CATARINA DE ALMEIDA MARQUES

MOLECULAR EFFECTS OF CONTINUOUS POSITIVE AIRWAY PRESSURE THERAPY IN PATIENTS WITH OBSTRUCTIVE SLEEP APNEA: A PROTEOMICS APPROACH

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Mestrado em Biotecnologia

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Assinatura:_____

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Abstract

Obstructive sleep apnea (OSA) is a common public health concern in many countries, including Portugal, causing deleterious effects on metabolic and cardiovascular health. There are still, however, gaps in scientific and clinical knowledge in this field.

Continuous positive airway pressure (CPAP) is considered the first line treatment in OSA, reducing co-morbidities and associated societal consequences such as accidents and cognitive impairment. However, residual sleepiness may persist in some patients and most chronic consequences of OSA may not be fully reversed by CPAP treatment. Therefore, studies are needed to estimate the reversible effects of CPAP (differences pre- to post-CPAP treatment) and irreversible effects of OSA, in other words, estimate the difference between patients with non-treated OSA and patients after effective treatment, when compared to controls.

The objective of this study was to evaluate, using a proteomic approach, the molecular effect of the therapeutic benefit and/or side effects of continuous positive airway pressure treatment in red blood cells of OSA patients with comorbidities, namely diabetes. Notwithstanding the focus on RBCs, plasma samples were also further analyzed to make the results more robust.

The results of the study have shown that PRDX2 is highly modulated in OSA patients with or without diabetes, and is associated with OSA's severity and metabolic status. We reported higher hyperoxidation of RBC PRDX2 oligomeric forms in diabetic OSA patients, and also higher hyperoxidation of plasma PRDX2 and PRX 1/4 in this same group.

CPAP benefits, including glycemic control, may correlate with RBC PRDX2 oligomeric redox-state associated with chaperone activity, and with decrease of plasma PRDXs stress-induced inflammation pathways.

PRDX2 is a promising biomarker candidate for OSA severity and metabolic status.

Key words: OSA-induced hypoxia, PRDX2, redox modulation, glycemic control, CPAP.

Resumo

O Síndrome da Apneia Obstrutiva do Sono (SAOS) é um problema de saúde pública emergente, comum em muitos países, incluindo Portugal. Esta doença é caracterizada por um colapso das vias aéreas superiores durante o sono, que provoca a diminuição ou interrupção do fluxo de ar, normalmente seguido de um despertar do sono. Como consequência, os pacientes sofrem de fragmentação do sono e sonolência diurna excessiva, tendo inúmeras repercussões no dia-a-dia. Além destas, a SAOS pode originar complicações graves, de natureza cardiovascular e metabólica. Entre outras complicações pode-se salientar hipertensão arterial, hipertensão pulmonar, insuficiência respiratória, arritmias e acidentes vasculares cerebrais. Os principais factores de risco para esta doença são a idade, obesidade, género, consumo de álcool e tabagismo.

Além de um distúrbio grave do sono, a SAOS é também uma doença de stress oxidativo. O stresse oxidativo surge quando ocorre uma diminuição da capacidade antioxidante, e um excesso de produção de espécies reactivas de oxigénio e/ou de nitrogénio. Apesar de os radicais livres possuírem um papel importante na regulação de vias de sinalização fundamentais para inúmeras funções celulares, quando há excesso de produção destas formas reactivas, estas podem causar danos em lípidos, proteínas e ácidos nucleicos, afectando diversos mecanismos celulares e fisiológicos. Assim, os repetidos eventos apneicos caracterizados pela hipóxia intermitente induzida pela SAOS estão na base de danos moleculares que, ao longo dos anos, podem revelar-se irreversíveis.

Existem várias medidas terapêuticas para a SAOS, contudo o tratamento de eleição é o CPAP (do inglês *Continuous Positive Airway Pressure*). Este método consiste na aplicação de uma pressão aérea positiva contínua por um ventilador, que é transmitida ao paciente através de uma máscara nasal, mantendo a via aérea permeável e permitindo um fluxo aéreo contínuo.

A utilização do CPAP possibilita uma melhoria clínica imediata, quando usado regularmente, melhorando assim a qualidade de vida dos pacientes. No entanto, as consequências crónicas derivadas da SAOS podem não ser totalmente revertidas pelo tratamento com CPAP. Assim, é importante compreender os mecanismos moleculares

que estão na base da SAOS, para poder melhor compreender os benefícios do CPAP, que podem contribuir para o desenvolvimento de novas terapias para esta doença.

Os glóbulos vermelhos, sendo as células responsáveis pelo transporte de oxigénio às diferentes regiões e órgãos do corpo, são considerados uma fonte de inflamação sistémica que pode conduzir a doenças metabólicas, tais como obesidade, resistência à insulina e hipertensão, patologias reconhecidas como inerentes à SAOS.

Estudos recentenmente desenvolvidos do Laboratório de Proteómica do INSARJ tiveram como objectivo analisar o proteoma de glóbulos vermelhos em doentes com SAOS, bem como os efeitos moleculares do CPAP nos glóbulos vermelhos destes doentes, com o intuito de encontrar potenciais biomarcadores para diagnóstico e/ou monitorização da doença. Foi reportado pelos autores que diversas proteínas se encontravam diferencialmente expressas nos doentes com SAOS e após o tratamento CPAP, sendo a Peroxirredoxina II (PRDX2) uma das consideradas mais relevantes para o estudo.

A PRDX2 é a terceira proteína mais abundante nos glóbulos vermelhos, sendo por isso fundamental para a sua sobrevivência. As PRDXs possuem funções importantes na célula. Além da sua função peroxidática, que ajuda a combater stress oxidativo através da degradação do peróxido de hidrogénio, actuam em vias de transdução de sinal, e possuem também actividade chaperone, dependendo da sua estrutura redox-oligomérica, sendo por isso importantes na protecção dos glóbulos vermelhos contra stresse oxidativo.

Estudos desenvolvidos no Laboratório de Proteómica do INSARJ anteriormente mencionados, mostraram que as formas monomérica e dimérica desta enzima antioxidante PRDX2 se encontravam significativamente hiperoxidadas nos glóbulos vermelhos de doentes com SAOS, principalmente durante a manhã, sugerindo a presença de stresse oxidativo severo que por sua vez leva a uma homeostasia antioxidante alterada, nos glóbulos vermelhos destes pacientes. Seis meses de tratamento com CPAP diminuíram esta hiperoxidação e originaram formas multiméricas hiperoxidadas, associadas à função chaperone da PRDX2, e descritas como estruturas protetoras que ajudam a aumentar a resistência ao stresse celular. Os níveis de PRDX2 oxidada correlacionaram-se com parâmetros polisomnográficos e metabólicos, nesses pacientes. Portanto, o estado redox / oligomérico da PRDX2 nos

glóbulos vermelhos tem sido proposto por esses autores como promissor candidato a biomarcador para gravidade da SAOS e monitorização do tratamento.

Assim, o objetivo deste trabalho consiste na validação da PRDX2 como potencial biomarcador, tendo como alvo de estudo, além da SAOS, a comorbidade diabetes, e os efeitos moleculares do tratamento CPAP nos glóbulos vermelhos destes pacientes. Para este efeito decidimos usar um cohort maior do que o utilizado nos estudos anteriores, composto por um grupo controlo (n = 18 em que dois são diabéticos), um grupo de pacientes SAOS não tratados (n = 18 em que três são diabéticos), e o mesmo grupo de doentes SAOS após seis meses de tratamento CPAP.

Os resultados deste estudo corroboram com os anteriores. Verificou-se que, no geral, a hiperoxidação da PRDX2 nos glóbulos vermelhos é mais elevada em pacientes SAOS, e diminui após seis meses de tratamento CPAP. Contudo, isto só acontece em pacientes SAOS não diabéticos. Após seis meses de tratamento CPAP, os pacientes SAOS com diabetes não revelam uma diminuição da hiperoxidação da PRDX2. Além disto, verificou-se o aparecimento de formas multiméricas hiperoxidadas após o tratamento CPAP, principalmente em pacientes não diabéticos. Estudos de correlação revelaram que os níveis de formas monoméricas e diméricas da PRDX2 correlacionam positivamente com prarâmetros do sono, nomeadamente RDI, ODI, T90, enquanto as formas multiméricas hiperoxidadas correlacionam positivamente com parâmetros metabólicos como a glicémia e hemoglobina glicada, sugerindo que na presença de hipóxia induzida pela SAOS não tratada, estes pacientes podem ter menor capacidade de produzir estruturas multiméricas potetoras na resposta a um aumento de níveis de glucose no sangue, capacidade que pode ser recuperada com o tratamento CPAP.

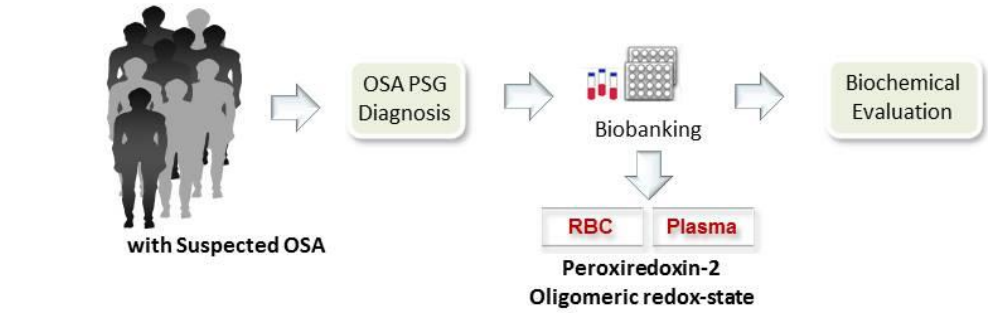
Analisando estes resultados, considerámos pertinente fazer uma experiência semelhante em amostras de plasma, utilzando o mesmo cohort, para robustecer o estudo da PRDX2 nestes doentes. O estudo revelou formas oligoméricas diferente das encontradas nos glóbulos vermelhos. Contudo, o estado oligomérico da enzima corrobora com os resultados anteriores: há um amento da quantidade de PRDX 2 em pacientes com SAOS, com ou sem diabetes, e uma diminuição após seis meses de tratamento CPAP, sendo que esta diminuição é significativa em pacientes não diabéticos. Além disto verificámos que os níveis das formas hiperoxidadas correlacionam positivamente com parâmetros relativos ao metabolismo glicolítico

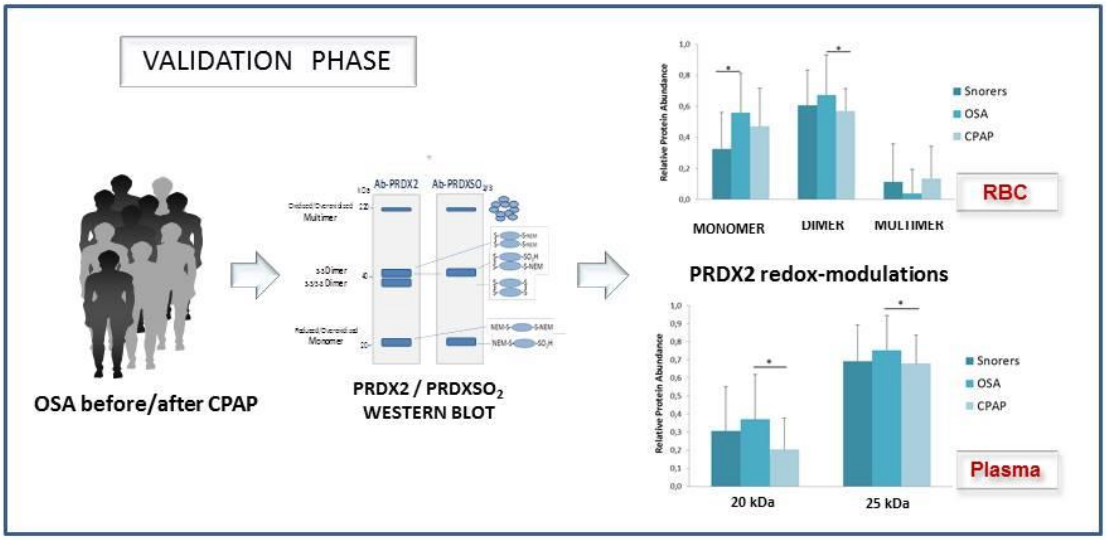
como glicémia, hemoglobina glicada e HOMA-IR. Após o tratamento CPAP estas correlações deixaram de se verificar, excluindo o grupo de pacientes SAOS com diabetes, que as manteve.

Assim, podemos dizer que o estado redox/oligomérico da PRDX2 pode ser considerado um potencial biomarcador para a severidade da SAOS e controlo do tratamento CPAP. Contudo, será necessário recorrer a outro tipo de abordagens que permitam estudar a fundo a PRDX2, nomeadamente as modificações pós traducionais desta enzima, e a sua relação com a SAOS e parâmetros metabólicos dos pacientes.

Palavras-chave: Hipóxia induzida pela SAOS, PRDX2, modulações redox, metabolismo glicolítico, CPAP.

Graphical Abstract





* Note - The boxed scheme represents the work carried out in this thesis project

Highlights

- PRDX 2 is highly modulated in OSA patients with or without diabetes, and is associated with OSA's severity and metabolic status
- PRDX2 and PRX 1/4 hyperoxidation is higher in OSA patients, especially in patients with diabetes, both in RBCs and plasma.
- CPAP benefits, including glycemic control, may correlate with RBC's PRDX2 oligomeric state associated with chaperone activity, and with a decrease of plasma PRDXs stress-induced inflammation pathways.
- PRDX2 is a promising biomarker candidate for OSA severity and metabolic status

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Abbreviations

1DE	One-Dimensional Electrophoresis
2D-DIGE	Two-Dimensional Differential Gel Electrophoresis
2DE	Two-Dimensional Electrophoresis
AHI	Apnea-Hypopnea Index
AHT	Arterial Hypertension
ATP	Adenosine Triphosphate
BMI	Body Mass Index
BP	Blood Pressure
BSA	Bovine Serum Albumin
CBC	Complete Blood Count
COPD	Chronic Obstructive Pulmonary Disease
CPAP	Continuous Positive Airway Pressure
ECG	Electrocardiogram
EEG	Electroencephalogram
EMG	Electromyography
EOG	Electrooculogram
EPW	Epworth Sleepiness Scale
FFA	Free Fatty Acid
GLUT4	Glucose Transporter Type 4
H_2O_2	Hydrogen Peroxide
Hb	Hemoglobin
Hb A1C	Glycated Hemoglobin
HOMA-IR	Insulin Resistance Index
INSARJ	Instituto Nacional de Saúde Doutor Ricardo Jorge
kDa	Kilodalton

LC	Liquid Chromatography
M	Molar Concentration
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
mL	Milliliter
mM	Millimolar
MS	Mass Spectrometry
NA	Not-applicable
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NEM	N-Ethylmaleimide
NS	Non-statistical meaning
ODI	Oxygen Desaturation Index
OSA	Obstructive Sleep Apnea
P95	95^{th} Percentile Pressure
PHT	Pulmonary Hypertension
PRDX2	Peroxiredoxin II
PRDXs	Peroxiredoxins
$PRDXSO_{2/3}$	Hyperoxidized Peroxiredoxin II
PSG	Polysomnography
PTMs	Post-translational modifications
PVDF	Polyvinylidene Fluoride
RBC	Red Blood Cell
RDI	Respiratory Disturbance Index
RDW	Red Cell Distribution Width
RNS	Reactive Nitrogen Species

ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
Srx	Sulfiredoxin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
T90	Time during which arterial O_2 saturation was inferior to 90%
Trx	Thioredoxin
WB	Western Blot
WBC	White Blood Cell
WHO	World Human Organization
µG	Microgram
µL	Microliter

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1 Introduction

Obstructive sleep apnea (OSA) is a common public health disease, inducing nocuous effects on metabolic and cardiovascular health, being thus intimately related to major consequences as arterial hypertension (AHT), stroke and diabetes. The continuous positive airway pressure (CPAP) is considered to be the most successful therapy for OSA, reducing associated co-morbidities and societal consequences. However, chronic consequences of OSA may not be fully reversed by CPAP treatment. Therefore, studies are needed in order to better understand metabolic changes associated to OSA, and to analyze CPAP's efficiency on these patients. The present thesis is focused on identifying means by which OSA can be identified and monitored. A proteomic approach was used to assess the molecular effect of the therapeutic benefit and/or side effects of CPAP treatment in red blood cells and plasma of OSA patients with comorbidities, namely diabetes. The thesis work targets a potential candidate biomarker – Peroxiredoxin II – reported as a promising marker for OSA severity and/or treatment monitoring. In the introduction a general overview of the disease is given followed by a brief consideration about biomarkers and then a more detailed description of proteomics and Peroxiredoxin II as a protein biomarker for OSA.

