Assessment of the redox status in patients with metabolic syndrome and type 2 diabetes reveals great variations

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Received November 25, 2015; Accepted January 5, 2016

DOI: 10.3892/etm.2016.2968

Abstract. The aim of the present study was to examine the effectiveness of a new redox status marker, the static oxidation reduction potential (sORP), for assessing oxidative stress in 75 patients with metabolic syndrome (MetS) and type 2 diabetes (T2D). A total of 35 normal subjects were used as the controls. Moreover, conventional markers of oxidative stress were assessed, such as thiobarbituric acid reactive substances (TBARS), protein carbonyls, the total antioxidant capacity in plasma, glutathione (GSH) levels and catalase (CAT) activity in erythrocytes. The results revealed that sORP was significantly higher (by 13.4%) in the patients with MetS and T2D compared to the controls, indicating an increase in oxidative stress. This finding was also supported by the significantly lower levels (by 27.7%) of GSH and the higher levels (by 23.3%) of CAT activity in the patients with MetS and T2D compared to the controls. Moreover, our results indicated a great variation in oxidative stress markers between the different patients with MetS and T2D, particarly as regards the GSH levels. Thus, the patients with MetS and T2D were divided into 2 subgroups, one with low GSH levels (n=31; GSH <3 μmol/g Hb) and another with high GSH levels (n=35; GSH >4 μmol/g Hb). The comparison of the markers between the 2 subgroups indicated that in the low GSH group, the GSH levels were significantly lower (by 51.7 and 52.9%) than those in the high GSH group and the controls, respectively. Furthermore, sORP in the low GSH group was significantly higher (by 8.1%) compared to the high GSH group, suggesting its sensitivity for assessing oxidative stress in patients wtih MetS and T2D. Moreover, this variation in oxidative stress levels between the different patients with T2D suggests that the assessment of the redox status may be important in prediabetic conditions, since there is evidence indicating that differences in the redox status in pre-diabetes may result in different outcomes.

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Abbreviations: CAT, catalase; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; H_2O_2, hydrogen peroxide; MetS, metabolic syndrome; ROS, reactive oxygen species; sORP, static oxidation reduction potential; TAC, total antioxidant capacity; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; T2D, type 2 diabetes

Key words: type 2 diabetes, metabolic syndrome, oxidative stress, oxidation reduction potential

Introduction

Free radicals are products of normal metabolism, including reactive oxygen species (ROS) such as superoxide anion radical (O2•-), hydroxyl radical (OH•) and peroxyl radical (RO_2•), and reactive nitrogen species (RNS), such as nitric oxide and the peroxynitrite radical (ONOO•) (1). Free radicals participate in several cellular functions, such as the regulation of signaling pathways and gene expression, and apoptosis (1,2). Endogenous sources of free radicals include the mitochondrial respiratory chain, inflammation, peroxisomes and cytochrome P450 (3). In addition, there are exogenous sources of ROS and RNS generation, such as smoking, air pollution, ultraviolet light and ionizing radiation (4). Free radicals are highly reactive species and can react with biological macromolecules (e.g., DNA, proteins and lipids), causing damage to these molecules (1). Living organisms have defense systems against free radicals, including antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) and

paraoxonase 1 (PON1), as well as non-enzymatic antioxidant compounds, such as glutathione (GSH), vitamins C and E, uric acid and ubiquinone (1). However, the overproduction of free radicals may lead to an imbalance in which the amount of ROS/RNS exceeds the antioxidant capacity, leading to oxidative stress associated with several pathophysiological conditions and diseases (1,5).

One of the pathophysiological conditions associated with oxidative stress is metabolic syndrome (MetS) (6). MetS is defined as a cluster of cardiovascular and type 2 diabetes (T2D) risk factors (7). MetS is diagnosed when a patient has at least three of the following risk factors: hyperglycemia, high blood pressure, high triglyceride levels, low high-density lipoprotein (HDL) cholesterol levels and obesity (7). There is evidence supporting the hypothesis that increased levels of oxidative stress may play an important role in MetS-related manifestations, including atherosclerosis and hypertension (8,9). Furthermore, oxidative stress is related with adiposity and insulin resistance in patients with MetS, suggesting that it is a crucial factor in the evolution of this pathological condition and not just a consequence (6,10,11).

As is already known, MetS may lead to the development of T2D, one of the most common metabolic disorders worldwide (12). T2D is characterized by hyperglycemia (i.e., high blood glucose levels) which occurs due to insulin resistance, that is, the cellular failure to respond normally to the insulin hormone (12). A number of studies have demonstrated that oxidative stress is associated with T2D, and particularly with its complications (12,13). In particular, some symptoms of T2D, such as hyperglycemia, insulin resistance and dyslipidemia induce oxidative stress through different mechanisms, such as increased advanced glycation end products (AGEs), inflammation, increased polyol pathway flux, increased hexosamine pathway flux and increased mitochondrial superoxide production (12-15). The increased levels of oxidative stress occurring in patients with T2D in turn aggravate some of the associated complications, particularly those involving the cardiovascular and neural system (12,14,16). Although the role of oxidative stress in diabetic complications has been established, its role as an etiological factor has not yet been fully elucidated (12,17).

