Aerobic Performance and Antioxidant Protection in Runners

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Key words
- antioxidant capacity
- exercise
- oxidative damage
- endurance runners

Abstract

Reactive oxygen species (ROS) production is known to increase as a result of muscular contractile activity and this phenomenon may perturb the fine-controlled cellular redox homeostasis within cells and tissues. We studied the possible correlations between individual aerobic performance-related factors and the oxidative stress markers profile in the serum of thirty-five endurance male runners that experienced a modified Bruce-based maximal graded exercise test. Our investigation assessed the systemic levels of malondialdehyde (MDA), protein carbonyl content (PCC) and total antioxidant status (TAS). We found that redox-related parameters and aerobic performance indicators were correlated. Indeed, significant negative associations between TAS and PCC (r-value −0.7, $p<0.001$) and between TAS and total protein content (r-value −0.4, $p=0.005$) were observed. A significant positive association between total protein and PCC (r-value 0.4, $p=0.012$) was also revealed. Only a trend of negative correlation between serum total protein and anaerobic threshold (r-value −0.3, $p=0.07$) was found. Interestingly, different responses in MDA levels were elicited by the ergometric test as a function of the individual anaerobic threshold. High aerobic capacities may be promising anthropometric factors indicative of adapted biochemical environments featuring enhanced protection against the oxidative challenge elicited by both regular endurance training and single intense exercise bouts.

Introduction

Although reactive oxygen species (ROS) are pro-oxidant molecules produced during physiological aerobic metabolism, they have been shown to be crucially involved in the pathogenesis of several diseases, such as cardiovascular disorders, neurodegenerations and cancer [20].

An imbalance between the pro-oxidants and anti-oxidant enzymatic and non-enzymatic systems can cause oxidative damages to lipids, proteins and DNA that, if unrepaired, may promote oxidative stress conditions, thus ultimately leading to cell function impairment [10, 26].

ROS cellular production is known to increase as a result of muscular contractile activity and this phenomenon may perturb the fine-controlled cellular redox homeostasis. As reviewed in detail by Deaton and Marlin [3], physical activity is known to promote ROS over-production through the following main pathways: 1) O_2 over-consumption-dependent increase in the mitochondrial leakage of oxygen-centred free radicals from the electron transport chain (ETC); 2) ischemia-reperfusion-induced activation of the enzyme xanthine oxidase (XO); 3) NAD(P)H-dependent superoxide release by activated neutrophils resulting from tissue damages and inflammatory processes.

Despite the increased ROS generation during exercise bouts, it has been established that regular and moderate physical activity has been linked to a reduced incidence of oxidative stress-associated pathologies; this apparent paradox is the result of exercise-induced hormetic adaptations which can involve the activation of specific redox-sensitive signalling pathways as well as the enhancement of ROS-scavenging defensive mechanisms and oxidative damage repairing systems, as reviewed by Radak and colleagues [20]. A number of exercise-induced adaptations of redox-related defensive systems seems to involve the activation of transcription factors, among which the nuclear factor-κB (NF-κB) is considered the most critical for the cell to cope with oxidative stress [9]. NF-κB has been shown to up-

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regulate the expression of several anti-oxidative enzymes such as the manganese-dependent superoxide dismutase (MnSOD), thus improving the overall protection against oxidative injury in the mitochondrion, one of the most vulnerable compartment of the cell against ROS-related damages [7,9].

On this basis, we hypothesized that higher aerobic capacities and O_2 utilization rate-related anthropometric parameters, such as VO_2max and anaerobic threshold (AT), may be associated to more efficient anti-oxidative mechanisms and, as a consequence, to enhanced scavenging activities towards the physical exercise-induced ROS over-production. This would point to high aerobic fitness levels as possible predictive factors indicative of good protection against oxidative challenges.

In order to verify our hypothesis, we analyzed the possible correlations between individual aerobic performance-related factors and the oxidative stress markers profile in the serum of endurance male runners that experienced a modified Bruce-based maximal graded exercise test.

