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Evening and morning peroxiredoxin-2 redox/oligomeric state changes in obstructive sleep apnea red blood cells: Correlation with polysomnographic and metabolic parameters

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ABSTRACT

We have examined the effects of Obstructive Sleep Apnea (OSA) on red blood cell (RBC) proteome variation at evening/morning day time to uncover new insights into OSA-induced RBC dysfunction that may lead to OSA manifestations. Dysregulated proteins mainly fall in the group of catalytic enzymes, stress response and redox regulators such as peroxiredoxin 2 (PRDX2). Validation assays confirmed that at morning the monomeric/dimeric forms of PRDX2 were more overoxidized in OSA RBC compared to evening samples. Six month of positive airway pressure (PAP) treatment decreased this overoxidation and generated multimeric overoxidized forms associated with chaperone/transduction signaling activity of PRDX2. Morning levels of overoxidized PRDX2 correlated with polysomnographic (PSG)-arousal index and metabolic parameters whereas the evening level of disulfide-linked dimer (associated with peroxidase activity of PRDX2) correlated with PSG parameters. After treatment, morning overoxidized multimer of PRDX2 negatively correlated with fasting glucose and dopamine levels.

Overall, these data point toward severe oxidative stress and altered antioxidant homeostasis in OSA RBC occurring mainly at morning time but with consequences till evening. The beneficial effect of PAP involves modulation of the redox/oligomeric state of PRDX2, whose mechanism and associated chaperone/transduction signaling functions deserves further investigation. RBC PRDX2 is a promising candidate biomarker for OSA severity and treatment monitoring, warranting further investigation and validation.

1. Introduction

Obstructive sleep apnea (OSA) syndrome is a sleep-associated respiratory disorder, characterized by recurrent episodes of apneas and hypopneas during sleep with resultant intermittent hypoxia and sleep fragmentation [1]. OSA affects about 22% of men (range, 9–37%) and 17% of women (range, 4–50%) but frequently is underdiagnosed [2]. OSA is associated with cancer [3] and cerebral, cardiovascular and metabolic pathologies [4], making this disorder a public health concern. The mechanisms responsible for these outcomes are oxidative stress and associated inflammatory, autonomic, vascular, and metabolic events [5]. The diagnosis of OSA is based on an overnight sleep exam, that can be a laboratory polysomnography (PSG) or, in some patients, a portable cardio-respiratory study [6]. Presently, positive airway pressure (PAP) devices are the first line treatment for OSA [7] acting on preventing obstructive episodes and thus inhibiting the cascade of deleterious OSA-associated pathologies. PAP improves daytime somnolence, cognitive function and quality of life and prevents cerebral, cardiovascular and metabolic consequences [7]. Additionally, PAP devices seem to decrease oxidative stress and inflammation [8,9].

The expense and time consuming nature of both sleep testing and PAP treatment contribute to under diagnosis and consequently to under treatment of OSA. Identification of predictive biomarkers of OSA concerning diagnosis, prognosis and response to treatment could be

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useful tool in its global strategy. Several approaches to biomarker discovery, such as advanced proteomics-based technologies, have gained importance in several pathologies, including OSA [10]. By using two-dimensional fluorescence difference gel electrophoresis (2-D DIGE) - based proteomics, we intended herein to investigate for the first time to our knowledge OSA red blood cell (RBC) proteome at two time points, before and after one night of sleep. Dysfunction in the RBC homeostasis has been described as a potential source of systemic inflammation that leads to metabolic diseases, such as obesity, insulin resistance and hypertension [11]. These disorders are also recognized as OSA associated sequelae [1,4,5]. The RBC redox-state may also play an important role in the physiology of circadian rhythms [12] and sleeplessness, hypoxia or oxidative-stress induced circadian disruption that may lead to tumorigenesis and metabolic syndrome [13,14]. Therefore, we are convinced that studies focused on the identification of dysregulated proteins in OSA RBC, before or after nocturnal intermittent hypoxia and sleep disruption could provide new insights into underlying pathological mechanisms and uncover new candidate biomarkers of OSA and OSA associated disorders. Additionally, studies evaluating the effect of PAP treatment in the modulation of these proteins will contribute to better understanding and monitoring of the effectiveness and/or side-effects of PAP therapy.

2. Material and methods

2.1. Patients

One hundred four consecutive male subjects with clinically suspected OSA syndrome were screened and evaluated through interviews for inclusion in this study. Before PSG study, all subjects underwent a restricted diet during three days, including no drugs to induce sleep. The PSG was performed overnight at the sleep laboratory according to standard procedures [6]. After diagnosis, PAP therapy with automatic devices (S9, Resmed, Australia) were prescribed for patients with OSA severe disease (respiratory disturbance index (RDI) ≥ 30/h) or with any severity (RDI ≥ 5/h) when associated with excessive diurnal sleepiness and/or cardio or cerebro vascular complications (hypertension, arrhythmia, coronary disease, cerebral or cardiac stroke) as described [15].

Patients were selected to participate in two phases of the study, namely discovery and validation phase. For the discovery phase, 12 subjects diagnosed with primary snoring (RDI < 5/h) and 12 subjects with moderate (RDI ≥ 15/h, but <30/h) ($n = 5$) or severe OSA (RDI ≥ 30/h) ($n = 7$) were selected (Cohort I, Table 1). For validation phase, 10 subjects with primary snoring (RDI < 5/h) and 10 subjects with mild (RDI ≥ 5/h, but <15/h) ($n = 4$) or moderate to severe OSA (RDI ≥ 15/h) ($n = 6$) that underwent six month of PAP treatment were selected (Cohort II, Table 2). Exclusion criteria were female gender (to avoid hormonal influence), shift workers, other sleep disorders, neuromuscular disease, heart failure, diabetes, neoplasia, acute disease and previous PAP treatment.

2.2. Sample collection & biochemical analysis

At the day of hospitalization, 24 h urine was collected for catecholamine determination, according to the hospital standard procedures. Blood samples were collected into EDTA-coated polypropylene tubes before and after PSG i.e., between 8:00 pm and 09:30 pm (referred to as 'evening') and between 7:30 am and 09:00 am.

(referred to as 'morning'). Patients who underwent PAP treatment, a second blood sample was collected after six months of treatment under the same conditions as above described but at morning time only. Hematological and metabolic analyses were immediately performed on morning samples according to the hospital procedures. For proteomics study, samples were kept no longer than 4 h at 4 °C until fractionation.

Table 1
Cohort I - discovery phase.