1.1 Obstructive Sleep Apnea

Obstructive sleep apnea (OSA) is a sleep disorder characterized by recurrent episodes of upper airway collapse during sleep. There is an incomplete obstruction of the pharynx during sleep, which causes cessation (apnea) or significant decrease (hypopnea) in airflow, in the presence of breathing effort [1]. OSA can be defined as a decrease in the oro-nasal airflow of at least 50% and at least 10 seconds, generally associated with reduced levels of arterial O_2 and increased blood levels of CO_2 [2]. These episodes of airway obstruction are usually followed by arousals. As a consequence, of OSA, patients have sleep fragmentation and excessive daytime sleepiness [3].

Hypopnea is defined as a 50 to 80% reduction in airflow, and originates from similar mechanisms to those that produce apnea. It may result in a reduction in

respiratory efforts due to a partial obstruction of the upper airway, and can also lead to increased blood levels of CO_2 and reduced levels of arterial O_2.

Snoring is also a form of partial airway obstruction, which results from a flow restriction and an increase in respiratory efforts, and just like apnea and hypopnea, leads to hypoventilation and/or sleep arousal [4].

The apnea/hypopnea index (AHI) describes the total number of apnea/hypopnea episodes per hour of sleep, which is usually <5 in normal individuals. The severity of OSA is indicated by AHI scores of 5–15 for mild, 15–30 for moderate and >30 for severe, and patients are characterized accordingly [5] as shown in table 1.1.

Table 1.1: Apnea/Hypopnea Index[6]

Mild	5 to 15 events per hour
Moderate	15 to 30 events per hour
Severe	More than 30 events per hour

1.2 Etiology and Pathogenesis

Sleep and its interaction with the respiratory control system destabilize the capacity of the upper airway to drive air into the lungs [4]. The loss of muscle tone promotes upper airway collapse during sleep, causing intermittent hypoxia, hypercapnia, sympathetic nervous system activation, and arousal from sleep [7].

Among the causes of apneic events, we may highlight a reduced excitatory stimulation, an active suppression of breathing due to inhibitory reflexes (coming either from the cardiovascular system, lungs and chest wall, or other somatic and visceral afferents), and loss of reflexes which normally ensure the maintenance of ventilation and do not rely on chemical control. Airway patency can also be compromised by a small or posterior placed mandible, redundant soft palate, tonsillar hypertrophy, and pharyngeal fat deposition. The recurrent apnea results from the

instability of the respiration feedback control, which causes the venting to occur in cycles, rather than remaining at a constant level [4,7].

The respiratory efforts against the obstruction cause large increases in cerebral blood flow and in blood pressure, since it increases the cardiac output [4].

It has been shown that sleep-disordered breathing is associated with daytime hypertension, so other pathogenic mechanisms must be active. For example, obesity causes pharyngeal fat deposition, which is a major factor. Increased SNS (Sympathetic Nervous System) activity, insulin resistance, systemic inflammation, altered oxidative stress and endothelial dysfunction are also factors that promote daytime hypertension, and are, therefore, intimately related to OSA's pathogenic mechanisms[7].

1.3 Epidemiology

Despite spending about a third of our lives asleep, it is only recently that the impact of the physiological state on breathing began to be better known. In fact, only in the 70s did the term " sleep apnea syndrome " emerge [8].

Obstructive sleep apnea is widely recognized as a major public health concern with numerous societal consequences, such as increased cardiovascular morbidity, heightened risk for metabolic dysfunction, and behavioral and cognitive deficits leading to impaired work performance and productivity [5,9]. The overall incidence is approximately 2% to 4%, with a predominance of males from 2:1 to 4:1. The prevalence of OSA varies widely, ranging from 14.7% to 36.5%, depending on gender and nationality. OSA also creates a huge economic burden when compared to other chronic diseases [9].

1.3.1 Risk Factors

The most common risk factors for OSA are: obesity, age over 50 and a neck circumference of more than 41 cm for females and more than 43 cm in males [10]. Gender, ethnicity, craniofacial abnormalities, genetic factors, as well as alcohol and smoking, are also important risk factors to be considered in OSA patients [11].

1.3.1.1 Age and Gender

Several epidemiologic studies show that more than 50% of adults over the age of 65 have some form of chronic sleep-related complaint. This is, in part, due to the variability in sleep parameters, being related with the high prevalence of OSA in advanced ages [11]. An early study reported that 70% of men and 56% of women aged between 65 and 99, had obstructive sleep apnea defined as an AHI ≥ 10 events per hour [12].

Despite OSA being more common in men than women, it is still not clear why [13]. In men, obstructive sleep apnea (AHI>10 events/h) is present in 3.2% of 20 to 44 year olds, 11.3% in 45 to 64 year olds, and 18.1% of those in the 61 to 100 years old age group. On the other hand, in women, the prevalence of OSA (AHI > 15 events/h) is 0.6% in ages of 20 to 44 years, 2.0% in 45 to 64 years, and 7.0% in groups of 61 to 100 years old [14,15]. Since men and women are anatomically different, this prevalence could be attributed to anatomical and functional properties of the upper airways, which might cause different responses in the ventilatory effort and/or arousals from sleep. For instance, imaging studies have revealed that men have increased fat deposition around pharyngeal airway when compared with women [13].

Also, hormonal differences play a role in the predisposition to abnormal breathing during sleep, and may help to explain the high prevalence of OSA in men, and women's susceptibility in the post- menopausal phase, to also develop this disease[11,13].

1.3.1.2 Obesity

Body weight has been identified as the major risk factor for obstructive sleep apnea by many epidemiologic studies around the world. More than 60% of patients referred for a diagnostic sleep evaluation exhibit, excessive body weight [11,16]. It is known that an increase in body weight may lead to the development of moderate to severe OSA, accelerating the progression of the disease [11]. For instance, fat deposition around the abdomen often leads to a reduction of the residual capacity. A reduced lung volume causes tethering effects on the upper airways, since it is associated with diminished oxygen stores, which may contribute to ventilator control instability. Finally, obesity has been associated with functional deterioration of the upper airway

muscles, also caused by fat deposition around the neck, which induces a reduction in functional residual capacity with a resultant decrease of the caudal traction on the upper airway [16].

Body weight is a critical factor, since the pathophysiology of OSA is directly linked with obesity, with an estimated 58% of moderate to severe cases being caused by a BMI greater than or equal to 25 kg/m^2. In order to minimize the current epidemic of obesity and, consequently, OSA, it is fundamental to find effective strategies to achieve long-term and sustainable weight loss along with life quality improvement [11].

1.3.1.3 Genetic and Anatomical factors

Studies using linkage analysis have provided a potential association between specific areas of the genome and OSA pathogenesis, also showing a common hereditary basis to the development of OSA, regardless of whether the patient is obese or not [16]. Still, no causative gene for OSA has been identified until now, but work is underway. Symptoms regarding apnea and the number of apneas per hour of sleep are present at a frequency twice as higher in families with affected members than in control families combined by age, gender and socio-economic level [17].

However, differences in craniofacial morphology may explain some of the changes in the risk of obstructive sleep apnea, in different ethnic groups [11]. Craniofacial dimorphism is associated with a subsequent positioning of the tongue base, which restricts the upper airways [18].

1.3.1.4 Smoking and drinking habits

Smoking and drinking habits have been suggested as possible risk factors for OSA[11]. Current smoking habits are associated with a higher prevalence of snoring and sleep-disordered breathing, and may also worsen the degree of these breathing disorders, when compared with those who have never smoked [13]. Even exposure to second-hand smoke has been independently linked with habitual snoring [11]. This can be explained by cigarette-induced airway inflammation and damage changing the structural and neural properties of the upper airway, increasing thus the risk of collapsibility during sleep [11,13]. On the other hand, alcohol relaxes upper airway dilator muscles, which can prolong apnea duration and worsen the hypoxemia severity.

Alcohol consumption may also suppress arousals and increase the frequency of occlusive episodes. However, the underlying mechanisms are still not well understood [13].

1.4 Symptoms

Symptoms for OSA include snoring, excessive daytime fatigue, apneas while sleeping, morning headaches, concentration problems, forgetfulness, depression, and sexual dysfunction, among others associated with metabolic disturbances. OSA remains mostly undiagnosed, since patients only come to the attention of a clinician when they complain of daytime sleepiness, or when their bed partners witness apnea episodes [19].

Table 1.2: Nocturnal and daytime symptoms for Obstructive Sleep Apnea [8]

Nocturnal symptoms	Daytime symptoms
• Nonrestorative sleep • Frequent loud snoring • Witnessed apneas, which often interrupt the snoring • Gasping and choking sensations that arouse the patient from sleep • Nocturia • Insomnia • Restless sleep, with experience of frequent arousals and tossing or turning during the night	• Morning headache • Dry or sore throat • Daytime fatigue/tiredness • Cognitive deficits; memory and intellectual impairment (short-term memory, concentration) • Decreased vigilance • Personality and mood changes, including depression and anxiety • Sexual dysfunction, including impotence and decreased libido • Gastroesophageal reflux

1.5 Consequences

The recurrent episodes of apnea-hypopnea and arousals during sleep may cause a series of metabolic changes, leading to cardiovascular and neuronal problems, which will condition a significant increase in morbidity and possibly mortality [8].

1.5.1 Arterial hypertension

OSA's association with AHT is frequent. An estimated 30-50% of patients with hypertension have OSA, while 20- 70% of patients with OSA have hypertension. It is known that obesity and age are risk factors for hypertension, and as these factors are very often present in patients with OSA, AHT was initially attributed to obesity and age. However, recent studies have shown that age, obesity and OSA are independent and additive risk factors of hypertension.

1.5.2 Heart function

In OSA, cardiac output decreases during sleep. This is due to negative intrathoracic pressure during the episodes of apnea, which can be significantly improved with CPAP treatment. OSA alone does not seem to cause heart failure. However, some studies showed that OSA may be an important risk factor for the development and aggravation of ischemic heart disease. Also, HTA, hypoxia and increased sympathetic activity during sleep seem to accelerate atherosclerosis. On the other hand, in the presence of an ischemic heart disease, hypoxia caused by apneas may lead to ischemia and cause angina, myocardial infarction or severe arrhythmias. CPAP treatment was shown to improve ischemic cardiopathy [8]. Essentially, ischemia, caused by hypoxia, might stimulate proteins which, in the presence of O_2 (in reperfusion), may overproduce free radicals, which directly cause tissue damage[20].

1.5.3 Cerebrovascular diseases

Patients with OSA have also increased morbidity and mortality of stroke. These patients cerebral blood flow decreases during sleep, whereas intracranial pressure increases in a cyclic manner, reaching its peak at the end of each apnea event.

1.5.4 Pulmonary hypertension

Pulmonary hypertension occurs in about 15% to 25% of patients with OSA. For PHT diagnosis, it is necessary to have a chronic respiratory failure due to a respiratory disease, most often Chronic Obstructive Pulmonary Disease (COPD) or major obesity [8].

1.6 Diagnosis

Several associated features should suggest the diagnosis of obstructive apnea-hypopnea sleep syndrome.

Table 1.3: Features commonly associated with OSA, useful for diagnosis [17]

Features commonly associated with apnea-hypopnea obstructive syndrome sleep
• Obesity • Mandibular/jaw hypoplasia • Systemic hypertension • Pulmonary hypertension • Tonsil hypertrophy • Sleep fragmentation • Sleep associated arrhythmias • Night angina • Gastroesophageal reflux • Compromised quality of life

Routine laboratory tests, such as pulmonary function tests, thyroid function, blood gas analysis, echocardiography or chest X-ray, are generally not helpful in making the diagnosis. Polysomnography is distinguished from sleep studies since it includes sleep staging, which is meant to include an Electroencephalogram (EEG), Electrooculogram (EOG), and a submental Electromyogram (EMG). Additional parameters of sleep include ECG, airflow, ventilation and respiratory effort, gas exchange by oximetry, extremity muscle activity, motor activity-movement, extended EEG monitoring, penile tumescence, gastroesophageal monitoring, snoring, body positions, etc. [21] Given the various symptoms and signs, clinical aspects alone do not allow the prediction with certainty of which patients will or not have OSAs. Thus, the clinical aspect allows only the selection of which patients are indicated for a polysomnographic recording, and only these recordings can truly diagnose OSA.

1.7 Treatment

Nasal continuous positive airway pressure (CPAP) therapy is undoubtedly the treatment of choice for symptomatic OSA patients and/or patients with associated heart or cerebrovascular diseases. However, despite its efficacy, treatment compliance is limited due to the side effects associated with the use of nasal CPAP and to the lack of perception of its benefit by affected individuals [22].

CPAP is effective both for the treatment of long-term obstructive sleep apnea and for the prevention of snoring. The effects depend on the positive level of pressure applied on the respiratory system, as the ideal pressure levels differ among patients.

Even if CPAP is suspended, its effects might last for days, so short interruptions of therapy for example, due to acute clinical diseases or even to have surgery, are well tolerated. However, some failures may occasionally occur: sometimes due to very low pressure, others due to an increase in the pressure required to prevent apnea. Factors such as alcohol consumption, hypothyroidism, and obesity can aggravate the stability of the airways.

Generally, patients accept CPAP therapy very well. However, most studies indicate that some patients (approximately 30%) do not accept it as well [4]. Side effects of the therapy may include a feeling of breathlessness, nasal dryness or rhinitis, ear pain and conjunctivitis. It is important to mention that CPAP's beneficial effects are controversial. Most studies show CPAP contributes to restore an OSA patient's health, by helping them reduce breathing efforts during the night, and this reduces daytime sleepiness and also improves the quality of life. On the other hand, OSA's patients with other major comorbidities and metabolic problems may have trouble using CPAP treatment. This is a controversial matter still under discussion [23,24,25]. Absolute contraindications to nasal CPAP therapy are complete nasal obstruction and fracture of the communicating skull base.

Other options for OSA treatment can be considered, such as intraoral devices, or even surgical interventions, depending on the causes underlying the disease [17].

1.8 Oxidative Stress and OSA

In living cells, oxidation-reduction (redox) reactions are present in fundamental processes of redox regulation, named 'redox signaling' and 'redox control'. Events like intermittent hypoxia often lead to a disturbance in this homeostatic balance, causing oxidative stress, and the consequent formation of reactive oxygen and nitrogen species (ROS/RNS) [26]. As mentioned before, OSA is characterized by a repeated obstruction of the upper airways, during nighttime sleep [27], causing episodes of intermittent hypoxia, which over time may lead to tissue injury in areas such as the cardiac muscle, lung and brain tissues, among others, causing direct damage [20].

Although several mechanisms are involved, such damage is mainly attributed to the production of reactive oxygen species (ROS) during re-oxygenation. These are highly reactive molecules, which interact with lipids, proteins, and nucleic acids, and as such, are responsible for the development of several pathologies [27,28].

Even though they cause oxidative damage, these molecules play a major role in cell signaling mechanisms, acting as "redox switches". For instance, ROS activate an inflammatory cascade, resulting in the increase of proinflammatory cytokines and the expression of adhesion molecules. Therefore, the exacerbated production of these reactive species may result in endothelial dysfunction and, thereby, cause the predisposition to several morbidities in patients with untreated OSA [29].

For this reason, oxidative stress is now considered a major contributor to the cardiovascular consequences observed in this group of patients [30].

Red blood cells (RBCs) are particularly exposed to oxidative stress, mainly due to hemoglobin autoxidation and subsequent constant flux of superoxide (O_2^-). Therefore, intraerythrocytic peroxidase activity is critically relevant, as it plays and important protective role against oxidative stress-induced damage [31].

1.9 Comorbidities in study: Diabetes Mellitus

Diabetes *mellitus* is an endocrine disease involving hormones, namely insulin, produced by the islets of Langerhans in the pancreas. This is a disease of great importance since at present it is a frequently seen disease, almost endemic, affecting

all countries worldwide, all ethnic groups and all social classes. The International Diabetes Federation (IDF) estimates that currently about 246 million people have diabetes worldwide, 46% of whom are aged between 40 and 50 years. By 2025, it is estimated that this number will increase up to 380 million. The most prevalent is type 2 and it currently affects 5.9% of the world adult population, with almost 80% coming from developing countries. The IDF estimates the prevalence of diabetes in Portugal to be about 6-8%.

Diabetes is considered an evolutionary disease affecting quality of life and life expectancy. Chronic complications caused by diabetes affect all organs and systems. It is considered the leading cause of blindness and renal failure in developed countries, and also the most important cause of lower limb amputations and heart and brain macrovascular disease. Neuropathy is another frequent complication which, together with the other outcomes, is highly disabling. The main cause of death in people with diabetes is cardiovascular complications (including stroke). About 3.8 million people die each year from diabetes, which is equivalent to the number of people dying from AIDS [32].

1.10 Classification of Diabetes Mellitus

The classification of Diabetes *Mellitus* adopted in Portugal is based on the standards published by the World Health Organization (WHO) in 1999, defining the classification of diabetes mellitus and other categories of glucose intolerance.

