OSAS

Oxidative stress in patients with obstructive sleep apnoea syndrome

Stress ossidativo nei pazienti con diagnosi di sindrome delle apnee ostruttive notturne

D. PASSALI[1], G. CORALLO[1], S. YAREMCHUK[2], M. LONGINI[3], F. PROIETTI[3], G.C. PASSALI[4], L. BELLUSSI[1]

[1] Department of Otolaryngology, University of Siena, Siena, Italy; [2] Institute of Otolaryngology NAMS of Ukraine, Kiev, Ukraine; [3] Department of Molecular and Developmental Medicine, University of Siena, Siena, Italy; [4] Department of Head and Neck Surgery-Institute of Otorhinolaryngology, Catholic University School of Medicine and Surgery, Rome, Italy

SUMMARY

Obstructive sleep apnoea syndrome (OSAS) is a disorder that leads to metabolic abnormalities and increased cardiovascular risk. The aim of this study was to identify early laboratory markers of cardiovascular disease through analysis of oxidative stress in normal subjects and patients with OSAS. A prospective study was designed to compare outcomes of oxidative stress laboratory tests in 20 adult patients with OSAS and a control group of 20 normal subjects. Laboratory techniques for detecting and quantifying free radical damage must be targeted to assess the pro-oxidant component and the antioxidant in order to obtain an overall picture of oxidative balance. No statistical differences in age, sex distribution, or BMI were found between the two groups ($p>0.05$). There were significant differences in the apnoea/hypopnoea index (AHI) between OSAS patients and the control group ($p<0.05$). Statistically significant differences in isoprostane, advanced oxidation protein products (AOPP) and non-protein bound iron (NPBI) levels were found between the study and control groups. No significant difference in the levels of thiol biomarkers was found between the two groups. The main finding of the present study was increased production of oxidative stress biomarkers in OSAS patients. The major difference between thiols and other oxidative stress biomarkers is that thiols are antioxidants, while the others are expressions of oxidative damage. The findings of the present study indicate that biomarkers of oxidative stress in OSAS may be used as a marker of upper airway obstructive episodes due to mechanical trauma, as well as a marker of hypoxaemia causing local oropharyngeal inflammation.

KEY WORDS: Obstructive Sleep Apnoea Syndrome • Oxidative damage • Biomarkers of oxidative stress • Polysomnography

RIASSUNTO

La Sindrome delle Apnee Ostruttive Notturne (OSAS) è una patologia caratterizzata da alterazioni metaboliche e da un elevato rischio di sviluppo di patologie cardiovascolari. Lo scopo dello studio è stato quello di identificare dei markers precoci predittivi di rischio cardiovascolare con la valutazione dello stress ossidativo misurato attraverso esami di laboratorio in soggetti normali e pazienti con diagnosi di sindrome delle apnee ostruttive notturne. È stato effettuato uno studio prospettico per confrontare i risultati di laboratorio ottenuti dalla valutazione dei biomarkers dello stress ossidativo in 20 pazienti adulti con OSAS e 20 soggetti sani. Le tecniche di analisi utilizzate avevano l'obiettivo di identificare e quantificare i danni dei radicali liberi attraverso la misurazione di anti-ossidanti e pro-ossidanti in modo da valutare l'equilibrio ossidativo presente nei due gruppi di studio. I due gruppi di pazienti sono risultati omogeni per sesso, età ed indice di massa corporea ($p < 0,05$). Una differenza statisticamente significativa è stata individuata tra i livelli di indice di apnea-ipopnea valutata alla polisonnografia e di isoprostani, produzione di proteine di ossidazione e proteine non legate al ferro nei due gruppi in esame. Nessuna differenza significativa è stata trovata nel livello dei tioli tra i soggetti sani e i pazienti con sindrome delle apnee ostruttive. I tioli, a differenza degli altri markers, sono molecole anti-ossidanti, i restanti sono invece espressione di danno ossidativo. I risultati dello studio indicano che i biomarkers potrebbero essere utilizzati come indici di ostruzione delle vie aeree superiori (VAS) e come marcatori precoci di ipossiemia causando processi flogistici ricorrenti e danno locale da radicali liberi a carico delle VAS.