Since oxidative stress is associated with MetS and T2D, its assessment in patients suffering from these disorders is useful for monitoring their progress and treatment, as well as for ameliorating the health-associated complications. Several biomarkers have been used for assessing oxidative stress levels in humans (18). However, the assessment of the redox status remains a time-consuming and impractical method in clinical settings, and thus there is a great need for developing new markers (19). In our previous studies, we measured a new marker, static oxidation reduction potential (sORP), in plasma using the RedoxSYS Diagnostic System for assessing oxidative stress induced by either physiological or pathophysiological conditions (20-23). sORP is the standard potential between a working electrode and a reference electrode with no driving current (or an extremely small current) which is proportional to the balance of reductants and oxidants and is what is classically termed ORP (i.e., a homeostatic parameter capturing the current balance of oxidants and reductants in a biological specimen). Low sORP values mean that the biological sample is in the normal range of oxidative stress, while higher than normal sORP values mean that the biological sample is in a higher state of oxidative stress.

The aim of the present study was to examine the effectiveness of sORP for assessing oxidative stress in patients having symptoms of both MetS and T2D. Moreover, conventional oxidative stress markers, such as thiobarbituric acid reactive substances (TBARS), GSH levels, CAT activity, protein carbonyl (CARB) levels and total antioxidant capacity (TAC) were measured in the blood of the patients in order to compare and correlate them with sORP.

Subjects and methods

Subjects. A total of 75 adult subjects manifesting both MetS and T2D, as well as 35 normal subjects participated in the present study. All experimental procedures were performed in accordance with the European Union Guidelines laid down in the 1964 Declaration of Helsinki and were approved by the Institutional Review Board of the University of Thessaly (Larissa, Greece).

Blood collection and handling. The participants visited the Standard Centre of Bioassays, 'Hartografoi Hygeias' in Athens (Greece) and blood samples were collected. Blood samples were drawn from a forearm vein of seated individuals and stored in ethylenediaminetetraacetic acid (EDTA; Becton-Dickinson, Franklin Lakes, NJ, USA) tubes for measuring the levels of TBARS, CARB and GSH, TAC, and CAT activity, and in heparin tubes for dertermining sORP. The samples were then centrifuged immediately at 1,370 x g for 10 min at 4˚C and erythrocytes were divided from the plasma. The erythrocytes were lysed with distilled water (1:1 v/v), inverted and centrifuged at 4,020 x g for 15 min at 4˚C, and the erythrocyte lysate was then collected for the measurement of CAT activity. A small amount of erythrocyte lysate (500 μl) was treated with 5% trichloroacetic acid (TCA; Sigma-Aldrich, Munich, Germany) (1:1 v/v), vortexed and centrifuged at 28,000 x g for 5 min at 4˚C. The supernatants were then removed and the procedure was repeated in the same way. Subsequently, the clear supernatants were transferred to new Eppendorf tubes and were used for the determination of GSH levels. Plasma and erythrocyte lysates were stored at -80˚C until further analysis.

Assesment of sORP using the Luoxis RedoxSYS diagnostic system. The sORP value was determined using the RedoxSYS diagnostic system (Luoxis Diagnostics, Inc., Englewood, CO, USA) as previously described (22). This marker exhibits the intergrated balance between oxidants and reductants in a specimen and is presented in mV. In this new and innovative method, 20 μl of plasma are applied to disposable sensors designed by Luoxis Diagnostics, Inc., which are then inserted into the RedoxSYS diagnostic system, and the sORP value is reported within 4 min.

Assessment of the levels of TBARS, GSH and CARB, TAC, and CAT activity. For the determination of the TBARS levels, the assay was based on the method described in the study by Keles *et al* (24). TBARS is a commonly and frequently

used method to determine lipid peroxidation. According to this method, 100 μl of plasma were mixed with 500 μl of 35% TCA (Merck KGaA, Darmstadt, Germany) and 500 μl of Tris-HCl (Sigma-Aldrich, St. Louis, MO, USA; 200 mM, pH 7.4) and incubated for 10 min at room temperature. One milliliter of 2 M sodium sulfate (Na_2SO_4) and 55 mM TBA solution were added and the samples were incubated at 95˚C for 45 min. The samples were cooled on ice for 5 min and were vortexed following the addition of 1 ml of 70% TCA. The samples were centrifuged at 15,000 x g for 3 min and the absorbance of the supernatant was read at 530 nm using a spectrophotometer (Hitachi U-1900; serial no. 2023-029; Hitachi, Tokyo, Japan). A baseline absorbance was taken into account by running a blank along with all samples during the measurement. The calculation of the TBARS concentration was based on the molar extinction coefficient of malondialdehyde.

