interest of analyzing the plasma membrane proteome of RBCs is due to the fact that modification of proteins at this localization could impact: (i) RBCs deformability needed for their flow and the delivery of oxygen to tissues; (ii) RBC prooxidant activities, and (iii) RBC removal through erytrophagocytosis [2]. Although RBCs play a key role in chronic vascular remodeling, no previous proteomic studies have been performed in RBCs from

patients with AAA. In the present study, we aimed to characterize the differential protein composition of RBC membrane extracts from AAA patients and controls.

For that purpose, RBCs were obtained from blood of 20 AAA patients (aortic diameter >3 cm) and ten controls (aortic diameter <3 cm confirmed by ultrasound without significant differences in sex, age, or risk factors). Seven AAA and four control RBC samples were analyzed by proteomics, while all RBC samples were used for final validation. The local IISEthical Committee approves the study and informed consent of all participating subjects was obtained. Membrane proteins isolated from erythrocytes were run in 3.5% stacking gels (Fig. 1A) and were analyzed by a label-free quantitative MS based strategy; using spectral count data (see Supporting Information). Data analysis based on spectral count data from MS/MS-based experiments provided us a list of 39 proteins with statistical significance between AAA patients and controls (changes equal/over 1.5-fold; $p$ 0.05, Table 1), from the 141 identified proteins. Differential quantitative analysis shown in Fig.1B by a volcano plot,where pointed lines limit the points corresponding to over- or downregulated proteins in AAA patients with respect to control subjects. Initial MS data show that the most abundant identified protein in all extracts was the band 3 anion transport (not shown), which is the highest abundant protein in plasma membrane from RBCs, suggesting the purity of our starting sample. However, we cannot discard the potential contribution of contaminants from other cell types (e.g. reticulocytes) [5]. Among others, MS data revealed altered levels of structural membrane proteins (e.g. ankyrin), components of the degradation machinery (e.g. proteasome subunits) and oxidative stress related proteins (e.g. catalase and peroxiredoxin-2 (Prdx-2)) in RBCs from AAA patients (Table 1). Due to the importance of oxidative stress in AAA [6,7], we validated catalase and Prdx-2 in samples from ten controls and 20 AAA patients by Western blot. Equal amounts of proteins (20 g) were resolved as previously reported [6]. As observed in Fig. 2, decreased catalase and Prdx-2 expression was observed in RBCs membrane extracts from AAA patients compared to controls, confirming the initial proteomic results. Catalase and Prdx-2 are mainly expressed in the cytosol, where no differences were found. It is important to note that whereas intracellular ROS are neutralized by the highly abundant cytosolic antioxidant systems in RBCs (catalase, glutathione peroxidase, and peroxiredoxin), ROS associated to hemoglobin oxidation (among them heme degradation products) are mainly located to the membrane [2].

Interestingly, the increase in heme degradation products has been found in the membrane fraction of senescent RBCs and of pathological RBCs (with less stable Hb) [8]. More recently, neutralization of membrane ROS by Prdx-2 was proposed as the underlying mechanism explaining the increase in heme degradation products in Prdx-2 KO mice [9]. All these data highlight the importance of a redox dysbalance at this localization.

The clinical relevance of our study could be associated to the potential role of RBC number and related anemia as a strong diagnostic/prognostic marker of AAA patients [3]. The recently described diminution of RBC number in AAA patients could be the result of both decreased RBC production and/or increased RBC clearance. In one hand, we recently observed decreased iron availability for RBC production associated to increase inflammatory conditions and tisular retention in AAA patients [3]. In the other hand, changes in RBC membrane composition could affect RBC clearance/elimination. Senescent/damaged RBCs are cleared by phagocytosis and it is generally assumed that aging-associated changes in membrane structure are caused by oxidative damage [10]. In addition to senescence, RBCs may die by eryptosis, a programmed suicidal death program characterized by cell membrane scrambling and cell shrinkage, with exposure of phosphatidylserine at the cell surface [11]. Oxidative stress activates Ca2+ sensitive K+ channels in the erythrocyte cell membrane presumably via generation of prostaglandins, which stimulate eryptosis [12]. We have shown herein altered expression in antioxidant proteins, along with structural proteins and several components of the proteasome complex, in RBC membrane of AAA patients. In the same line, when recent studies have analyzed the interactome of RBCs to address main pathways involved in RBC vitality, oxidative stress along with proteins of the proteasome machinery has been included [13]. Thus it could be speculated that the decrease in RBC number and associated anemia in AAA patients could be due, at least in part, to the increased clearance of "damaged" RBCs (or RBCs with altered membrane protein composition). In this respect, a decreased activity of membrane bound antioxidant systems has been suggested to potentially decrease RBC lifespan [14]. Accordingly, Prdx-2 KO mice display haemolytic anemia [15]. Finally, in addition to the important role of altered RBC membrane composition associated to the role of RBC number as diagnostic/prognostic biomarker in AAA, our study could help to understand pathological mechanisms involved in AAA of potential therapeutic interest. Among them, decreased