PAROLE CHIAVE: *Sindrome delle apnee ostruttive notturne • Danno ossidativo • Biomarkers dello stress ossidativo • Polisonnografia*

Acta Otorhinolaryngol Ital 2015;35:420-425

Introduction

Obstructive sleep apnoea syndrome (OSAS) is a disorder that leads to metabolic abnormalities and increased cardiovascular risk [1]. It is characterised by obstruction of the upper airways with repetitive pauses in breathing during sleep and daytime sleepiness, despite efforts made to breath, and is usually associated with a reduction in blood oxygen saturation. Patients with OSAS are usually unaware of this sleep disruption, but changes in sleep patterns contribute significantly to the prominent symptom of chronic daytime sleepiness typical of these patients.

OSAS has many organic sequelae. Excessive daytime sleepiness impairs quality of life, cognitive performance and social activity [2]. Cardiovascular consequences, such as systemic arterial hypertension, coronary artery disease, heart failure and stroke, are the greatest risks [3-9]. The mechanisms hypothesised to explain the association between OSAS and cardiovascular disease are varied and probably interconnected. In fact, repeated episodes of airway occlusion during sleep determine hypoxaemia, hypercapnia and rapid recurrent changes in intrathoracic pressure, triggering a wide variety of autonomic and haemodynamic responses [4 5]. During episodes of OSAS, intermittent hypoxia determines an increase in oxidative stress that may be involved in the development of cardiovascular disease, vascular injury and endothelial dysfunction. There is much evidence that inflammatory markers, such as oxidative stress, play important roles in atherogenesis and arterial thrombus formation. Patients with OSAS are subject to oxidative stress, due for example to elevated production of reactive oxygen species correlated with high levels of soluble circulating inflammatory factors, such as adhesion molecules [6]. Intermittent apnoea-related hypoxia and post-apnoeic reoxygenation probably contribute to production of reactive oxygen species and inflammatory mediators, triggering upper airway and systemic inflammation. Upper airway inflammation, aggravated by mechanical injury caused by repeated pharyngeal collapse, increases airway obstruction. The systemic inflammatory process also increases release of oxygen-free radicals beyond physiological antioxidant capacity, generating oxidative stress. Various diseases and/or anatomical conditions of the upper airways play a significant role in the ethiopathogenesis of OSAS [7-11]. The diagnostic technique of Sleep Endoscopy represents the gold standard for the diagnosis of the anatomical sites involved in the pathogenesis of OSAS [10]. The treatment of these particular conditions rely to some extent on surgical interventions [12-16].

The adverse effects of obstructive apnoea on the cardiovascular system are not limited to sleep. Daytime sympathetic nervous activity and systemic blood pressure also increase in these patients. Although the mechanism is uncertain, intermittent apnoea-related hypoxia may be involved, since hypoxia causes sympathetic activation and blood pressure elevations that persists after removal of the hypoxic stimulus. The aim of this study was to identify early laboratory markers of cardiovascular disease through analysis of oxidative stress in subjects without pathological obstruction of the upper airways and patients diagnosed with OSAS.

Materials and methods

A prospective study was designed to compare outcomes of oxidative stress laboratory tests in 20 adult patients with OSAS and a control group of 20 normal subjects. The inclusion criteria for the study group were: a) age 18-60 years; b) no previous treatment for OSAS; c) polysomnogram AHI (Apnoea-Hypopnoea Index) > 30. The exclusion criteria were: a) smoking; b) no comorbidities that increase oxidative stress (diabetes, obesity, asthma, nasal polyposis and hypertension). All subjects underwent in the same day: ENT (Ear, Nose and Throat) examination with endoscopy, polysomnography, BMI (Body Mass Index) assessment and evaluation of oxidative stress in plasma sample from fasting venous blood and in urine's exam. Evaluation of oxidative stress in healthy subjects and patients on pharmacotherapy is indispensable to exclude tissue damage and monitor response to treatment in all conditions involving reactive oxygen species. Laboratory techniques for detecting and quantifying free radical damage must be targeted to assess the pro-oxidant component and the antioxidant in order to obtain an overall picture of oxidative balance. Oxidative stress is now recognised to be involved in the pathogenesis of at least 100 different diseases, including atherosclerosis, emphysema/bronchitis, Parkinson's disease, muscular dystrophies, preeclampsia, cervical cancer, alcoholic liver damage, diabetes, nephropathy with renal impairment, Down's syndrome, aging, retrolental fibroplasia, cerebrovascular disorders, ischaemia-reperfusion damage and rheumatoid arthritis.