The concentration of CARB, an index of protein oxidation, was determined based on the method described in the study by Patsoukis *et al* (25). In this assay, 50 μl of 20% TCA were added to 50 μl of plasma and this mixture was incubated in an ice bath for 15 min and centrifuged at 15,000 x g for 5 min at 4˚C. The supernatant was discarded and 500 μl of 10 mM 2,4-dinitrophenylhydrazine (DNPH; Sigma-Aldrich, Munich, Germany) (in 2.5 N HCl) for the sample, or 500 μl of 2.5 N HCl for the blank, were added to the pellet. The samples were incubated in the dark at room temperature for 1 h with intermittent vortexing every 15 min and were centrifuged at 15,000 x g for 5 min at 4˚C. The supernatant was discarded and 1 ml of 10% TCA was added, vortexed and centrifuged at 15,000 x g for 5 min at 4˚C. The supernatant was discarded and 1 ml of ethanol-ethyl acetate (1:1 v/v) was added, vortexed and centrifuged at 15,000 x g for 5 min at 4˚C. This washing step was repeated twice. The supernatant was discarded and 1 ml of 5 M urea (pH 2.3) was added, vortexed and incubated at 37˚C for 15 min. The samples were centrifuged at 15,000 x g for 3 min at 4˚C and the absorbance was read at 375 nm. The calculation of the CARB concentration was based on the molar extinction coefficient of DNPH. Total plasma protein was assayed using the Bradford protein assay.

The GSH levels were measured based on the method previously described in the study by Reddy *et al* (26). A total of 20 μl of erythrocyte lysate was treated with 5% TCA, mixed with 660 μl of 67 mM sodium potassium phosphate (pH 8) and 330 μl of 1 mM 5,5-dithiobis-2 nitrobenzoate (DTNB; Sigma-Aldrich, Munich, Germany). The samples were incubated in the dark at room temperature for 45 min and the absorbance was read at 412 nm using a spectrophotometer (Hitachi U-1900; serial no. 2023-029; Hitachi). The GSH concentration was calculated relative to a calibration curve made using commercial standards.

The measurement of CAT activity was based on the method described by Aebi (27). In particular, 4 μl of erythrocyte lysate (diluted 1:10) were added to 2,991 μl of 67 mM sodium potassium phosphate (pH 7.4) and the samples were incubated at 37˚C for 10 min. A total of 5 μl of 30% hydrogen peroxide (H_2O_2) was added to the samples and the change in absorbance was immediately read at 240 nm using a spectrophotometer (Hitachi U-1900; serial no. 2023-029; Hitachi) for 130 sec. The determination of CAT activity was based on the molar extinction coefficient of H_2O_2.

Finally, the determination of TAC was based on the method described in the study by Janaszewska and Bartosz (28). In this assay, 20 μl of plasma were added to 480 μl of 10 mM sodium potassium phosphate (pH 7.4) and 500 μl of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. The samples were then incubated in the dark for 30 min at room temperature and then centrifuged at 20,000 x g for 3 min. The absorbance was read at 520 nm using a spectrophotometer (Hitachi U-1900; serial no. 2023-029; Hitachi). TAC is presented as mmol of DPPH reduced to 2,2-diphenyl-1-picrylhydrazine by receiving one hydrogen atom from the antioxidants of plasma.

Statistical analysis. For statistical analysis, data were analyzed by one-way ANOVA followed by Dunnett's test for multiple pairwise comparisons. The level of statistical significance was set at $P<0.05$. For all statistical analyses, SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA) was used. Data are presented as the means + standard error of the mean (SEM).

Results

The results revealed that the sORP values in plasma were significantly ($P<0.05$) higher by 13.4% in the patients with MetS and T2D compared to the controls, indicating an increase in oxidative stress (Fig. 1A). No statistically significant differences were observed in the CARB and TBARS levels, and TAC in the plasma between the patients with MetS and T2D and the controls (Fig. 1B-D). The GSH levels in erythrocytes were significantly ($P<0.05$) lower by 27.7% in the patients with MetS and T2D compared to the controls (Fig. 2A). CAT activity in erythrocytes was significantly ($P<0.05$) higher by 23.3% in the patients with MetS and T2D compared to the controls (Fig. 2B).

