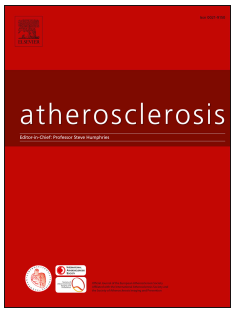
Accepted Manuscript



Blood hydrogen peroxide break-down activity in healthy subjects and in patients at risk of cardiovascular events

Roberto Carnevale, Cristina Nocella, Pasquale Pignatelli, Simona Bartimoccia, Lucia Stefanini, Stefania Basili, Marta Novo, Alessandra D'Amico, Vittoria Cammisotto, Daniele Pastori, Francesco Violi

PII: S0021-9150(18)30210-7

DOI: 10.1016/j.atherosclerosis.2018.04.025

Reference: ATH 15478

To appear in: *Atherosclerosis*

Received Date: 28 December 2017

Revised Date: 22 March 2018

Accepted Date: 24 April 2018

Please cite this article as: Carnevale R, Nocella C, Pignatelli P, Bartimoccia S, Stefanini L, Basili S, Novo M, D'Amico A, Cammisotto V, Pastori D, Violi F, Blood hydrogen peroxide break-down activity in healthy subjects and in patients at risk of cardiovascular events, *Atherosclerosis* (2018), doi: 10.1016/j.atherosclerosis.2018.04.025.

Blood hydrogen peroxide break-down activity in healthy subjects and in patients at risk of cardiovascular events

Roberto Carnevale[1*], Cristina Nocella[1*], Pasquale Pignatelli[2], Simona Bartimoccia[2], Lucia Stefanini[2], Stefania Basili[2], Marta Novo[2], Alessandra D'Amico[1], Vittoria Cammisotto[2], Daniele Pastori[2**], Francesco Violi[2**]

***These authors contributed equally to this work**

**** Joint authorship**

(1) Department of Medical-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Latina, Italy.

(2) I Clinica Medica, Atherothrombosis Centre, Department of Internal Medicine and Medical Specialties, Sapienza University of Rome

Correspondence to: Roberto Carnevale, PhD, Department of Medical-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Latina, Italy. Corso della Repubblica 79. Phone: +3907731757231; fax +3907731757231; e-mail: roberto.carnevale@uniroma1.it

ABSTRACT

Background and aims: Antioxidant status has been shown to be associated with cardiovascular events (CVEs). Aim of the study was to develop an assay measuring serum hydrogen peroxide (H_2O_2) break-down activity (HBA) of healthy subjects (HS) and to validate it in a cohort of patients affected by atrial fibrillation (AF).

Methods: We developed the HBA assay in 121 HS and validated it in 842 AF patients. The occurrence of CVEs was registered and correlated with HBA in AF during a median follow-up of 30.6 months (3226 patient-years). A combined endpoint of CVEs included fatal/non-fatal ischemic stroke and myocardial infarction, cardiovascular death and transient ischemic attack.

Results: In HS, median HBA was 61.2% [IQR: 52.9-69.4]. AF patients disclosed lower HBA than 30 HS balanced for age and sex (48.6% [IQR: 24.7-65.1] *vs.* 59.4% [IQR: 49.2-66.2], $p<0.001$). During a mean follow-up of 30.6 months (3226 patient-years), 168 CVEs occurred (5.2%/year). A multivariable Cox's proportional hazards regression analysis showed that age group 3 (71-80 years, HR:5.419, $p=0.020$), age group 4 (>80 years, HR:9.783, $p=0.002$), diabetes (HR:1.464, $p=0.049$), previous cardiac events (HR:1.887, $p=0.001$) events and HBA (below median, HR:2.313, $p<0.001$) predicted CVEs.

Conclusions: We developed an easy assay to measure serum HBA, which was associated with CVEs in AF patients. This assay may represent an additional useful tool for cardiovascular risk stratification and should be validated in other high-risk populations.

Keywords: hydrogen peroxide, assay, antioxidant, atrial fibrillation, cardiovascular events.

INTRODUCTION

Oxidative stress plays a key role in the mechanism of atherogenesis by favouring the formation of oxidized low-density lipoproteins (oxLDL) and ensuing accumulation in the artery wall, where oxLDL contribute to foam cell formation and eventually atherosclerotic plaque. Formation of oxLDL stems from LDL interactions with reactive oxidant species (ROS) such as superoxide anion,

OH radicals, hydrogen peroxide (H_2O_2) [1]. Among ROS, H_2O_2 plays an important role not only in the atherosclerotic but also in the thrombotic process, as it is a potent stimulus for activation of platelets. In particular, H_2O_2 serves to amplify the platelet response to agonists and to propagate formation of thrombus growth via formation of thromboxane A_2, which is a powerful aggregating molecule derived from arachidonic interaction with COX1 [2,3]. Humans possess an enzymatic armamentarium to counteract the deleterious effects of H_2O_2, namely catalase and glutathione peroxidase (GPx) [4]. In absence of an appropriate ROS scavenger activity, experimental and clinical studies documented a tendency to thrombotic complications [5]. Thus, animals deficient of GPx3 displayed enhanced platelet activation, platelet-rich thrombi and occluded vessels compared to wild type ones [6]. Furthermore, in patients with stable coronary disease impaired GPx1 was associated with an enhanced risk of cardiovascular complications [7].

