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ANALYSIS OF THE CORRELATIONS BETWEEN OXIDATIVE STRESS, GELATINASES AND THEIR TISSUE INHIBITORS IN THE HUMAN SUBJECTS WITH OBSTRUCTIVE SLEEP APNEA SYNDROME

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Obstructive sleep apnea syndrome (OSAS) is commonly associated with endothelial dysfunction, atherosclerosis and cardiovascular disorders. On the basis of this observation, our aim was to examine the oxidative status and the matrix metalloproteases (MMP) profile in a group of subjects with OSAS. We enrolled 48 subjects with OSAS defined after a 1-night cardiorespiratory sleep study, who were subsequently subdivided in two subgroups according to the severity of OSAS (low grade = L-OSAS; high grade= H-OSAS). We measured the parameters of oxidative stress, such as lipid peroxidation, protein oxidation, total antioxidant status (TAS), nitric oxide metabolites (NOx), and the plasma concentrations of the gelatinases (MMP-2 and MMP-9) and their tissue inhibitors (TIMP-1 and TIMP-2). We found a significant impairment of oxidative status in H-OSAS compared to L-OSAS and higher plasma levels of MMP-9 and TIMP-1 in H-OSAS compared to L-OSAS. In this study we observed a positive correlation between TBARS and MMP-9, a positive correlation between PC and MMP-9, and a negative correlation between NOx and MMP-9, especially in the whole group of OSAS subjects. These data underline how strong interrelationships among some parameters of the oxidative stress, in particular those reflecting lipid peroxidation, protein oxidation and NOx, and MMP-9 are evident in OSAS subjects. All these information may be useful in the clinical practice keeping in mind the cardiovascular complications generally accompanying the obstructive sleep apnea syndrome.

Key words: *obstructive sleep apnea syndrome, oxidative stress, matrix metalloproteases, tissue inhibitors of metalloprotease, lipid peroxidation, nitric oxide*

INTRODUCTION

Obstructive sleep apnea syndrome (OSAS) is a sleep disorder characterized by repeated partial or complete obstructions of upper airways during sleep with consequent apnea or hypopnea, intermittent arterial oxygen desaturation and sleep disruption (1). OSAS affects especially middle-aged and elderly subjects and its prevalence is increasing worldwide (2).

OSAS is significantly and independently associated with an increased risk of cardiovascular diseases, cerebrovascular events and all-cause mortality and some studies have demonstrated that the incidence of cardiovascular events is related to its severity (3-5). Atherosclerosis is common in OSAS (6), and the elevated mortality is associated with the severity of the atherosclerosis (7). The mechanisms leading to the development and the progression of atherosclerotic plaques involve multiple factors, including oxidative stress, endothelial dysfunction, and inflammatory factors. The continued hypoxia-reoxygenation episodes have a key role in the pathogenesis of the endothelial dysfunction: the intermittent hypoxia may induce the production of reactive oxygen species (ROS) that contribute to the generation of adhesion molecules, leukocyte activation, and to an enhanced systemic inflammation (8).

In particular, evaluating the oxidative/antioxidant status of subjects with OSAS, several authors have observed an increase in lipid (9-11) and protein oxidation (9, 12) and a decrease in nitric oxide (NO) metabolites (13), and in antioxidant defenses (9, 10, 14), even if other authors did not find any difference in plasma lipid peroxidation, total antioxidant capacity and protein carbonyl levels between OSAS subjects and controls (15). In addition, an altered expression of some matrix metalloproteases (MMPs) and their tissue inhibitors (TIMPs) has been described in subjects with OSAS (16-22). MMPs, and in particular gelatinases (MMP-2 and MMP-9), are involved in the atherosclerotic lesion development and progression (23, 24). MMPs and oxidative stress seem to be strongly correlated in subjects with high cardiovascular risk (25, 26, 28-31). The link between oxidative stress and MMPs has been demonstrated in several experimental models (32-34): peroxynitrite, in the presence of gluthation, activates some MMPs *via* the S-glutathiolation of the cystein in the propeptide domain (26, 34) but, at higher concentrations, can lead to the inactivation of MMP-2 (34). Also hydrogen peroxide (H_2O_2) activates MMP-2 and promotes the expression of MMP-2 and MMP-9 in human venous endothelial cells (35). The activation of MMPs *via* S-nitrosylation is still unclear, even if a role of NO has been suggested by some authors (27). In addiction, ROS can influence

MMP transcription influencing the activity of the mitogen-activated protein kinase (MAPK), of the MAPK phosphatase or of the histone deacetylase (36).