Phase demographic, polysonographic and analytical characterization			
Demographic and PSG parameters	Screened subjects		
	snorer($n = 12$)	OSA ($n = 12$)	p value
Age (years)	46.8 (10.0)	45.8 (7.2)	NS
Habits			
Current smoking (n)	6	4	NS
Current drinking (n)	7	6	NS
EPW Score	9.1 (4.3)	11.7 (6.5)	NS
Observational features			
Morning arterial pressure (mmHg)	141.3 (23.1)/ 88.8 (14.6)	129.8 (13.1)/ 78.9 (8.3)	NS
BMI (kg/m^2)	26.9 (2.7)	30.8 (1.9)	0.0002
Abdominal perimeter (cm)	97.5 (6.9)	107.4 (8.6)	0.005
Comorbidities			
Hypertension (n)	6	4	NS
Respiratory diseases (n)	3	1	NS
Dyslipidemia (n)	8	10	NS
Polysomnographic parameteres			
RDI (events/h)	2.7 (1.5)	41.9 (20.5)	5.96E-07
Arousal Index (events/h)	12.6 (4.8)	34.6 (18.4)	0.0003
Minimum arterial saturation (%)	87.4 (4.1)	80.5 (7.6)	0.006
T90 (%)	0.1 (0.2)	19.4 (24.6)	0.006
ODI (events/h)	3.0 (4.4)	41.2 (27.0)	3.79E-05
Sleep efficiency (%)	76.7 (14.1)	82.1 (6.8)	NS
Analytical parameteres			
Glycemic profile			
Glucose (70–110 mg/dl)	95.2 (8.5)	96.7 (12.0)	NS
Hb A1C (4–6%)	5.7 (0.4)	5.8 (0.4)	NS
Insulin (3–25 mU/l)	11.0 (6.2)	20.6 (12.5)	0.013
HOMA-IR (≥3.8)	2.6 (1.6)	5.0 (3.0)	0.012
Lipid profile			
Cholesterol (<190 mg/dl)	206.2 (44.3)	193.0 (30.8)	NS
Triglycerides (<150 mg/dl)	127.3 (69.7)	217.1 (127.7)	0.022
Cardiovascular marker			
Homocysteine (3.7–13.9 μmol/l)	15.6 (4.3)	15.1 (2.9)	NS
Urinary catecholamines			
Adrenalin (1.7–22.4 μg/24 h)	15.0 (15.5)	10.8 (9.3)	NS
Nor-adrenalin (12.1–85.5 μg/24 h)	73.5 (34.0)	75.0 (37.4)	NS
Dopamine (0–498 μg/24 h)	389.4 (218.4)	367.3 (169.5)	NS
Complete hemogram			
RBC (4.5–5.9 × 10^{12}/l)	5.0 (0.4)	5.1 (0.5)	NS
Hemoglobin (13–17.5 g/dl)	15.4 (0.8)	15.2 (1.6)	NS
Hematocrit (40–50%)	45.9 (2.1)	45.1 (4.1)	NS
MCV (80–97 fl)	91.9 (6.4)	88.9 (4.1)	NS
MCH (27–33 pg)	30.9 (1.8)	30.0 (1.6)	NS
MCHC (31.5–35.5 g/dl)	33.6 (1.0)	33.7 (1.1)	NS
RDW (11.5–14.5)	13.4 (0.7)	13.5 (0.9)	NS
Leukocytes (4.0–11 × 10^9/l)	6.7 (1.2)	7.8 (2.3)	NS
Platelets (150–450 × 10^3 μl)	237.8 (40.1)	261.7 (77.2)	NS

NS: non-statistical meaning.
EPW: Epworth Sleepiness Scale; BMI: body mass index; RDI: respiratory disturbance index; T90%: time spent with saturation < 90%; ODI: oxygen desaturation index; HbA1C: hemoglobi n glycated; HOMA-IR: model assessment of insulin resistance; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin; RDW: red cell distribution width.

The obtained RBC pellets were aliquoted and stored at −80 °C until analysis.

2.3. 2D-DIGE based proteomics discovery phase

RBC sample preparation, analysis by 2D-DIGE approach followed by mass spectrometry for protein identification and protein annotation and classification using dedicated bioinformatics tools are described in detail in [16].

2.4. Validation phase

The validation of proteomics data was performed for peroxiredoxin 2 (PRDX2) on samples from Cohort II (Table 2).

Table 2
Cohort II - validation phase.

Demographic, polysonographic and analytical characterization					
Demographic and PSG parameters	Screened subjects				
	Snorer ($n = 10$)	OSA ($n = 10$)	PAP ($n = 10$)	*p value* Snorer vs OSA	*p value* OSA vs PAP
Age (years)	43.6 (11.9)	43.0 (7.1)	–	NS	NS
Habits					
Current smoking (n)	4	2	–	NS	n/a
Current drinking (n)	5	6	–	NS	n/a
EPW score	11.7 (4.8)	11.5 (4.5)	6.6 (4.7)	NS	0.0003
Observational features					
Morning arterial pressure (mmHg)	138.6 (21.7)/85.1 (13.5)	124.2 (18.3)/77.8 (12.3)	–	NS	n/a
BMI (kg/m^2)	27.5 (3.9)	29.6 (2.3)	–	NS	n/a
Abdominal perimeter (cm)	98.0 (9.3)	105.0 (8.8)	–	NS	n/a
Comorbidities					
Hypertension (n)	5	5	–	NS	n/a
Respiratory diseases (n)	0	0	–	NS	n/a
Dyslipidemia (n)	7	7	–	NS	n/a
Polysomnographic parameteres					
RDI (events/h)	3.3 (1.2)	34.8 (26.5)	–	0.001	n/a
Arousal index (events/h)	12.8 (4.8)	34.7 (19.8)	–	0.002	n/a
Minimum arterial saturation (%)	87.4 (3.4)	81.0 (7.4)	–	0.012	n/a
T90 (%)	0.1 (0.1)	12.6 (19.5)	–	0.028	n/a
ODI (events/h)	5.2 (5.9)	28.8 (27.4)	–	0.008	n/a
Sleep efficiency (%)	79.8 (14.3)	80.4 (12.7)	–	NS	n/a
PAP record					
Number of days without use	–	–	54.4 (63.7)	–	n/a
Total of recording days	–	–	200.0 (21.5)	–	n/a
Use per night (min)	–	–	269.7 (123.4)	–	n/a
Residual AHI (events/h)	–	–	1.6 (1.1)	–	n/a
Analytical parameteres					
Glycemic profile					
Glucose (70–110 mg/dl)	96.1 (8.1)	95.1 (11.9)	95.3 (11.2)	NS	NS
Hb A1C (4–6%)	5.6 (0.4)	5.7 (0.4)	5.7 (0.3)	NS	NS
Insulin (3–25 mU/l)	11.5 (6.9)	20.7 (13.5)	28.9 (40.9)	0.036	NS
HOMA-IR (≥3.8)	2.8 (1.8)	5.0 (3.2)	7.2 (10.8)	0.036	NS
Lipid profile					
Cholesterol (<190 mg/dl)	200.9 (39.8)	181.1 (23.6)	178.9 (28.1)	NS	NS
Triglycerides (<150 mg/dl)	128.5 (70.7)	169.6 (90.7)	153.4 (69.9)	NS	NS
Cardiovascular marker					
Homocysteine (3.7–13.9 μmol/l)	14.7 (4.3)	14.1 (2.8)	15.4 (2.0)	NS	NS
Urinary catecholamines					
Adrenalin (1.7–22.4 μg/24 h)	17.3 (15.3)	9.9 (9.1)	20.0 (7.7)	NS	0.010
Nor-adrenalin (12.1–85.5 μg/24 h)	56.9 (15.9)	64.1 (36.9)	54.3 (25.6)	NS	NS
Dopamine (0–498 μg/24 h)	301.7 (117.7)	244.8 (105.5)	258.8 (123.3)	NS	NS
Complete hemogram					
RBC (4.5–5.9 × 10^{12}/l)	5.0 (0.5)	5.1 (0.4)	4.9 (0.3)	NS	0.018
Hemoglobin (13–17.5 g/dl)	15.4 (0.9)	15.5 (1.2)	15.1 (1.0)	NS	0.041
Hematocrit (40–50%)	45.7 (2.4)	45.7 (3.3)	44.1 (3.1)	NS	0.008
MCV (80–97 fl)	91.5 (5.3)	89.5 (3.3)	89.7 (3.4)	NS	NS
MCH (27–33 pg)	30.7 (1.2)	30.2 (1.3)	30.7 (1.2)	NS	NS
MCHC (31.5–35.5 g/dl)	33.6 (1.1)	33.8 (0.9)	34.3 (0.8)	NS	0.051
RDW (11.5–14.5)	13.3 (0.5)	13.2 (0.9)	13.8 (0.6)	NS	0.030
Leukocytes (4.0-11 × 10^9/l)	7.1 (1.0)	7.6 (1.8)	7.2 (1.8)	NS	NS
Platelets (150–450 × 10^3 μl)	241.5 (31.1)	238.5 (46.2)	203.4 (47.8)	NS	0.012