In a previous study, we found that the induction of oxidative stress exhibited great variability between different individuals, since the outcome of an oxidant stimulus may be affected by several different factors (e.g., genetic, physiological and biochemical) (21,29). Based on this observation, the individual variability of the tested oxidative stress markers within the patients with MetS and T2D was examined (Fig. 3). Among these markers, the GSH marker exhibited the greatest variability, since there was a 6-fold difference between the lowest value and the highest value (Fig. 3A). GSH was also one of the three markers that exhibited a significant difference in its levels between the patients with MetS and T2D and the controls. In addition, GSH is considered one of the most important endogenous antioxidant molecules and a major contributor to the cellular redox status of living organisms (30). Thus, the patients with MetS and T2D were divided into 2 subgroups, the first one with low GSH levels (n=31; GSH <3 μmol/g Hb) and the second one with high GSH levels (n=35; GSH >4 μmol/g Hb). Nine patients had intermediate GSH values, that is, between 3.1 and 3.9 μmol/g Hb, and thus they were not included in any of the 2 subgroups, so as to have a clear distinction of patients as regards the GSH levels. Between the average values of these 2 GSH groups, there was a statistically significant ($P<0.05$) difference (by 51.7%) in GSH levels in erythrocytes (Fig. 4A). Moreover, the GSH levels were significantly ($P<0.05$) lower (by 52.9%) in the low GSH group compared with the controls (Fig. 4A). In addition, in these 2 GSH groups, the differences between the other oxidative

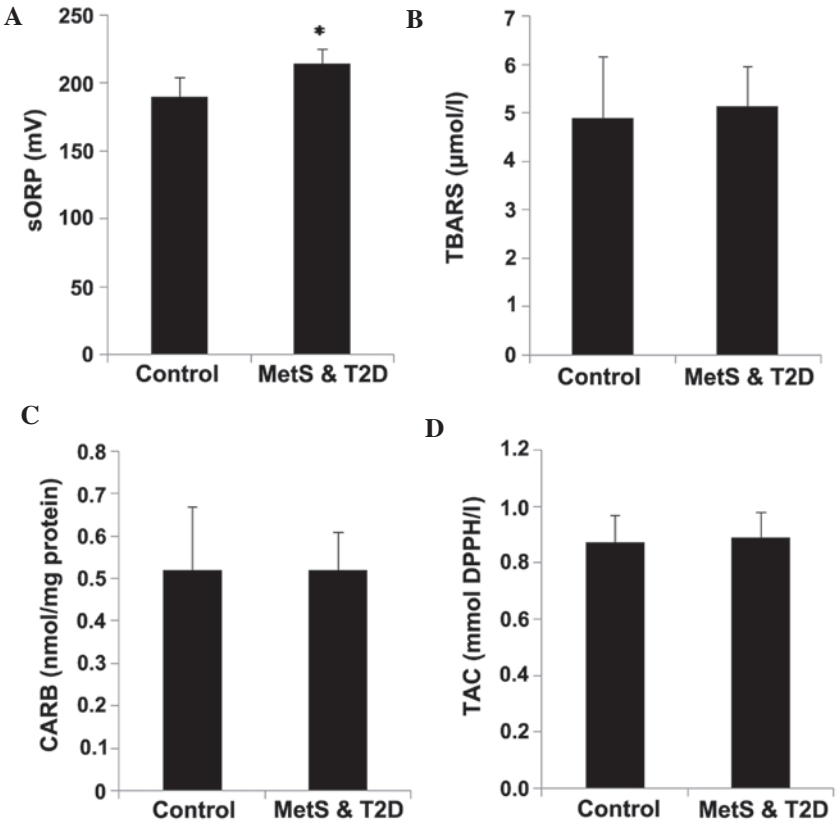

Figure 1. Markers of the redox status in the plasma of patients with metabolic syndrome (MetS) and type 2 diabetes (T2D) and normal controls. (A) Static oxidation reduction potential (sORP), (B) thiobarbituric acid reactive substances (TBARS), (C) protein carbonyl (CARB), (D) total antioxidant capacity (TAC). *P<0.05, significantly different compared to the controls.

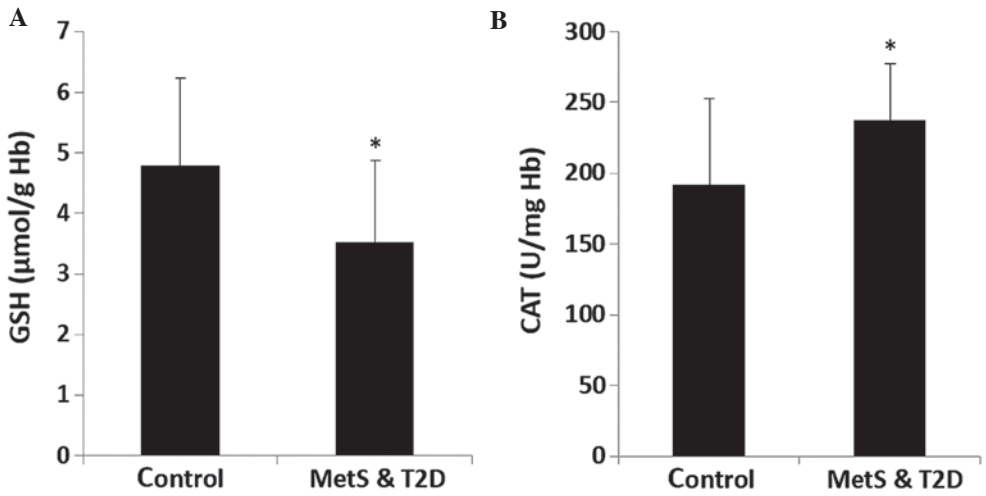

Figure 2. Markers of the redox status in erythrocytes of patients wiht metabolic syndrome (MetS) and type 2 diabetes (T2D) group and normal controls. (A) Glutathione (GSH), (B) catalase (CAT). *P<0.05, significantly different compared to the controls.