Previously we have evaluated the behavior of lipid peroxidation and protein oxidation (37), the nitric oxide metabolites and the erythrocyte deformability (38) and also the gelatinases and their inhibitors in OSAS subjects (in press); the aim of this research was to examine some parameters of the oxidative status and their possible relationships with gelatinases and TIMPs in the same group of subjects with OSAS.

MATERIALS AND METHODS

Patients

We consecutively recruited 48 subjects (36 men and 12 women; mean age 50.3 ± 14.68 years) with obstructive sleep apnea syndrome from those with suspected OSAS referred to our center. OSAS was diagnosed after a 1-night cardiorespiratory sleep study: apneas were defined as the cessation of airflow for ≥10 seconds and hypopneas were defined as a transient reduction of breathing ≥ 50% with an oxygen desaturation of ≥3% or as a reduction of breathing ≥ 30% with an oxygen desaturation of ≥ 4% for ≥10 seconds. Obstructive apneas and hypopneas were distinguished from central events by the detection of respiratory efforts during the event. AHI was defined as the number of obstructive apneas and hypopneas per hour of sleep. Patients with an AHI ≥ 5 were considered as affected by OSAS and then they were subdivided according to the AHI value in two subgroups: Low (L = 21 subjects with AHI < 30) and High (H = 27 subjects with AHI > 30). Therefore the Low subgroup included subjects with mild to moderate OSAS, while the H subgroup included the subjects with severe OSAS. Means and S.D. of age, BMI, waist and neck circumference, AHI, oxygen desaturation index (ODI), and mean nocturnal SO_2 (mSO_2) are reported in *Table 1*. Twenty-three of the OSAS subjects had arterial hypertension, 10 had diabetes mellitus and 6 had cardiovascular disease (history of myocardial infarction or stroke). Each subject gave the informed consent and the study was approved by the Ethical Committee.

On fasting venous blood, collected by puncture from the antecubital vein of each subject after the night of cardiorespiratory sleep study and immediately transferred to glass tube anticoagulated with EDTA-K3, we evaluated lipid peroxidation, protein carbonyl (PC) groups, total antioxidant status (TAS), nitric oxide metabolites (NOx), gelatinases (MMP-2 and -9) and their tissue inhibitors (TIMP-1 and -2).

Lipid peroxidation

Lipid peroxidation was evaluated in plasma by detection of thiobarbituric acid-reactive substances (TBARS), generated by peroxidative processes, which include lipid peroxides and malonildialdehyde. The evaluation of TBARS was made by fluorimetry, using 1,1,3,3-tetramethoxypropane as standard.

Protein carbonyl (PC) groups

The PC groups were measured by an enzyme-linked immunosorbent assay (ELISA) kit (BioCell PC test kit, Enzo Life Sciences AG, Switzerland).

Total antioxidant status (TAS)

TAS was obtained using an Assay kit (Calbiochem, La Jolla, USA) which relies on the ability of plasma antioxidant substances

Table 1. Means ± S.D. of age, anthropometric characteristic and OSAS parameters in the two subgroups of OSAS patients.

	L-OSAS (n = 21)	H-OSAS (n = 27)
Males/Females	12 / 9	25 / 2
Age (years)	45.3 ± 14.4	52.8 ± 14.2
BMI (kg/m^2)	35.72 ± 8.49	35.10 ± 6.47
Waist circumference (cm)	114.2 ± 14.5	122.5 ± 16.6
Neck circumference (cm)	41.50 ± 3.25	46.62 ± 4.15***
AHI	15.13 ± 8.15	56.63 ± 18.90***
mSO_2 (%)	93.4 ± 2.68	89.50 ± 3.45***
ODI	14.28 ± 9.39	55.38 ± 25.75***

***P < 0.001 versus L-OSAS (Student's 't' test for unpaired data). BMI, body mass index; mSO_2, mean oxygen saturation; AHI, apnea/hypopnea index; ODI, oxygen desaturation index.