expression of antioxidant systemsinRBCsmembranefromAAApatientscouldimpact: (i) prooxidant reactions in cells near them in AAA tissue (e.g. neutrophils in ILT and vascular smooth muscle cells/VSMCs in wall) and (ii) phagocyte recruitment to AAA tissue for removal of redox-induced senescent RBCs. Thus, increasing the expression or activity of these antioxidant systems (e.g. Prdx-2, catalase) could be a therapeutic option in the treatment of AAA. In agreement, specific catalase overexpression in VSMCs has been recently demonstrated to prevent experimental AAA formation [16].

On the whole, our proteomic study shows that RBC membrane protein composition is altered in AAA, although future studies should be performed to understand the functional consequences of the modification of proteins in the membrane of RBC in AAA patients.

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## References

[1] Michel, J. B., Martin-Ventura, J. L., Egido, J., Sakalihasan, N. et al., Novel aspects of the pathogenesis of aneurysms of the abdominal aorta in humans. *Cardiovasc. Res.* 2011, *90*, 18–27.

[2] Mohanty, J. G., Nagababu, E., Rifkind, J. M., Red blood cell oxidative stress impairs oxygen delivery and induces red blood cell aging. *Front. Physiol.* 2014, *5*, 84. doi: 10.3389/fphys.2014.00084.

[3] Martinez-Pinna, R., Lindholt, J. S., Madrigal-Matute, J., Blanco-Colio, L. M. et al., From tissue iron retention to low systemic haemoglobin levels, new pathophysiological biomarkers of human abdominal aortic aneurysm. *Thromb Haemost.* 2014, *112*, 87–95.

[4] Pasini, E. M., Lutz, H. U., Mann, M., Thomas, A. W., Red blood cell (RBC) membrane proteomics–Part II: comparative proteomics and RBC patho-physiology. *J. Proteomics* 2010, *73*, 421–435.

[5] Barasa, B., Slijper, M., Challenges for red blood cell biomarker discovery through proteomics. *Biochim. Biophys.Acta* 2014, *1844*, 1003–1010.

[6] Ramos-Mozo, P., Madrigal-Matute, J., Martinez-Pinna, R., Blanco-Colio, L. M. et al., 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. *Arterioscler. Thromb. Vasc. Biol.* 2011, *31*, 3011–3019.

[7] McCormick, M. L., Gavrila, D., Weintraub, N. L., Role of oxidative stress in the pathogenesis of abdominal aortic aneurysms. *Arterioscler. Thromb. Vasc. Biol.* 2007, *27*, 461–469.

[8] Nagababu, E., Mohanty, J. G., Bhamidipaty, S., Ostera, G. R., Rifkind, J. M., Role of the membrane in the formation of heme degradation products in red blood cells. *Life Sci.* 2010, *86*, 133–8.

[9] Nagababu, E, Mohanty, J. G., Friedman, J. S., Rifkind, J. M., Role of peroxiredoxin-2 in protecting RBCs from hydrogen peroxide-induced oxidative stress. *Free Radic Res.* 2013, *47*, 164–171.

[10] Ghashghaeinia, M., Cluitmans, J. C., Akel, A., Dreischer, P. et al., The impact of erythrocyte age on eryptosis. *Br. J. Haematol.* 2012, *157*, 606–14.

[11] Lang, F., Abed, M., Lang, E., Foller, M., Oxidative stress and suicidal erythrocyte death. *Antioxid. Redox Signal.* 2014, *21*, 138–153.

[12] Cimen, M. Y., Free radical metabolism in human erythrocytes. *Clin. Chim. Acta* 2008, *390*, 1–11.