These data lead to hypothesize that impaired systemic scavenging capacity is a potential risk factor for cardiovascular events. However, all available assays did not focus on a specific pathway involved in ROS detoxification nor measured specifically H_2O_2 scavenger capacity, thus providing only a non-specific measure of global antioxidant status. This approach, while taking into account several different sources of ROS, does not allow the identification of any specific enzymatic pathway underlying the oxidative imbalance [8].

To address this issue, we developed a methodology to measure serum Hydrogen peroxide (H_2O_2) Break-down Activity (HBA) in healthy subjects (HS). Then, we tested the predictive ability of this new assay for cardiovascular events (CVEs), in patients at high cardiovascular risk, such as those suffering from atrial fibrillation (AF), during a follow-up of about 3 years.

PATIENTS AND METHODS

The study consisted of three phases: 1) assessment of HBA in blood samples from HS; 2) comparison of HBA from HS to that from AF patients; 3) predictive value of the assay toward CVEs in AF patients.

Samples collection

Blood samples without anticoagulant were collected between 8:00 and 9:00 am and centrifuged for 10 minutes at 300 g. The supernatant was stored at -80 °C until use.

Spectrophotometric Method Optimization

Linearity study

Hydrogen peroxide 30% was diluted with distilled water to get final concentrations 0.35, 0.7, 1.4, 2.8, 5.6, and 11.2 mg/ml. The solutions were scanned on a spectrophotometer in the UV range 190–1100nm (See Supplemental Figure 1 Panel A). The spectrum was recorded between 225-230 nm (See Supplemental Figure 1 Panel A). The calibration plot was constructed as concentration *vs.* amplitude.

Precision

Precision was evaluated as repeatability and reproducibility intra-day and inter-day. Repeatability intra-day was determined by analysing the H_2O_2 solution (1.4 mg/ml) for 20 times on the same day. Inter-day precision was determined by analyzing the same concentration of H_2O_2 20 times daily for 3 days over the period of a week. For the evaluation of intra-day and inter-day variability, the mean of replicates, standard deviation (SD) and coefficient of variation (CV) was calculated. Reproducibility was evaluated by comparing intra-day and inter-day values using two different spectrophotometers: 1) UV-visible spectrophotometer (A380, AOE Instruments Shanghai Co., Ltd., Shanghai, China), single beam optical system, length range Wave 190-1100 nm; 2) UV-visible

spectrophotometer (8453 Agilent Technologies, Santa Clara (California), USA), single beam, wavelength range 190-1100 nm.

Stability study

Samples stability was evaluated by measuring the absorbance up to 60 minutes. The final value is expressed as a percentage variation, that is the difference between initial absorbance values (T0) and absorbance after 60 minutes (T60).

Method optimization and standardization for determination of % HBA *in serum*

The HBA was measured after incubating different amounts of serum sample (2.5, 5, 10, 20, 40 µl) with 200 µl of H_2O_2 and 800 µl of sample diluent (Hank's Balanced Salt Solution, HBSS) at 37°C for 30 minutes. At the end of 30 minutes, to avoid proteins that absorb at 230nm, 200 µl of stop solution (Trichloroacetic Acid, TCA) was added and the samples were then centrifuged at 3000 rpm for 5 minutes. Finally, the supernatant was read at 230 nm by UV-Visible Single-beam Spectrophotometer (A380, AOE Instruments Shanghai Co., Ltd, Shanghai, China).

The % of HBA was calculated according to the following formula: % Of HBA = [(Ac-As) / Ac] X 100 where Ac is the absorbance of H_2O_2 1.4 mg/ml and is the absorbance in the presence of the serum sample.

Stability and precision study of H_2O_2 solutions in presence of serum samples

To verify the stability of serum samples, three aliquots of serum samples were subjected to three freezing/thawing cycles and then absorbance of 1.4 mg/ml H_2O_2 in presence of serum was evaluated.

The repeatability of the measurements of H_2O_2 solutions in presence of serum samples was evaluated by measuring the intra-day and inter-day variation coefficient (CV). For the evaluation of intra-day variability, absorbance of replicates (n=10) was evaluated on the same day. The mean, SD and CV% were calculated. For the evaluation of inter-day variability, the mean of 10 replicates on 3 successive days, SD and CV% were calculated. Reproducibility was verified by comparing intra-day CVs (n=10 replicates) and inter-days (n=10 replicates for n=3 days) obtained using two different instruments as previously described.