Pro-oxidant and antioxidant assays were performed at the Oxidative Stress laboratory of the Neonatal Unit of Siena Hospital.

NPBI (non-protein bound iron)

Iron is a versatile and highly reactive element. Having two valencies, iron (II) (ferrous) and iron (III) (ferric), it has access to a wide range of redox potentials spanning the standard redox potential range from +300 to -500 mV. Normally, iron is safely sequestered in transport proteins such as transferrin and lactoferrin and stored in proteins such as ferritin and haemosiderin. As iron ions cannot exist in plasma, the term "free iron" was introduced to indicate a low molecular mass iron form without high-affinity binding to transferrin. A lowering of plasma pH, as occurs during ischaemia (a frequent event in preterm newborns), releases iron, producing free radicals [17-22],which release even more iron by mobilising zinc from ferritin. The resulting cascade of iron release and free radical production may cause extensive cell damage.

The method of detection of NPBI in small samples of biological fluids and tissues [23] is based on preferential chelation of NPBI by a large excess of NTA (nitrilotriacetic acid, low affinity ligand). NTA captures all iron bound to low molecular weight proteins and non-specifically bound to serum proteins. It does not remove iron bound to transferrin or ferritin. A two-step filtration procedure was used to separate NPBI: 1) filtration with a 100 kDa MWCO (Molecular Weight Cut-Off) Vecta-Spin Micro-

Whatman ultracentrifuge filter; 2) filtration with a 20 kDa MWCO Vecta Spin Micro-Whatman ultracentrifuge filter at RCF 16.1 and 4°C. The filtrate was injected directly into an isocratic reverse-phase liquid chromatograph after precolumn derivatisation with the high affinity iron ligand 3-hydroxy-1,2-dymethyl-4(1H)pyridone. All glassware and plastic ware was treated to ensure minimum iron contamination.

Protein carbonyls are formed by a variety of oxidative mechanisms and are sensitive indices of oxidative injury. Reactive oxygen species (ROS) cause cell damage, such as oxidation of amino acid residues on proteins to protein carbonyls, leading to alterations in protein structure and amino acid sequence, formation of protein-protein cross-linking and fragmentation of the protein backbone. Carbonyl groups form during normal aging, as well as in neonates receiving oxygen ventilation, and are increased by oxidative stress. Protein carbonyl groups are formed by oxidation of the side chains of lysine, proline, arginine and threonine residues; they are produced as a consequence of oxidative cleavage of the peptide backbone via the amidation pathway or by cleavage associated with oxidation of glutamyl residues. Carbonyl groups can also be formed as a result of secondary reactions of certain amino acid side chains with lipid oxidation products, such as 4-hydroxy-2-nonenal (HNE) or with reducing sugars or their oxidation products.

The quantity of protein carbonyls in a protein sample can be determined by derivatising with dinitrophenylhydrazine (DNP) and measuring protein-bound DNP with an anti-DNP antibody. Protein carbonyl concentrations were determined by enzyme-linked immunosorbent assay (ELISA), by which carbonyls can be quantified with microgram quantities of protein. The assay was set up so that about 1 mg of derivatised protein was applied to each well of the ELISA plate.

Isoprostanes are a series of prostaglandin-like compounds formed by direct ROS attack on arachidonic acid (AA), an unsaturated fatty acid component of cell membranes. Unlike prostaglandins, which are enzymatic products of this fatty acid, isoprostanes are initially formed in situ in cell membranes, from which they are subsequently cleaved by phospholipase [24]. The different pathways for the formation of F2-isoprostanes during oxidation of AA lead to four series of regioisomers (5, 8, 12 and 15 series), which can comprise eight racemic diasteromers. An isoP, 8-iso-$PGF_{2\alpha}$, is formed in abundance in vivo in human diseases correlated with free radical production [25 26].