[13] D'Alessandro, A., Righetti, P. G., Zolla, L., The red blood cell proteome and interactome: An update. *J. Proteome Res.*2010, *9*, 144–63.

[14] Tsantes, A. E., Bonovas, S., Travlou, A., Sitaras, N. M., Redox imbalance, macrocytosis, and RBC homeostasis. *Antioxid. Redox Signal.* 2006, *8*, 1205–1216

[15] Lee, T. H., Kim, S. U., Yu, S. L., Kim, S. H. et al., Peroxiredoxin II is essential for sustaining life span of erythrocytes in mice. *Blood* 2003, *101*, 5033–5038.

[16] Parastatidis, I., Weiss, D., Joseph, G., Taylor, W. R., Overexpression of catalase in vascular smooth muscle cells prevents the formation of abdominal aortic aneurysms. *Arterioscler. Thromb. Vasc. Biol.* 2013, *33*, 2389–2396.

[17] Piersma, S. R., Fiedler, U., Span, S., Lingnau, A. et al., Workflow comparison for label-free, quantitative secretome proteomics for cancer biomarker discovery: Method evaluation, differential analysis and verification in serum. *J. Proteome Res.* 2010, *9*, 1913–1922.

**Table 1.** List of proteins differentially expressed in membrane fractions from human erythrocytes

| SwissProt Code[a)] | *p*-values[b)] | FC (patients versus CT)[b)] | Protein ID[c)] |
|---|---|---|---|
| Q13439 | $1.24 \times 10^{-4}$ | Unique in patients | Golgin subfamily A member 4 |
| P17612 | $113 \times 10^{-3}$ | Unique in patients | cAMP-dependent protein kinase catalytic subunit alpha |
| Q5JWF2 | $8.40 \times 10^{-3}$ | Unique in patients | Guanine nucleotide-binding protein G(s) subunit alpha isoforms XIas |
| Q9UPS8 | $1.46 \times 10^{-2}$ | Unique in patients | Ankyrin repeat domain-containing protein 26 |
| P62879 | $1.47 \times 10^{-2}$ | Unique in patients | Guanine nucleotide-binding protein G(I)/G(S/G(T) subunit beta-2 |
| P35580 | $1.24 \times 10^{-3}$ | Unique in patients | Myosin-10 |
| P21583 | $1.53 \times 10^{-4}$ | Unique in control | Kit Ligand |
| Q09666 | $3.65 \times 10^{-2}$ | Unique in patients | Neuroblast differentiation-associated protein AHNAK |
| O00231 | $1.16 \times 10^{-2}$ | Unique in patients | 26S proteasome non-ATPase regulatory subunit 11 |
| P11277 | $2.68 \times 10^{-3}$ | 1.8 | Spectrin beta chain, erythrocyte |
| P27105 | $4.61 \times 10^{-4}$ | −1.9 | Erythrocyte band 7 integral membrane protein |
| P02549 | $6.05 \times 10^{-4}$ | 2.5 | Spectrin alpha chain, erythrocyte |
| P68871 | $6.64 \times 10^{-3}$ | −1.8 | Hemoglobin subunit beta |
| P69905 | $2.10 \times 10^{-2}$ | −1.6 | Hemoglobin subunit alpha |
| P32119 | $4.89 \times 10^{-3}$ | −1.7 | Peroxiredoxin-2 |
| Q14254 | $1.36 \times 10^{-3}$ | 1.7 | Flotillin-2 |
| A2TKE6 | $1.74 \times 10^{-2}$ | −1.8 | Cellular titin isoform PEVK variant 4 |
| P35527 | $9.43 \times 10^{-3}$ | 4.5 | Keratin, type I cytoskeletal 9 |
| A8MZ70 | $2.23 \times 10^{-2}$ | −2.2 | Moesin |
| B8Q185 | $2.89 \times 10^{-2}$ | 1.7 | Glycophorin A MNS blood group |
| P18577 | $9.42 \times 10^{-4}$ | 2.7 | Blood group Rh(CE) polypeptide |
| P17931 | $1.23 \times 10^{-2}$ | −2.1 | Galectin-3 |
| P04040 | $3.68 \times 10^{-3}$ | −16.2 | Catalase |
| A8MTT4 | $7.02 \times 10^{-4}$ | 4.7 | Nebulin |
| Q02224 | $2.07 \times 10^{-2}$ | −6.1 | Centromere-associated protein E |
| P09543 | $2.12 \times 10^{-4}$ | 12.7 | 2′,3′-cyclic-nucleotide 3′-phosphodiesterase |
| P25786 | $1.76 \times 10^{-2}$ | −3.3 | Proteasome subunit alpha type-1 |
| P68133 | $1.38 \times 10^{-2}$ | −3.8 | Actin, alpha skeletal muscle |
| Q5CZC0 | $2.33 \times 10^{-2}$ | 3.3 | Fibrous sheath-interacting protein 2 |
| P15311 | $5.11 \times 10^{-2}$ | −13.0 | Ezrin |
| O75326 | $9.37 \times 10^{-3}$ | −3.1 | Semaphorin-7A |
| Q6NW34 | $4.76 \times 10^{-2}$ | 3.1 | Uncharacterized protein C3orf17 |
| P25789 | $3.47 \times 10^{-2}$ | −4.0 | Proteasome subunit alpha type-4 |
| P49720 | $1.51 \times 10^{-2}$ | −4.0 | Proteasome subunit alpha type-3 |
| P28074 | $3.05 \times 10^{-2}$ | −4.4 | Proteasome subunit beta type-5 |
| P28066 | $8.50 \times 10^{-3}$ | −19.0 | Proteasome subunit alpha type-5 |
| Q9H444 | $2.94 \times 10^{-2}$ | −8.3 | Charged multivesicular body protein 4b |
| P28072 | $1.21 \times 10^{-2}$ | −4.7 | Proteasome subunit beta type-6 |
| P25788 | $4.36 \times 10^{-3}$ | −10.4 | Proteasome subunit alpha type-3 |