In vitro study

To evaluate the role of H_2O_2 scavenger enzymes, we incubated serum samples with mercaptosuccinic acid (5 mM, Sigma Aldricht, St Louis, Missouri, USA) that is one of the strongest and specific inhibitors of glutathione peroxidase (GPx) [9] or with sodium azide (1 mM, Sigma Aldricht, St Louis, Missouri, USA) that selectively inhibits catalase [10]. The inhibitors were used alone or in combination and the percentage of HBA was evaluated and compared to untreated serum.

Study population

We performed a prospective single-centre cohort study including 860 patients with non-valvular AF were treated with oral vitamin K antagonists (INR target 2.5), referring to the Atherothrombosis Centre of the Department of Internal Medicine and Medical Specialties of Sapienza-University of Rome, from September 2007 to October 2015. Eighteen patients were excluded from the analysis for missing data and 842 composed the final cohort.

Exclusion criteria included: presence of prosthetic heart valves, cardiac stent placement or cardiac revascularization in the previous year, severe cognitive impairment, chronic infectious diseases, autoimmune systemic diseases and active cancer. We also excluded patients taking antioxidants.

Baseline medical history and anthropometric data were recorded, and blood and urine samples were collected. Cardiovascular risk factors were defined as previously described [11].

The primary endpoint of the study was a composite of CVEs including fatal/non-fatal myocardial infarction and ischemic stroke, cardiac revascularization, cardiovascular death and transient ischemic attack, as previously described [11]. Data on CVEs were prospectively collected during follow-up, and only the first cardiovascular event was used for the analysis.

All patients provided a written informed consent before being included in the study. The study protocol was approved by the local ethical board of Sapienza-University of Rome and was conducted according to the principles of the Declaration of Helsinki.

Statistical analysis

Categorical variables were reported as counts (percentage). The normal distribution of parameters was assessed by Kolmogorov–Smirnov test. Continuous variables were expressed as mean ± standard deviation, or median and interquartile range (IQR). Correlations were made using Pearson's linear correlation test or Spearman's rank correlation coefficient, rS, respectively. Independence of categorical variables was tested with the $\chi 2$ test. Student unpaired t test was used to compare means, and ANOVA test with post-hoc Bonferroni correction was used to compare groups. A reference value for HBA was investigated in HS and then compared to that from a subgroup of AF patients balanced for age and sex. For the analysis, AF population was divided according to the median value of H_2O_2 scavenger activity. Then, multivariable logistic regression analysis was used to assess factors associated with reduced (below median) H_2O_2 scavenger activity after adjustment for potential confounders. The cumulative incidence of cardiovascular events in AF patients was estimated using a Kaplan–Meier product-limit estimator for patients above and below the median. Survival curves were formally compared using the log-rank test. Cox's proportional hazards regression analyses were used to calculate the adjusted relative hazards of cardiovascular events by each clinical variable. The multivariable analyses were performed entering

the following pre-specified variables as covariates, representative for demographic characteristics and for the most common cardiovascular risk factors present in AF patients: age groups, female sex, body mass index (BMI) smoking, arterial hypertension, diabetes, history of cardiac events, history of cerebrovascular events, HF, treatment with statins and antiplatelet drugs.

To further investigate the relationship between HBA and age we divided the cohort into 4 age groups: age group 1 (50-60 years, n=50), age group 2 (61-70 years, n=248), age group 3 (71-80 years, n=353), age group 4 (>80 years, n=191).

Statistical significance was set at a *p* value <0.05. All tests were two-tailed, and analyses were performed using computer software packages (SPSS-18.0, SPSS Inc.).

RESULTS

Method optimization

Stability and precision study of HBA

The calibration curve was obtained by plotting the absorbance versus the concentration data. The linear regression data for the calibration curves showed good linear relationship over the concentration from 0.35 mg/ml to 1.4 mg/ml. The linear regression equation was y = 0.2655x + 0.0594 with correlation coefficient 0.9972. HBA shows λ_{max} at 230nm. The validation parameters of the method are reported in Supplemental Table 1.

The next step was to determine the reaction kinetics with respect to the time period prior to the spectrophotometric measurement. The absorbance of each solution (0.35 mg/ml to 1.4 mg/ml) relative to the time periods of 10, 20, 30, 40, 50 and 60 min was determined. The results showed that 1.4 mg/ml was the most stable concentration with as the percentage variation between 0 and 60 minutes was -2.9% (Supplemental Table 2).

Repeatability was determined by analyzing concentrations of H_2O_2 solutions (1.4 mg/ml) for 20 times in the same day (intra-day) and for 20 times for 3 days in a week (inter-day) and

reproducibility was evaluated by comparing intra-day and inter-day values using two different spectrophotometers. The % CV values < 10 indicates that measurements of H_2O_2 was repeatable and reproducible (Table 1).