Table 2. Means ± S.D. of oxidative parameters, nitric oxide metabolites, gelatinases and their inhibitors in the two subgroups of OSAS patients.

	L-OSAS	H-OSAS
TBARS (nmol/ml)	5.247 ± 0.469	7.351 ± 1.629***
PC (nmol/mg prot)	0.230 ± 0.088	0.382 ± 0.099***
TAS (mmol/l)	1.370 ± 0.162	1.237 ± 0.112**
NOx (micromol/l)	33 .47 ± 10.05	22.84 ± 7.79***
MMP-9 (ng/ml)	89.22 ± 11.07	106.8 ± 14.78***
TIMP-1 (ng/ml)	64.87 ± 5.53	70.40 ± 5.09***
MMP-2 (ng/ml)	37.90 ± 10.44	34.12 ± 7.39
TIMP-2 (ng/ml)	104.8 ± 8.35	106.6 ± 10.19

**P < 0.01 ***P < 0.001 versus L-OSAS (Student's 't' test for unpaired data).

Table 3. Values of r for linear correlations between oxidative parameters, nitric oxide metabolites, gelatinases and their inhibitors in the two subgroups and in the whole group of OSAS patients.

	L-OSAS	H-OSAS	All OSAS patients
TBARS vs. MMP-9	–0.138	0.375#	0.541***
TBARS vs. MMP-2	0.044	–0.248	–0.243
TBARS vs. TIMP-1	0.085	–0.403 *	0.121
TBARS vs. TIMP-2	0.072	–0.227	–0.064
PC vs. MMP-9	–0.377	0.294	0.395**
PC vs. MMP-2	–0.014	0.073	–0.111
PC vs. TIMP-1	0.088	–0.186	0.249
PC vs. TIMP-2	0.147	–0.046	0.001
TAS vs. MMP-9	0.483*	–0.166	–0.157
TAS vs. MMP-2	–0.085	0.401*	0.185
TAS vs. TIMP-1	0.245	0.353	0.021
TAS vs. TIMP-2	0.043	0.211	0.068
NOx vs. MMP-9	0.506*	–0.358	–0.283 #
NOx vs. MMP-2	–0.300	0.103	0.001
NOx vs. TIMP-1	–0.099	0.057	–0.262
NOx vs .TIMP-2	0.090	0.074	0.016

#P = 0.05, *P < 0.0,5 **P < 0.01, ***P < 0.001 (linear regression).

to inhibit the oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline sulfonic acid) (ABTS) to the radical cation ABTS·+ by a peroxidase (39). The radical concentration was measured by spectrophotometry.

Nitric oxide metabolites (NOx)

Considering that *in vivo* NO has a very short life (less than 0.1 s) and it is converted into nitrite (NO_2^-), which has a half-life of few minutes, and into the more stable nitrate (NO_3^-), NOx represents almost only the nitrate concentration. In the laboratory method adopted by us at first nitrate was converted into nitrite by a nitrate reductase, and then nitrite was assessed by spectrophotometry after addition of Griess reagent.

Gelatinases and their inhibitors

Plasma concentrations of gelatinases (MMP-2 and MMP-9) and their inhibitors (TIMP-1 and TIMP-2) were evaluated using respectively the Human MMP-2 ELISA and Human MMP-9 ELISA kit (Boster Biological Technology, LTD) and the Human TIMP-1 ELISA and Human TIMP-2 ELISA kit (Boster Biological Technology, LTD).

Statistical analysis

Data were expressed as means ± S.D. The statistical difference between the L subgroup and the H subgroup of OSAS subjects was estimated using the Student's "t" test for unpaired data; the correlations were performed employing the linear regression test. The null hypothesis was rejected for P values < 0.05.

RESULTS

First of all, the L and H subgroup of OSAS subjects are significantly different regarding the neck circumference, the mean oxygen saturation and the oxygen desaturation index (*Table 1*).