Several biomarkers are available for researchers in order to quantify the oxidatively-modified macromolecules in biological samples, although no single measurement can adequately describe oxidative damage; therefore, a battery of assays has been proposed in order to reliably monitor oxidative stress/damages in biological specimens [6]. As extensively reviewed by Hwang and Kim, the assessment of malondialdehyde (MDA) and protein carbonyl content (PCC) are among the most widely employed assays used to determine lipid peroxidation and oxidative damage to proteins, respectively [8].

Furthermore, since the ROS removal rate is mostly controlled by a variety of low molecular weight antioxidants, there is a great interest in determining the total antioxidant status (TAS) in tissues and body fluids, thus considering the cumulative action resulting from the cooperation of all the different enzymatic and non-enzymatic compounds with anti-oxidative properties [21].

Blood carries a number of substances that are considered markers of oxidative damage, thus changes in the circulating concentrations of these markers are thought to reflect the corresponding variations in many highly-perfused tissues and organs (e.g. skeletal muscles) [14,19]. In addition, the blood has a crucial role in maintaining the redox homeostasis in tissues against pro-oxidant biochemical shifts (e.g. during physical activity), since it redistributes and deliveries reducing equivalents to all body areas [18].

Materials and Methods

Human subjects

Thirty-five diabetes-, smoking- and cardiovascular disease-free long distance male runners participated to the study. Subjects were all middle aged (42±5 years) with similar body mass index (24±3 Kg/m2) and with similar training histories (11±4 years, 4±1 h/wk, 45±7 Km/wk). Individual resting cardiac frequency, diastolic and systolic arterial pressure values were determined (54±9 bpm, 77±5 and 121±5 mmHg, respectively). Individual fat-free mass percentage was also recorded (83±5). The usual dietary habits of each participant in the study were assessed and the nutrient analysis showed an average daily energy intake of 14000 kJ with 60% from carbohydrate, 25% from fat, and 15% from protein (mediterranean-style diet). Participants were excluded if they had used vitamin or mineral supplements in the four weeks prior to the study. Furthermore, the participation in athletic competitions was forbidden during the four weeks prior to the laboratory test. The experimental protocol was approved by the ethical committee of the University "G. d'Annunzio" of Chieti-Pescara and all participants provided their written informed consent before the study. Subjects were not allowed to take anti-inflammatory drugs nor to participate in any intense physical effort 48 h before the blood withdrawals. The terms of the latest version of the Declaration of Helsinki for Medical Research involving Human Subjects have been adhered.

Cardiopulmonary exercise testing

All participants underwent a physician-supervised maximal treadmill test, according to the modified Bruce protocol [4]. Briefly, the test included seven stages (3 min each) with increasing speeds (2.74–8.05 Km/h) and grades (0–18%) of the treadmill.

All the tests were performed in the morning hours. Ventilation, oxygen uptake and carbon dioxide output were measured by a computer-controlled breath-by-breath analyzer (Schiller CS-200 Ergo-Spiro), combined with an electrocardiograph for heart rate and rhythm control. Maximal oxygen uptake (VO_2max) (62±6 mL/Kg/min), was determined on the basis of the spirometric analysis of VO_2 and of VCO_2. Predicted maximal heart rate and anaerobic threshold (40±8 mL/Kg/min) were determined by using the 220-age and V-slope methods, respectively. Fasting blood samples were collected immediately before and 30 min after the maximal test. Capillary whole-blood samples were taken from the earlobe before start, at the end of each stage and 1, 3, 5, 7 and 10 min after cessation of exercise and were analyzed for lactate concentrations (Greiner BioChemica, Flacht, Germany). During the exercise protocol the liquid assumption was strictly controlled.

Storage of the samples

All the redox markers studied are sensitive to storage conditions thus isolated serum specimens were immediately frozen at −80 °C. Preserving additive-free samples were processed as described below within one week.