Samples for evaluation of oxidative stress were collected with butylated hydroxytoluene (BHT) to prevent oxidation during processing, centrifuged at 1000 rpm and the supernatant was stored at a temperature of -80°C. F2-isoprostanes in plasma and urine were quantified after purification and derivatisation using the method of Morrow [27]; the method used was selected ion monitoring gas chromatography/negative ion chemical ionisation-mass spectrometry (GC/NICI-MS) employing 2H4] 8-iso-prostaglandin F2α as internal standard. 1 ng of deuterated 15-F2t-IsoP was added to the sample as an internal standard. The sample was then acidified to pH 3 with 1 M HCl and diluted to 3 ml with H_2O. The mixture was vortexed and applied to a C18 Sep-Pak column preconditioned with 5 ml methanol and 5 ml water (pH 3). The column was then washed sequentially with 10 ml water (pH 3) and 10 ml heptane. Samples were eluted with 10 ml ethyl acetate/heptane (50:50, v/v). The ethyl acetate/heptane eluate from the C18 Sep-Pak was then dried in a stream of nitrogen and applied to a silica Sep-Pak. The cartridge was washed with 5 ml ethyl acetate and samples were eluted with 5 ml ethyl acetate/methanol (50:50, v/v). The ethyl acetate/methanol eluate was evaporated under a stream of nitrogen. Ssamples were then converted to pentafluorobenzyl ester with a mixture of 40 µl 10% pentafluorobenzyl bromide in acetonitrile and 20 µl 10% *N,N*-diisopropylethylamine in acetonitrile at 37°C for 30 min**.** The reagents were dried under nitrogen and the residue eluted on TLC with 50 µl methanol. Compounds migrating in the region of the methyl ester of PGF2a (Rf 0.22) and the adjacent area 1.1 cm above were scraped and extracted from the silica gel with ethyl acetate. The ethyl acetate was dried under nitrogen and the isoprostanes converted to a trimethylsilyl ether derivative by adding 20 µl BSTFA (N,O-bis trimethylsilyl trifluoroacetamide) and 10 µl dimethylformamide, and incubating at 37°C for 20 min. The reagents were dried under nitrogen and the isoprostanes redissolved in 20 µl undecane for analysis by GC/MS (Gas chromatography–mass spectrometry). For quantification purposes, we compared the height of the peak containing the derivatised 15-F2t-IsoP (m/Z 569) with the height of the deuterated internal standard peak.

Thiol production was measured with the -SHp test (Diacron International, Italy) [28 29]. Thiols are a qualitatively significant component of the antioxidant plasma barrier. Indeed, thiol groups (-SH) of plasma compounds (e.g. proteins, P-SH) oppose the propagation step of radical chain reactions by inactivating alkoxyl radicals (RO*), according to the reaction:

2 P-SH + 2 RO* → 2 PS* + 2 ROH → P-S-S-P + 2 ROH

They also neutralise the tissue-damaging action of hydroxyl radicals (HO*), according to the reaction:

2 P-SH + 2 HO* → 2 PS* + 2 H_2O → P-S-S-P + 2 H_2O

From a merely stoichiometric point of view, a pair of thiol groups reduces a pair of alkoxyl (RO*) or hydroxyl (*OH) radicals by exchanging two electrons (as hydrogen atoms), inactivating the radicals. Indeed, alkoxyl and hydroxyl radicals are transformed to alcohol and water, respectively, while the thiol groups, now oxidised, react among themselves, forming disulphide bonds. The -SHp test is based on the ability of thiol groups in a biological sample to develop a photometrically-detectable coloured

Table I. Demographic and clinical data of the study and control groups.

	Study group	Control group	p value
Number of subjects	20	20	
Age	47.3 ± 10.44	39 ± 12.12	p > 0.05
Sex (Male/Female)	14/6	12/8	p > 0.05
BMI (Body Mass Index)	25.11 ± 3.01	23.22 ± 2.39	p > 0.05
AHI (Apnoea – Hypopnoea Index)	35.89 ± 16.27	1.3 ± 1.53	p < 0.0001

complex (maximum peak of absorbance, 405 nm) in an adequately buffered solution (R_1 reagent of the kit) by reacting with 5,5-dithiobis-2-nitrobenzoic acid (DTNB), which is dissolved as a chromogenic mixture (R_2 reagent of the kit). The intensity of photometrically-detected colour is directly proportional to the concentration (or title) of thiols, according to the Lambert-Beer law. Continuous variables were analysed using a Student's t test. All tests were two-tailed, and p values of <0.05 were taken as statistically significant. Data is presented as mean ± standard deviation or as mean/median with range, as appropriate.