In the H subgroup of OSAS subjects we found a significant increase in lipid peroxidation and protein oxidation and a significant decrease in total antioxidant status and in NO metabolites in comparison with the L subgroup (*Table 2*). Similarly, in the H subgroup of OSAS subjects we observed a significant increase in the plasma concentration of MMP-9 and TIMP-1 in comparison with the L subgroup, while regarding the plasma concentration of MMP-2 and TIMP-2 no statistical difference was observed between the two subgroups (*Table 2*).

Considering the aim of this research, we examined all the correlations among the parameters of oxidative status and the parameters of the metalloproteinases profile. From this statistical evaluation was evident that in the L subgroups MMP-9 was positively correlated with TAS and NOx (*Table 3, Figs. 3* and *4*) while in the H subgroup we found a positive correlation between MMP-9 and TBARS (*Table 3, Fig. 1*), a positive correlation between MMP-2 and TAS and a negative correlation between TIMP-1 and TBARS (*Table 3*). In the whole group of OSAS subjects only MMP-9 was positively correlated with TBARS and carbonyl groups while it was negatively correlated with NOx (*Table 3, Figs. 1, 2* and *4*).

In addition we evaluated the correlations among the indicators of oxidative stress, the MMPs profile, and the parameters of OSAS severity in the entire group of OSAS subjects. We found a positive correlation between TBARS and AHI and between TBARS and ODI and a negative correlation between TBARS and mSO_2 (*Table 4*). PC were positively correlated with AHI and ODI and negatively correlated with mSO_2 (*Table 4*), while TAS was negatively correlated with AHI and ODI and positively correlated with mSO_2 (*Table 4*). Regarding the NOx, we noted a negative correlation with AHI and ODI and a positive correlation with mSO_2 (*Table 4*). We also observed a positive correlation between MMP-9 and AHI and between MMP-9 and ODI and a negative correlation between MMP-9 and mSO_2 (*Table 4*); no significant correlation among

MMP-2, TIMP-1, TIMP-2 and polysomnographic parameters was found.

DISCUSSION

The data of this study confirm the results previously described by us and in fact lipid peroxidation, protein oxidation, total antioxidant status and NO metabolites are significantly influenced by the degree of severity of this syndrome (37, 38). The behavior of oxidative status is dependent in particular on the hypoxia-reoxygenation episodes that characterize OSAS (8, 40). An increased mitochondrial ROS synthesis in endothelial cells exposed to hypoxia has been proved (8). As it is known, *in vitro* hypoxia induces leukocyte activation (41) and ROS production