Total antioxidant status

Because of difficulty in measuring each antioxidant component separately and interactions among antioxidants, methods have been developed to assess the total antioxidant status of serum or plasma. The 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox)-equivalent antioxidant capacity (TEAC) assay is a widely used kit-based commercial method. This assay is based on the suppression of the absorbance of radical cations of 2,2′-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS®) by antioxidants in the test sample when ABTS incubates with a peroxidase (metmyoglobin) and hydrogen peroxide [21]. Briefly, 10 µL of serum were added in duplicate to 10 µL of metmyoglobin and 150 µL of the chromogen solution supplied by the manufacturer. Then, reactions were initiated by the addition of 40 µL of hydrogen peroxide, as indicated by the manufacturer (cat. 709001; Cayman Chemical, Ann Arbor, USA). Reaction mixtures were incubated for 3 min at room temperature and then read by a Victor3 microplate reader (PerkinElmer, Waltham, USA). A linear calibration curve was computed from pure Trolox-containing reactions (range: 0–0.33 mM).

Thiobarbituric acid-reactive substances

The measurement of thiobarbituric acid-reactive substances (TBARS) is a well-established method in order to detect lipid peroxidation [28]. We used TBARS Assay Kit (cat. 10009055; Cayman Chemical) which allows a rapid photometric detection at 532 nm of the thiobarbituric acid-malondialdehyde (TBA-MDA) adduct. In brief, 100 µL of serum were added in duplicate to 100 µL of sodium dodecyl sulfate (SDS) and 4 mL of colour reagent, as suggested by the manufacturer. Then reaction mixtures were incubated for one hour in boiling water and centrifuged at 1 600 g for 10 min at 4 °C. Samples were, then, warmed for 5 min at 25 °C and read by a Lambda25 spectrophotometer (PerkinElmer). A linear calibration curve was computed from pure MDA-containing reactions (range: 0–50 µM).

Protein carbonyls

Cayman Chemical's Protein Carbonyl Assay Kit (cat. 10005020) was used in order to evaluate colorimetrically oxidized proteins [13]. In brief, each serum sample was treated in duplicate with 2,4-dinitrophenylhydrazine (DNPH) and dissolved in 2.5 M HCl. The formation of a Schiff base between protein carbonyls and DNPH produced the corresponding hydrazones which could be isolated by 20% trichloroacetic acid and ethanol-ethyl acetate (1:1)-based centrifugations at 10 000 g for 10 min at 4 °C. Then, – pellets containing hydrazone were redissolved in guanidine hydrochloride and read at 370 nm, by a Victor3 microplate reader (PerkinElmer), as described in detail by the manufacturer. The absorbance of 2.5 M HCl-treated samples were substracted from the DNPH-treated samples and the obtained corrected values were used to determine the concentrations of the protein carbonyls ($\varepsilon = 22\,000\ M^{-1}cm^{-1}$). Later values were normalized to the total protein concentration in the final pellet (absorbance reading at 280 nm) in order to consider protein loss during the washing steps, as suggested in the kit's user manual.

Total protein assay

A highly sensitive and selective colorimetric bicinchoninic acid-based commercial kit was used in order to determine the serum total protein content (cat. 23225; Pierce Biotechnology, Rockford, USA). Bovine serum albumin was used as the standard [23].

Statistical analysis

All data were processed through SigmaStat and Statistica 7 softwares. The analysis of ergometric test-induced changes was performed by the paired t-test, whereas correlation studies were carried out by using the Pearson product moment method. Student's t-test was computed in order to assess the dependence of the redox changes induced by the ergometric test as a function of the aerobic fitness level. p-values less than 0.05 were always considered statistically significant.

Results

Cardiopulmonary exercise bout-induced effects

The analysis of blood lactate levels revealed a marked increase of this analyte as a consequence of the cardiopulmonary exercise bout (5.3 ± 0.9 fold over basal level). The subjects recovered lactate basal levels during the defatigation period (1.4 ± 0.3 fold over basal level).

Table 1 Effect of exercise on the main redox-related serum parameters.

	Pre-exercise Mean ± SD (n = 35)	Post-exercise Mean ± SD
total proteins (mg/mL)	62 ± 6	62 ± 4
TBARS (µM)	22 ± 10	20 ± 10
PCC (nmol/mg protein)	0.5 ± 0.2	0.5 ± 0.2
TAS (mM)	0.64 ± 0.13	0.66 ± 0.12

TBARS, thiobarbituric acid-reactive substances; PCC, protein carbonyl content; TAS, total antioxidant status.

Our statistical analyses showed that the cardiopulmonary exercise bout did not alter any individual redox-related parameter studied significantly, as shown in **Table 1**.