NS: non-statistical meaning; n/a: not-applicable.
EPW: epworth sleepiness scale; BMI: body mass index; RDI: respiratory disturbance index; T90%: time spent with saturation < 90%; ODI: oxygen desaturation index; AHI: apnea-hypopnea index; HbA1C: hemoglobin glycated; HOMA- IR: model assessment of insulin resistance; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin; RDW: red cell distribution width.

PRDX2 was evaluated by a non-reduced 4–12% SDS-PAGE mini gels followed by western blotting (WB) using 1:20,000 rabbit anti-PRDX2 or 1:3000 rabbit anti- PRDX-$SO_{2/3}$ antibody (Ab; Abcam) to investigate PRDX-2 redox-oligomeric state as described [17]. Before lyses, 100 mM of N-ethylmaleimide (NEM) was added to the samples to prevent exogenous-induced oxidation [17].

2.5. Statistical analysis

Descriptive analyses for clinical and analytical data were expressed as mean ± standard deviation (SD), and frequency (% values) was used to characterize the groups. One-way ANOVA (analysis of variance) was used to compare statistically more than two groups; Paired Student-*t*-test was used to compare evening and morning condition in the same group set (Snorer or OSA), and to evaluate the effect of (before/after) PAP treatment in OSA group set. Student-*t*-test for independent samples was used to compare Snorer and OSA groups at evening or morning condition. Correlation of variables was carried out using Pearson Correlation test. The level of statistical significance was set at 5% (p-value < 0.05).

3. Results

3.1. Patients: clinical, biochemical and metabolic characteristics

Results are summarized in Tables 1 and 2. In both, Cohort I (Discovery phase) and Cohort II (Validation phase), significant differences were observed regarding the PSG parameters and the insulin and insulin

resistance, determined by homeostatic model assessment of insulin resistance (HOMA-IR) levels, which are higher in OSA patients compared with Snorers ($p < 0.05$ Student t-test). No significant differences were observed for the other parameters, except that in Cohort I, significant differences were observed in the body mass index (BMI) and triglycerides level, both were higher in OSA patients compared to Snorers (Student t-test, $p < 0.05$) (Table 1). No significant differences were found in these parameters between OSA and Snorer groups participating in Cohort II (Table 2).

After six month of PAP treatment (compliance with mean usage >4 h.night^{-1}), patients reported a significant decrease in excessive daytime somnolence, evaluated by the Epworth Sleepiness Scale (EPW) score (Paired Student t-test, $p < 0.05$) (Table 2).

The urinary catecholamine, adrenalin, was significant higher, while no differences were observed for nor-adrenaline and dopamine after treatment. The Hemogram data, although showing clinical normal reference values, it revealed a small, but significant, decrease in the RBCs and platelets count, haemoglobin concentration and hematocrit in patients after PAP treatment. The mean corpuscular hemoglobin (MCH) and red cell distribution width (RDW) were significantly higher in response to treatment (Paired Student t-test, $p < 0.05$). There were no significant changes in glucose and lipid profile and cardiovascular marker after treatment (Table 2).

3.2. Day or night variations in OSA RBC proteome

950 protein spots were visualized on 2DIGE images. 76 of these exhibited significant differences in abundance (fold change ≥ 1.2; Anova $p < 0.05$) between OSA and Snorer's at evening or morning times (Fig. 1 - Data in Brief [16]).

From these spots, 31 were identified by MS, corresponding to 21 unique proteins suggesting the existence of post-translational modification (PTM) regulations (Table 1-Data in Brief [16]).

Some of these proteins/proteoforms showed abundance changes exclusively in OSA morning or OSA evening compared with the ones from Snorers or between morning and evening whatever is OSA or Snorers (Fig. 2-Data in Brief [16]). Functional analysis by DAVID Bioinformatics Resources [18] showed that most identified proteins are associated with catalytic, oxidoreductase, peroxidase, hydrolase, ATPase and antioxidant activity and nucleoside binding. Concerning biological process, OSA morning differential proteins were mainly associated with negative regulation of metabolic, cellular and biological processes and response to chemical stimulus. OSA evening differential proteins were mostly associated with positive regulation of catalytic activity and molecular function and catabolic and metabolic process. Morning compared with evening in OSA showed a larger numbers of differential proteins thus a larger number of associated biological processes, which included response to chemical stimulus, oxidation reduction, regulation of catalytic activity and response to stress (Fig. 2-Data in Brief [16]).