stress markers were also examined. There were no significant differences observed in CAT activity in erythrocytes between the 2 GSH groups (Fig. 4B). However, CAT activity was significantly (P<0.05) higher in the low and high GSH groups by 20.4 and 26.7%, respectively than in the controls (Fig. 4B). The sORP values in the plasma of the patients in the low GSH group were significantly (P<0.05) higher (by 8.1%) compared with those of the patients in the high GSH group (Fig. 5A). Moreover, the sORP values were significantly (P<0.05) higher in the patients in the low and high GSH groups (by 15.6 and 6.9%, respectively) compared with the controls (Fig. 5A). In addition, the CARB levels in plasma were significantly (P<0.05) higher (by 16.7%) in the low GSH group compared with the high GSH group (Fig. 5B). There were no significant differences observed in TBARS and TAC levels in plasma between the 2 GSH groups (Fig. 5C and D).

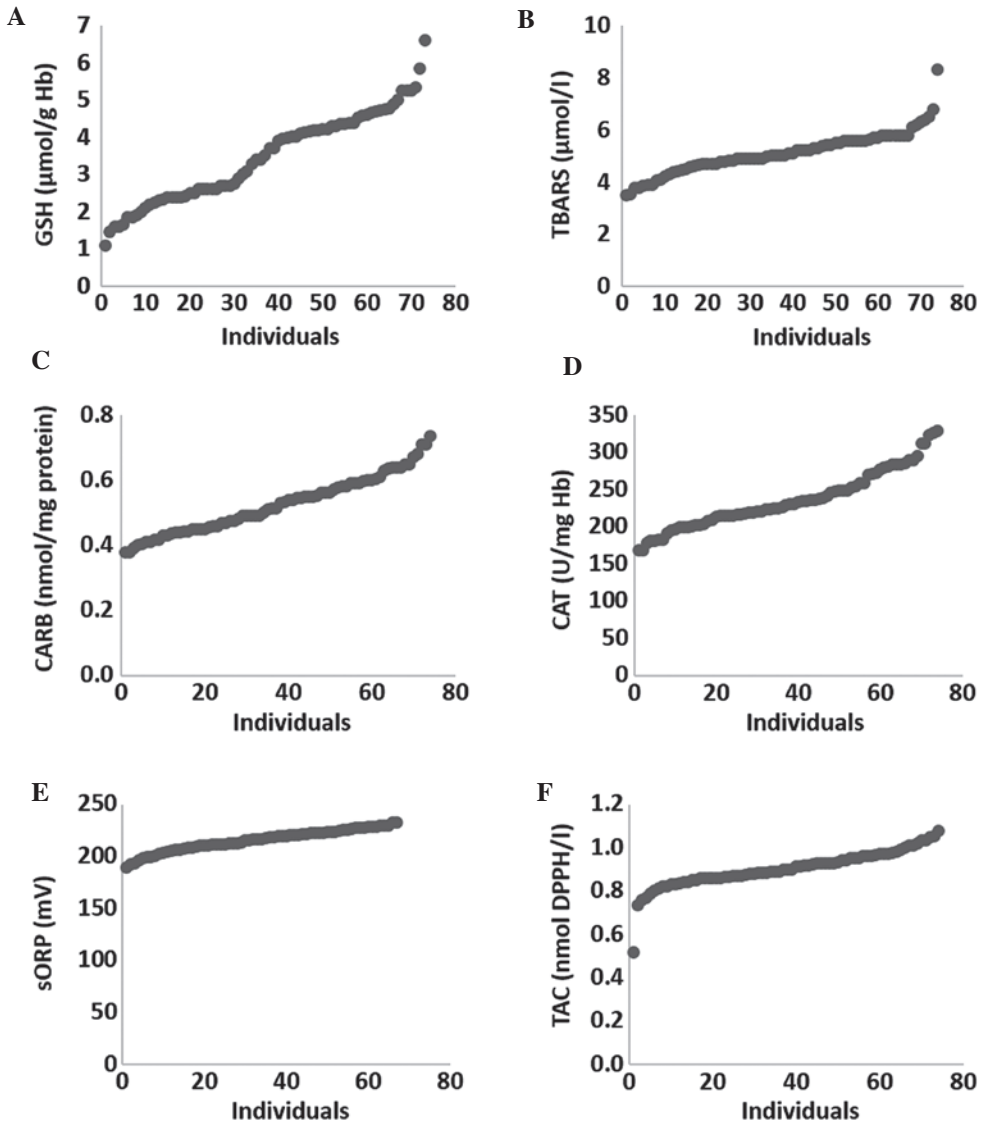

Figure 3. Values of redox biomarkers of each patient with metabolic syndrome and type 2 diabetes. (A) Glutathione (GSH) (in erythrocytes), (B) thiobarbituric acid reactive substances (TBARS) (in plasma), (C) protein carbonyls (CARB) (in plasma), (D) catalase (CAT) (in erythrocytes), (E) static oxidation reduction potential (sORP) (in plasma), (F) total antioxidant capacity (TAC) (in plasma).