Statistical associations between redox-related and anthropometric parameters

Pearson product moment-based pre-exercise vs. post-exercise data processing was performed in the initial phase of the result analysis; significant positive associations between all the studied parameters were found: in particular, pre- vs. post-total protein content, r-value 0.77 with $p < 0.001$; pre- vs. post-TBARS, r-value 0.60 with $p < 0.001$), pre- vs. post-PCC, r-value 0.85 with $p < 0.001$ and pre- vs. post-TAS, r-value 0.96 with $p < 0.001$ (not shown).

Significant associations between some markers both in pre- and post-exercise samples were revealed after the Pearson correlation analysis. We found a significant ($p < 0.001$) negative association between total antioxidant status and protein carbonyl content (r-values −0.73 for pre-exercise and −0.70 for post-exercise, respectively), as reported in **Fig. 1**. In addition, the correlation analysis between serum total protein and anaerobic threshold detected r-values of -0.29 for pre-exercise and −0.31 for post-exercise, thus approaching the critical absolute value of 0.34 for significant weak associations with n = 35, as shown in **Fig. 2**. Moreover, a negative correlation ($p < 0.01$) between overall antioxidant status and total protein content was observed (r-values −0.46 for pre-exercise and −0.42 for post-exercise, respectively) (**Fig. 3**). The correlation analysis showed also a positive association ($p < 0.05$) between total protein and protein carbonyl content (r-values 0.42 for pre-exercise and 0.35 for post-exercise, respectively) (**Fig. 4**). The correlation study between overall antioxidant status and anaerobic threshold revealed a close but not statistically significant positive association, both for pre- and post-exercise ($p = 0.07$) (not shown).

Dependence of acute exercise-induced changes upon anthropometric parameters

We analyzed the possible relationship between the level of aerobic performance and the oxidative stress markers changes observed after the ergometric test. We found a significant dependence of acute exercise bout-induced variations of serum TBARS upon individual anaerobic thresholds ($p < 0.05$), as reported in **Fig. 5**. The selected AT threshold (40 mL/kg/min) represented the average AT value in our samples. No other significant relationships were found.