Type 1 diabetes *mellitus* (T1DM) is a specific autoimmune disease of the pancreas. It can appear at any age, but is most common in the first decades of life, with a peak of incidence at 13-15 years. This form of diabetes occurs in about 5-10% of all people who suffer from diabetes. It is believed that there is a genetic predisposition and a precipitating factor for this type of diabetes to develop. This association results in the activation of various immune mechanisms leading to progressive autoimmune damage of the beta cells of the pancreatic islets, starting with a progressive decrease, until exhaustion, of insulin production [32].

Type 2 diabetes *mellitus* (T2DM) is a heterogeneous disease that results from genetic, environmental and metabolic changes, which contribute to a key defect:

insulin resistance and compensatory, but insufficient, secretion of insulin by pancreatic beta cells [32].

Concerning the cellular mechanisms of insulin resistance in type 2 diabetes, there is defective glycogen synthesis, as a consequence of multiple factors, such as the level of glucose transport (GLUT4), hexokinase II and glycogen synthetase activity. Also free fatty acids (FFA) play a key role in the induction of insulin resistance, in obesity and type 2 diabetes. Obesity and physical inactivity, which follow type 2 diabetes in over 80% of cases, are factors which worsen insulin resistance and are thus involved in the growing epidemics of type 2 diabetes.

Signs and symptoms of type 2 diabetes result either from the lack of insulin or from the lack of its action, and include polyuria, polydipsia, polyphagia and weight loss. These classic signs may not be very obvious, and the disease might only be diagnosed late, through the manifestation of chronic complications.

Type 2 diabetes can be prevented or delayed in people at risk (obese, elderly or with diabetic first-degree relatives). Prevention is based on an appropriate and continued exercise program, and a careful diet. Early diagnosis and timely effective treatment are crucial. Nowadays there are a set of oral drugs (oral antidiabetics) that can be used alone or in combination, in order to improve their efficiency. Insulin should also be used whenever oral drugs are unable to correct the disease. Since diabetes is often associated with hypertension and dyslipidemia, these changes must be treated in order to reduce cardiovascular risk [32].

1.10.1 OSA and Type2-Diabetes

OSA is common in people with type 2 diabetes. Given the high prevalence of OSA and type 2 diabetes in older adults, the presence of one of these conditions prompt the evaluation of the other, since up to 50% of patients with OSA have type 2 diabetes, and approximately 50% of patients with type 2 diabetes have moderate to severe OSA. It is known that as age increases, the predisposition for developing type 2 diabetes also increases, as there is a decrease in insulin sensitivity and insulin secretion by the pancreatic beta-cells. This is mainly attributed to fat accumulation and also a reduction in mitochondrial function in muscle [33].

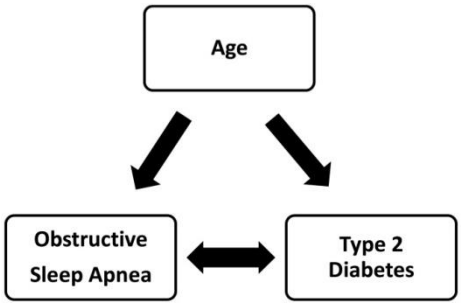

Figure 1.1: Interactions between age, OSA and type 2 diabetes [33]

Age seems to be the main common factor behind both OSA and type 2 diabetes development (Figure 1.1). An increase in age is associated with impairments in sleep quality, and thus increase susceptibility to OSA. Also, it is more likely for older people to develop insulin resistance, glucose intolerance and type 2 diabetes, since intermittent hypoxia and sleep fragmentation play a fundamental role in its development [33]. The mechanisms through which intermittent hypoxia and sleep fragmentation may affect glucose metabolism are illustrated in figure 1.2.

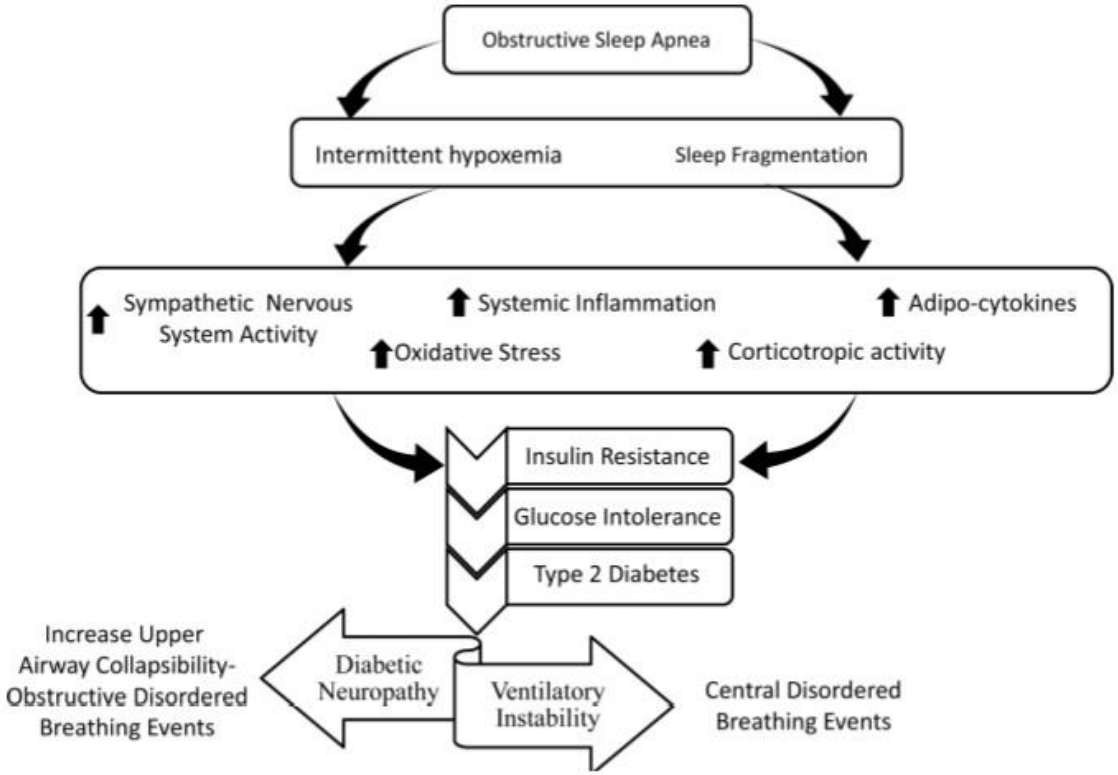

Figure 1.2: Association and causal links for OSA and metabolic abnormalities, leading to type 2 diabetes [33].

OSA may accelerate the age-related progression of glucose intolerance, which eventually leads to type 2 diabetes. Also, OSA may cause an increase in oxidative stress, as well as in the sympathetic nervous system activation, which can lead to a decrease in insulin sensitivity and secretion.

OSA's severity has, concomitantly, been associated with higher fasting glucose and glucose levels, reinforcing the idea that patients with OSA seem to have a higher prevalence of type 2 diabetes and metabolic syndrome. Surprisingly, some studies on CPAP treatment for OSA fail to demonstrate consistent improvements neither in metabolic function, nor in glycemic control. But it is important to notice that these studies may have methodological flaws such as small sample size, limited duration of treatment or even poor adherence to CPAP therapy, and are therefore conclusions concerning the metabolic effects of CPAP in OSA patients may require further investigation [33].

1.11 Biomarkers Discovery

The definition of a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological response to therapeutic intervention (*The Biomarker Definition Working Group, 2001*).

The term "biomarker" was first mentioned in 1973 by the journal *Space Life Science*, but its definition has suffered many alterations until 1980, when biomarkers were employed as a parameter to assess a given biological or clinical process. Still, an incorrect understanding of this definition often leads to ambiguity and its common misuse [34].

Biomarkers are used daily in a clinical setting to assess disease diagnosis, prognosis and risk, as well as for monitoring therapeutic efficiency. For example, the diagnosis of diabetes is given through an evaluation of serum glucose levels, one of the most widely known examples of a clinical biomarker in use, nowadays. In a clinical perspective, biomarker refers to a broad subcategory of medical signs, which are characteristics of biological processes that can be measured accurately and reproducibly [35].

Biomarkers can be placed in different categories, depending on their utility: diagnostic, early detection (screening), monitoring, prognostic, safety, and efficacy biomarkers [36]. In regards to the stages of biomarker discovery and establishment, this is distinguished by three different ranges: exploratory biomarkers, probable valid biomarkers and known valid biomarkers [37]. The process of biomarker development, i.e., discovery, validation, and regulatory approval, is a long, arduous and complex process.

A wide range of technologies can be utilized for detection of biomarkers, including genomic, epigenomic, proteomic and metabolomic approaches [38]. In the present thesis validation of a protein biomarker, Peroxiredoxin II, is described and so a more extensive consideration of this type of biomarker is now provided. In disease biomarker discovery, proteomics have been popular in blood, either in plasma, serum or blood cells. Despite proteomics technologies being in constant development, the most popular procedures are two-dimensional gel electrophoresis (2-D gel) and liquid chromatography (LC) for protein separation, both followed by tandem mass spectrometry (MS/MS) for protein identification [19].

Proteomics refers to the set of technologies applied to explore the proteome, allowing evaluation of hundreds to thousands of proteins at specific timings and under specific conditions. A proteomics approach has innumerous advantages such as gathering information regarding changes in protein abundance, post-translational protein modifications (PTMs), as well as protein–protein cell and tissue interactions, which can be crucial to understanding the true molecular phenotype of a disease, and helping uncover disease biomarkers and disease targets for new drug development [19].

1.11.1 Discovery phase

The first stage of this study was an early biomarker discovery phase, in which RBC samples from OSA patients were analyzed by a 2D-DIGE based proteomic technique, followed by mass spectrometry, for protein identification and protein annotation and classification using bioinformatics tools [39]. Results from this early study led to several proteins to be identified and noted as differentially expressed, one of which, PRDX2, will go through a rigorous validation phase.

1.12 Biomarker Validation

Peroxiredoxin II has been reported to be a good candidate biomarker for OSA severity and treatment monitoring [39]. However, further validation is required. Herein we will proceed for PRDX2 validation as candidate biomarker, regarding its association with OSA associated with comorbidities, namely diabetes, and also study CPAP's molecular implications on these patients, to test its true efficiency.

1.12.1 Biomarkers in OSA

Previous studies reported several differentially expressed proteins in OSA patients, before and after CPAP treatment, one of which was PRDX2, and it was considered to be the most relevant for further validation as candidate biomarker for OSA severity and/or treatment monitoring [39].

(PRDXs) are a large family of thiol-containing peroxidases. Their primary function relies on their peroxidase activity, which contributes to the control of endogenously produced peroxides, in eukaryotes [40]. Besides their role in antioxidant activity, PRDXs are shown to be involved in other cellular functions, such as proliferation, differentiation [41], intracellular signaling [42], and chaperone activity [40], and have also been shown to play an important role in the circadian cycle [43]. Mammalian cells express six PRDX isoforms, which can be found at several cellular compartments [41] – PRDX1, 2 and 6 can be found in the cytosol, PRDX3 in the mitochondrial matrix, PRDX4 in the endoplasmic reticulum, and PRDX5 can be found in mitochondria, peroxisomes and in the cytosol [44]. In human erythrocytes, Peroxiredoxin II (PRDX2) is known to be the third most abundant protein [40].

1.12.1.1 Peroxiredoxin II

2-Cys Peroxiredoxins (PRDXs) are typically present as non-covalent homodimers with a disulfide formation bound between the peroxidatic cysteine of one monomer with the resolving cysteine of another monomer [44].

Reduced PRDXs can be oxidized by a molecule of hydrogen peroxide that bind to their peroxidatic cysteine (SpH) sites, allowing the resolving cysteines (SrH) to form a disulfide bond (S-S) with them (Figures 1.3).

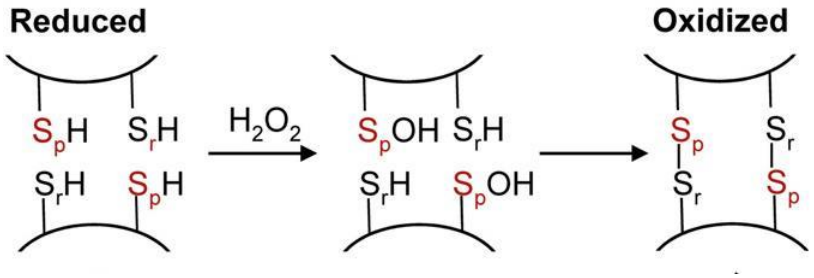

Figure 1.3: The peroxidatic cysteine (SpH) reacts with hydrogen peroxide to form a sulfenic acid. The oxidized cyseine SpOH can then condense with the resolving cysteine (SrH) on the opposing PRDX to form a intermolecular disulfide bond [44].

The thioredoxin system (Trx/NADPH) is responsible for reduction of the 2-Cys PRDXs. The reduction of the disulfide is considered to be the rate-limiting step in the PRDXs catalytic cycle, since both thioredoxin (Trx) and thioredoxin reductase are present at lower concentrations in cells than the PRDXs. Thereby, cells under oxidative stress are expected to intermediately accumulate oxidized PRDXs [44].

A second oxidation may occur in the intermediate Sulfenic form of 2-Cys PRDXs. The sulfenic cysteine can be further oxidized by another hydrogen peroxide molecule, to a sulfinic form (S-SO_2H). This sulfinic form can be recycled by the Srx/ATP cycle (Figure1.4). Sulfiredoxin (Srx) was found to bind to hyperoxidized PRDXs, making it possible to reduce hyperoxidized forms. However, this reduction is very slow. Hyperoxidized forms can still be present in the cell several hours after oxidative stress exposure, making it possible to detect by Western Blotting [43,44]. Also, a further, but irreversible oxidation may occur, when another molecule of H_2O_2 reacts with the sulfinic cysteine (Cys-SO_2H) of a PRDX, turning it into a sulfonic form (S-SO_3H) (Figure 1.4).This reaction effectively removes PRDX from the redox cycle[43].

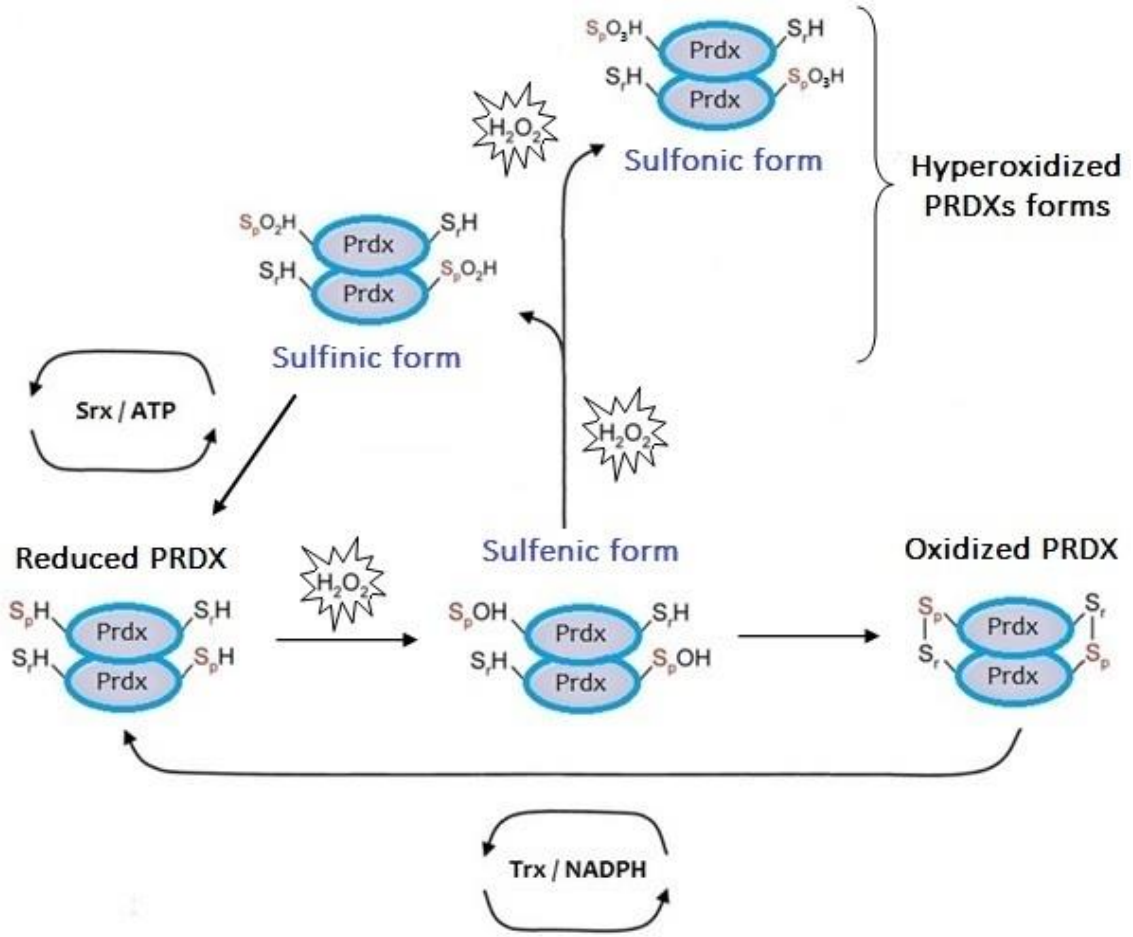

Figure 1.4: Adapted [44] Redox interconversions of typical 2-Cys PRDX, and their recycling cycles.