Stability and precision study of serum samples

To verify stability, 3 aliquots of serum were subjected in the same day to 3 cycles of freezing followed by thawing at 37°C. H_2O_2 solution in the presence of serum was found to be stable under stress (See Supplemental Table 3).

The repeatability of measurement obtained with serum samples taken from HS was established by measuring the intra- and inter-day CV. For the evaluation of intraday variability, 10 measurements were performed and mean, SD and CV% were calculated. For the evaluation of inter-day variability, the average of 10 measurements in 3 days, SD and CV% were calculated. Reproducibility was established by comparing intraday and inter-day values obtained using two different instruments. The developed method was repeatable and reproducible as CV% values were < 10% (Table 1).

Moreover, measurement of HBA in presence of different amounts of serum samples showed 2.5 µl of serum as the most reliable quantity to assess HBA.

In vitro study

Treatment of serum with mercaptosuccinic acid, the inhibitor of GPx, and sodium azide, the inhibitor of catalase, resulted in a significant reduction in HBA (-45% and -28%, respectively). Compared to single agents, combined treatment with both inhibitors reduced the percentage of HBA by 67% (See Supplemental Figure 2).

Application of HBA in HS and AF patients

To find out a value of HBA in HS, we tested our method in 121 HS; 72.7% were men with a mean age of 56.5±11.9 years (See Supplemental Table 4). The median value of HBA was 61.2% [IQR: 52.9-69.4].

Median HBA was significantly lower in AF compared to 30 out of 121 HS balanced for age and sex 48.6% [IQR: 24.7-65.1] *vs.* 59.4% [IQR: 49.2-66.2], $p<0.001$) (See Supplemental Figure 1 panels B and C).

Patients with a HBA below median were more frequently in AF rhythm and had a higher prevalence of HF and history of cerebrovascular events; they were also less likely to use statins (Table 2).

In AF patients, HBA was not correlated with lipid profile (R=0.046, $p=0.178$ for total cholesterol, R=-0.022, $p=0.531$ for HDL cholesterol, R=0.051, $p=0.154$ for LDL cholesterol and rS=0.030, $p=0.397$ for triglycerides).

HBA decreased from age of fifty to sixty years, was stable between sixty and seventy years, thereafter abruptly declined (ANOVA test $p=0.007$, age group 4 *vs.* 1, $p=0.001$; (Figure 1, Panel A). Analysis of factors associated with low HBA showed that persistent/permanent AF (*vs.* paroxysmal) (OR:1.340, $p=0.045$), arterial hypertension (OR:0.602, $p=0.029$), HF (OR:1.506, $p=0.043$), previous cerebrovascular events (OR:2.227, $p<0.001$) were independently associated with a reduced HBA (below median); also, a trend for age was found (Table 3).

H_2O_2 break-down activity and cardiovascular events

During a mean follow-up of 30.6 months (3226 patient-years), 168 CVEs were registered (5.2%/year). Of these, 20 fatal and 17 non-fatal MI, 19 cardiac revascularizations, 67 cardiovascular deaths, 22 non-fatal and 16 fatal ischemic strokes and 7 TIAs. Patients experiencing CVEs during follow-up had significantly lower HBA compared to patients free from events 27.7% [4.7-51.2] *vs.* 51.3% [31.6-68.1], respectively $p<0.001$.

After dividing the cohort according to the median of HBA, we found that patients below median had increased risk of CVEs compared to those above (log-rank test $p<0.001$), with 46 CVEs occurring in the group above and 122 in the group below median (Figure 1, Panel B).

A multivariable Cox's proportional hazards regression analysis (Table 4), showed that age group 3 (*vs.* group 1, HR:5.419, $p=0.020$), age group 4 (*vs.* group 1, HR:9.783, $p=0.002$), diabetes (HR:1.464, $p=0.049$), previous cardiac events (HR:1.887, $p=0.001$) events and HBA (below median, HR:2.313, $p<0.001$) independently predicted CVEs.

DISCUSSION

The study reports a novel assay to measure blood ability to break-down H_2O_2 (HBA) in HS and in patients at risk of CVEs, such as those with AF. Herewith, we show that HBA is lower in AF patients compared to HS and independently predicts CVEs suggesting its potential use to screen for patients at risk of CVEs.

Hydrogen peroxide is physiologically present in blood and is produced via superoxide dismutase conversion of superoxide anion to hydrogen peroxide [12]. Hydrogen peroxide is stable oxidant molecule, which is implicated in atherothrombosis as evidenced by increased risk of CVEs in patients with elevated hydrogen peroxide in the blood [13]. Detoxification of hydrogen peroxide is devolved to antioxidant enzymes such as catalase or glutathione peroxidases [14,15]. In case of down-regulation of these antioxidants an increased risk of thrombosis has been reported [11].