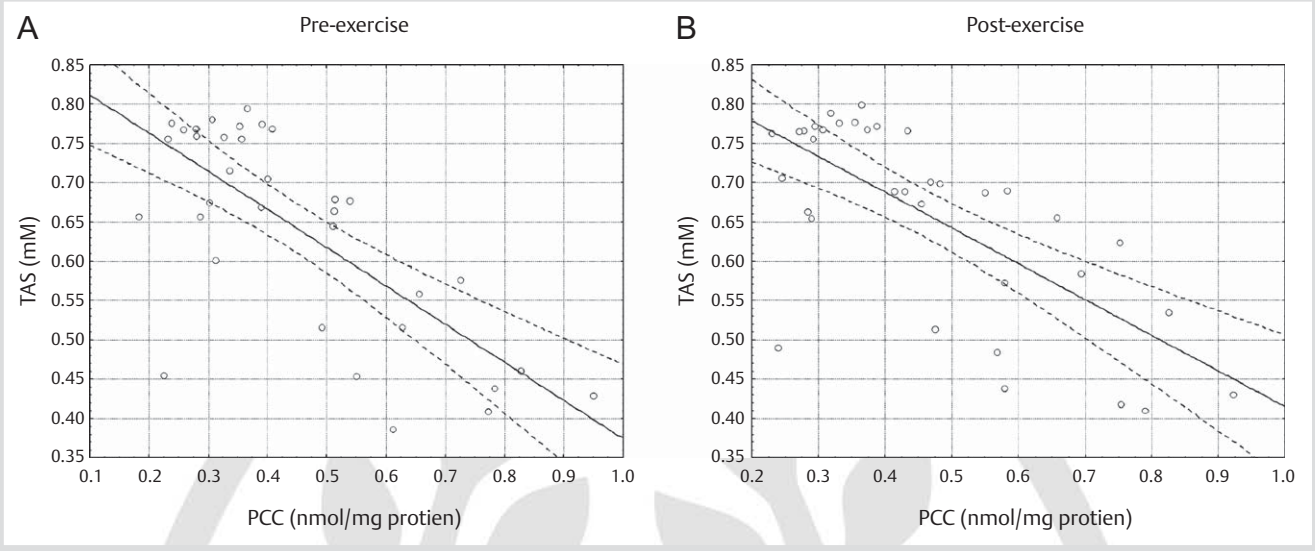

Fig. 1 Pearson product-moment correlation between total antioxidant status (TAS) and protein carbonyl content (PCC) in endurance runners' serum before (**A**) and after (**B**) a maximal treadmill-based ergometric test. The analysis showed significant negative associations both pre- and post-exercise (r-values −0.73 and −0.70, $p<0.001$, respectively). The continuous and dotted lines show the linear regression function and the 95% confidence interval, respectively.

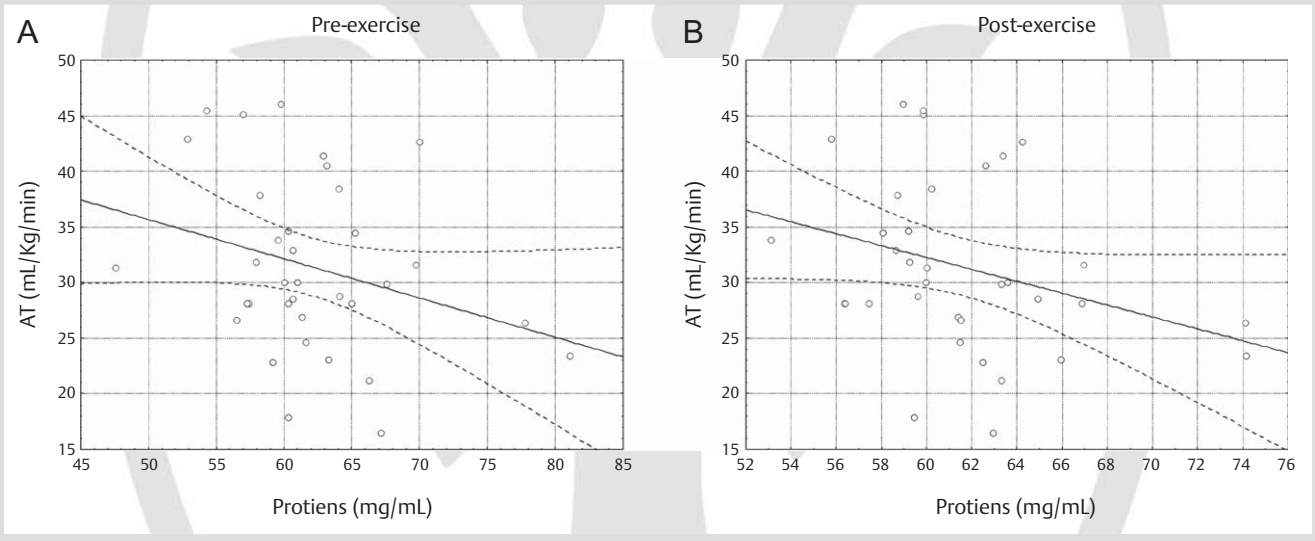

Fig. 2 Pearson product-moment correlation between anaerobic threshold (AT) and total protein content in endurance runners' serum before (**A**) and after (**B**) a maximal treadmill-based ergometric test. The analysis revealed a tendency to negative correlations both pre- and post-exercise (r-values −0.29 and −0.31, respectively). The continuous and dotted lines show the linear regression function and the 95% confidence interval, respectively.

Discussion

Despite the fact that the ergometric test did not cause any post-exercise effect, several interesting correlations were detected between some markers, in both pre- and post-exercise conditions. Significant positive associations between all the pre- vs. post-exercise studied parameters were detected. All of these highly positive linear statistical correlations were not surprising since, as stated above, our study did not reveal any significant cardiopulmonary exercise-derived effect on the redox-related indexes. Thus, it was not unexpected to observe higher post-exercise values in those runners who showed also higher pre-exercise values of the parameters studied.