In human cells, PRDX is in the form of a reduced and oxidized dimer, and can exchange subunits together, to form decamers or dodecamers. The hyperoxidation of PRDX interferes with these changes, causing the formation of hyperoxidized decameric molecules. These hyperoxidized structures can be converted into high molecular mass (oligomers and multimers) which are associated with increased chaperone activity [43].

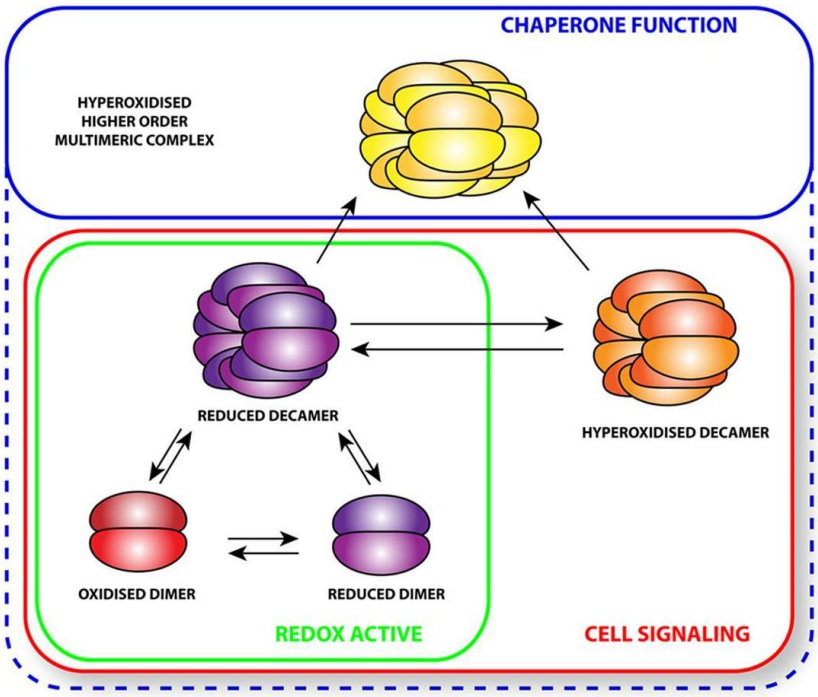

Figure 1.5: Reduced PRDX can exist as decamers or dodecamers that dynamically exchange subunits with a cellular pool of oxidized and reduced dimers [43].

PRDXs have a dynamic quaternary structure within the cell, thus each conformational state is thought to have its own functionality. For instance, as oxidized (S-S/S-S) PRDXs tend to stay in a decameric form, reduced, hyperoxidized or disulfide-linked (S-S) PRDXs can adopt decameric or dodecameric structures. These reduced decameric structures seem to be the most stable, under physiological conditions. The dodecamers rapidly turn over, release oxidized dimers, which are quickly reduced, in order to re-enter the oligomeric state [43].

However, when hyperoxidation into the sulfinic form occurs, this dynamic cycle could be frozen, which leads to the formation of larger complexes, like multimers, promoting functionality changes of PRDX within the cell (Figure 1.5).Therefore, as PRDX function changes according to different oligomeric forms, it is essential to study the oligomeric-redox state of these proteins, since post-translational modifications, such as hyperoxidation, may play a crucial role in oxidative stress-related molecular mechanisms.

The redox-oligomeric state of PRDX2 can be studied by non-reducing SDS-PAGE followed by Western Blotting (WB) with specific antibodies for PRDX2 and PRDX $SO_{2/3}$, to evaluate the amount of oligomeric forms in different reduced, oxidized and hyperoxidized states.

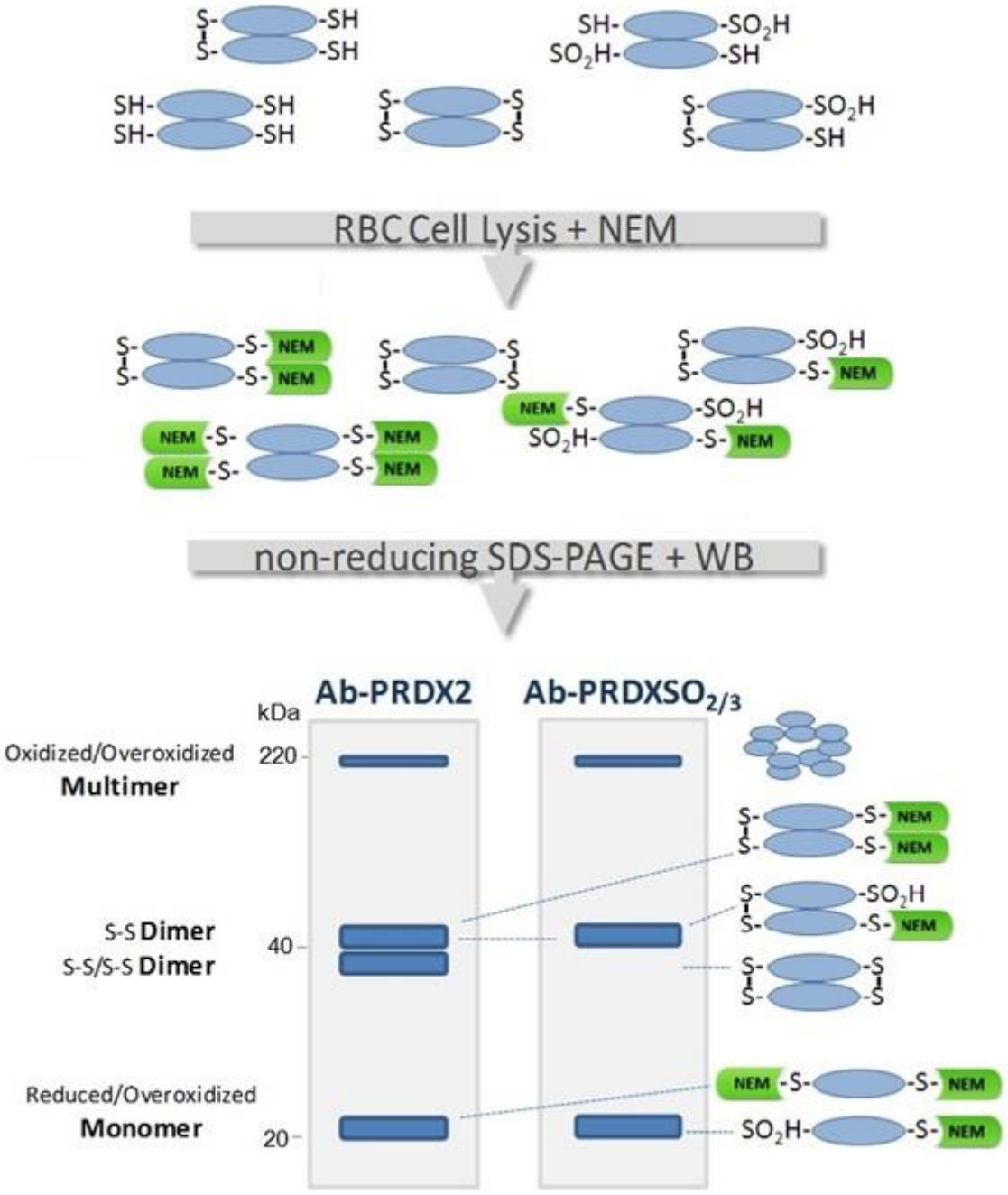

Figure 1.6: Adapted [39]. Measurement of PRDX2 redox/oligomeric states in RBC with NEM analyzed by SDS-PAGE under non-reducing condition followed by Western blotting with Ab-PRDX2 or Ab-PRDX$SO_{2/3}$

In sample preparation, it is very important to add some sort of alkylating agent to help prevent external oxidation or re-oxidation (for example N-Ethylmaleimide – NEM). This compound binds to the dimer/monomer reduced cysteines, preventing external oxidations that naturally occur throughout the study process [44].

All monomeric, dimeric and multimeric forms can be distinguished in different separated protein bands, each one with a different molecular mass, as shown in Figure 1.6. By analyzing all these bands, each one corresponding to a specific protein form, we intend to unveil some answers about PRDX2 oxidation status, and its correlation with clinical and polysomnographic data, trying to understand how it might relate with OSA and OSA severity, with regards to the effects of CPAP treatment, in diabetic and non-diabetic patients.

2 Objective

Recent studies [39] by INSARJ Proteomics Laboratory showed alterations in several proteins, mainly related with stress response and redox regulation, namely, PRDX2, in OSA patients. Previous validation assays confirmed that in the morning, monomeric and dimeric forms of PRDX2 were hyperoxidized in OSA RBCs when compared to evening samples. Also, after six months of CPAP treatment, these oligomeric PRDX2 forms showed a significant decrease in dimer/monomer hyperoxidation, adding a multimeric form that is thought to be related to signaling and chaperone activities. Concerning the correlation between PRDX2 oligomeric forms and the patient's clinical parameters, differences have been shown in insulin and insulin resistance, which were significantly higher in OSA patients than in snorers [39].

Taking these results into account, the aim of this study endeavors to unveil the mechanisms behind the pathogenesis of OSA, and its relationship with the therapeutic response in patients with OSA, with a special emphasis on diabetic patients, as diabetes seems to be intimately linked with OSA severity. The aim was achieved through attainment of the following objectives:

1) Analysis of redox-oligomeric forms of PRDX2 in Control and non-treated OSA groups, in diabetic and non-diabetic patients, both in RBCs and plasma;
2) Analysis of redox-oligomeric forms of PRDX2 in OSA group after six months of CPAP treatment, in diabetic and non-diabetic patients, in both RBCs and plasma.
3) Correlation of efficiency of CPAP treatment and the impact of diabetes on these patients' health and metabolic status.

3 Material and Methods

3.1 Samples and Patients

A total of 104 patients with suspected OSA were clinically analyzed in the Department of Pulmonology, in order to create a set of medical records which collated demographic data, lifestyle, nutritional status, body mass index, blood pressure, heart rate, metabolic profile, and polysomnographic parameters (PSG) for each patient. All the data was further introduced into a database – Biobank. This database included only male patients, with ages between 25 and 55 years of age.

The diagnosis was made by the polysomnographic exam. Before the PSG, patients underwent a restricted diet for three days. This exam was carried during the night, in a sleep laboratory. All these data are archived in a Database associated with a Biobank (plasma and erythrocytes) under informed consent, approved by the national data protection and Ethics Commission of the Northern Lisbon Hospital Center and by the National Institute of Health Dr. Ricardo Jorge.

3.1.1 Sample selection

In this study, three groups of individuals were selected from the Biobank: a group of 18 individuals without OSA (control group – snorers), in which two were diabetics, a second group of 18 individuals with OSA, in which three were diabetics and a third group of 18 individuals (same individual of the second group) after six month CPAP treatment, making a total of 54 different samples. Demographic, polysomnographic and analytical characterization for this cohort is shown in annex III.

3.1.2 Sample preparation

All stock samples (total 54), both RBCs and plasma needed to be previously prepped before running the gel. They were prepared using NEM (*N- Ethylmaleimide*) to prevent eventual external oxidation (Figure 3.1A and 3.1B). The NEM solution was first prepared with ethanol (96%) to a final concentration of 2M, and from this solution we prepared another one, to a final concentration of 100mM, using a physiological saline solution (NaCl 0.9%). Sample preparation is described in Figure 3.1.

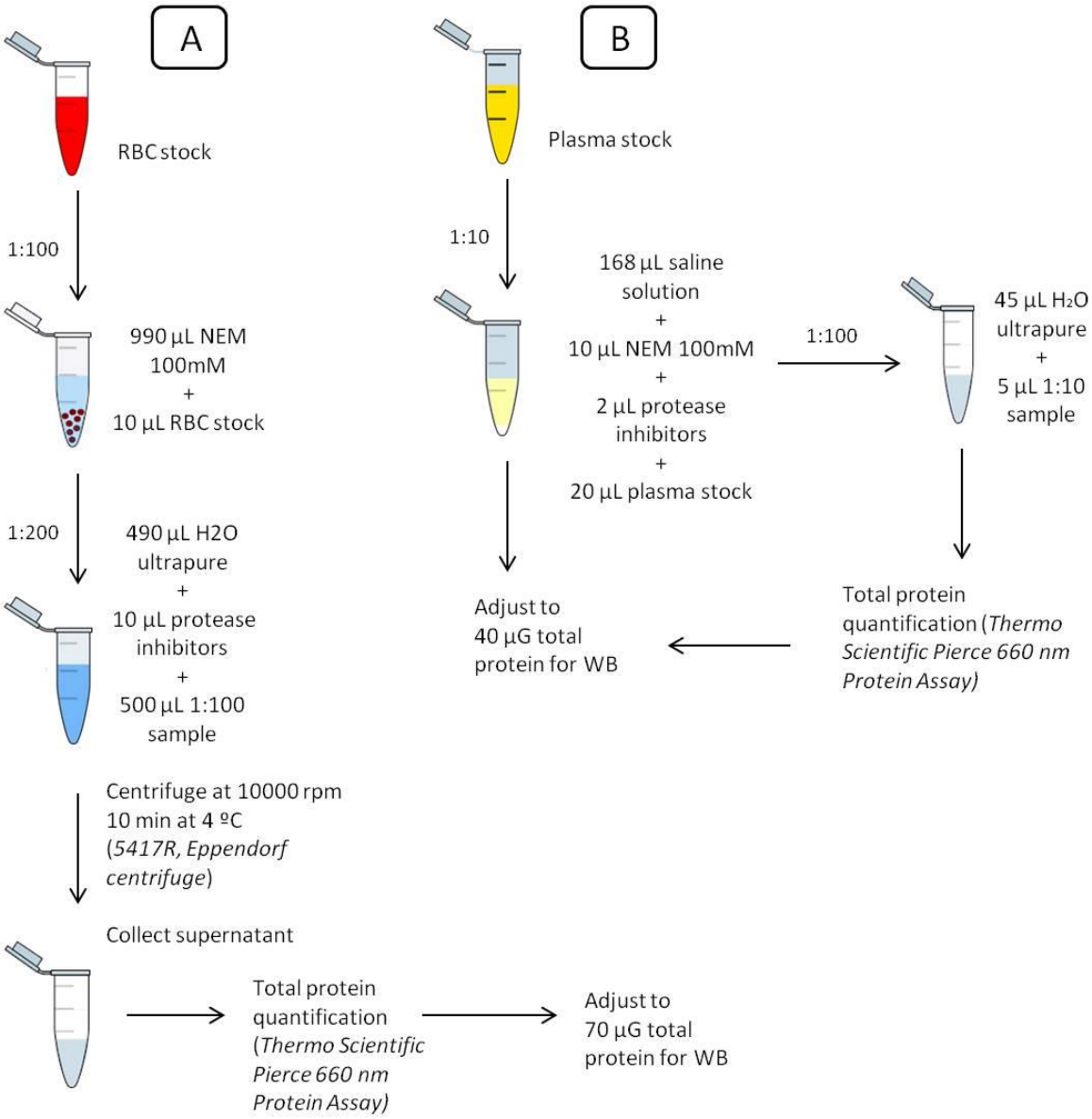

Figure 3.1: RBCs (A) and Plasma (B) sample preparation for WB. Preparation starts with sample dilution using NEM 100 mM and ethanol 96%, with addition of protease inhibitors. RBC and Plasma samples are further diluted with ultrapure water for total protein quantification. Diluted samples are then adjusted for specific total protein concentration for WB – 1:200 dilution is used in RBC sample adjustment for 70µG (A) and 1:10 dilution is used in Plasma sample adjustment for 40 µG total protein (B).

3.1.3 Total protein quantification

All the previously prepared samples were quantified by a colorimetric method – *Thermo Scientific Pierce 660nm Protein Assay* – which includes pre-diluted standards of bovine serum albumin (BSA) and a reagent which is responsible for color changing – Ionic Detergent Compatibility Reagent (IDCR). This is a quick and easy test to use for

total protein quantification and is more linear than coomassie-based Bradford assays, as it is compatible with higher concentrations of most detergents, reducing agents and other commonly used reagents.