Notably, recent evidence suggested that low concentrations of hydrogen peroxide, mostly produced by Nox4 at endothelial site, may exert a vascular protective action [16]. This finding is not in contrast with our report; thus, hydrogen peroxide concentration increase may have deleterious consequences when an oxidative imbalance occurs as in case of atherosclerotic disease.

Hence, rapid and reliable analysis of HBA property may be useful for clinical purpose to identify patients at risk of CVEs.

Thus, we developed a new assay to measure HBA property, which could be easy to perform, rapid and not expensive. The method here reported allows measuring the blood HBA property by a spectrophotometric method at 230 nm using small amounts of blood**.**

The assay reflects maximally the activity of glutathione peroxidase and catalase without being specific for any of these enzymes. However, it permits to clear-cut differentiate HS from patients at risk of cardiovascular events such as AF ones, who, in fact, are at increased risk of cardiovascular events [17]. In particular, patients with AF showed lower HBA property compared to controls and were at increased risk of CVEs if HBA property was <30% during a follow-up of approximately 3 years. Cox's proportional hazards regression analysis confirmed that values of HBA below median independently predicted CVEs along with age, HF and prior cardiovascular and cerebrovascular events.

Among the factors affecting blood HBA, we found that HF and previous cerebrovascular events were associated with below median values. Of interest was also the fact that a progressive decline of blood HBA by aging was found. This is in keeping with previous data showing an age-related decline of GPx3 activity in AF patients [11].

We found also an inverse association between arterial hypertension and HBA, which may be explained by the fact that anti-hypertensive drugs may favourably affect antioxidant status. For instance, ACE inhibitors inhibit angiotensin II pathway, which is a strong inducer of ROS-producing enzymes such as NADPH oxidase [18].

The study has implications and limitations. Differently from previous reports, which pointed to a total, non-specific antioxidant blood property, we focused on H_2O_2 blood antioxidant property, which is known to be a risk for thrombosis. This novel approach based on a specific antioxidant pathway instead of a non-specific total antioxidant capacity would provide novel insights on the contribution of each antioxidant enzyme in causing the oxidative imbalance in different specific clinical settings (i.e. cardiovascular disease, cancer, or autoimmunity), and also would allow the

planning of targeted interventional trials. For example, patients with impaired HBA should be advised for nutritional counseling, as a high adherence to Mediterranean Diet was found to be associated with an enhanced antioxidant status, specifically a high activity of circulating GPx3 [19]. Thus, a nutritional intervention would represent a first approach to modulate the oxidative imbalance of patients with low HBA.

The assay is simple, rapid and cheap and allowed to discriminate patients at risk of CVEs. The assay is limited by a low specificity towards the enzymes, which specifically breakdowns hydrogen peroxide in blood such as glutathione peroxidase and catalase but it provides an indirect measure of their combined activity. Moreover, it does not take into account other potential systemic sources of oxidative stress.

Even if we did not directly evaluate all possible interactions with concomitant medications, most of drugs prescribed to AF patients do not interfere with spectrophotometric assay at 230 nm. Moreover, blood samples were taken in the morning after overnight fasting and before administration of any drug, so that trough plasma concentrations of drugs were expected.

Finally, the study has been performed in a single centre and in elderly Caucasian AF patients, thus the assay should be evaluated also in other high-risk populations.

In conclusion, we developed a simple assay to measure blood HBA, which may be useful to identify patients at higher risk for cardiovascular disease.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Author contributions:

R.C., F.V. designed the study and wrote the manuscript. C.N. analyzed the data and performed the experiments. P.P. recruited patients. L.F., M.N., V.C. performed in vitro study. D.P. performed statistical analysis.

References

[1] Levitan, I, Volkov, S and Subbaiah, PV, Oxidized LDL: diversity, patterns of recognition, and pathophysiology, Antioxidants & redox signaling, 2010;13:39-75.

[2] Pignatelli, P, Pulcinelli, FM, Lenti, L, et al., Hydrogen peroxide is involved in collagen-induced platelet activation, Blood, 1998;91:484-490.

[3] Freedman, JE, Oxidative stress and platelets, Arteriosclerosis, thrombosis, and vascular biology, 2008;28:s11-16.

[4] Violi, F, Loffredo, L, Carnevale, R, et al., Atherothrombosis and Oxidative Stress: Mechanisms and Management in Elderly, Antioxidants & redox signaling, 2017;27:1083-1124.

[5] Pastori, D, Carnevale, R and Pignatelli, P, Is there a clinical role for oxidative stress biomarkers in atherosclerotic diseases?, Internal and emergency medicine, 2014;9:123-131.

[6] Jin, RC, Mahoney, CE, Coleman Anderson, L, et al., Glutathione peroxidase-3 deficiency promotes platelet-dependent thrombosis in vivo, Circulation, 2011;123:1963-1973.

[7] Blankenberg, S, Rupprecht, HJ, Bickel, C, et al., Glutathione peroxidase 1 activity and cardiovascular events in patients with coronary artery disease, The New England journal of medicine, 2003;349:1605-1613.