We found that subjects with higher TAS values showed lower protein carbonyl contents. As previously established, the ROS removal rate is mostly controlled by a variety of compounds and chemical species with anti-oxidative properties such as glutathione, vitamins and redox-active enzymes; thereby, the negative association between TAS and PCC is in accordance with the protective role of overall circulating reducing power in the systemic protection against the oxidative damage to proteins. We further observed that runners with higher anaerobic thresholds seemed to show the tendency, though not statistically significant in our experimental set, to exhibit lower total protein serum contents; the same runners showed as well more efficient total antioxidant capacities. The potential significance of our results might be explained when taking into account literature data which

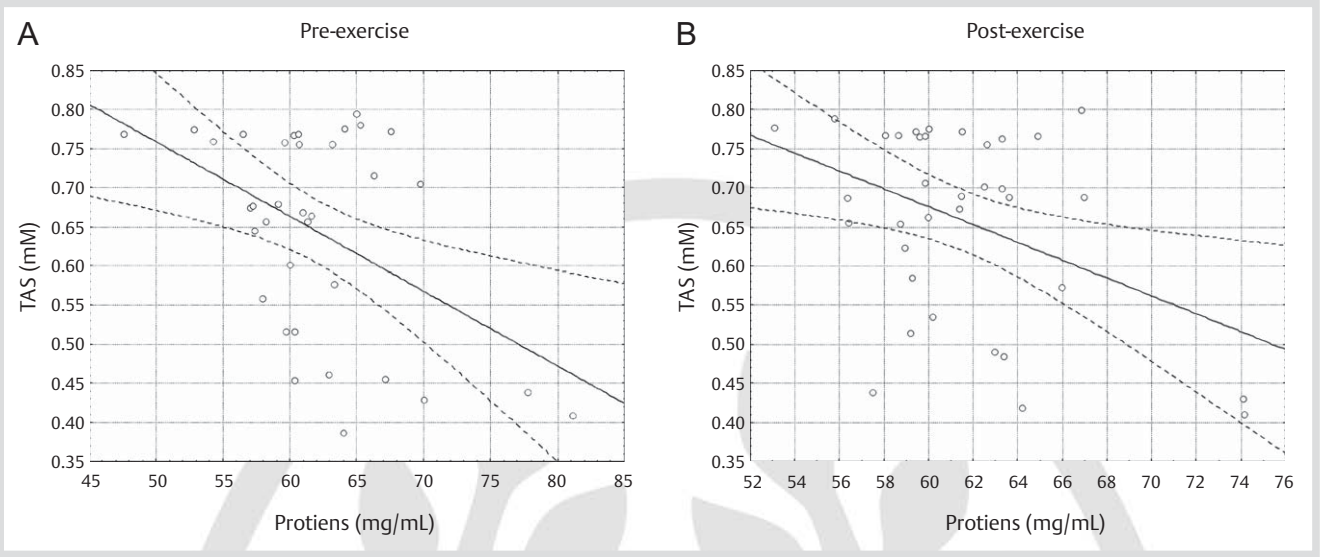

Fig. 3 Pearson product-moment correlation between total antioxidant status (TAS) and total protein content in endurance runners' serum before (**A**) and after (**B**) a maximal treadmill-based ergometric test. The analysis showed negative associations both pre- and post-exercise (r-values −0.46 and −0.42, $p<0.01$, respectively). The continuous and dotted lines show the linear regression function and the 95% confidence interval, respectively.