The samples were applied one to each microplate well, the reagent is then added and the microplate is stirred for five minutes, or until the color is uniform. The reading of the absorbance was performed in a spectrophotometer – *Spectra max 340, Molecular Devices* – at a wave-length reading of 660nm. Standards absorbance was used to provide a calibration curve, which then allows us to calculate the protein concentration of each sample simply by replacing the absorbance value in the line equation. It is important to note the need to dilute the stock samples to a concentration of 1:200 for RBCs and to 1:100 for plasma. This is absolutely necessary to assure that the absorbance values are in the range of the calibration curve values.

From previous testing, it was decided to adjust the final concentration of RBCs samples to 70 µG, and plasma samples to 40 µG of total protein – results shown in annex II.

3.2 Western Blotting

3.2.1 1DE SDS-PAGE

The nature of the samples used in this study makes them a complex mixture of proteins, thus it is necessary to separate the protein in the sample using their molecular weight. For this purpose, we used 1DE SDS-PAGE.

SDS is an anionic detergent which contains molecules with a negative charge in a wide pH range when dissolved. This detergent destroys the complex structure of proteins (provokes denaturation) and binds to the polypeptide chains, giving them a negative charge. Thus, the negatively charged proteins only depend upon its molecular mass to move along the gel. All gels used in this study were *NuPAGE 4-12% Bis-Tris Mini Gels (1.0mm x 15 well) – NOVEX by life Technologies*.

These precast gels are composed by two different layers, which have different functions. The first layer (stacking gel) is needed to concentrate all proteins in one band, allowing them to migrate at the same time. The second layer (running gel) effectively allows proteins to separate, according to their molecular weight.

3.2.2 Sample preparation for 1DE

The previously prepared samples were adjusted to a final total protein concentration of 70 µG for RBCs (from 1:200 samples) and 40µG for plasma (from 1:10 samples). In order to homogenize the final volume for the race (10µL/lane), it was necessary to freeze-dry RBC samples using different volumes for each sample (see annex II). After lyophilization, it was added 2.5 µL LDS (*Lithium Dodecyl Sulfate*) of *NuPAGE LDS Sample Buffer 4X NOVEX* by *Life Technologies*, and 7.5 µL ultrapure water (type II) was added to each sample, followed by stirring at 70 ° C for 10 minutes, in order to denature the sample proteins.

It was not necessary to freeze-dry plasma samples, since the volume needed to adjust for 40µG of total protein was less than 10 µL. In order to have a final volume of 10L/lane, it was added 1,5 µL LDS and 1 µL β -mercaptoethanol – reduced conditions – it was added ultrapure water and 1:10 diluted plasma was added in different volumes for each sample (see annex II). 2 µL marker (*Magic Mark XP Western Protein Standard, NOVEX by Life Technologies*) was also applied to the gel.

The electrophoretic run was performed under a potential of 150V, for approximately one hour, using a denaturing concentrated running buffer – *NuPAGE® MES SDS Running Buffer* (20X) – 30 mL of this buffer diluted in ultrapure water, to a final volume of 600 mL.

3.2.3 Membrane Transfer

After the proteins were separated by molecular weight, using SDS-PAGE, they were transferred from the gel to PVDF – *polyvinylidene fluoride* – membranes (*Immobilon- P Membrane*, PVDF, 0.45 µm, 26.5 cm x 3.75 m roll, for *Western Blotting* – Millipore*, Fisher Scientific*). PVDF membranes were pre-activated with 100% methanol (v/v) (*Merck*) for 15 minutes with some agitation, and then washed with ultrapure water, before they were incubated with complete transfer buffer (composition in annex I) for 10 minutes, with stirring.

This is a wet transfer, meaning it occurs immersed in transfer buffer, at a low temperature. For this purpose, it was necessary to hold the gel next to the membrane, in constant contact with the buffer, pressured between sponges – in a sandwich. Thus,

the sandwiches were assembled in the following order: pad, 3MM papers, gel, membrane, 3MM papers and pad. Subsequently, the sandwiches were placed in a cuvette with transfer buffer, as shown in figure 3.2.

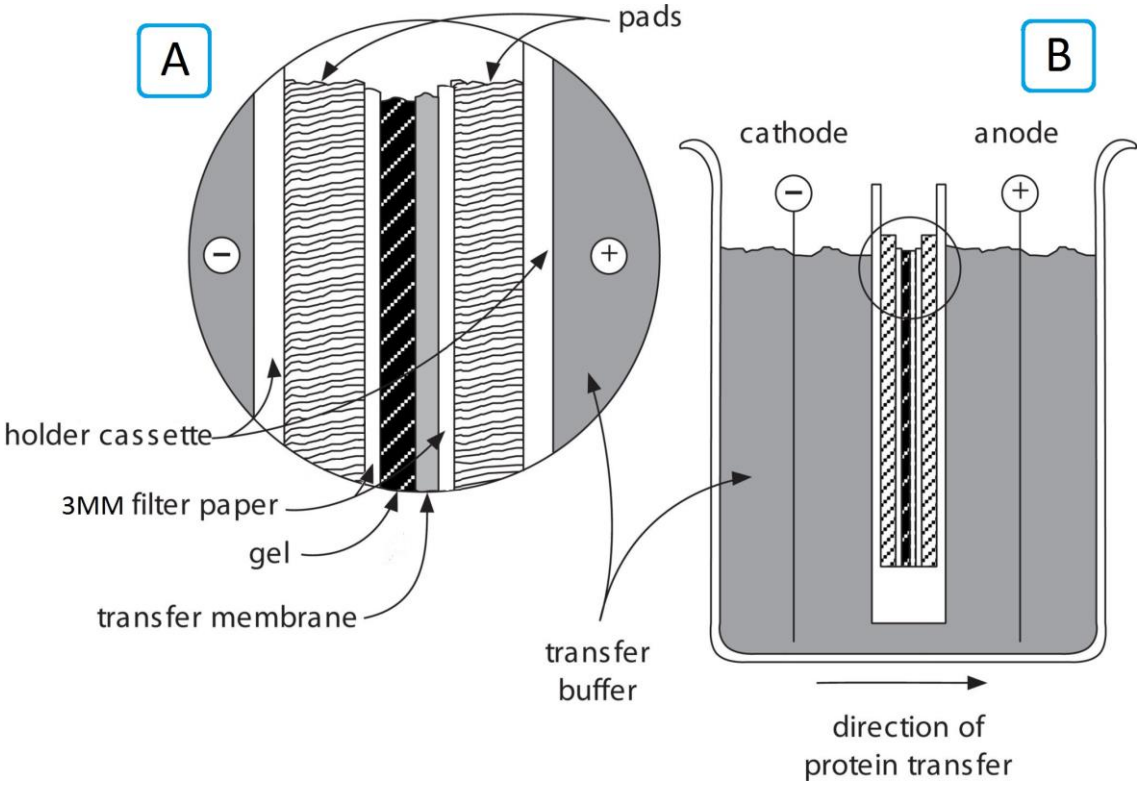

Figure 3.2: Adapted [45]. Electroblotting using a tank transfer unit. After the cassette is assembled (A) it is then placed in a tank containing transfer buffer (B). The membrane is positioned on the anode side of the gel and charged proteins are transferred from the gel onto the membrane.

The 3MM papers and pads used to make pressure and increase contact between the gel and the membrane were also soaked in full transfer buffer. The transfer took place at 10 ° C for 1 hour and 30 minutes at 400 mA.

3.2.4 Verification of protein transfer from SDS-PAGE gels to PVDF membranes

After transference, the membranes were incubated in a 0.1% (p/v) Ponceau solution in 5% (v/v) acetic acid (composition in annex I) over five minutes with stirring, and then discolored for five minutes with 5% (v/v) acetic acid, in order to visualize the transferred protein bands. After reddening, the membranes were scanned, and the images obtained were stored for later analysis. This staining was then removed through several successive washings using PBS-T buffer 1x (Phosphate Buffered Saline

Tween20 solution – composition indicated in annex I) with vigorous stirring, until the dye was completely removed from the membranes. At this stage, the membranes were dried, between 3 MM papers, and vacuum stored at 4 ° C until use.

3.2.5 Membrane Blocking

To avoid non-specific binding (between antibody and membrane), the membranes were blocked with a solution of 5% (p/v) nonfat dry milk (*Nestlé*) diluted in PBS-T buffer, for 1 hour, with some stirring, at room temperature.

3.2.6 Membrane incubation with primary antibody

After the blocking stage the membranes were washed twice with PBS-T, and then incubated with a primary antibody (concentration used is shown in table), with slow stirring, overnight at 4 ° C. The antibodies used in this study were: Anti-peroxiredoxin-2 (Ab-15572, rabbit polyclonal, *Abcam*) and Anti-peroxiredoxin-$SO_{2/3}$ (Ab-16830, Rabbit polyclonal, *Abcam*). All antibodies were diluted in a solution of 5% milk powder in PBS-T.

3.2.7 Membrane incubation with secondary antibody

After incubating the membranes with the primary antibody, a washing cycle was initiated with two brief washes, a 15 minutes wash and ending with three washings of 5 minutes stirring with PBS-T buffer, to remove the excess primary antibody.

The secondary antibody used was anti-rabbit IgG HRP (*GE Healthcare*, UK) which was also diluted in 5% (p/v) milk powder in 1x PBS-T (concentrations in Table 3.1). This incubation was performed for 1 hour with slow agitation, at room temperature. Then began a new washing cycle, identical to the primary antibody wash cycle, differing during the last 5 minutes of washing, where a 1x PBS solution (Phosphate Buffered Saline solution – composition in annex I) was used, instead of PBS-T buffer.

Table 3.1: Dilutions of primary and secondary antibodies used in this study.

RBCs analysis		Plasma analysis	
Antibodies	**Dilutions**	**Antibodies**	**Dilutions**
Anti-PRDX2	1:20000	Anti-PRDX2	1:8000
IgG Anti-rabbit HRP	1:10000	IgG Anti-rabbit HRP	1:3500
Anti-PRDXSO$_{2/3}$	1:3000	Anti-PRDXSO$_{2/3}$	1:2000
IgG Anti-rabbit HRP	1:5000	IgG Anti-rabbit HRP	1:3500

3.2.8 Protein Detection

This step was performed in a dark room, where the membranes were covered with a chemiluminescence detection solution from Amersham ECL Western blotting detection reagents, *GE Healthcare*. The membrane is placed in a cassette and then covered with detection solution (about 1 ml per membrane) for 2 minutes. Then an x-ray film (*AGFA*) was placed on top of the membranes and the cassette was shut in order to impress the sheet. It remained closed for a period of time depending on the luminous intensity of bands for each protein studied – 15 minutes for PRDX2 and 30 minutes for PRDXSO$_{2/3}$. After exposure the x-ray film was revealed in a processor (Medical X-Ray Processor - *Kodak*).

3.2.9 Determination of Protein Relative Abundance

After results for each protein (PRDX2 and PRDX SO$_{2/3}$) identification were obtained, the x-ray films (sheets) were scanned and images were saved in JPEG (16 bit) format. Subsequently, we analyzed the protein bands, using ImageJ – through densitometry. This software allows the selection of an area surrounding the protein/band, and the relative amount of protein can be detected by desitometry.

As mentioned previously, colored membranes (with *Ponceau S*) were scanned and saved as JPEG images. We used these images to quantify the hemoglobin band in RBCs and in plasma we quantified the Albumin band, and we used these values to normalize PRDX2 and PRDX SO$_{2/3}$ values. Further details regarding normalization can be found in annex V.

With the normalized areas obtained by densitometry, graphs were elaborated, with different peaks and areas related to the pixel density of each band corresponding to each protein in study.

The data acquired from ImageJ were analyzed using Excel (*Microsoft Office 2010* version). Means and standard deviations were calculated of all samples – Snorers, OSA and CPAP, diabetic and non-diabetic. These results were used for graphing and statistical analysis.

3.3 Statistical Analysis

Descriptive analysis for clinical and analytical data was expressed as mean ± standard deviation (SD), and frequency (% values) was used to characterize the groups. One-way ANOVA (analysis of variance) was used to statistically compare more than two groups; Student-T for independent samples test was used to compare diabetic and non-diabetic conditions in the same group set (Snorer or OSA). To evaluate the effect of CPAP treatment (before/after) in the OSA group set, Paired Student-T test was used. The level of statistical significance was set at 5% (p value < 0.05).

Correlations of PRDX2 and PRDX $SO_{2/3}$ (RBCs and Plasma) results with polysomnographic and clinical parameters were performed with *IBM SPSS Statistics 24.0* software, using Pearson's correlation tests.

4 Results

4.1 Peroxiredoxin II in RBCs

To analyze the different forms PRDX2, reduced, oxidized and hyperoxidized, WB method was performed under non-reducing conditions, using an antibody against PRDX2 and against the hyperoxidized form, PRDX $SO_{2/3}$, as shown in figures 4.1 and 4.6.

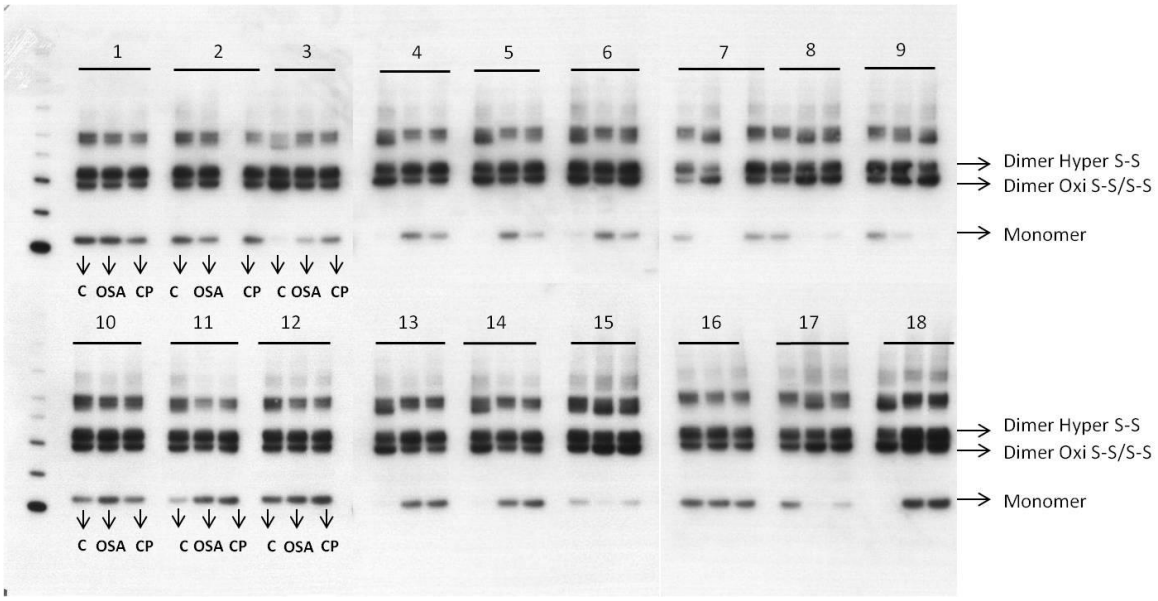

Figure 4.1: RBCs results of PRDX2 Western blot detection using the ECL system and imaging by X-ray processor. Samples were separated on SDS-PAGE gels before transfer to PVDF membranes. Detection of proteins was via the Ab-PRDX2 (dilution 1:20000 – methods, see page 29). The samples were grouped into 18 different groups for ease of analysis. The order of samples in each group is always the same and is indicated in samples 1, 2, 3, 10, 11 and 12 – Control (C), non-treated OSA (OSA) and post CPAP treatment (CP) samples, n = 54. Monomeric – 20 kDa – and dimeric – 40 kDa – PRDX2 forms can be observed.

Figure 4.1 shows different oligomeric protein forms of PRDX2 having different molecular masses that migrated along the gel. These oligomeric forms relate mainly to monomeric and dimeric forms (-S-S).

The results of the quantification of PRDX2 bands (Figure 4.2) reveal differences concerning protein abundance and also concerning its structural forms. In general, we can say the monomeric form of this protein is found in less abundance when compared with dimeric, oxidized and hyperoxidized forms. It is also possible to say that there is an increase in the amount of protein in OSA – CPAP samples compared to the Control group, regardless of its structural form.