[8] Huang, D, Ou, B and Prior, RL, The chemistry behind antioxidant capacity assays, J Agric Food Chem, 2005;53:1841-1856.

[9] Chaudiere, J, Wilhelmsen, EC and Tappel, AL, Mechanism of selenium-glutathione peroxidase and its inhibition by mercaptocarboxylic acids and other mercaptans, The Journal of biological chemistry, 1984;259:1043-1050.

[10] Vetrano, AM, Heck, DE, Mariano, TM, et al., Characterization of the oxidase activity in mammalian catalase, The Journal of biological chemistry, 2005;280:35372-35381.

[11] Pastori, D, Pignatelli, P, Farcomeni, A, et al., Aging-Related Decline of Glutathione Peroxidase 3 and Risk of Cardiovascular Events in Patients With Atrial Fibrillation, Journal of the American Heart Association, 2016;5.

[12] Loscalzo, J, Oxidant stress: a key determinant of atherothrombosis, Biochem Soc Trans, 2003;31:1059-1061.

[13] Freedman, JE, Loscalzo, J, Benoit, SE, et al., Decreased platelet inhibition by nitric oxide in two brothers with a history of arterial thrombosis, The Journal of clinical investigation, 1996;97:979-987.

[14] Dayal, S, Wilson, KM, Motto, DG, et al., Hydrogen peroxide promotes aging-related platelet hyperactivation and thrombosis, Circulation, 2013;127:1308-1316.

[15] Rhee, SG, Yang, KS, Kang, SW, et al., Controlled elimination of intracellular H(2)O(2): regulation of peroxiredoxin, catalase, and glutathione peroxidase via post-translational modification, Antioxidants & redox signaling, 2005;7:619-626.

[16] Schroder, K, Zhang, M, Benkhoff, S, et al., Nox4 is a protective reactive oxygen species generating vascular NADPH oxidase, Circulation research, 2012;110:1217-1225.

[17] Violi, F, Soliman, EZ, Pignatelli, P, et al., Atrial Fibrillation and Myocardial Infarction: A Systematic Review and Appraisal of Pathophysiologic Mechanisms, Journal of the American Heart Association, 2016;5.

[18] Nguyen Dinh Cat, A, Montezano, AC, Burger, D, et al., Angiotensin II, NADPH oxidase, and redox signaling in the vasculature, Antioxidants & redox signaling, 2013;19:1110-1120.

[19] Pastori, D, Carnevale, R, Menichelli, D, et al., Is There an Interplay Between Adherence to Mediterranean Diet, Antioxidant Status, and Vascular Disease in Atrial Fibrillation Patients?, Antioxidants & redox signaling, 2016;25:751-755.

380 **Figure legend**

381 **Figure 1.** (A) Blood H_2O_2 break-down activity (HBA) according to decades of age (ANOVA test
382 p=0.007, age group 4 *vs.* 1, p=0.001). (B) Kaplan-Meier curves according to the median value of
383 HBA (black line: below median; grey line: above median).

384

385
386
387

Table 1. Repeatability and reproducibility of H_2O_2 solution and of H_2O_2 solution in presence of serum samples.

The repeatability of 1.4 mg/ml H_2O_2 solution was evaluated for n=20 replicates (intra-day) and n=20 replicates for 3 days (inter-day). Reproducibility was evaluated by comparing intra-day and inter-day values using two different spectrophotometers.

The repeatability of H_2O_2 solution in presence of sample was evaluated for n=10 replicates (intra-day) and n=10 replicates for 3 days (inter-day). Reproducibility was evaluated by comparing intra-day and inter-day values using two different spectrophotometers.

	1.4 mg/ml H_2O_2			
	Intra-day (n=20)		**Inter-day (n=20)**	
	Mean absorbance ±SD	% CV	Mean absorbance ±SD	% CV
Repeatability	0.480±0.002	0.35	0.478±0.005	1.08
Reproducibility	0.469±0.003	0.73	0.481±0.026	0.27
	H_2O_2 solution in presence of 2.5 µl of serum sample			
	Intra-day (n=10)		**Inter-day (n=10)**	
	Mean absorbance ±SD	% CV	Mean absorbance ±SD	% CV
Repeatability	0.192±0.002	0.86	0.189±0.004	2.13
Reproducibility	0.185±0.010	5.6	0.191±0.013	6.7

Table 2. Baseline characteristics of the study cohort according to the median value of hydrogen peroxide (H_2O_2) break-down activity (HBA) in atrial fibrillation patients