a) Accession code according to SwissProt database. b) Fold change (FC) and its associated p-value in each group (CT, control subjects group). Negative FC values indicates downregulation in patients. If FC is unique, this indicates black-and-white regulation, as previously described [17]. c) Protein description.

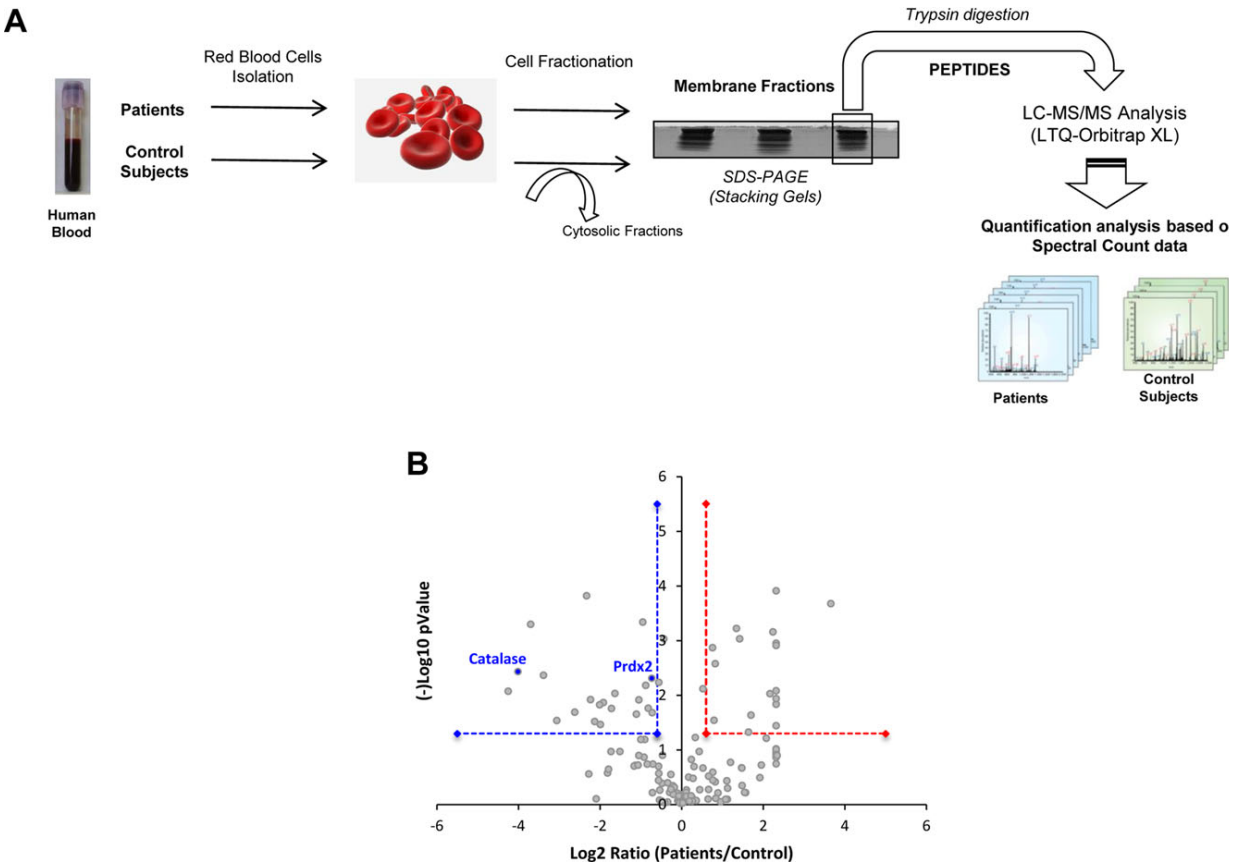


**Figure 1.** (A) General schedule showing the procedure followed. (B) Volcano plot provided by the proteomic quantitative analysis. Red and blue pointed lines represent the protein candidates' thresholds (over- and downregulated