3.3. Peroxiredoxin-2 redox/oligomeric states in OSA RBC

Acidic PRDX-2 spot, reported as hyper/oxidized proteoforms of PRDX2 [17] was one of those proteins identified higher abundant in 2DIGE maps of OSA morning samples (Anova $p < 0.05$) compared to the ones from Snorers (Fig. 1).

To validate whether the redox/oligomeric states of PRDX-2 are differentially modulated in OSA as indicated by the proteomics data, samples were analysed by a non-reducing WB using an antibody (Ab) against PRDX2 or against sulphinylated/sulfonylated ($-SO_{2/3}H$) forms of PRDXs ($PRDXSO_{2/3}$) [17]. A new Cohort of Snorer and OSA patients, before and after six month of PAP treatment, was used (Table 2).

As illustrated in Fig. 2A, in non-reducing SDS-PAGE condition, dimer or multimer of PRDX2 present in cells that are fully reduced or fully overoxidized are denatured and visualized as ~21 kDa monomers by WB using Ab-PRDX2. In contrast, dimeric/multimeric oxidized forms that have two disulfides (S-S/S-S) linked peroxidatic cysteins or one disulfide linked whereas the other is either reduced or overoxidized (S-S/SH or S-S/SO_2H) are denatured and visualized as ~50 kDa (denominated here as S-S/S-S dimer) and 52KDa (S-S dimer), respectively [19]. Multimers fully or partially disulfide linked (>200 kDa) that are not denatured are also eventually observed [17]. However, the short exposure time used to minimize film saturation (WB detection), limited visualization and quantification of multimeric forms detected by the Ab-PRDX2 antibody. Using the Ab-$PRDXSO_{2/3}$ on RBC lysates analysed by a non-reducing SDS-PAGE condition, PRDX2 that contain sulphinylated (SO_2H) cysteins is exclusively detected [20].

The WB results using Ab-PRDX2 showed higher abundance of PRDX2 monomers in OSA morning compared to the ones from Snorers (Figs. 3A and 4A). After PAP treatment, these levels were much increased (Fig. 4A). The WB using Ab-$PRDXSO_{2/3}$ confirmed that the higher levels of PRDX2 monomers in OSA were mostly overoxidized (Figs. 3B and 4B). Interestingly, the increased levels of PRDX2 monomers after PAP treatment might mostly correspond to reduced forms since their overoxidation level showed significant decrease after treatment (Fig. 4B).

In OSA, although the morning level of PRDX2 monomer was not significantly different compared with evening, its overoxidation level was higher than evening (Fig. 3B). In Snorers, the amount of PRDX2 monomer as its overoxidation level were, in contrast, significantly lower in morning compared with evening ones (Fig. 3B).

In OSA, the level of evening PRDX2 S-S/S-S oxidized dimer presented a tendency, although not significant, to be slightly higher compared with Snores ones, corroborating our Proteomics data (spot 19 in Fig. 1

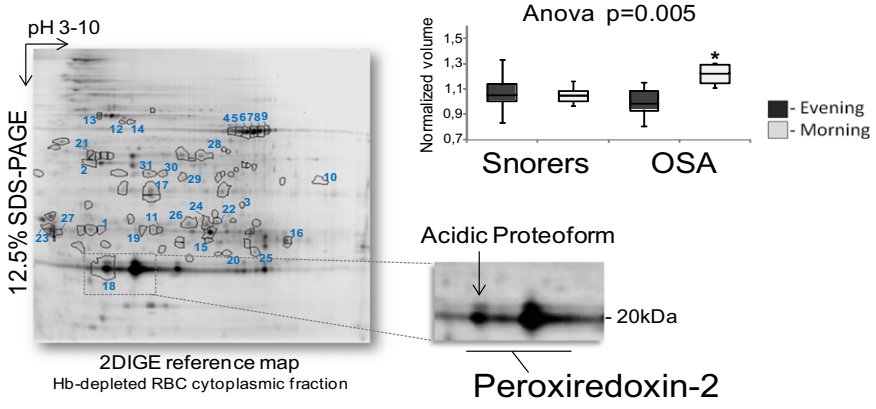

Fig. 1. 2DIGE reference map of Hb-depleted RBC from OSA and Snorers patients collected at evening or morning day time. Differentially abundant protein/proteoforms spots are numbered and indicated with circles on the 2D–gel reference image displayed on the left. Their identity is fully described in Table 3. The acidic form of PRDX2 is one of these differentially proteins showing significantly higher levels of abundance in OSA morning samples as shown on the right.