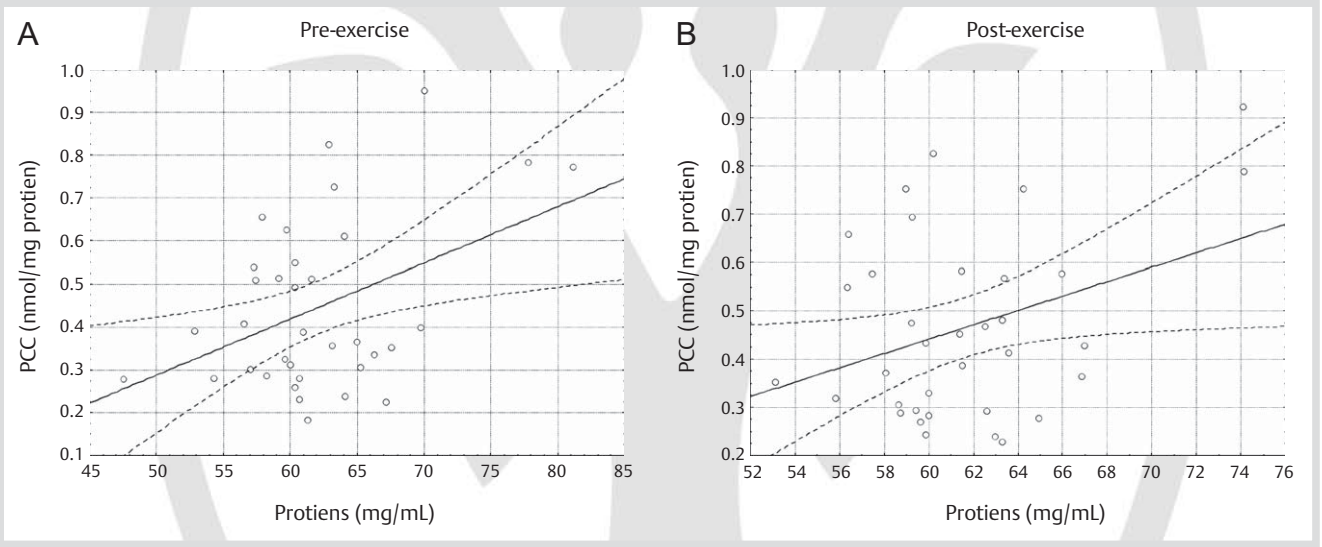

Fig. 4 Pearson product-moment correlation between protein carbonyl content (PCC) and total protein content in endurance runners' serum before (**A**) and after (**B**) a maximal treadmill-based ergometric test. The analysis showed positive associations both pre- and post-exercise (r-values 0.42 and 0.35, $p<0.05$, respectively). The continuous and dotted lines show the linear regression function and the 95% confidence interval, respectively.

report that, under oxidative stress-promoting conditions, the most abundant plasma protein, albumin, undergoes chemical oxidative modifications which, in turn, induce its rapid elimination from the circulating blood [2,18], thus reducing the plasmatic total protein content. Others researchers have previously shown that the redox-active amino acids of albumin go through chemical oxidation about twice as fast as those of other proteins [1]. Oxidative modifications of many proteins results in a significant decrease in their physiological activity, and the chemical alteration frequently acts as a marking stage for degradation, thus playing an important role in protein removal and turnover [22,24]. Some researchers suggested that the proteasome complex could play a crucial role in the regular exercise-induced enhancement of the removal mechanisms of oxidatively-modified proteins [20]. Despite the fact that our results seem to show adaptive responses involving metabolic changes and enhanced mechanisms aimed at neutralizing oxidative damages in subjects familiar to physical activity programs, other possible explanations for the observed inverse correlation between TAS and total plasma protein should not be excluded; in particular, it might also be possible to relate the low plasma protein levels in the high AT athletes with high protein utilization as fuel available to extend the individual aerobic phase. Nevertheless, our results seem to suggest that specific adaptive responses involving metabolic changes and enhanced mechanisms aimed at neutralizing oxidative damages may characterize subjects familiar to physical activity programs. Our interpretation of the obtained results is supported by the positive correlation detected between protein carbonyl content and total plasma proteins. Indeed, some authors have suggested that the exercise-induced PCC increase should be mainly derived from albumin oxidation [11,15]. In summary, our data seem to support the notion that