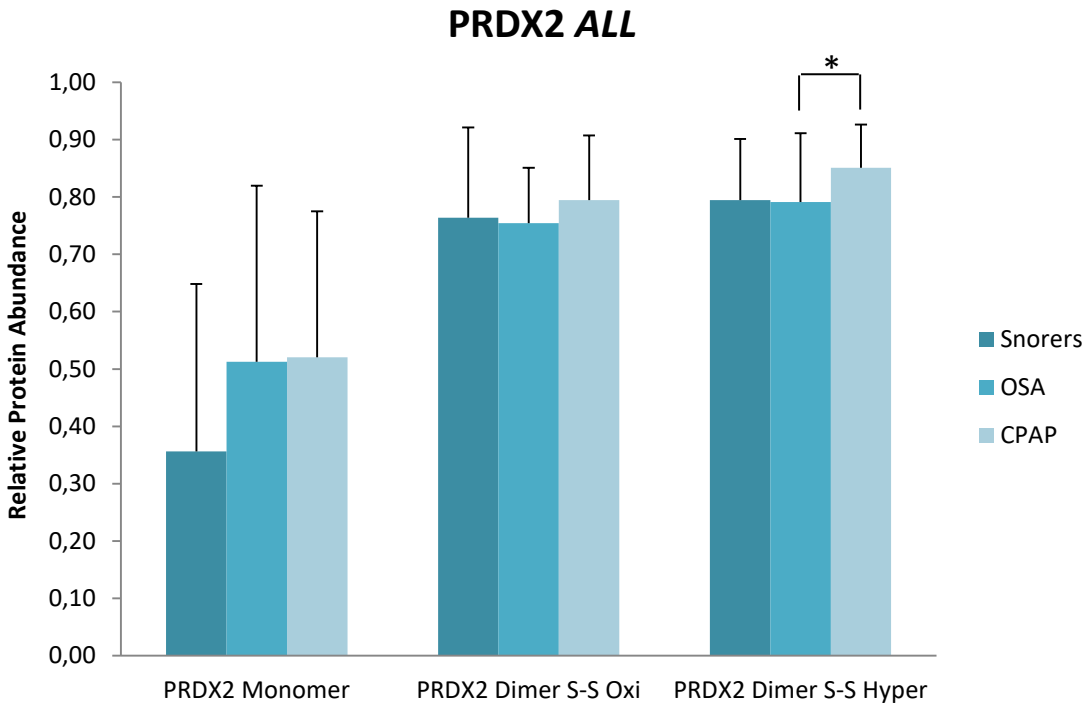

Figure 4.2: RBCs relative protein abundance of PRDX2 monomeric, oxidized and hyperoxidized dimeric forms in samples, in Snorers (n=18) and OSA (n=18) groups, and after CPAP (n=18) treatment. Significant differences between groups are denoted by "*" $p < 0.05$.

Regarding the presence of the monomeric PRDX2 form in the RBCs, it is noticeable a higher abundance of this form in OSA patients when comparing to the control group. After six months of CPAP treatment, this abundance seems to increase in non-diabetic OSA patients and decrease in diabetics (Figure 4.3). These differences are, however, not significant. Only when analyzing the oxidized dimeric PRDX2 form, the increase its abundance after six months of CPAP treatment is statistically significant, in non-diabetic OSA patients (Figure 4.4).

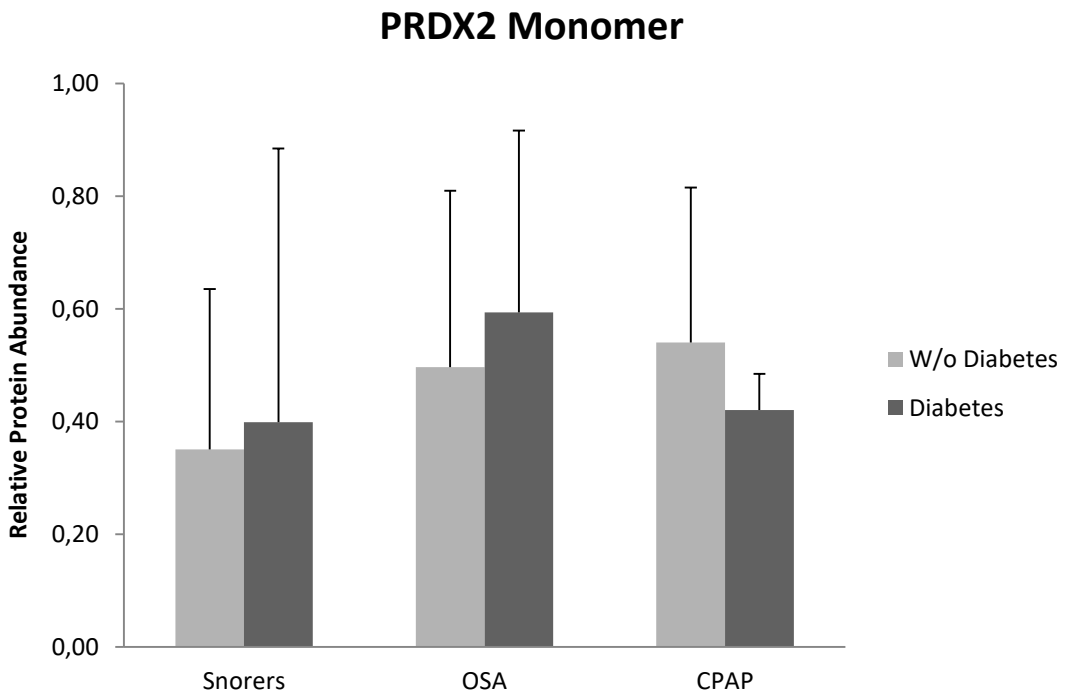

Figure 4.3: RBCs relative protein abundance of PRDX2 monomeric form in samples, in Snorers (n = 18; 16 samples from non-diabetic snorers and 2 samples from diabetic snorers) and OSA group (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients), and after CPAP treatment (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients). No significant differences were found.

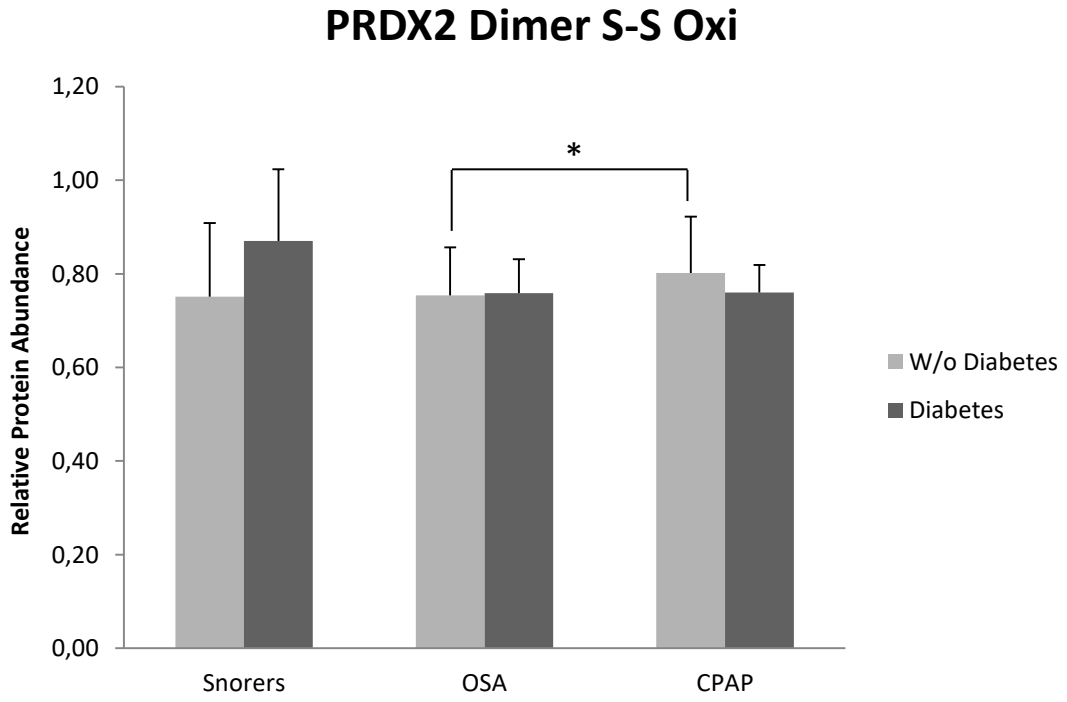

Figure 4.4: RBCs relative protein abundance of PRDX2 oxidized dimeric form in samples, in Snorers (n = 18; 16 samples from non-diabetic snorers and 2 samples from

diabetic snorers) and OSA group (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients), and after CPAP treatment (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients). Significant differences between groups are denoted by "*" $p < 0.05$.

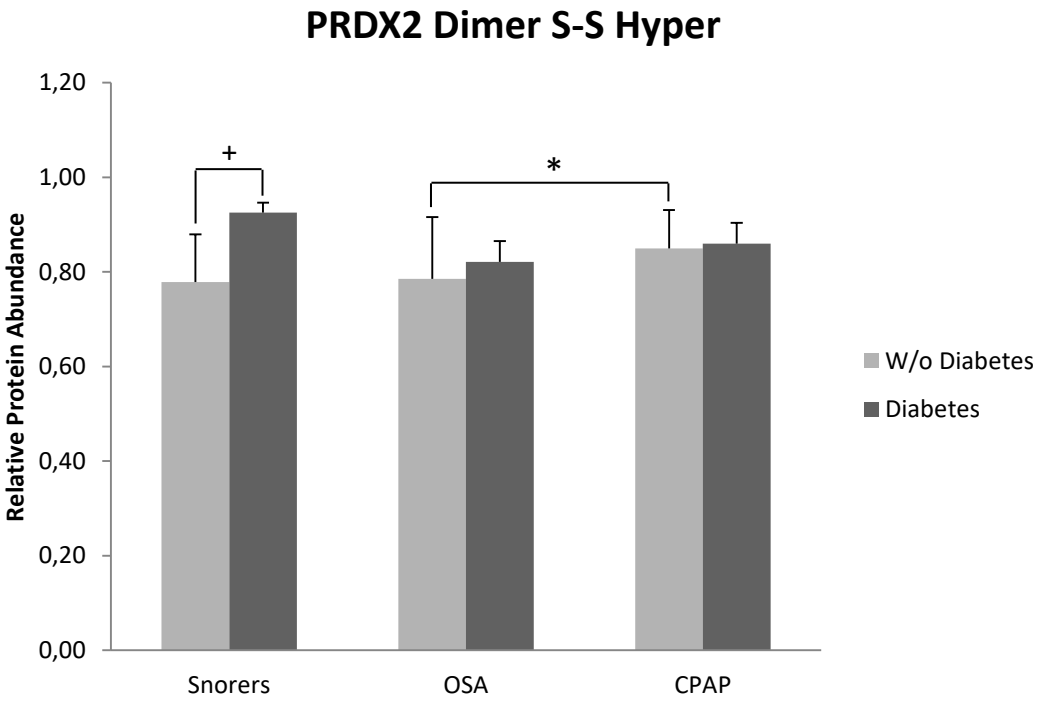

Figure 4.5: RBCs relative protein abundance of PRDX2 hyperoxidized dimeric form in samples, in Snorers (n = 18; 16 samples from non-diabetic snorers and 2 samples from diabetic snorers) and OSA group (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients), and after CPAP treatment (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients). Significant differences between groups are denoted by "*" $p < 0.05$, and inner group differences are denoted by "+" $p < 0.05$.

Concerning the hyperoxidized PRDX2 dimeric form found in the RBCs, it is notable a significantly higher abundance of this protein form in diabetic snorer patients, comparing to non-diabetics. Hyperoxidized dimeric forms seem to decrease in diabetic OSA patients and increase again after six months of CPAP treatment. This increase is significant, however, only among non-diabetic OSA patients (Figure 4.5).

Results for PRDX2 oligomeric forms are grouped and properly marked in Fig. 4.6.

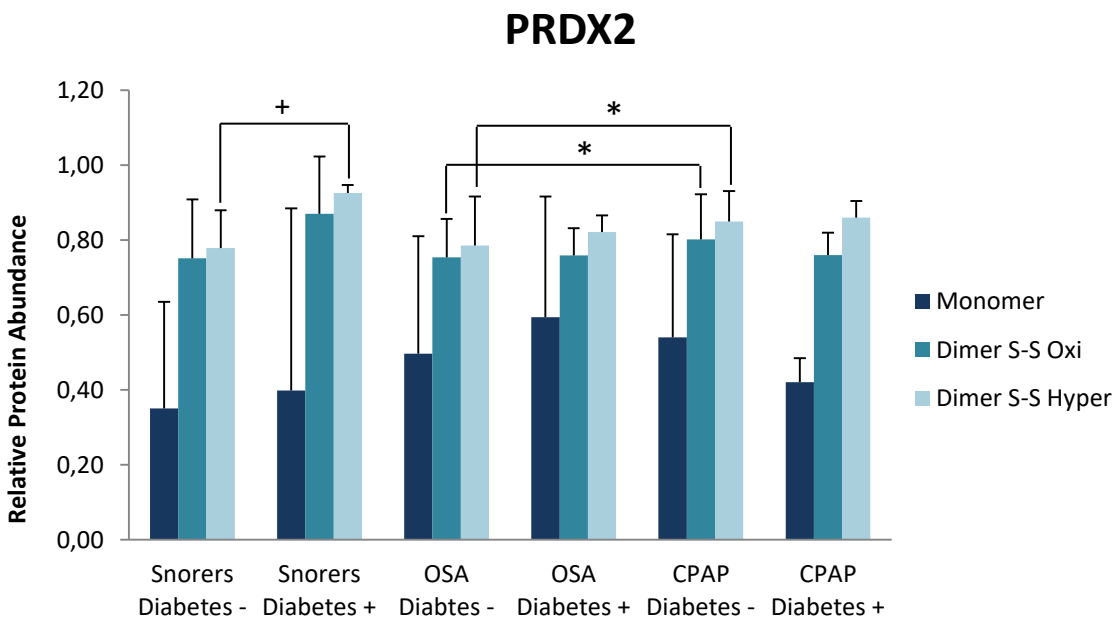

Figure 4.6: RBCs relative protein abundance of PRDX2 monomeric, oxidized and hyperoxidized dimeric forms for diabetic (Diabetes+) and non-diabetic (Diabetes-) patients, in Snorersl (n = 18; 16 samples from non-diabetic snorers and 2 samples from diabetic snorers) and OSA groups (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients), and after CPAP treatment (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients). Significant differences between groups are denoted by "*" $p < 0.05$, and inner group differences are denoted by "+" $p < 0.05$.

To better understand this protein action in patients with OSA and after CPAP treatment, we decided to study hyperoxidation in PRDX2 (PRDX $SO_{2/3}$) through WB technique. Results are shown in figure 4.7.

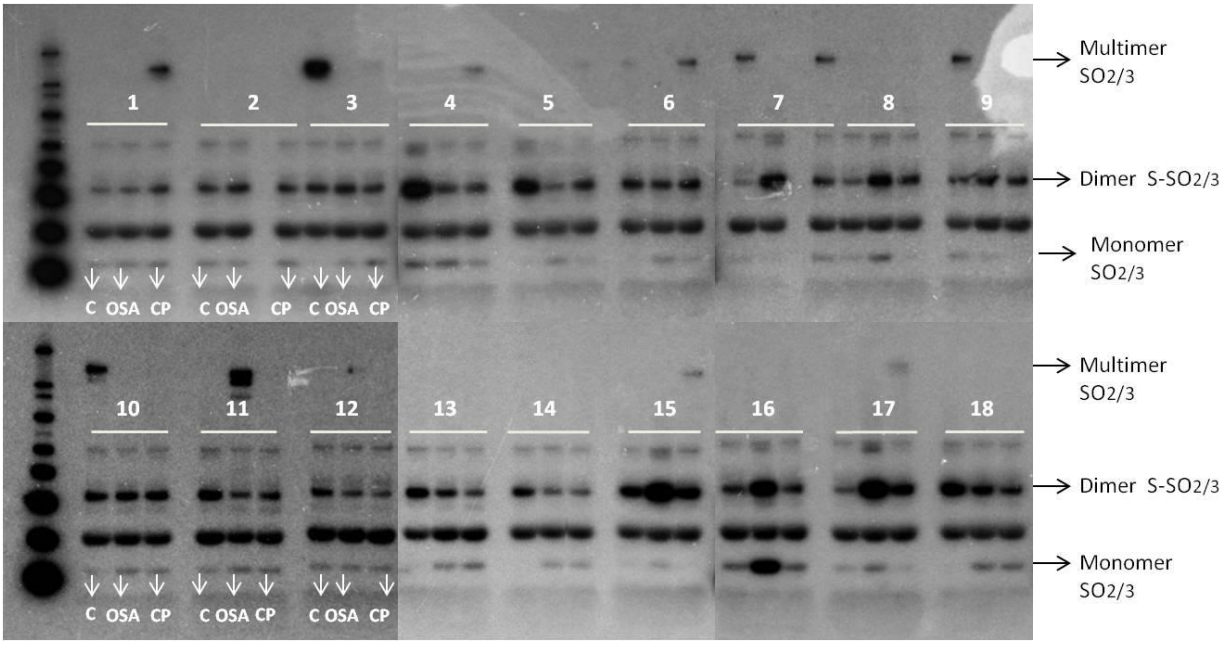

Figure 4.7: RBCs results of PRDX $SO_{2/3}$ Western blot detection using the ECL system and imaging by X-ray processor. Samples were separated on SDS-PAGE gels before transfer to PVDF membranes. Detection of proteins was via using the Ab-PRDX $SO_{2/3}$ (dilution 1:3000 – methods, see page 29). The samples were grouped into 18 different groups for ease of analysis. The order of samples in each group is always the same and is indicated in samples 1, 2, 3, 10, 11 and 12 – Control (C), non-treated OSA (OSA) and post CPAP treatment (CP) samples, n = 54. Monomeric – 20 kDa – dimeric – 40 kDa – and multimeric – 220 kDa – forms can be observed.