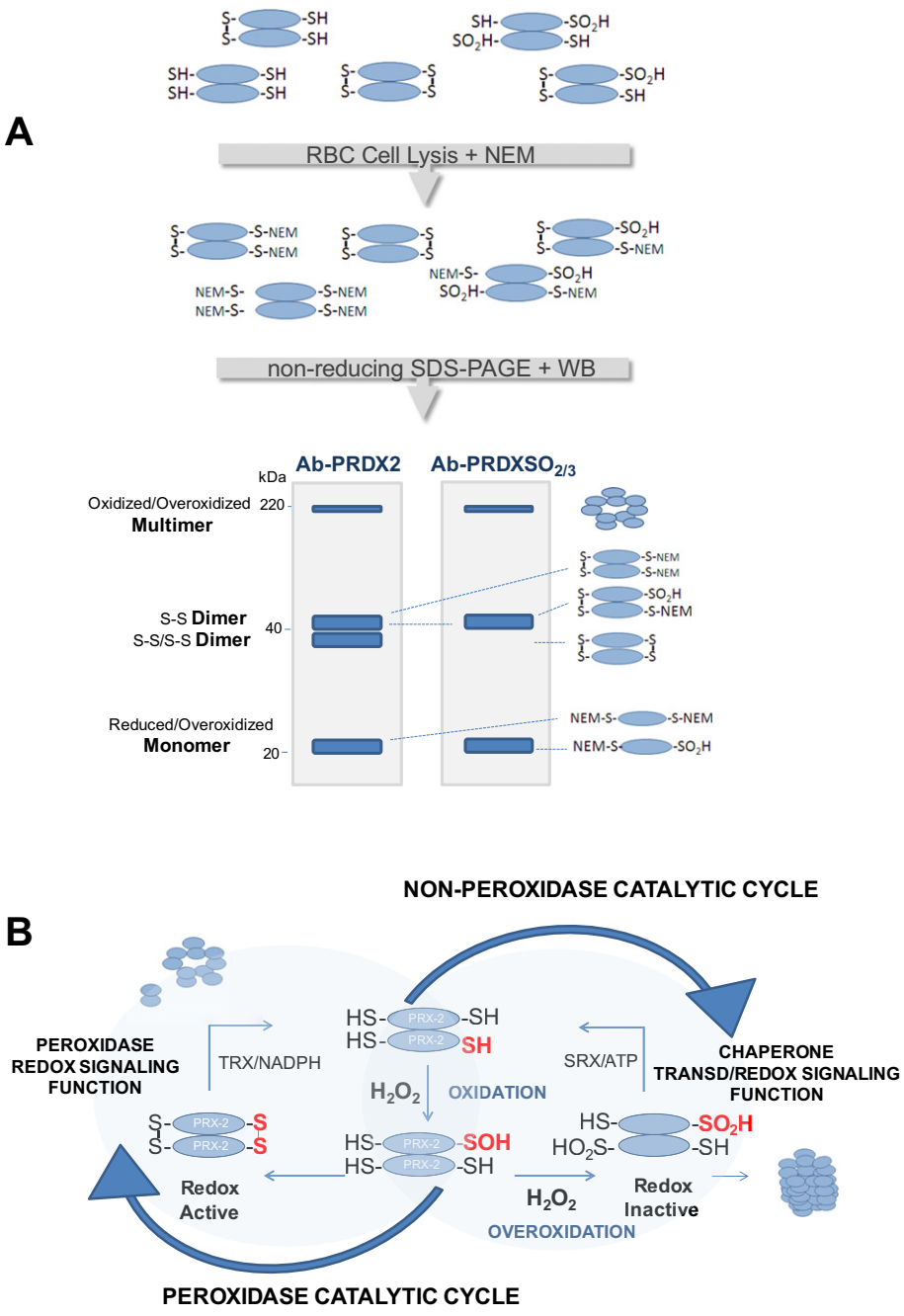

Fig. 2. Monitoring the redox/oligomeric states of PRDX2 as classical 2-Cys PRDX. (A) Measurement of PRDX2 redox/oligomeric states in RBCs with NEM analysed by SDS-PAGE under non-reducing condition followed by Western blotting with Ab-PRDX2 or Ab-PRDXSO$_{2/3}$ (adapted from [17]). NEM is added before and during RBC lyses to prevent peroxidatic cysteines from exogenous-induced oxidation. At non-reducing SDS-PAGE condition, one/two cysteine disulphide bonds linking two PRDX2 monomers into a dimer are kept and migrate at 40 kDa bands, respectively; while PRDX2 dimers composed by fully reduced or fully overoxidized cysteines are denatured into monomers migrating at a unique 20 kDa band (in other cells reduced and overoxidized monomers migrate as two close separated bands). In addition to dimers, PRDXs can form higher order oligomers, predominantly decamers, which are made from reduced, oxidized and/or overoxidized dimers. The four redox/oligomeric states are identified by the Ab-PRDX2. The Ab-PRDXSO$_{2/3}$ detects those PRDXs forms whose peroxidatic cysteines are overoxidized to SO$_{2/3}$H acids. Dimers with two dissulfide cysteine linked are thus not detected by this antibody as represented in the Fig. (B) Summarized catalytic cycles of 2-Cys PRDXs adopting different redox/conformation associated functions . Under peroxidase catalytic cycle, 2Cys-PRDX efficiently controls H_2O_2 concentration through a canonical (re)cycle via thioredoxin(TRX)/NADPH system. Peroxidase activity of 2-Cys PRDXs is not only for oxidant defence but also for H_2O_2 signaling sensing and/or transduction. In presence of high levels of H_2O_2, 2-Cys PRDX undergoes a non-peroxidase catalytic cycle or sulphinic (SO_2H) or overoxidized cycle that is recycled to active enzyme by sulfiredoxin in the presence of ATP. Reversible oxidation is coupled to circadian rhtythm to 2-Cys PRDX function. Overoxidation of 2-Cys PRDXs allows substantial accumulation of H_2O_2 for signalling purposes or to confer chaperone function to 2-Cys PRDX under severe oxidative stress. Chaperone activity is attributted to multimeric decameric rings of 2Cys-PRDXs.

and Table 1- Data in Brief [16]). The level of dimeric S-S form detected by Ab-PRDX2 showed no significant differences between OSA and Snorers whatever was evening or morning (Fig. 3A). However, higher level of S-S/SO_2 dimeric forms detected by Ab-PRDX SO$_{2/3}$ was observed in OSA compared to Snorers (Fig. 3B). Interesting, these higher levels of S-S/SO_2 dimer at morning decreased significantly after PAP treatment