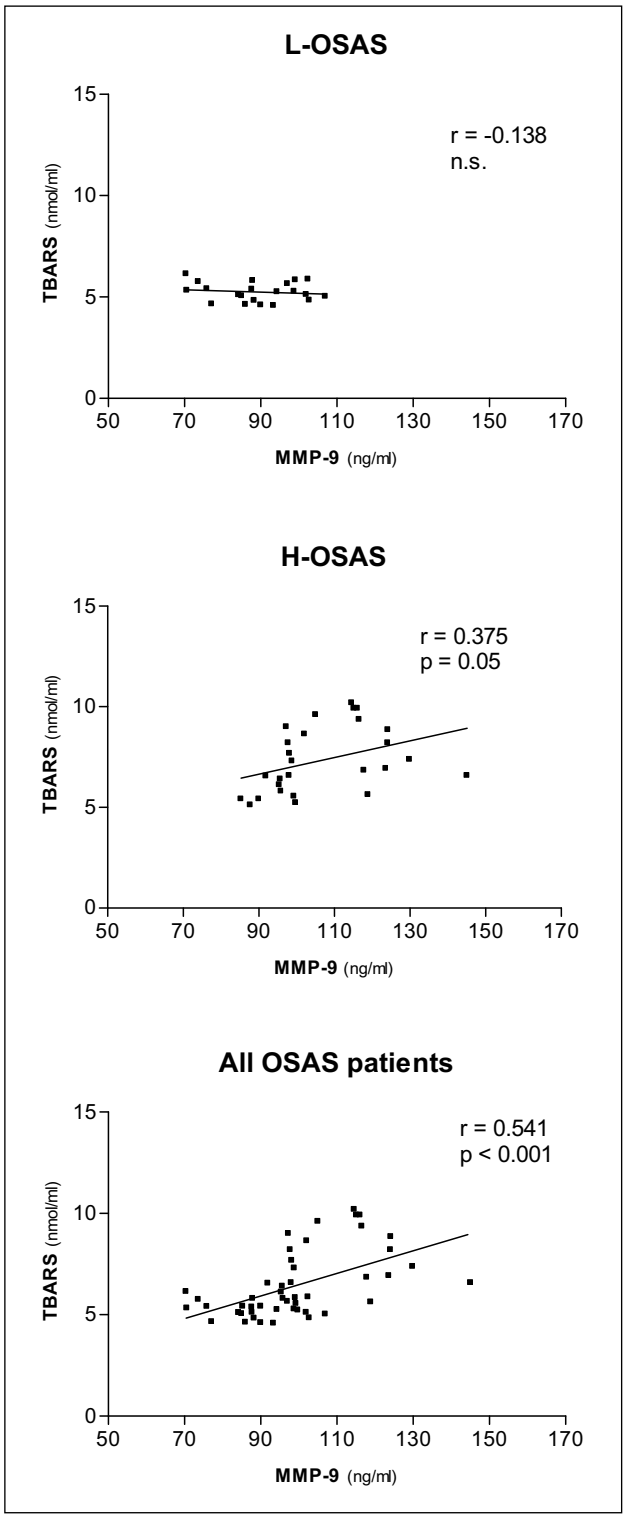

Fig. 1. Correlations between MMP-9 and TBARS in the two subgroups and in the whole group of OSAS patients.