Ethical standards

The present study was approved by the Ethics Committee of the University of Siena and were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and later amendments. All persons gave their informed consent prior to inclusion in the study.

Results

Demographic and clinical data of the study population and control group are shown in Table I. No statistical differences in age, sex distribution, or BMI were found between the two groups (p > 0.05). There were significant differences in AHI between OSAS patients and the control group (p < 0.05). Statistically significant differences in isoprostane levels were found between the study and control groups (67.35±40.02 vs 21.195±14.71, p < 0.0001; Fig. 1). AOPP (Advanced Oxidation Protein Products) levels were significantly higher in the OSAS group (111.97±24.44 vs 19.53 ±6.85, p < 0.0001; Fig. 2). Statistical differences in NPBI were found between the two groups of patients (3.01±2.21 vs 0.69±0.83, p = 0.0014; Fig. 3). No significant difference in the levels of thiol biomarkers was found between the two groups (488.80 ±65.27 vs 489.1±58.15, p = 0.6733; Fig. 4). Significantly higher urine isoprostane levels were found in OSAS group (216.78±244.22 vs 1.13±0.12, p < 0.0001; Fig. 5).

Discussion

OSAS is a major risk factor for cardiovascular and cerebrovascular disease. The mechanisms involved in the pathogenesis of OSAS include oxidative stress, systemic inflammation and endothelial dysfunction. The main finding of

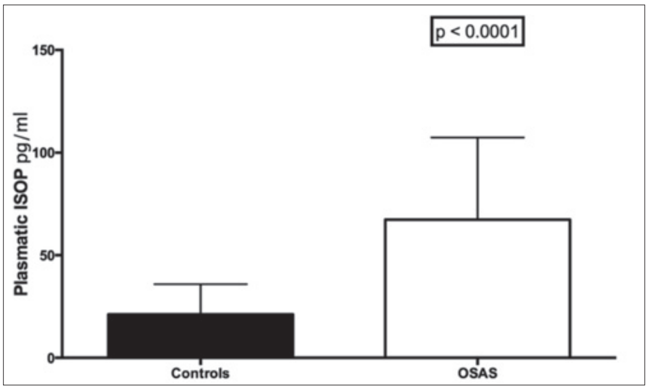

Fig. 1. Isoprostane levels in the two study groups.

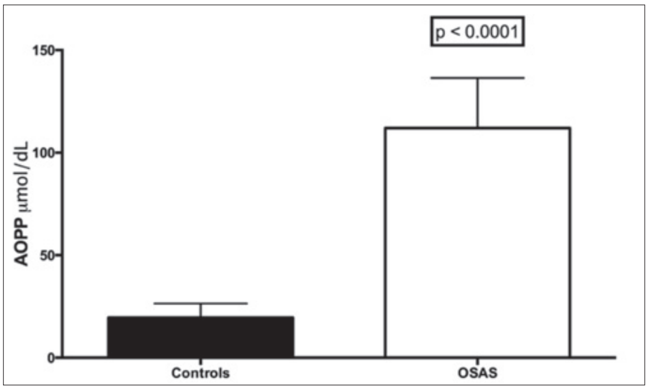

Fig. 2. AOPP levels in OSAS patients and the control group.

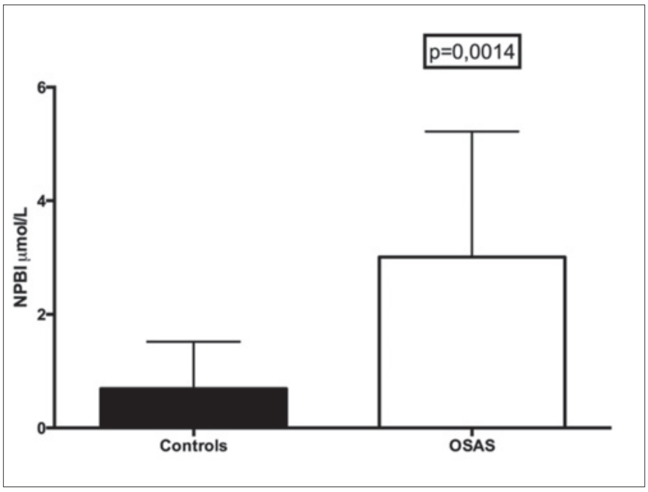

Fig. 3. NPBI levels in the two study groups.