Variables	**Overall**	**HBA**		*p* value
	(n=842)	Above median (n=421)	Below median (n=421)	
Age (years)	73.5±8.1	72.9±8.0	74.0±8.2	0.047
Persistent/permanent AF (vs. paroxysmal)	53.3	48.9	57.7	0.013
Women (%)	41.7	42.3	41.1	0.780
Body mass index (Kg/m^2)	27.4±4.7	27.5±4.7	27.3±4.6	0.473
Smokers (%)	10.1	11.9	8.3	0.109
CHA_2DS_2-VASc score	3.5±1.5	3.4±1.4	3.6±1.6	0.023
Arterial hypertension (%)	88.6	90.5	86.7	0.103
Diabetes mellitus (%)	19.9	20.5	19.2	0.666
Heart failure (%)	16.0	13.3	18.8	0.039
Previous cerebrovascular events (%)	14.3	9.7	18.8	<0.001
Previous cardiac events (%)	24.3	25.2	23.5	0.630
Statins (%)	41.9	45.4	38.5	0.050
Antiplatelets (%)	19.5	18.9	20.0	0.723

AF: Atrial fibrillation; HBA: hydrogen peroxide break-down activity.

Table 3. Multivariable logistic regression analysis of factors associated with reduced (below median value) hydrogen peroxide (H_2O_2) break-down activity (HBA) in atrial fibrillation patients.

	***p* value**	**Odds ratio**	**95% confidence interval**	
Persistent/permanent AF (*vs.* paroxysmal)	0.045	1.340	1.006	1.784
Female sex	0.512	0.908	0.680	1.212
Arterial hypertension	0.029	0.602	0.382	0.949
Diabetes	0.897	0.976	0.681	1.401
Smoking	0.106	0.676	0.420	1.087
Heart failure	0.043	1.506	1.013	2.239
Previous cerebrovascular events	<0.001	2.227	1.469	3.376
Previous cardiac events	0.266	0.812	0.562	1.172
Statin use	0.120	0.789	0.586	1.063
Body mass index	0.951	1.001	0.969	1.034
Antiplatelet drugs	0.603	1.105	0.758	1.611
Age group 2 (*vs.* group 1)	0.071	1.824	0.950	3.504
Age group 3 (*vs.* group 1)	0.079	1.794	0.934	3.445
Age group 4 (*vs.* group 1)	0.057	1.962	0.981	3.923

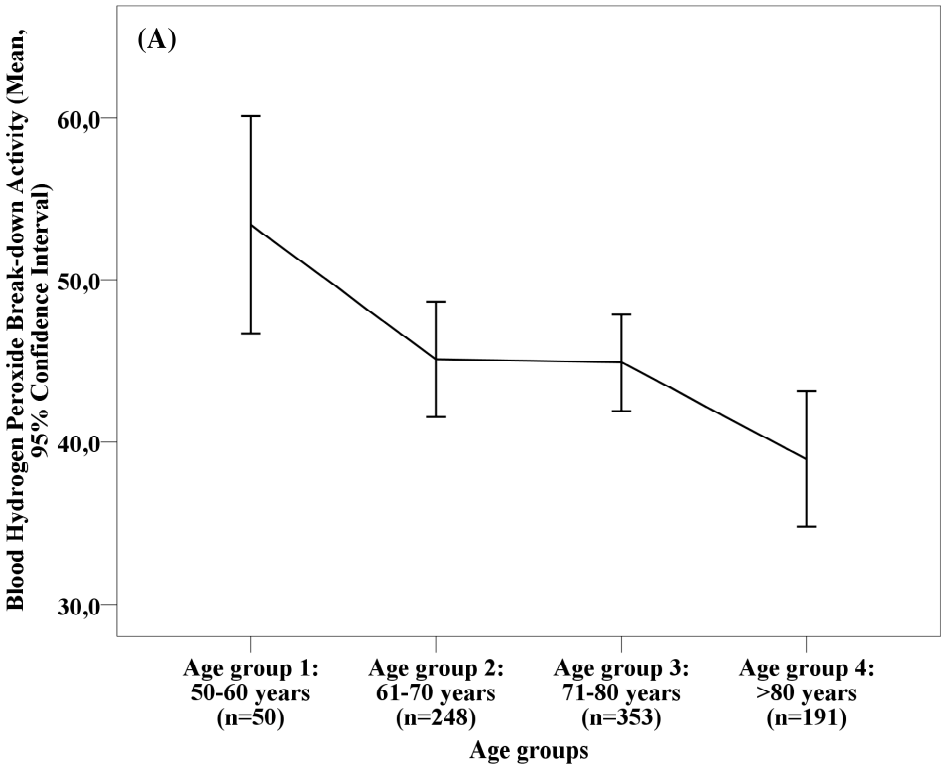
AF: atrial fibrillation

Table 4. Multivariable Cox's proportional hazards regression analysis of factors associated with the occurrence of cardiovascular events during follow-up in atrial fibrillation patients