In this study, hyperoxidized multimeric forms were detected in some Control samples, OSA samples, and also after 6 months of CPAP (Figures 4.7 and 4.8).

Regarding the quantification obtained from the standardization of the dimeric and monomeric hyperoxidized bands (PRDX $SO_{2/3}$), it is important to note a significant increase of the hyperoxidized monomeric form in the OSA group, when compared with the control, and also a significant decrease of the dimeric form after CPAP treatment. Furthermore, it is possible to observe that, despite being found in lower abundance when compared to the remaining protein forms present in the cells, multimeric forms are present in major quantity in control and CPAP groups, rather than in OSAs (Figure 4.8).

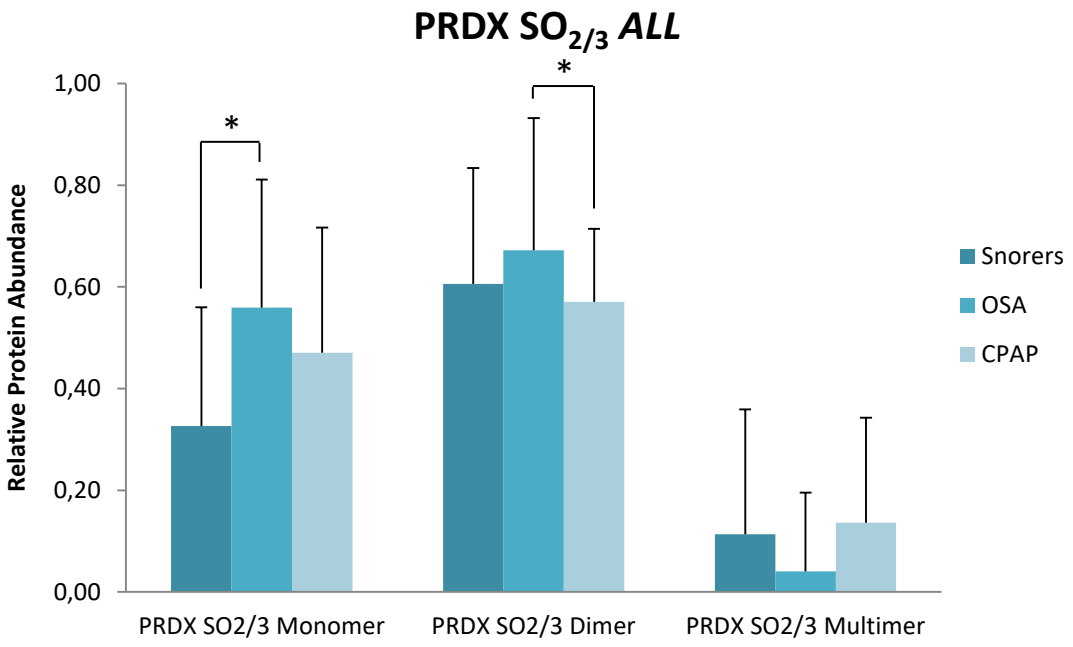

Figure 4.8: RBCs relative protein abundance of PRDX $SO_{2/3}$ monomeric, dimeric and multimeric hyperoxidized forms in samples, in Snorers (n=18) and OSA (n=18) groups, and after CPAP treatment (n=18). Significant differences between groups are denoted by "*" $p < 0.05$.

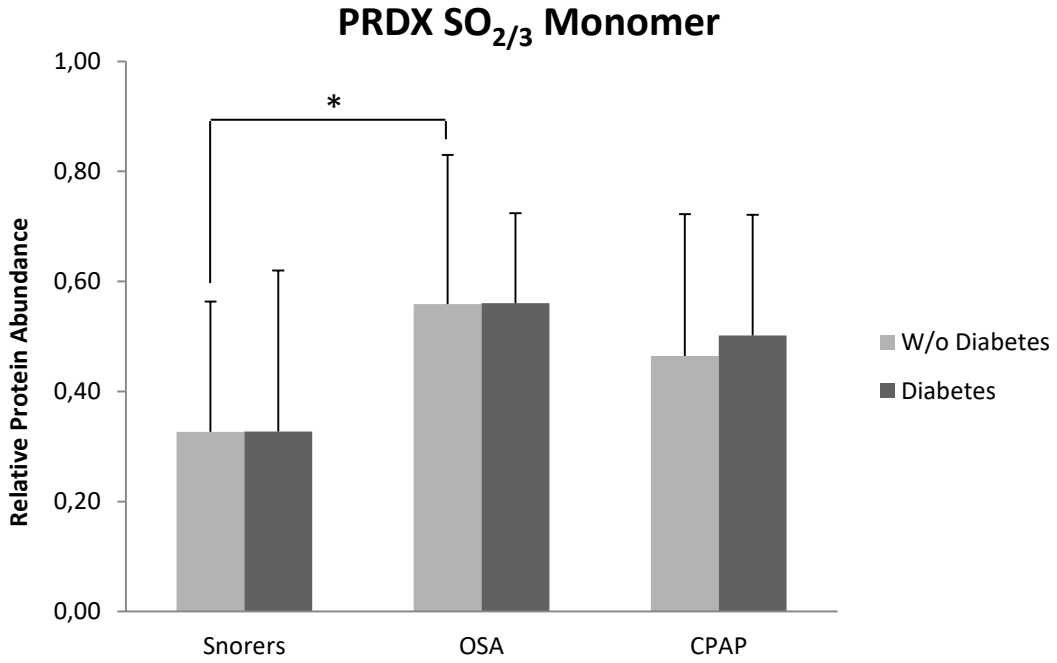

Figure 4.9: RBCs relative protein abundance of PRDX $SO_{2/3}$ hyperoxidized monomeric form in samples, in Snorers (n = 18; 16 samples from non-diabetic snorers and 2 samples from diabetic snorers) and OSA group (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients). Significant differences between groups are denoted by "*" $p < 0.05$.

Looking more closely at the results of the monomeric band PRDX $SO_{2/3}$ (Figure 4.9), and comparing diabetic groups with non-diabetic, no differences are seen, except for a slight increase in the OSA diabetes group after six months of CPAP treatment, however this has no statistical relevance. Only upon analyzing the non-diabetic group, is a significant increase of hyperoxidized monomeric form seen in the OSA group compared to Control.

Regarding the hyperoxidized dimeric form PRDX $SO_{2/3}$ (Figure 4.10), the most significant change relies on an accentuated decrease of relative protein abundance of this form only in the non-diabetic group, after six months of CPAP treatment.

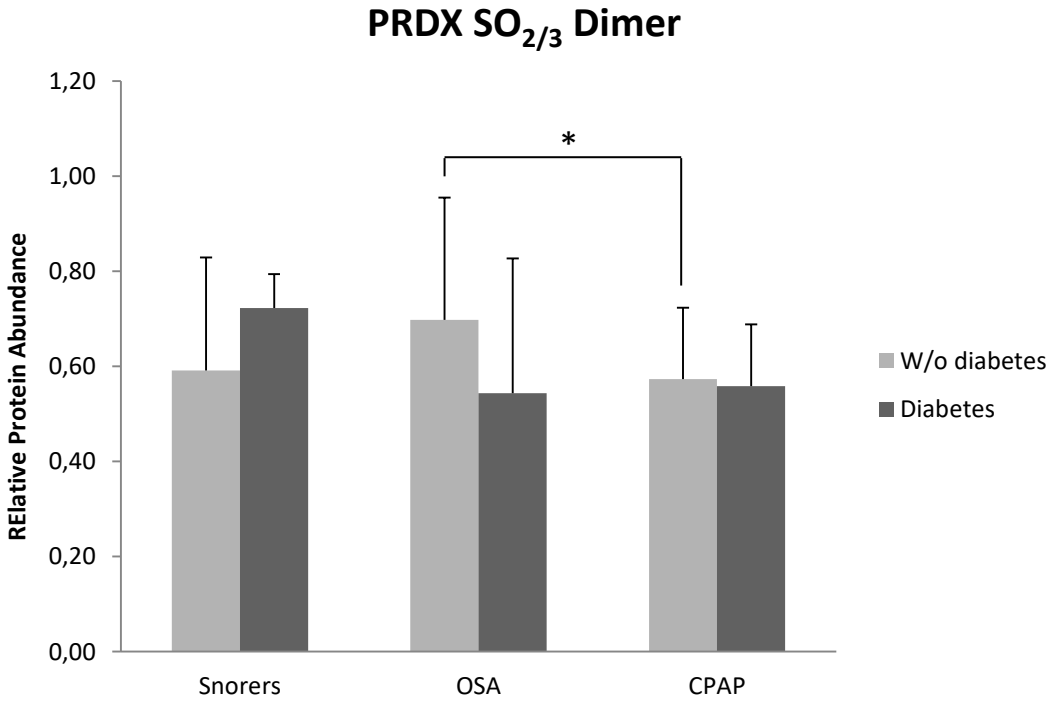

Figure 4.10: RBCs relative protein abundance of PRDX $SO_{2/3}$ hyperoxidized dimeric form in samples, in Snorers (n = 18; 16 samples from non-diabetic snorers and 2 samples from diabetic snorers) and OSA group (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients). Significant differences between groups are denoted by "*" $p < 0.05$.

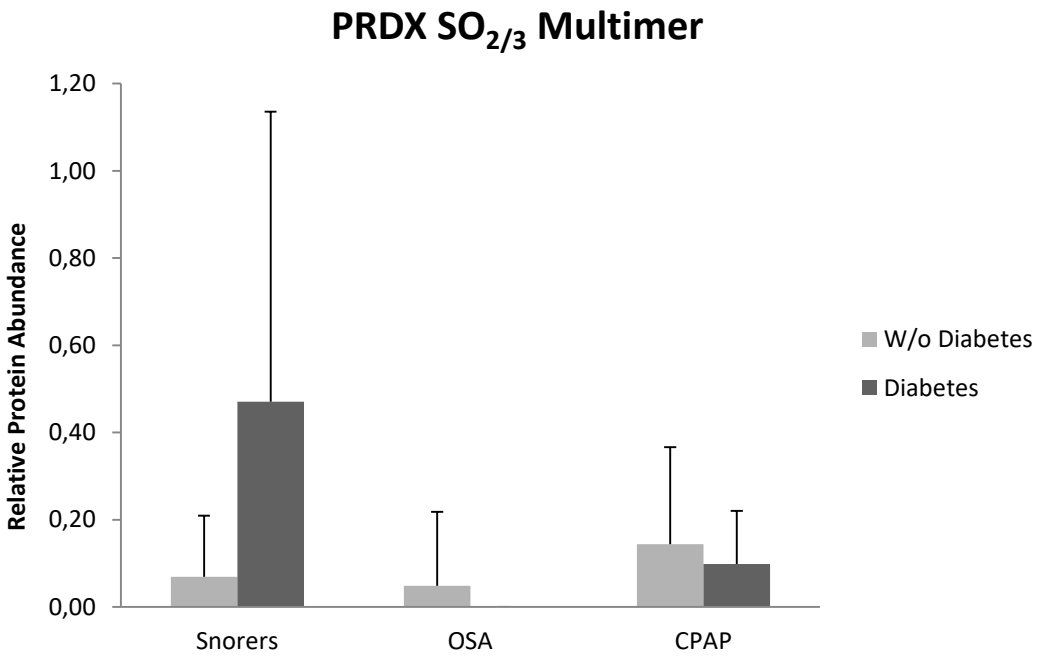

Figure 4.11: RBCs relative protein abundance of PRDX $SO_{2/3}$ hyperoxidized multimeric form in samples, in Snorers (n = 18; 16 samples from non-diabetic snorers and 2 samples from diabetic snorers) and OSA group (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients). Significant differences between groups are denoted by "*" $p < 0.05$.

Finally, in regards to the multimeric hyperoxidized form, it is possible to note lower levels in control and OSA groups, and a tendency to increase after six months of CPAP treatment in non-diabetic patients. In diabetics, this multimeric form is much more abundant in the Control group (however, only one diabetic individual revealed de presence of these multimeric structures), it is absent in the OSA group, and shows a slight increase after CPAP treatment (Figure 4.11).

In short, the results for oligomeric forms of PRDX $SO_{2/3}$ are summarized below, in figure 4.12.

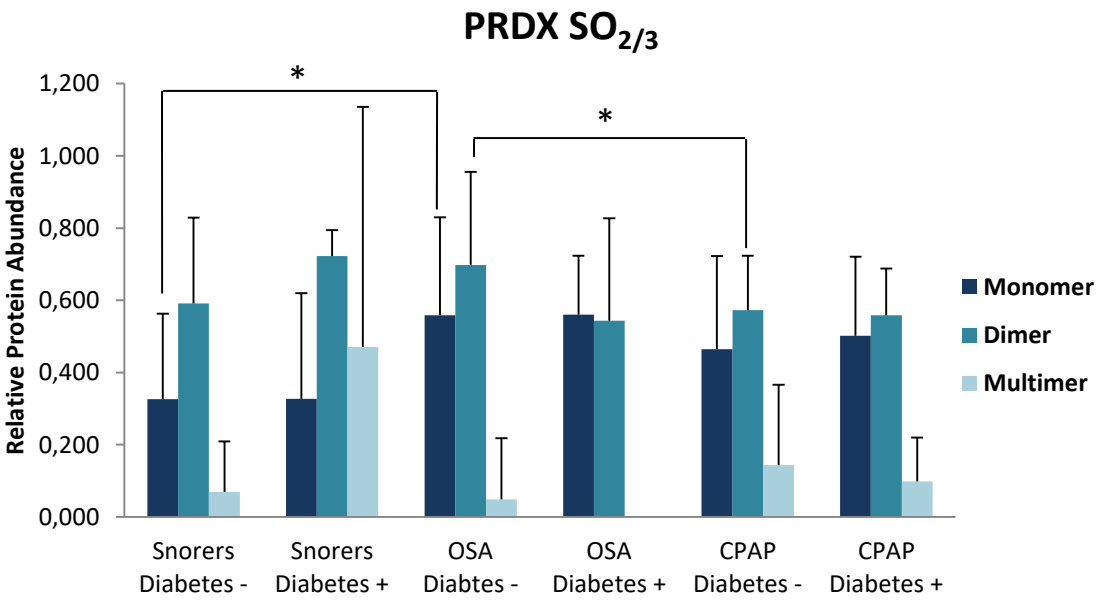

Figure 4.12: RBCs relative protein abundance of PRDX $SO_{2/3}$ monomeric, dimeric and multimeric hyperoxidized forms for diabetic (Diabetes+) and non-diabetic (Diabetes-) patients, in Snorers (n = 18; 16 samples from non-diabetic snorers and 2 samples from diabetic snorers) and OSA (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients) groups, and after CPAP treatment (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients). Significant differences between groups are denoted by "*" $p < 0.05$.

4.2 Peroxiredoxin II in Plasma

Taking into consideration the previous results obtained from RBCs, we thought it pertinent to study the same oligomeric forms of Peroxiredoxin II (PRDX2) in plasma samples, with the same cohort used previously. In order to do this, we used the same antibodies against PRDX2 and against the hyperoxidized form PRDX $SO_{2/3}$, as can be seen in figures 4.13 and 4.18. Regarding PRDX2, unlike what was seen in RBCs, we could only observe two distinct bands: one with a molecular weight of 20 kDa – monomer – and another about ≈25 kDa – unknown monomeric form. This same band of ≈25 kDa also reacts with PRDX $SO_{2/3}$ antibody. This hyperoxidation antibody allows us to identify besides the ≈25 kDa form, another specific reactive band of about 50 kDa (figure 4.18).