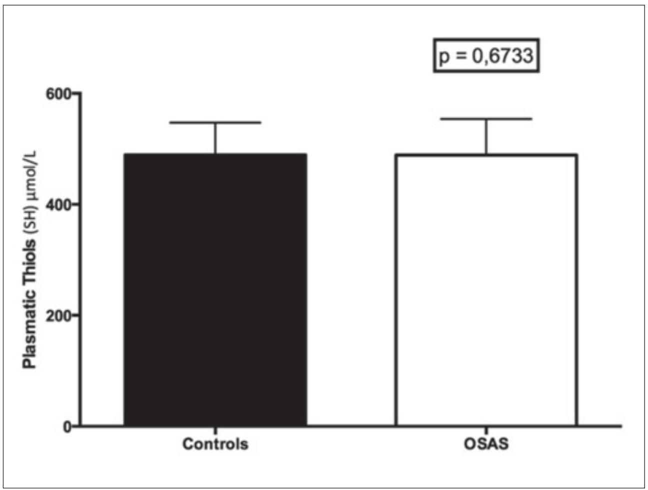

Fig. 4. Levels of thiol biomarkers in the two study groups.

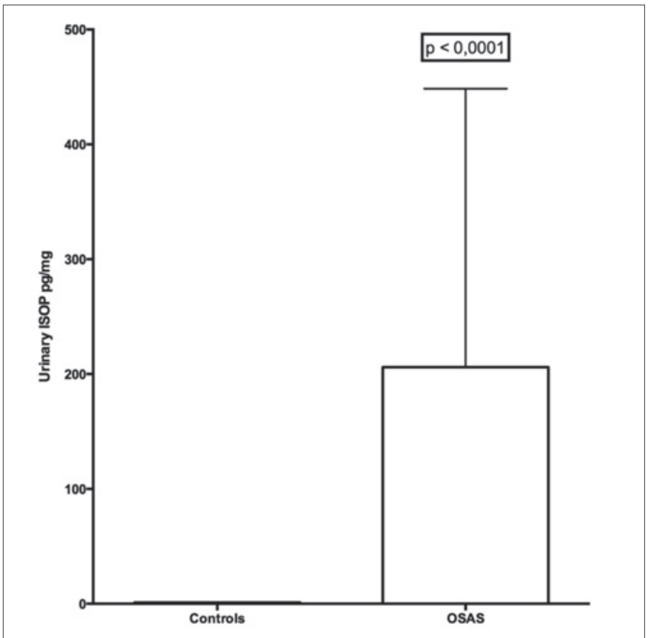

Fig. 5. Urine isoprostane levels in OSAS patients.

the present study was increased production of oxidative stress biomarkers in OSAS patients. Since biomarkers are inflammatory markers, this finding is in line with previous studies showing inflammation of the pharynx, uvula, soft palate and oral cavity of OSAS patients [30 31].

Various studies have outlined the role of inflammation markers as indirect markers of upper airway obstructive episodes. A correlation has been demonstrated between oral nitric oxide and OSAS severity due to local oropharyngeal inflammation caused by mechanical trauma and hypoxaemia. Oral inflammation in OSAS is thought to originate in the upper airways, where repetitive closing and opening during apnoeic episodes leads to increased production of inflammatory cytokines. Oral inflammation in OSAS may also be a consequence of intermittent hypoxia and reperfusion. The increased levels of oxidative stress biomarkers may arise from the recurrent episodes of hypoxia, while subsequent inflammatory status and reperfusion damage are cofactors that may lead to a further increase in these biomarkers.

Our results showed significant differences in the levels of the biomarkers studied in the two groups of patients, apart from thiols, which did not show any difference between patients and controls. The main difference between thiols and the other oxidative stress biomarkers is that thiols are antioxidants, while the others are expressions of oxidative damage. Thiols are a qualitatively significant component of the antioxidant plasma barrier. Indeed, thiol groups of plasma compounds (e.g. proteins, P-SH) oppose the propagation of radical chain reactions by inactivating alkoxyl radicals. The similar levels of thiols found in the two groups confirm the homogeneity of the two study populations, since no patient was taking antioxidant drugs or had manifest cardiovascular disease that may have led to reduced expression of antioxidants. Furthermore, thiol levels may be a late rather than an early marker of oxidative stress.