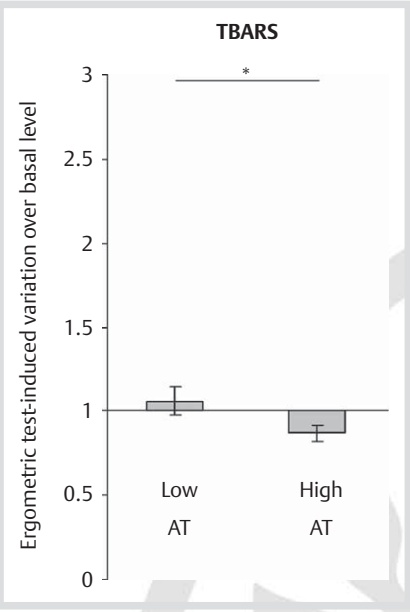

Fig. 5 Dependence of maximal treadmill-based ergometric test-induced variations of thiobarbituric acid-reactive substances (TBARS) serum levels upon individual anaerobic threshold (AT) in endurance runners. Low AT, subjects with AT below 40 mL/kg/min (n = 19); high AT, subjects with AT above 40 mL/kg/min (n = 16). Values are given as X-fold over basal levels (means ± s. d.). $^{*}p < 0.05$ vs. low AT (Student's t-test).

high AT subjects may present, as a factor related to improved antioxidative protection systems, more efficient mechanisms aimed at removing oxidatively-modified proteins from the systemic blood flow. This, in turn, may be crucial to avoid the potentially harmful accumulation of oxidized enzymes and regulatory polypeptides which often show altered physiological activities.

Looking for further evidence to confirm our hypothesis, the possible associations between anaerobic threshold and the treadmill test-dependent variation of the oxidative stress markers were investigated. We revealed that subjects with lower aerobic performances showed a clear trend to increase their TBARS levels after the ergometric test, whereas in individuals with better aerobic fitness the exercise bout did not trigger significant variations of serum MDA levels. This result seems to confirm that a better anti-oxidative capacity might be speculated to be an important molecular feature characterizing the biochemical environment in subjects with higher aerobic fitness. Indeed, it is well known that cell membranes are among the biological structures most readily oxidatively-damaged by acute whole-body exercise bouts [16, 17, 27]. An efficient ROS-scavenging defensive system may be crucial in order to ensure a rapid protection against the exercise-induced lipid peroxidative damage. The different responses in TBARS levels elicited by the ergometric test in the two groups of runners (low vs. high anaerobic thresholds) strongly supports our hypothesis concerning a significant relationship between the efficiency of the major anti-oxidative defensive systems and the athletic performance level.

Our findings are supported by those obtained in other studies in which high maximal oxygen consumptions were found to be associated with elevated plasma or erythrocyte antioxidant powers [5, 25].

The observed lower plasma protein content/PCC and higher TAS in higher AT athletes should also be discussed taking into account the procedure used for protein oxidative damage detection. Protein carbonyls may derive primarily from metal-catalyzed oxidation reactions and aldehyde-mediated attack towards aminogroups of proteins; thereby, the observed levels of carbonyl derivatives may in part originate also from lipid peroxidation-related processes. In this context, the decreased levels of post-exercise TBARS in high AT subjects may be strictly linked to their low PCC. The direct comparison of TAS and AT showed a close but not statistically significant positive correlation. This might indicate that in endurance runners the association between physical fitness and overall antioxidant defence efficiency may be rather complex, thereby not allowing to rule out the possibility of unknown variables playing a role in their interplay; with the above considerations in mind, we can argue that a larger sample size could help in revealing the direct positive relationship between antioxidant capacity and aerobic power. A larger sample size may also allow analyses with enough statistical power to detect as significant the correlation between AT and serum total protein, since, as already mentioned, our thirty five subjects-based study revealed only an indicative trend in the association between those two parameters.

Future experiments aimed at confirming and expanding the results of the present study will include larger sample size and datasets.

Although important improvements of the aerobic performance can be achieved through training programs, there are several indications for an influence of the anaerobic threshold and maximal oxygen uptake by genetic factors, as recently reviewed by Levine [12]. Thereby, it could be argued that within each training-dependent variability, inter-individual differences may result from genetic predisposition. Work is in progress to establish also whether known polymorphisms of specific redox-related genes can affect individual response to physical activity.

It could be interesting to verify whether the conclusions arising from this long distance runners-based study may be extended to non trained subjects featuring low AT and poor aerobic power. Future experiments would extend and improve our knowledge about the biochemical and molecular responses that may be evoken by acute physical bouts in habitual exercisers and in sedentary individuals.

In conclusion, we suggest that high aerobic capacities could be promising anthropometric factors indicative of adapted biochemical environments featuring enhanced protection against the oxidative challenge elicited by both regular endurance training and single intense exercise bouts.

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