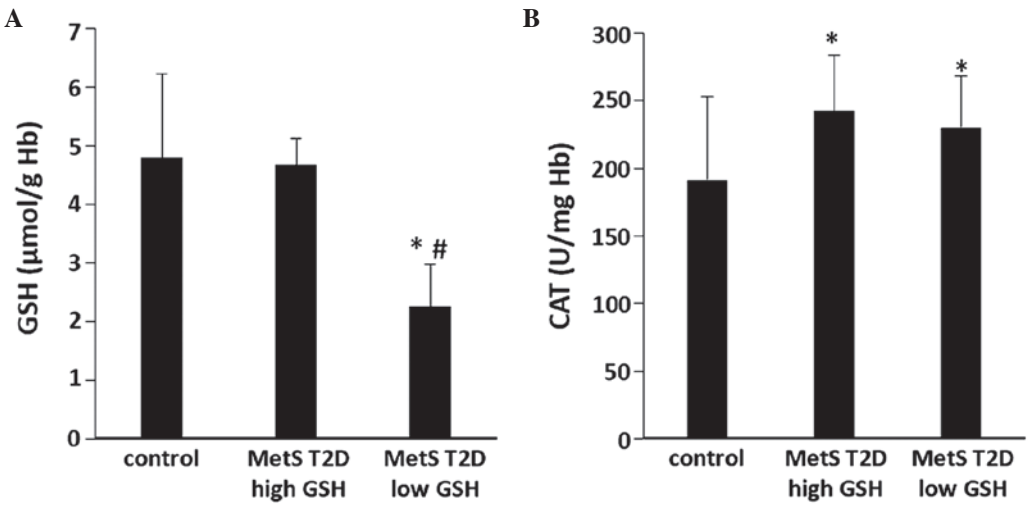

Figure 4. Markers of the redox status in erythrocytes of patients with metabolic syndrome (MetS) and type 2 diabetes (T2D) exhibiting low and high glutathione (GSH) levels, and the normal controls. (A) GSH, (B) catalase (CAT). *P<0.05, significantly different compared to the controls. #P<0.05, significantly different compared to the high GSH group.