proteins in patients versus controls, respectively). Catalase and peroxiredoxin-2 (Prdx2) are two of the differential proteins validated in an additional group of human samples.

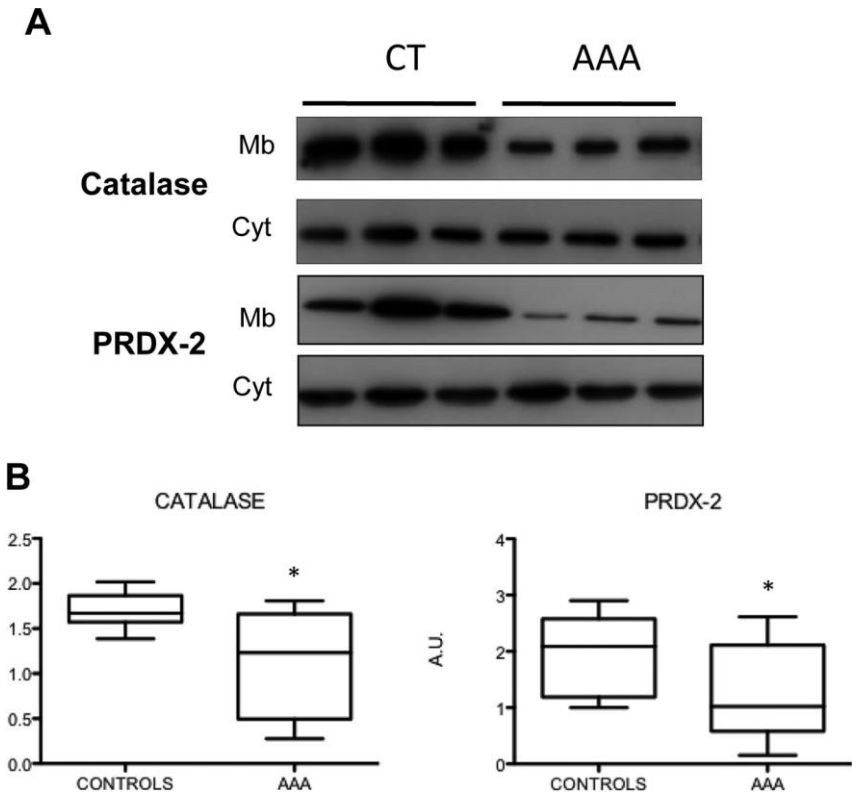


**Figure 2.** RBCs membrane composition from AAA patients and controls. (A) Representative Western blot using anticatalase (abcam) and antiperoxiredoxin 2 (santa cruz) antibodies on RBC membrane and cytosolic extracts isolated from controls (CT) and patients (AAA). (B) Densitometric analysis from ten controls and 20 AAA patients (*$p <$ 0.05 versus CT).