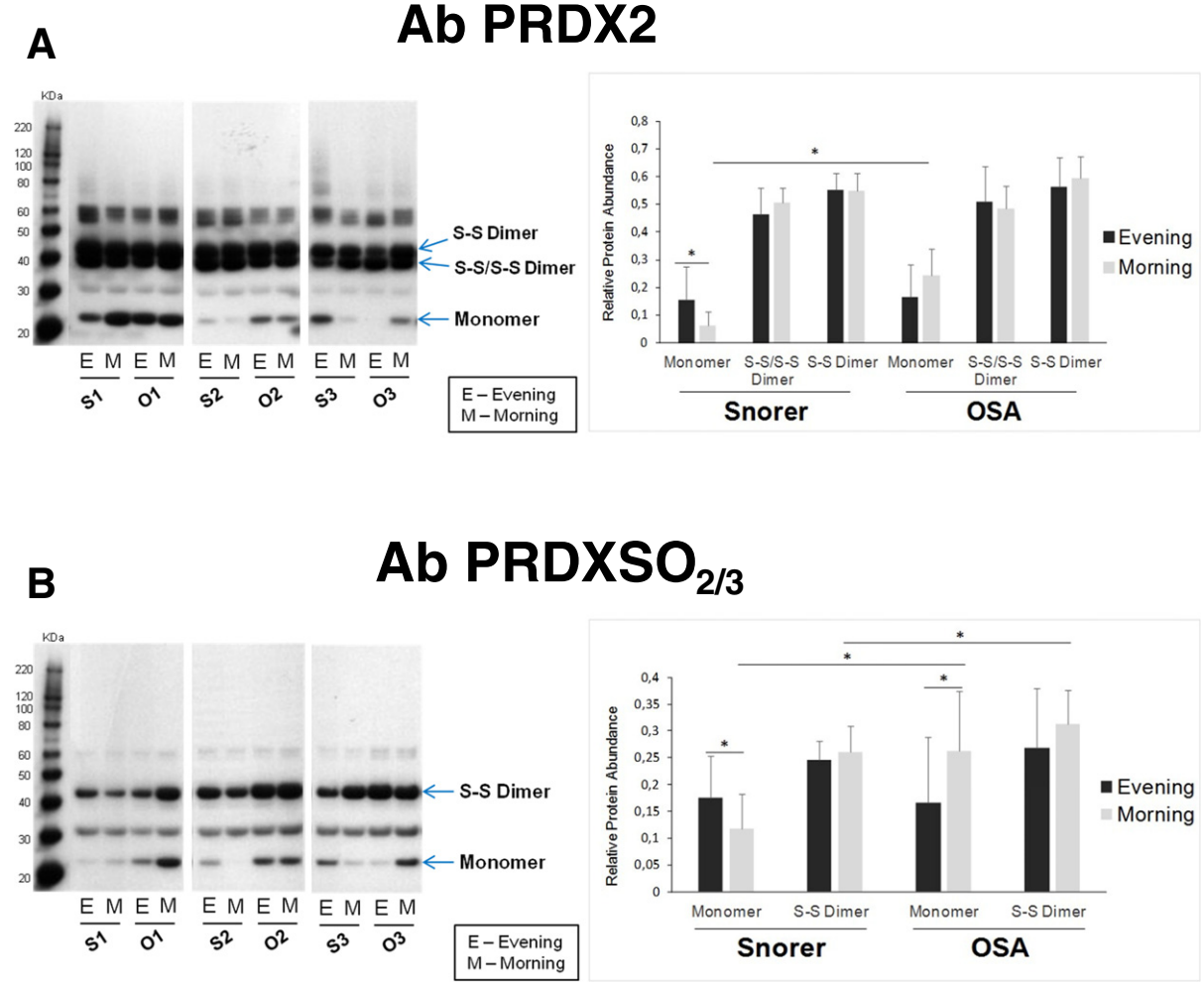

Fig. 3. Western blot validation of 2DIGE proteomics data indicating redox/oligomeric changes in the PRDX2 from OSA evening/morning RBC *versus* Snorers'. Representative western blots (on the left) of non-reducing SDS-PAGE incubated with Ab PRDX2 (A) or Ab PRDXSO$_{2/3}$ (B) and graphic representations (on the right) of the relative normalized protein abundance of the different redox/oligomeric forms of PRDX2 identified in the different groups/conditions [Snorers (S) or OSA/evening (E) or morning (M)] calculated from densitometric analysis (see Material & methods). Statistically significant differences ($p < 0.05$) between groups/conditions are indicated (*).

(Fig. 4A and B). Moreover, multimeric overoxidized forms of PRDX2 were also detected mostly exclusively in OSA patients that underwent six month of PAP treatment (Fig. 4B).

3.4. PRDX2 correlation with OSA clinical parameters

The correlation between the different redox/oligomeric state of PRDX2 at evening or morning time and PSG and biochemical variables were studied. To facilitate interpretation, Table 3 showed the correlations obtained for S-S/S-S dimer and overoxidized (SO_2) monomers and overoxidized (S-S/SO_2) dimer, since they are unequivocally associated with peroxidase catalytic cycle and non-peroxidase (overoxidized) cycle of PRDX2, respectively as represented in Fig. 3B. At evening, the level of S-S/S-S dimer positively correlated to the PSG parameters [RDI, oxygen desaturation index (ODI) and time spent with saturation <90% (T90%)] whereas the level of SO_2 monomer or S-S/SO_2 dimer negatively correlated to RDI, ODI and diurnal oximetry (Table 3).

In morning samples, the level of SO_2 monomer positively correlated with PSG-Arousal Index while the level of S-S/SO_2 dimer positively correlated with glucose metabolism parameters (HbA1C, insulin and HOMA-IR) and negatively with minimum oxygen saturation. No correlation was observed for morning level of S-S/S-S dimer with clinical variables under study (Table 4). After PAP treatment, the level of morning overoxidized monomer and dimer positively correlated with triglycerides. Overoxidized dimer and multimer negatively correlated with dopamine and fasting glucose parameters and positively with MCH hematological parameter (Table 4).

4. Discussion

This study examined for the first time the effect of OSA on the RBC proteome homeostasis, which dysregulation may lead to OSA severity and outcomes. Samples collected at two time points ('evening' and 'morning'), i.e., before and after night PSG, were investigated by 2DIGE-based proteomics. Proteins/proteoforms associated with RBC stress response and antioxidant regulatory system were the most changed in OSA compared with Snorers when investigated in evening or morning samples [16]. Since RBCs are devoid of any translational machinery, these day-night changes might result from PTM regulations.

PRDX2, a member of six thiol peroxidases playing a key role in the antioxidant defense and redox signaling [17], is one of those proteins changed in OSA RBC. PRDX2 is the third most abundant protein in RBC, which circadian redox events has been reported as involved in the mechanism for timekeeping [21]. PRDXs have been demonstrated to regulate several diseases related to redox-dependent process [22]. Modulations in the redox state or oligomeric structure of PRDXs can have a significant impact on their function and thus in the signaling cascades of events in which it participates [21]. In our proteomics study, acidic forms of PRDX2 described as overoxidized [17] were significantly increased in OSA morning RBCs.