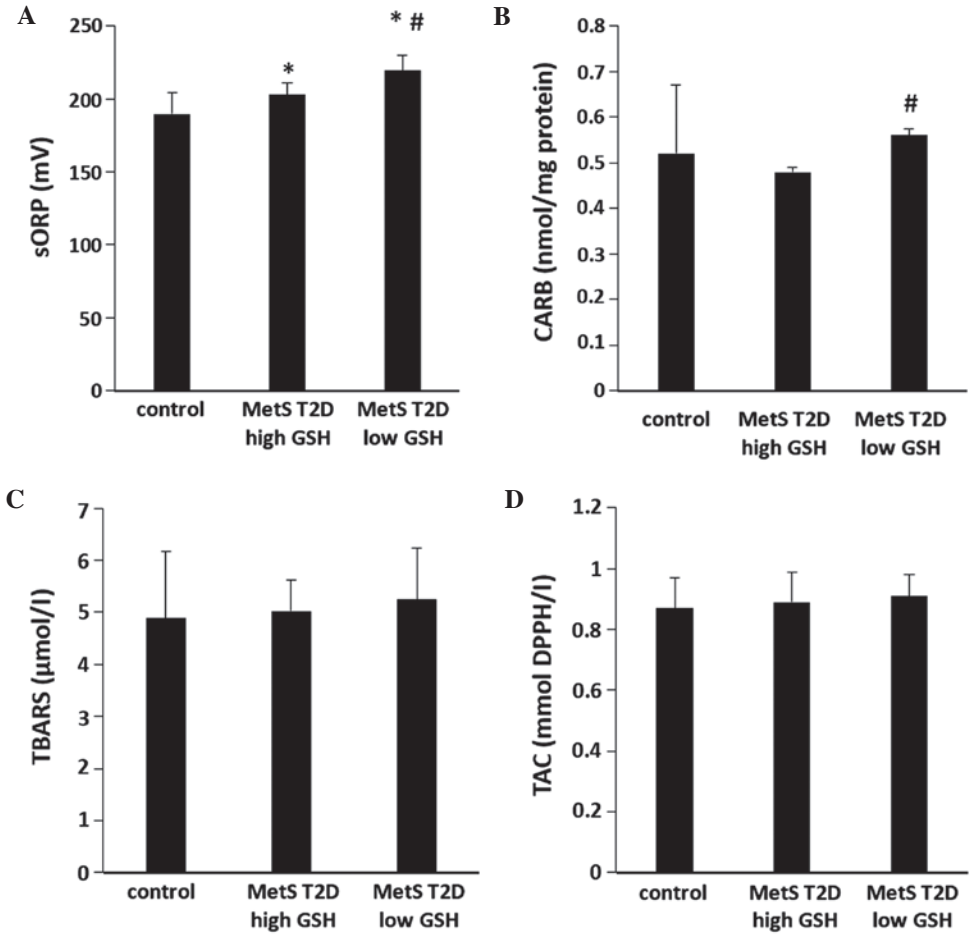

Figure 5. Markers of the redox status in plasma of patients with metabolic syndrome (MetS) and type 2 diabetes (T2D) exhibiting low and high glutathione (GSH) levels, and the normal controls. (A) Static oxidation-reduction potential (sORP), (B) protein carbonyls (CARB), (C) thiobarbituric acid reactive substances (TBARS), (D) total antioxidant capacity. *P<0.05, significantly different compared to the controls. #P<0.05, significantly different compared to high GSH group.

Discussion

MetS is a cluster of medical conditions, including abdominal obesity and insulin resistance, plus any two of the following four factors: i) increased triglyceride levels, ii) decreased HDL cholesterol levels, iii) increased blood pressure, and iv) increased fasting blood glucose levels (7). The prevalence of MetS is approximately 22.9% in the US population and up to 36% of Europeans aged between 40-55 suffer from the disease (31,32). MetS is also a risk factor for developing T2D, another type of metabolic disorder, characterized basically by elevated blood glucose levels due to insulin resistance and affects approximately 380 million individuals worldwide (12). Both of these disorders are also associated with oxidative stress, a pathophysiological condition in which there is an overbalance of free radicals production against antioxidant mechanisms (12,13,33,34). Oxidative stress occurring in patients with MetS and T2D may further aggravate the associated complications, particularly those involving the cardiovascular system (14,16,35,36). Thus, the assessment of oxidative stress in patients with MetS and T2D is considered useful for monitoring their health status (37-39). In previous studies, we demonstrated that the determination of the sORP values in plasma, a new marker of oxidative stress, was effective for assessing the redox status in different physiological conditions and diseases (20-23). Thus, the aim of the present study was to examine the effectiveness of sORP for assessing oxidative stress in patients manifesting both MetS and T2D.

The results revealed that the sORP values in plasma were significantly higher in the patients with MetS and T2D compared with the controls, suggesting the induction of oxidative stress in the patients affected by these two metabolic disorders. In our previous studies, we observed increased sORP values in patients with sepsis and in conditions of strenuous exercise-induced oxidative stress (21-23).

The significantly lower GSH levels in the erythrocytes of the patients with MetS and T2D compared with the controls also supported the induction of oxidative stress in the patients with MetS and T2D. Another study also reported decreased GSH levels in patients with MetS (40). Likewise, a decrease in GSH levels in human erythrocytes and serum has been demonstrated in other studies on patients with T2D (41-43). GSH is one of the most important antioxidant mechanisms in living organisms, and thus low GSH levels are associated with oxidative stress and the manifestation of various diseases (30,44,45). As regards the mechanisms through which T2D is associated with low GSH levels, it has been suggested that in hyperglycemia, glucose is used in the polyol pathway, resulting in a decrease in nico-

tinamide adenine dinucleotide phosphate-oxidase (NADPH), which is necessary for the GSH reductase enzyme to regenerate GSH from oxidized glutathione (GSSG) (46).

In this study, the patients with MetS and T2D exhibited a significant increase in CAT activity compared to the controls. CAT is the main regulator of hydrogen peroxide metabolism, which is associated with diabetes mechanisms, such as the expression of glucose receptor and insulin secretion (47). Other studies have demonstrated conflicting results, reporting either a decrease (47), increase (48) or no change (49) in CAT activity in hyperglycemic conditions. It has been proposed that an organism may increase CAT activity in some cells, such as erythrocytes in order to protect itself from free radical-induced cell damage in diabetic conditions, particularly in cells with low CAT activity, such as pancreatic beta cells (50). Thus, increase in CAT activity may also indicate the induction of oxidative stress in patients with MetS and T2D, as similarly shown by sORP and GSH markers.