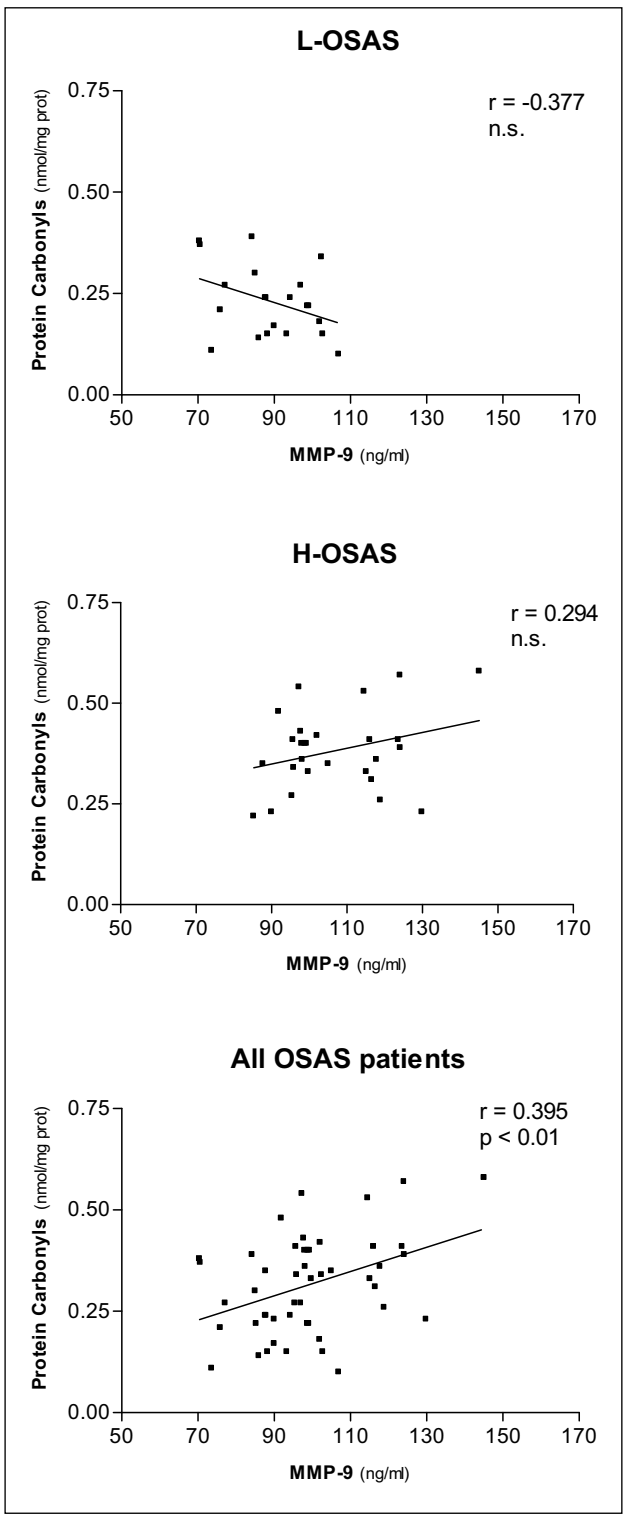

Fig. 2. Correlations between MMP-9 and PC in the two subgroups and in the whole group of OSAS patients.

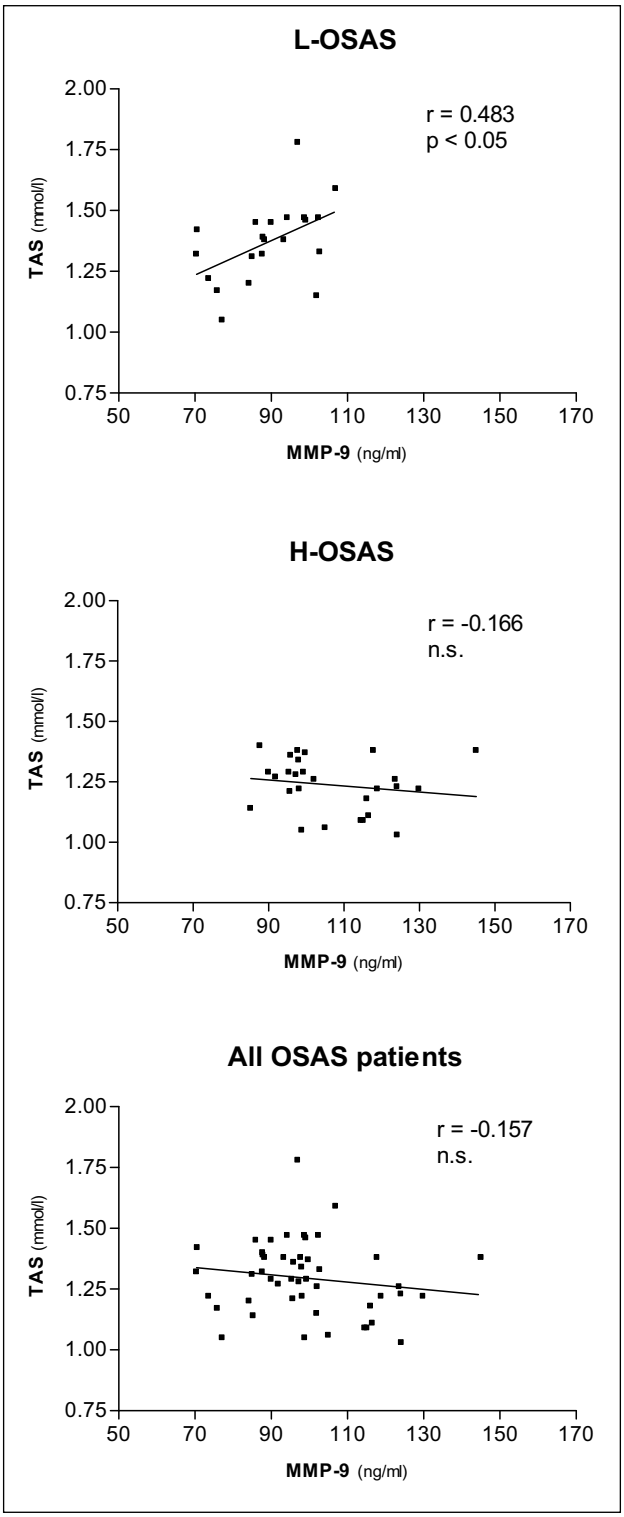

Fig. 3. Correlations between MMP-9 and TAS in the two subgroups and in the whole group of OSAS patients.