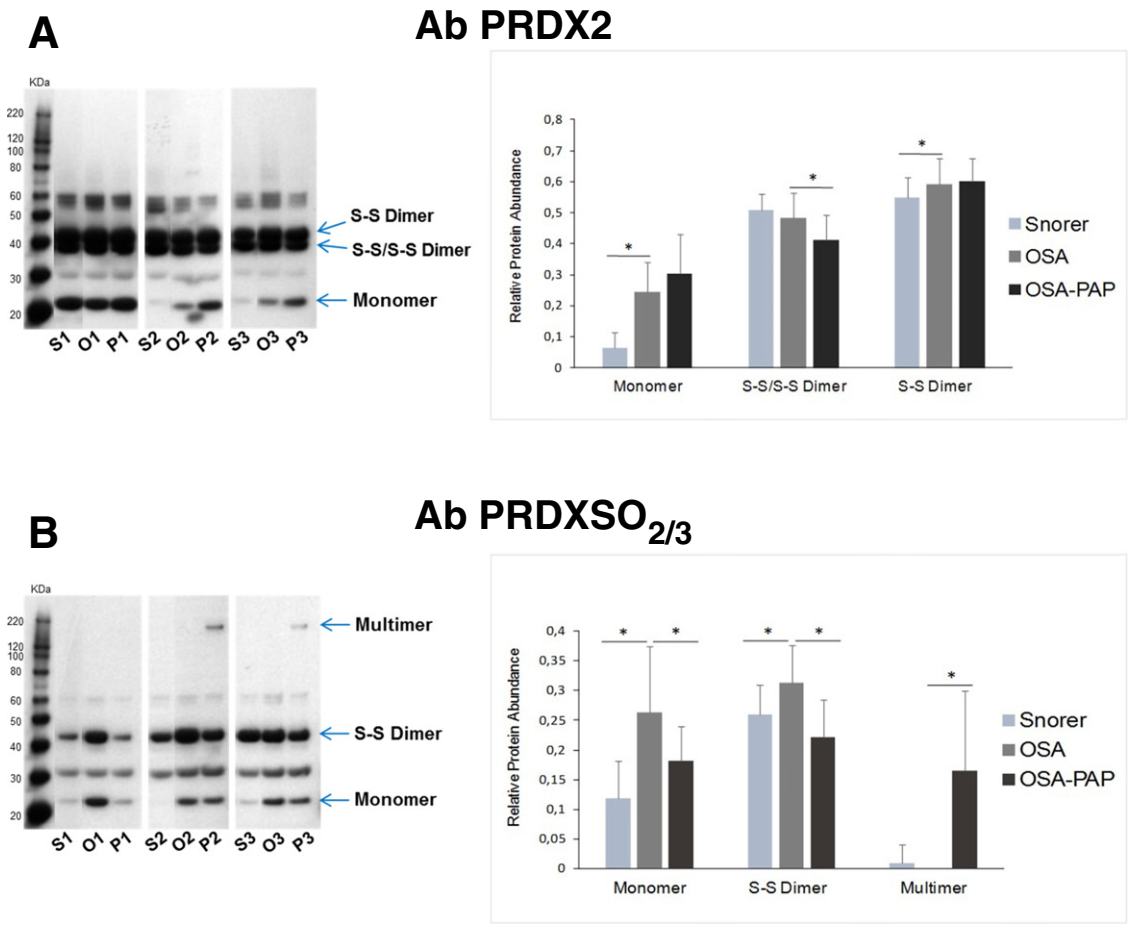

Fig. 4. Effect of six month of PAP treatment on the redox/oligomeric state of PRDX2 from morning RBC. Representative western blots images (on the left) of non-reducing SDS-PAGE incubated with Ab PRDX2 (A) or Ab PRDXSO$_{2/3}$ (B) and graphic representations (on the right) of the relative normalized protein abundance of the different redox/oligomeric forms of PRDX2 identified in the different patients group (Snorers (S), OSA before and after PAP (P) calculated from densitometric analysis (see Material & methods). Statistically significant differences ($p < 0.05$) between groups/conditions are indicated (*).

Table 3
Correlation of redox/oligomeric state of PRDX2 to PSG and metabolic variables under study.

	Evening			Morning		
PRDX2	Correlate	Pearson r value	*p* value	Correlate	Pearson r value	*p* value
S-S/S-S dimer	RDI	0.633*	0.049	No significant correlation observed		
	RDI	0.633*	0.049			
	ODI	0.657*	0.039			
	T90%	0.727*	0.017			
SO_2 monomer	RDI	−0.399	0.081	PSG-arousal index	0.482*	0.031
	RDI	−0.399	0.081			
	ODI	−0.474*	0.035			
	Diurnal oximetry	−0.823**	0.000			
S-S/SO_2 dimer	Diurnal oximetry	−0.467*	0.038	Min O_2 saturation	−0.586**	0.007
				Hb A1C	0.452*	0.045
				Insulin	0.448*	0.048
				HOMA-IR	0.448*	0.048

RDI, respiratory disturbance index; ODI, oxygen desaturation index; T90%, the time spent with saturation < 90%; PSG, polysomnography; Min O_2 saturation, minimum oxygen saturation HbA1C, glycated hemoglobin; HOMA-IR, homeostatic model.
** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

PRDX2 are obligate homodimers as minimal functional unit. As typical 2-Cys PRDX, the PRDX2 peroxidatic cystein of one subunit reacts with hydrogen peroxide (H_2O_2) and is oxidized to sulfenic acid derivatives (-SOH) that form a disulfide (-S-S-) bound with the resolving cystein of another subunit. Oxidized dimeric form is subsequently reduced by thioredoxin (Trx) system to enable further peroxidase catalytic cycle of H_2O_2. Occasionally and with increasing H_2O_2 concentration, 2-Cys PRDXs can be overoxidized to sulphinic and sulphonic acids (Cp-

Table 4
Correlation of redox/oligomeric state of PRDX2 to metabolic variables under study after PAP treatment.

	Morning		
PRDX2	Correlate	Pearson r value	*p* value
SO_2 monomer	Fasting glucose	−0.810**	0.005
	Triglycerides	0.713*	0.021
S-S/SO_2 dimer	Triglycerides	0.770**	0.009
	Dopamine	−0.666*	0.035
SO_2 multimer	Fasting Glucose	−0.842**	0.002
	Dopamine	−0.697*	0.025
	MCH	0.713*	0.021

MCH, mean corpuscular hemoglobin.
** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

$SO_{2/3}H$), resulting in the inactivation of its peroxidase activity as explained by the "floodgate" hypothesis [23]. Overoxidation to sulphinic acids may then be recycled to peroxidatic active forms by sulfiredoxin in an ATP-dependent reaction [17]. These two alternative catalytic cycles of 2-Cys PRDXs, the peroxidase one and the non-peroxidase, i.e., the sulphinic acid (overoxidized) one, involves dramatic changes in its quaternary structure and function (Fig. 2B). Reduced 2-Cys PRDXs can exist as decamer or dodecamer forms and dynamically exchange subunits with cellular pool. Upon oxidation, decamer dissociates into dimers to recover peroxidase activity through Trx-reduction recycling. Overoxidation and probably other PTMs interfere with subunit exchange and stabilized the decameric structure into higher-order of multimerization [24]. Reversible overoxidation of 2-Cys PRDXs has been seem as an evolutionary adapted mechanisms to allow H_2O_2-dependent cell signaling purposes, circadian rhythm to PRDXs recycling/function and chaperone function in response to severe oxidative stress [25].

To investigate whether the redox-oligomeric state of PRDX2 is differentially modulated in OSA RBC as indicated by our proteomics data, we performed non-reduced WB assays on these cells using AbPRDX2 and Ab-PRDX$SO_{2/3}$. The data confirmed our proteomics results by showing that in morning samples the level of both PRDX2 monomers and dissulfide linked (S-S) dimers resulting from fully and/or partially overoxidized dimers/oligomers, respectively, were significantly increased and higher overoxidized to SO_2H acid in OSA compared to Snorers. Evidence from human studies has shown that untreated OSA causes oxidative stress [5]. Therefore, our findings strongly suggest that in OSA RBC the higher overoxidation of PRDX2 at morning might be a consequence of overnight hypoxia/sleep fragmentation-induced oxidative stress response.