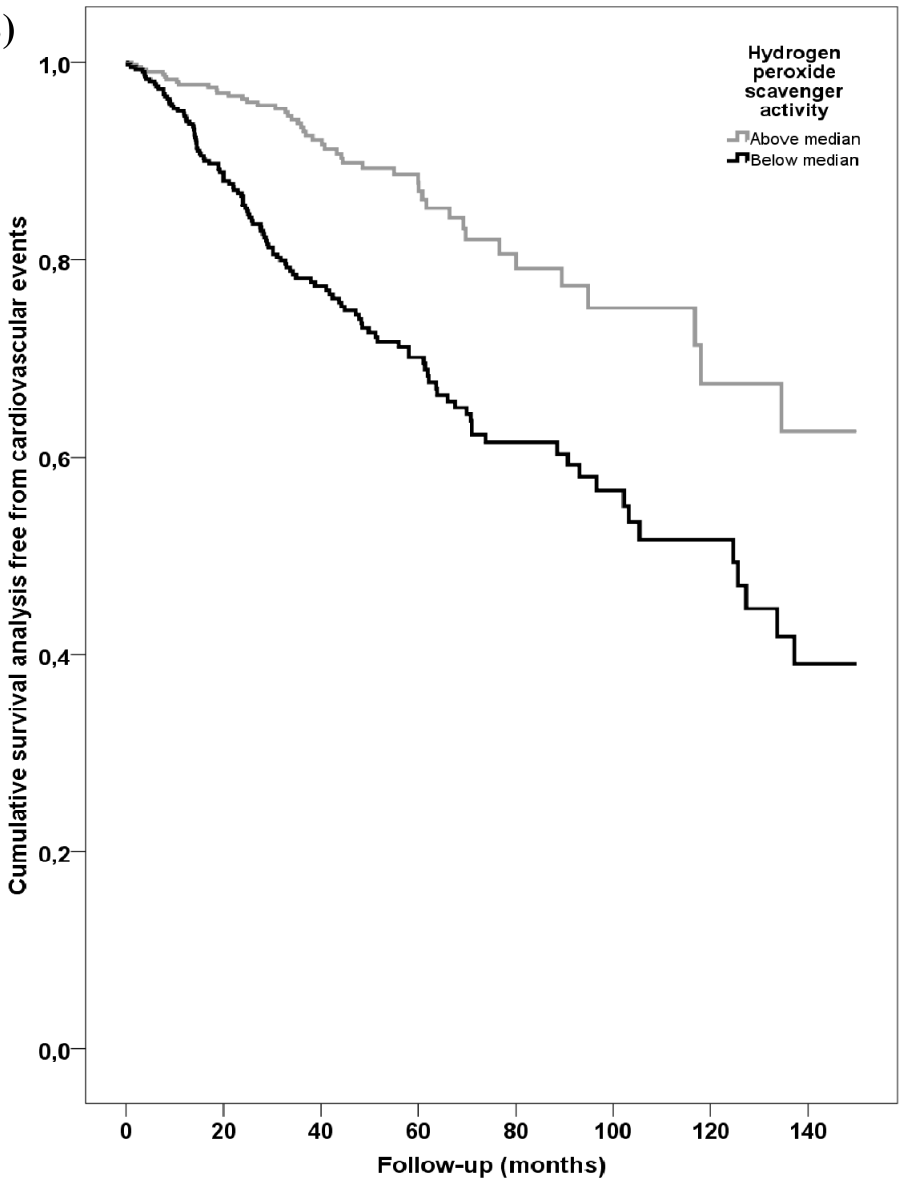
	***p* value**	**Hazard Ratio**	**95% confidence interval**	
Persistent/permanent AF (*vs.* paroxysmal)	0.436	0.875	0.624	1.225
Female sex	0.421	0.868	0.615	1.225
Age group 2 (*vs.* group 1)[a]	0.180	2.691	0.633	11.445
Age group 3 (*vs.* group 1)[a]	0.020	5.419	1.300	22.581
Age group 4 (*vs.* group 1)[a]	0.002	9.783	2.285	41.881
HBA (below median)	<0.001	2.313	1.616	3.311
Arterial hypertension	0.329	1.413	0.706	2.827
Diabetes	0.049	1.464	1.001	2.141
Smoking	0.961	1.014	0.585	1.757
Heart failure	0.073	1.425	0.968	2.098
Previous cerebrovascular events	0.101	1.384	0.939	2.040
Previous cardiac events	0.001	1.887	1.316	2.707
Statin use	0.761	0.947	0.666	1.346
Body mass index	0.734	1.007	0.968	1.047
Antiplatelet drugs	0.947	1.013	0.696	1.474

[a]Global *p* value <0.001.

AF: atrial fibrillation; HBA: hydrogen peroxide (H_2O_2) break-down activity.



(B)



Above median	421	334	203	105	51	28	16	12
Below median	421	299	191	118	63	37	24	13

Highlights:

- We developed an easy assay to measure serum hydrogen peroxide break-down activity (HBA) 121 healthy subjects
- We validated the HBA assay on 842 atrial fibrillation patients
- The HBA assay may represent an additional useful tool for cardiovascular risk stratification