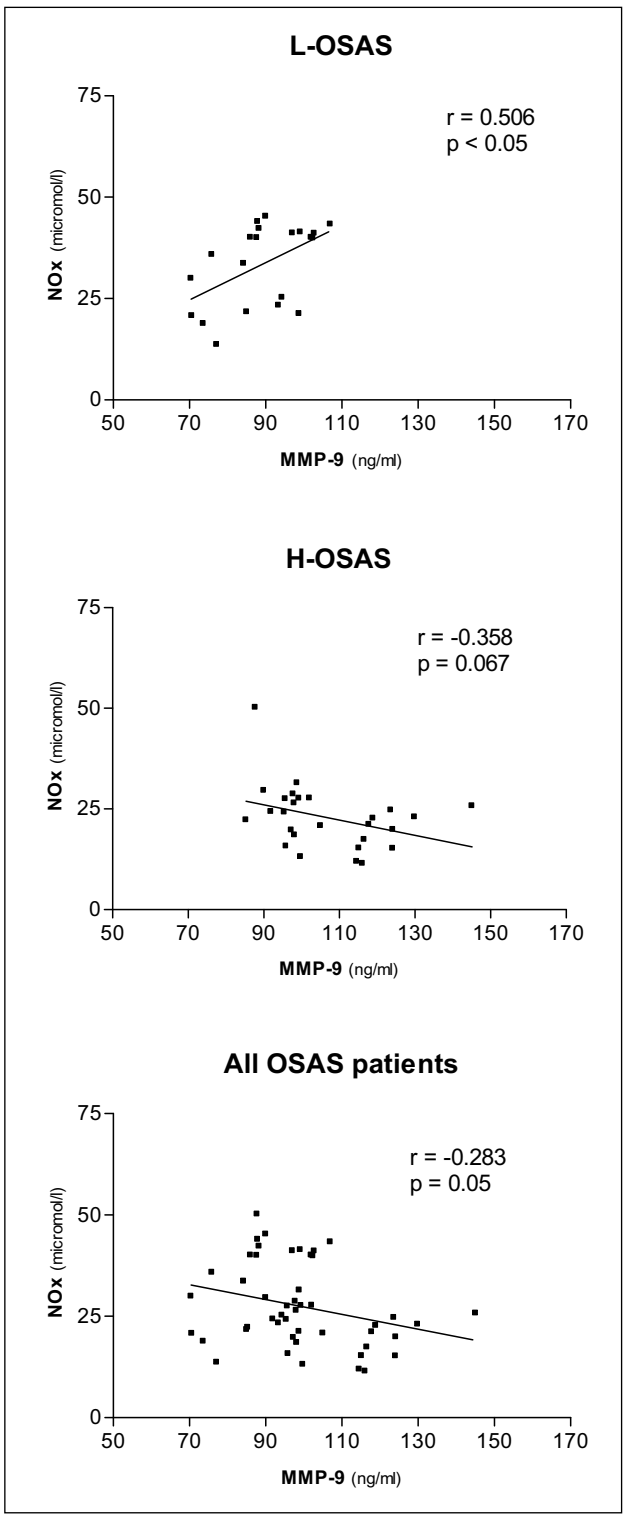

Fig. 4. Correlations between MMP-9 and NOx in the two subgroups and in the whole group of OSAS patients.

and some authors (8) have also described an increased ROS synthesis by monocytes and granulocytes from OSAS subjects. ROS indirectly influence several nuclear transcription factors such as NF-κB that leads to an increased production of cytokines and adhesion molecules, and the hypoxia-inducible factor-1α (HIF-1α), that increases the sympathetic activity (8, 40). All these considerations seem to find an equilibrium point when we observe the close positive correlation between TBARS and carbonyl groups (data not shown) as well as the strong negative correlation between TBARS and TAS (data not shown) and between carbonyl groups and TAS (data not shown), especially in the entire group of OSAS subjects.