The results showed 100% specificity for all biomarkers analysed, and 100% and 95% sensitivity for protein carbonyl and urine isoprostanes, respectively (Tab. II). Plasma isoprostanes and NPBI showed 60-70% sensitivity. In conclusion, the findings of the present study indicate that biomarkers of oxidative stress in OSAS may be used as a precursor marker of upper airway obstructive episodes due to mechanical trauma, as well as a marker of hypoxaemia causing local oropharyngeal inflammation. Increased oxidative stress may have important clinical implications in OSAS patients in terms of diagnostic, therapeutic and prognostic aspects. The aim of the present study was to evaluate the levels of oxidative stress indicators in OSAS patients and a control group. It will be important investigate whether the levels and activities of these markers are correlated with demographic, biochemical, metabolic and polysomnographic parameters. By amplifying oxidative and nitrosative stress, oxidative biomarkers may have a pathogenic role in OSAS.

Conclusions

The findings of the present study indicate that biomark-

Table II. Sensitivity and specificity of oxidative stress biomarkers in OSAS subjects.

	Sensitivity	Specificity
Plasma isoprostanes	70%	100%
Protein carbonyls	100%	100%
NPBI (non-protein bound iron)	60%	100%
Thiols	0%	100%
Urine isoprostanes	95%	100%

ers of oxidative stress in OSAS may be used as a marker of upper airway obstructive episodes due to mechanical trauma, as well as a marker of hypoxemia causing local oropharyngeal inflammation.

References

1 Bonsignore MR, Zito A. *Metabolic effects of the obstructive sleep apnea syndrome and cardiovascular risk.* Arch Physiol Biochem 2008;114:255e60.

2 Engleman HM, Douglas NJ. *Sleep. 4: Sleepiness, cognitive function, and quality of life in obstructive sleep apnoea/hypopnoea syndrome.* Thorax 2004;59:618-22.

3 McNicholas WT, Bonsigore MR. *Sleep apnoea as an independent risk factor for cardiovascular disease: current evidence, basic mechanisms and research priorities.* Eur Respir J 2007;29:156-78.

4 Bradley TD, Floras JS. *Sleep apnea and heart failure. Part I: Obstructive sleep apnea*. Circulation 2003;107:1671-8.

5 Lanfranchi P, Somers VK. *Ostructive sleep apnea and vascular disease*. Respir Res 2001;2:315-9.

6 Rifai N, Ridker PM. *Inflammatory markers and coronary heart disease*. Curr Opin Lipidol 2002;13:3383-9.

7 Salamanca F, Costantini F, Bianchi A, et al. *Identification of obstructive sites and patterns in obstructive sleep apnoea syndrome by sleep endoscopy in 614 patients.* Acta Otorhinolaryngol Ital 2013;33:261-6.

8 Tarsitano A, Marchetti C. *Unusual presentation of obstructive sleep apnoea syndrome due to a giant mandible osteoma: case report and literature review.* Acta Otorhinolaryngol Ital 2013;33:63-6.

9 Passàli D, Tatti P, Toraldo M, et al. *OSAS and metabolic diseases: Round Table, 99th SIO National Congress, Bari 2012.* Acta Otorhinolaryngol Ital 2014;34:158-66.

10 De Corso E, Fiorita A, Rizzotto G, et al. *The role of drug-induced sleep endoscopy in the diagnosis and management of obstructive sleep apnoea syndrome: our personal experience.* Acta Otorhinolaryngol Ital 2013;33:405-13.

11 Poje G, Zinreich JS, Skitarelić N, et al. *Nasal septal deformities in chronic rhinosinusitis patients: clinical and radiological aspects.* Acta Otorhinolaryngol Ital 2014;34:117-22.

12 *Salamanca* F, Costantini F, Mantovani M, et al. *Barbed anterior pharyngoplasty: an evolution of anterior palatoplasty.* Acta Otorhinolaryngol Ital 2014;34:434-8.