However, in this study, no differences were observed in the TBARS and CARB levels (indicating lipid peroxidation and protein oxidation, respectively), in plasma between the patients with MetS and T2D and the controls. Although studies have demonstrated that T2D is accompanied by increased lipid peroxidation, the latter is not a prerequisite for MetS (42,51). On the contrary, it seems that for some unclear reason, lipid peroxidation may even be decreased in MetS (52). Thus, the co-occurrence of both MetS and T2D in the patients may explain the absence of increased TBARS levels in their plasma. Moreover, no increase was observed in the CARB levels in the patients with MetS and T2D compared with the controls, although protein oxidation is considered a characteristic of either MetS or T2D (51,53). This absence of increase in CARB levels may be explained by the fact that advanced oxidation protein products (AOPPs) instead of CARB have been shown to be the most appropriate marker for protein oxidation in MetS (51). AOPPs have also been reported to be increased in T2D (54). AOPPs are generated by the action of chloraminated oxidants (e.g., hypochlorous acid and chloramines) produced by myeloperoxidase in activated neutrophils during oxidative stress (55). CARB are produced on protein side chains (particarly of Pro, Arg, Lys and Thr) when they are oxidized (18).

Furthermore, TAC marker did not differ significantly between the patients with MetS and T2D and the controls. Since TAC is considered a marker of the total redox status, this finding was in contrast to the induction of oxidative stress indicated by other markers (actually, TAC would be expected to be reduced). However, this result may be explained when considering that TAC is based on the assessment of the reductant compounds, which along with the antioxidant enzymes constitute the antioxidant defense mechanisms. Although some antioxidants (e.g., GSH) are reduced in MetS and T2D disorders, some others such as uric acid have been reported to be increased (51). Uric acid accounting for approximately 60% of the antioxidant activity in human plasma is believed to be increased in MetS subjects as insulin may reduce uric acid elimination in the urine (51,56). Thus, although TAC may remain unchanged due to this parallel increase and decrease in different antioxidants in MetS and T2D conditions, oxidative stress occurs as oxidant compounds are increased more than the antioxidants. For this reason, and as we have suggested previously (21,23), the sORP marker may be a better marker than TAC for assessing the total redox status, since the former is based on the evaluation of the difference between oxidants and reductants while the latter only on the reductants (i.e., antioxidants).

In this study, the patients with MetS and T2D exhibited great variations in the values of oxidative stress markers, particularly those of GSH, and thus the patients were divided into 2 subgroups, one with low GSH (<3 μmol/g Hb) and the other with high GSH (>4 μmol/g Hb) levels. The statistical comparison of the average values of oxidative stress markers between the two subgroups indicated that the low GSH group had significantly higher sORP levels than the high GSH group, suggesting greater oxidative stress in the former group compared to the latter. This finding was also supported by the higher protein oxidation levels as shown by CARB in the low GSH group compared with the high GSH group. There were no significant differences observed in TAC, and in the CAT and TBARS levels between the two GSH groups. Since oxidative stress has been associated with the severity of complications in patients with either MetS or T2D (33-36), the observed variation of the induction of oxidative stress in such subjects emphasizes the need for assessing their redox status. Namely, higher oxidative stress levels in patients with MetS and T2D may be an alarming sign for applying appropriate interventions (e.g., antioxidant supplementation), so as to reduce the aggravation of complications (12,37). Among the two oxidative stress markers assessing total redox status (i.e., sORP and TAC), sORP seems to be a suitable marker for assessing oxidative stress levels in patients with MetS and T2D, since it was associated with lower GSH and higher CARB levels.

Moreover, the assessment of the redox status may be important in prediabetic conditions. According to a new theory suggested by Watson (57) and Sharoff *et al* (58), there may be a close association between T2D and the redox status. According to this theory, a main cause of diabetes is a reductive environment in the endoplasmic reticulum, impairing disulphide bond formation needed to stabilize the 3D conformation of physiologically active proteins (57). Namely, an oxidative environment seems to be required for the proper folding and the normal function of proteins. Major evidence supporting this theory is that the membranous sacs of the endoplasmic reticulum of insulin-resistant rodents contain higher amount of unfolded polypeptides and many fewer S-S bonds than normal endoplasmic reticulum (59,60). Moreover, it has been demonstrated that supplementation with antioxidant decreased the ability of exercise to make cells more sensitive to insulin (61). In addition, subjects carrying mutations impairing the synthesis of antioxidant molecules manifested increased insulin sensitivity (62). Based on this theory, our findings showing that oxidative stress levels varied greatly among MetS and T2D subjects emphasize the need for the assessment of redox status in prediabetic subjects, which may help to discern those with reductive redox status from those with oxidative one, and so to make the appropriate interventions. It has often been suggested without distinction the antioxidant supplementation in prediabetic subjects, although as explained above this may be harmful for those having a reductive redox status. In

future studies, we will investigate the association between the redox status and clinical signs of prediabetic subjects.

In conclusion, the present results suggest that sORP may be an effective marker for assessing oxidative stress in MetS and T2D patients, since it was higher in these subjects compared to control ones. Moreover, sORP was effective for discerning the oxidative stress levels among MetS and T2D patients, since it was associated with low GSH and high CARB levels. Thus, the use of such a marker may be useful for identifying eagerly high oxidative stress levels in MetS and T2D patients, and consequently reducing complications by making the appropriate interventions. Moreover, sORP may be useful for discerning high from low oxidative stress levels in prediabetic subjects, which may also determine the type of intervention.

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