Although between morning and evening the level of overoxidized PRDX2 monomer changed in both OSA and Snorers, probably a behavior coupled to circadian rhythm, in OSA, the level of this overoxidized form was higher in morning than evening, while in Snorers the opposite was observed, i.e., much lower in morning than evening. Whether this change in OSA causes or results from an imbalance in the circadian system of PRDX2, need to be investigated.

The evening level of PRDX2 S-S/S-S dimer positively correlated with OSA PSG diagnostic parameters such as RDI, ODI and T90% while morning levels of overoxidized forms showed negative correlation with some of these PSG parameters (Table 3). Since this dimeric form results from peroxidase enzymatic activity of the protein (Fig. 2B), these data suggest that at evening the peroxidase cycle of PRDX2 predominates in OSA and is associated with OSA severity.

At morning, no correlation was found for S-S/S-S dimer to any clinical parameters. However, the level of PRDX2 SO_2 monomer showed positive correlation with PSG-arousal index while S-S/SO_2 dimer showed positive correlation with metabolic parameters such as HbA1C, insulin and HOMA-IR (Table 3). These data suggest that at morning the non-peroxidase (overoxidation) cycle of PRDX2 predominates in OSA and is mainly associated with the metabolic status of the patients.

In RBCs, the circadian oscillation of PRDX2 overoxidation is determined by hemoglobin autoxidation [20]. Oxidative stress can induce oxidative PTMs in hemoglobin [26]. PRDX2 bind and stabilize hemoglobin against excessive oxidative stress-induced aggregation [27]. High levels of plasma glucose produce glycated hemoglobin (HbA1C), a marker of diabetes progression, making hemoglobin more amenable to oxidation [28]. Although not diagnosed as diabetics, OSA patients in this study presented higher level of insulin and HOMA-IR compared to Snorers. The HbA1C levels correlated positively ($r = 0.561$, $p = 0.01$) with the glucose levels in these patients (data not shown). This pre-diabetic condition associated with OSA-induced oxidative stress might explain the correlation of morning level of RBC PRDX2 overoxidation with glycemic indicators.

Differential associations of circulating PRDXs (PRDX1,2,4 and 6) levels with indicators of glycemic control and triglycerides in type 2 diabetes mellitus have been reported [29,30]. However, intracellular PRDX2 is a peroxidase and chaperone/transduction signaling protein whereas plasma PRDX2 that is stress-induced secreted from different cells such as activated macrophages presented chemokine-like actions enabling induction of inflammatory responses [31]. More studies will be needed to better define the role(s) of PRDX2 proteoforms in different cells and subcellular location under disease associated with redox imbalance oxidative condition such as OSA and OSA-associated metabolic disorders.

The existence of morning RBC samples of OSA patients after six months of PAP treatment allowed us to investigate the therapeutic effect of PAP in the modulation of RBC PRDX2 at least, at this day time. After treatment, the morning higher level of overoxidation of both PRDX2 SO_2 monomer as S-S/SO_2 dimer showed a significant decrease. Since the total level of PRDX2 monomers showed increases after PAP the decrease in its overoxidation state might be followed by an increase of reduced (-SH) forms of the protein. Interesting, however was that, overoxidized (SO_2) multimer of PRDX2 (>200 kDa) was identified almost exclusively in the majority OSA patients after PAP treatment and in two Snorers patient's controls. Multimeric overoxidized PRDXs is associated with chaperone function, although chaperone function can be independently modulated by protein PTM regulation. Any redox state or multimeric structure of PRDXs is also associated with redox signaling functions of the protein [17].

The therapeutic effect of PAP in the reduction of PRDX2 overoxidation associated with putative activation of chaperone and/or transduction signaling function of PRDX2 as suggested by these findings warrants further investigation of its mechanisms of action and its relation with oxidative stress and/or inflammation control by PAP treatment.

After PAP treatment, the morning level of RBC overoxidized PRDX2 multimer negatively correlated with the level of serum glucose and urine catecholamine dopamine (Table 4). OSA-induced sympathetic activity following an increased catecholamines secretion is associated with cardiovascular disorders and disturbance in glucose metabolism [32]. The effects of PAP on sympathetic activity and glucose homeostasis control are still debated [33,34]. In this study, although no significant changes in the catecholamine and glycemic profiles were observed after PAP, except in adrenalin that slightly increased after treatment, the level of dopamine positively correlated ($r = 0.796$, $p = 0.006$) with glucose level after PAP (data not shown).

Interestingly, RBCs are capable of transport, accumulation and metabolization of catecholamines, whose biological significance remains unclear [35]. Under physiological conditions catecholamines, in particular dopamine, are higher concentrated in RBCs than in plasma via an active transport regulated by insulin [36]. The putative association of RBC PRDX2 to this process as suggested by its positive correlation with insulin and negative correlation with dopamine and glucose in OSA before and after PAP, respectively, deserves further elucidation.

There are some limitations of this study. Sample size, female gender exclusion, OSA group constituted by non-diabetics with higher levels of blood insulin and HOMA-IR and Snorers group constituted by non-completely healthy subjects limit the data generalization. Although patients were instructed to follow a restricted diet for three days before urine/blood collection to minimize its impact in patient's antioxidant status and catecholamine determination, their dietary habits were not fully controlled. The evaluation of two time points (evening and morning) can provide important insights to a sleep disease like OSA but are not sufficient to study circadian rhythmicity of protein expression or activity. Since no evening samples were collected after treatment, further studies on PRDX2 redox-oligomeric state at this day time will be necessary to complete the data. Proteomics and validation analysis were performed on −80 °C stored samples, which could introduce some bias in the molecular events of both control and disease samples.

5. Conclusions

In summary, the present findings demonstrated evening and morning differential alterations in OSA RBC proteome probably in consequence of nocturnal apnea-induced intermittent hypoxia/sleep disruption experienced by these patients. The antioxidant defense and/or redox-signaling involving PRDX2 seemed highly modulated and correlated with OSA severity and/or metabolic status of the patients. The observed proteome changes point toward oxidative stress and/or decreased antioxidant capacity in OSA mainly at morning time but with consequences along the day. Six months of PAP significantly increased chaperone/transduction signaling function of RBC PRDX2 that might be linked with beneficial effects of PAP in improving oxidative-stress and metabolic status of the patients. The redox/oligomeric state of RBC PRDX2 is promising candidate biomarker for OSA disease severity and PAP therapeutic management that warrants further investigation and validation in larger population.

Conflict of interest

The authors have declared no conflict of interest.

Transparency Document

The Transparency document associated with this article can be found, in online version.

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