The increase in NF-κB is associated with the endothelial dysfunction, confirmed by decreased levels of activated

Table 4. Values of r for linear correlations between the OSAS parameters and oxidative parameters, nitric oxide metabolites, gelatinases and their inhibitors in the whole group of OSAS patients.

	vs. AHI	vs. mSO_2	vs. ODI
TBARS	0.885***	–0.524***	0.881***
PC	0.684***	–0.462**	0.631***
TAS	–0.544***	0.423**	–0.472**
NOx	–0.615***	0.418**	–0.523***
MMP-9	0.450**	–0.482***	0.360*
MMP-2	–0.278#	0.149	–0.393*
TIMP-1	0.255	–0.238	0.235
TIMP-2	–0.049	–0.131	–0.104

#P = 0.05, *P < 0.05, **P < 0.01, ***P < 0.001 (linear regression).

endothelial NO synthases (eNOS) (42). This last datum contributes to explain the behavior of NO metabolites in OSAS subjects and in particular why its trend is dependent on its severity degree. As the oxygen is a substrate of NOS, the frequent episodes of desaturation decrease NOS activity; in addition, hypoxia is also responsible for alterations in gene regulation, so it could suppress the transcription of the eNOS gene (43).

On cultured human umbilical vein endothelial cells, an intermittent hypoxia causes significant lower levels of NO, NOS activity and NOS mRNA expression (44), while in animal models it has been proved that the intermittent hypoxia down-regulates the eNOS expression inducing NF-κB activity and the consequent overproduction of TNF-α, which inhibits eNOS expression (45). In OSAS an increased NF-κB may also reduce the levels of activated eNOS and all these premises seem to be confirmed by the negative correlation between TBARS and NOx (data not shown) and between carbonyl groups and NOx (data not shown) in the entire group of OSAS subjects.

As well as for the parameters of the oxidative status, also MMP-9 and TIMP-1 are influenced by the degree of severity of this syndrome; this finding agrees with the data obtained by some authors (16, 18, 19, 22) in adults with OSAS, although it differs from what found by other authors in children with OSAS (17).

The activity of MMPs is regulated by the four TIMPs: TIMP-1 inhibits in particular MMP-9 while TIMP-2 inhibits especially MMP-2 (46) and this prerequisite explains easily the positive correlation between MMP-9 and TIMP-1 and between MMP-2 and TIMP-2 observed in the entire group of OSAS subjects (data not shown). The trend of the gelatinases and their tissue inhibitors may be imputable to their cosecretion or to a compensatory effect (47) and it influences the extracellular matrix remodeling (48, 49).

However, the principal aim of this study has regarded the possible interrelationships between the parameters reflecting the oxidative stress and the gelatinases in OSAS subjects. The intermittent hypoxia that induces the ROS overproduction may contribute to the generation of mediators of inflammation and at the same time may activate, together with other proteases, the MMPs (25, 26, 50). We believe that in OSAS the behavior of the gelatinases is dependent especially on their overproduction stimulated by the hypoxia-reoxygenation events and by some cytokines, such as IL-6 and TNF-α (21, 51-53) and this physiopathological consideration substantiates the significant positive correlation found among TBARS, carbonyl groups and MMP-9 in the whole group of OSAS subjects.

Bearing in mind that OSAS is a clinical condition accompanied by different complications, such as arterial hypertension, coronary disease and cerebrovascular events (3-5, 54), it should be considered if and how the oxidative stress and the MMPs might play a role in the development of these complications. At the same time the literature data underline how the use of cPAP may reduce lipid peroxidation and protein oxidation (55-60) and may increase TAS (61) and NO (58, 62-67) as well as the same treatment may reduce the plasma levels or the production of MMP-9 (18, 19).

Considering the prognosis of these subjects, especially of those with severe OSAS, another aspect that deserves to be underlined is if oxidative stress and gelatinases may be contemplated as pharmacological target in this clinical condition.

In conclusion, we found an alteration of the parameters of the oxidative status and of the MMP profile in OSAS subjects that seems to be more evident in the subgroup of subjects with a severe degree of the disease evaluated according to the AHI. The data of this study moreover show interesting statistical correlations among lipid peroxidation, protein oxidation and MMP-9.

Conflict of interests: None declared.

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