13 Mantovani M, Minetti A, Torretta S, et al. *The "Barbed Roman Blinds" technique: a step forward.* Acta Otorhinolaryngol Ital 2013;33:128.

14 Scarano E, Della Marca G, De Corso E, et al. *Hyoid myotomy without suspension: a surgical approach to obstructive sleep apnoea syndrome.* Acta Otorhinolaryngol Ital 2014;34:362-7.

15 Milano F, Mondini S, Billi MC, et al. *The impact of a multidisciplinary approach on response rate of mandibular advancing device therapy in patients with obstructive sleep apnoea syndrome.* Acta Otorhinolaryngol Ital 2013;33:337-42.

16 Giarda M, Brucoli M, Arcuri F, et al. *Efficacy and safety of maxillomandibular advancement in treatment of obstructive sleep apnoea syndrome.* Acta Otorhinolaryngol Ital 2013;33:43-6.

17 Shouman BO, Mesbah A, Aly H. *Iron metabolism and lipid peroxidation products in infants with hypoxic ischemic encephalopathy*. J Perinatol 2008;28:487-91.

18 Sävman K, Nilsson UA, Thoresen M, et al. *Non-protein-bound iron in brain interstitium of newborn pigs after hypoxia.* Dev Neurosci 2005;27:176-84.

19 Bracci R, Perrone S, Buonocore G. *Red blood cell involvement in fetal/neonatal hypoxia.* Biol Neonate 2001;79:210-2.

20 Dorrepaal CA, Van Bel F, Steendijk P, et al. *Influence of inhibition of nitric oxide synthesis on cardiac function in the newborn lamb after hypoxic-ischemic injury.* Biol Neonate 2000;78:98-105. Erratum in: Biol Neonate 2001;79:20.

21 Shadid M, Van Bel F, Steendijk P, et al. *Effect of deferoxamine on post-hypoxic-ischemic reperfusion injury of the newborn lamb heart.* Biol Neonate 1999;75:239-49.

22 Shadid M, Buonocore G, Groenendaal F, et al. *Effect of deferoxamine and allopurinol on non-protein-bound iron concentrations in plasma and cortical brain tissue of newborn lambs following hypoxia-ischemia.* Neurosci Lett 1998;248:5-8.

23 Paffetti P, Perrone S, Longini M, et al. *Non-protein-bound iron detection in small samples of biological fluids and tissues.* Biol Trace Elem Res 2006;112:221-32.

24 Yin H, Gao L, Tai HH, et al. *Urinary prostaglandin F2alpha is generated from the isoprostane pathway and not the cyclooxygenase in humans.* J Biol Chem 2007;282:329-36.

25 Reich EE, Montine TJ, Morrow JD. *Formation of novel D-ring and E-ring isoprostane-like compounds (D4/E4-neuroprostanes) in vivo from docosahexaenoic acid.* Adv Exp Med Biol 2002;507:519-24.

26 Fam SS, Morrow JD. *The isoprostanes: unique products of arachidonic acid oxidation-a review.* Curr Med Chem 2003;10:1723-40.

27 Milne GL, Sanchez SC, Musiek ES, et al. *Quantification of F2-isoprostanes as a biomarker of oxidative stress.* Nat Protoc 2007;2:221-6.

28 Ellman GL. *Tissue sulfhydryl groups.* Arch Biochem Biophys 1959;82:70.

29 Carratelli M, Porcaro L, Ruscica M, et al. *Reactive oxygen metabolities and prooxidant status in children with Down's syndrome.* lnt J Clin Pharmacol Res 2001;21:79-84.

30 Friberg D, Ansved T, Borg K, et al. *Histological indications of a progressive snorers disease in an upper airway muscle.* Am J Respir Crit Care Med 1998;157:586-93.

31 Sekosan M, Zakkar M, Wenig BL, et al. *Inflammation in the uvula mucosa of patients with obstructive sleep apnea.* Laryngoscope 1996;106:1018-20.

Received: November 22, 2015 - Accepted: November 30, 2015

Address for correspondence: Desiderio Passali, Department of Otolaryngology, University of Siena, viale Bracci 16, 53100 Siena, Italy, Tel. +39 0577 585470, E-mail: d.passali@virgilio.it