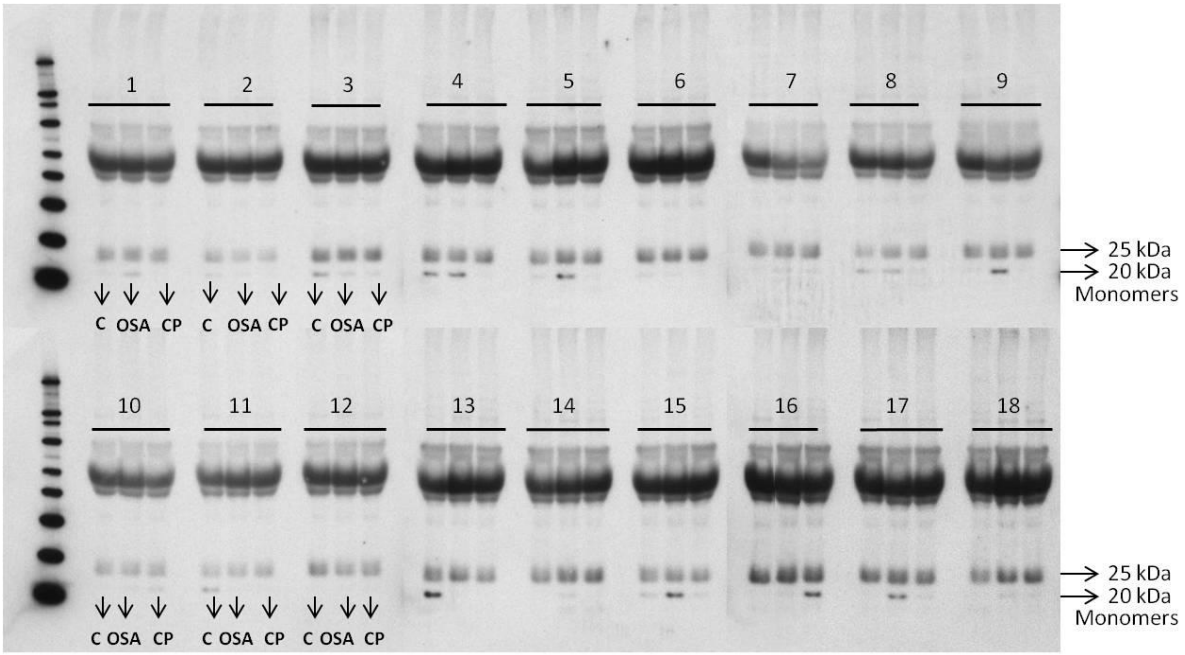

Figure 4.13: Plasma results of PRDX2 Western blot detection using the ECL system and imaging by X-ray processor. Samples were separated on SDS-PAGE gels before transfer to PVDF membranes. Detection of proteins was via using the Ab-PRDX2 (dilution 1:8000 – methods, see page 29). The samples were grouped into 18 different groups for ease of analysis. The order of samples in each group is always the same and is indicated in samples 1, 2, 3, 10, 11 and 12 – Control (C), non-treated OSA (OSA) and post CPAP treatment (CP) samples, n=54. Two monomeric PRDX2 forms can be observed.

Regarding the presence of PRDX2 in plasma, in general we can state that there is an increase of protein abundance in the OSA group when comparing with the Control group, and a decrease after six months of CPAP treatment, this being significant ($p<0.05$) in both the monomeric 20 kDa band and in the ≈25 kDa band (Figure 4.14).

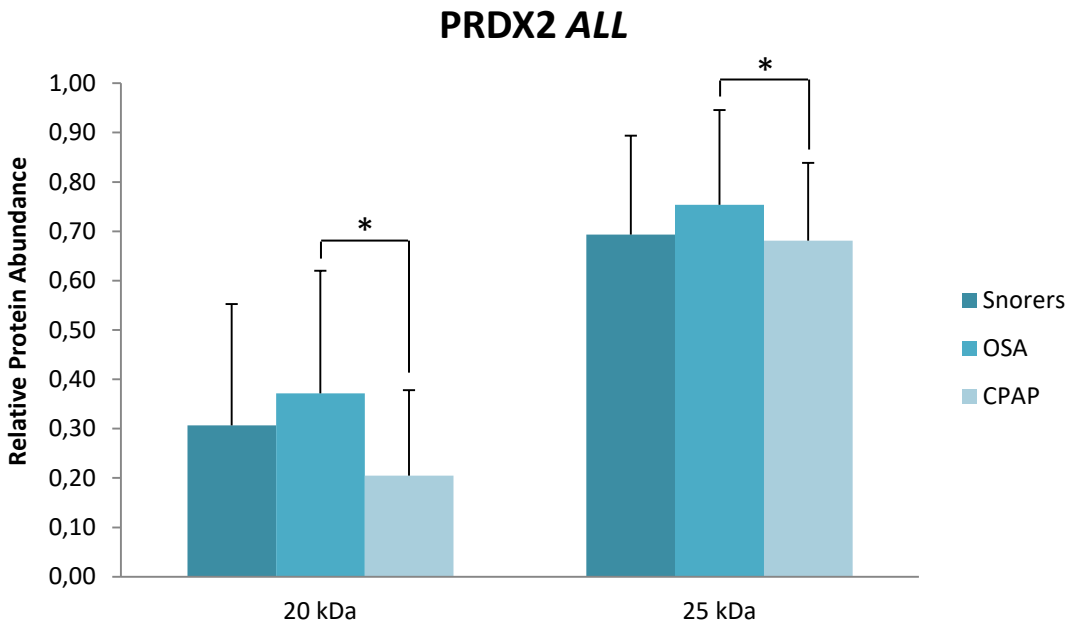

Figure 4.14: Plasma relative protein abundance of PRDX2 20kDa and 25kDa monomeric forms in samples, in Snorers (n = 18) and OSA (n = 18) groups, and after CPAP (n = 18) treatment. Significant differences between groups are denoted by "*" $p<0.05$.

Analyzing the ≈25 kDa form, it is possible to note once more, in both diabetic and non-diabetic patients, a slight increase of protein abundance in the OSA group, compared to the control, and a decrease after CPAP treatment which is considered significant ($p < 0.05$) in both diabetic and non-diabetic group, as can be seen in the graph above (Figure 4.15). However, when looking at the PRDX2 monomeric form (20 kDa), despite revealing the same tendencies as shown before, i.e. an increase in OSA compared with control and a decrease after treatment, in both diabetic and non-diabetic groups, we can notice a higher abundance of this protein form in diabetic controls and non-treated OSA patients, compared to non-diabetics. Nevertheless, after six months of CPAP treatment, diabetic patients reveal decrease of protein abundance attaining lower levels that those registered among non-diabetics (figure 4.16). Results regarding the relative quantification of PRDX2 bands (20 and 25 kDa) in plasma are summed up and properly marked in figure 4.17.

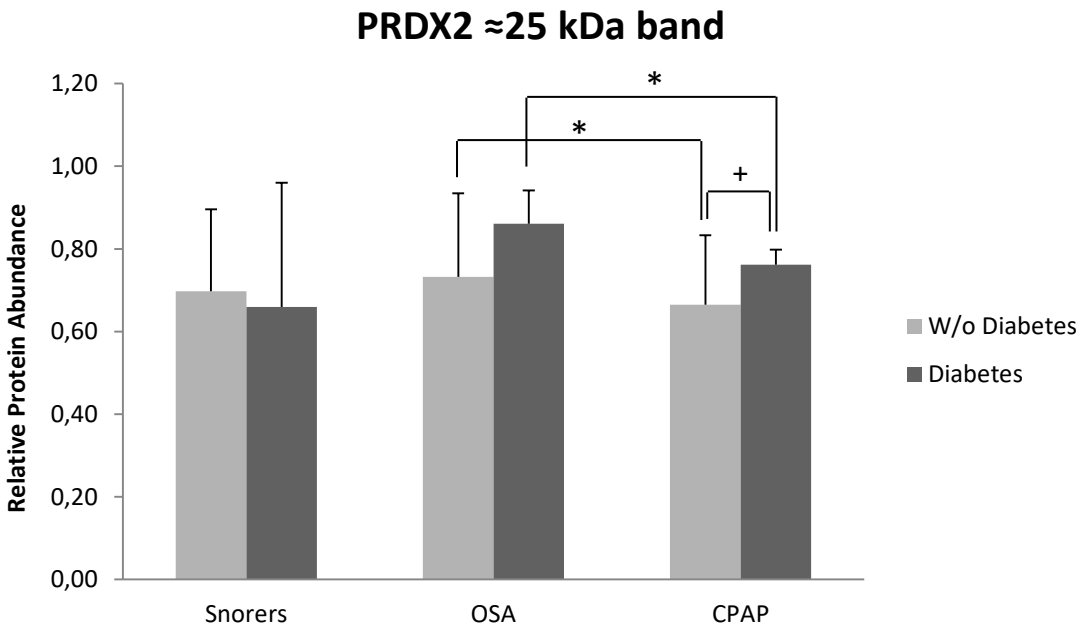

Figure 4.15: Plasma relative protein abundance of PRDX2 25 kDa monomeric form in samples, in Snorers (n = 18; 16 samples from non-diabetic snorers and 2 samples from diabetic snorers) and OSA group (n = 18; 15 samples from non-diabetic OSA patients and 3 from diabetic OSA patients, and after CPAP treatment (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients). Significant differences between groups are denoted by "*" $p < 0.05$, and inner group differences are denoted by "+" $p < 0.05$.

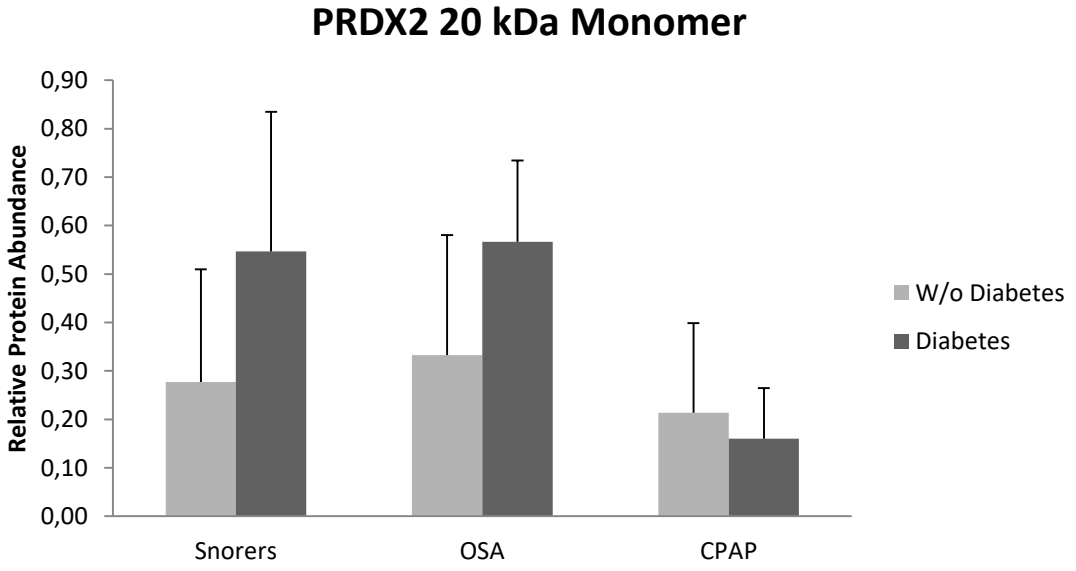

Figure 4.16: Plasma relative protein abundance of PRDX2 20 kDa monomeric form in samples, in Snorers (n = 18; 16 samples from non-diabetic snorers and 2 samples from diabetic snorers) and OSA group (n = 18; 15 samples from non-diabetic OSA patients and 3 from diabetic OSA patients, and after CPAP treatment (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients). Significant differences between groups are denoted by "*" $p < 0.05$.

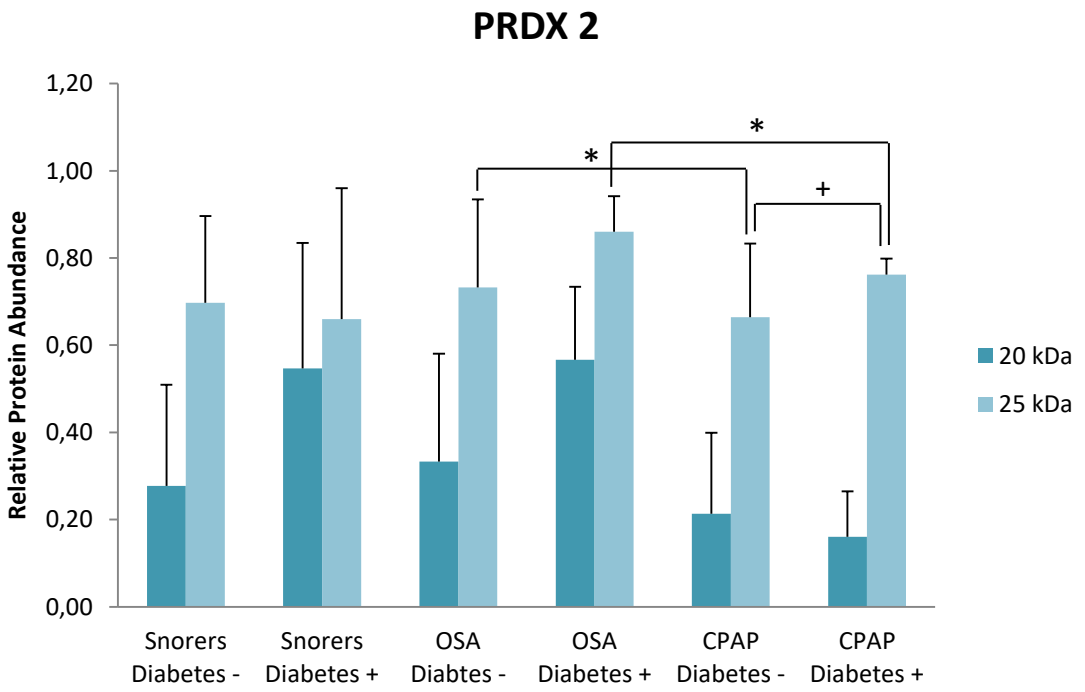

Figure 4.17: Plasma relative protein abundance of PRDX2 20kDa and 25 kDa monomeric forms for diabetic (Diabetes+) and non-diabetic (Diabetes-) patients, in Snorers (n = 18; 16 samples from non-diabetic snorers and 2 samples from diabetic snorers) and OSA (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients) groups, and after CPAP treatment (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients). Significant differences between groups are denoted by "*" $p < 0.05$, and inner group differences are denoted by "+" $p < 0.05$.

As previously stated, we studied the hyperoxidized forms of PRDX2 (PRDX $SO_{2/3}$) by WB technique, in order to, like in RBCs, try to better understand the function of the protein in OSA patients, diabetics and non-diabetics, and after CPAP treatment. Results are shown in figure 4.18.

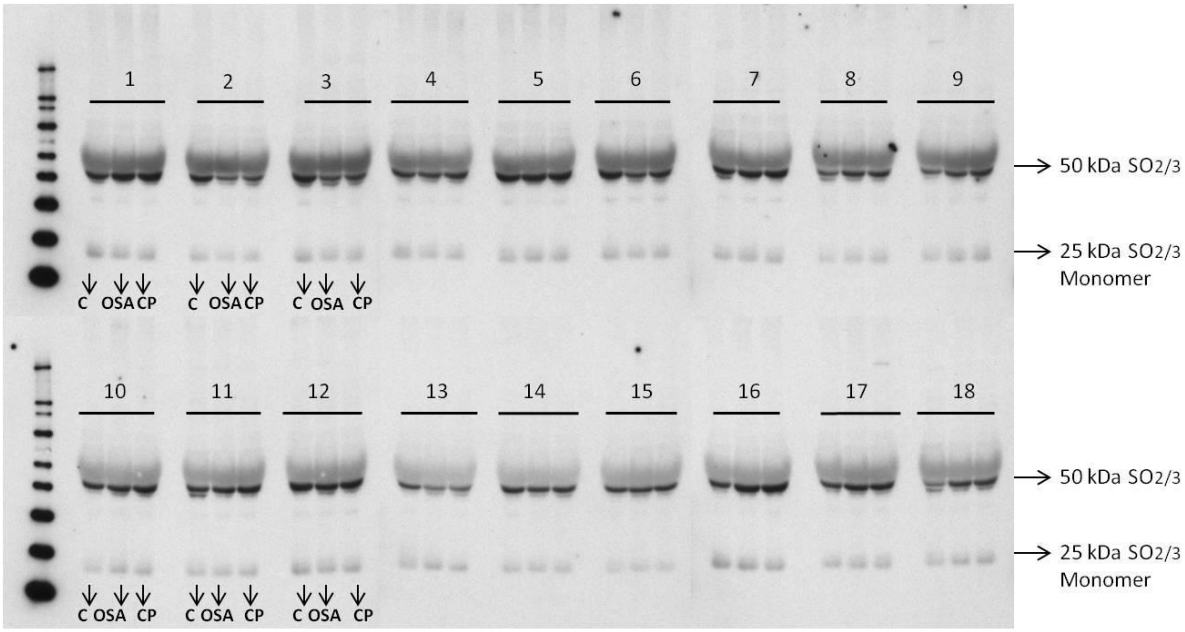

Figure 4.18: Plasma results of PRDX $SO_{2/3}$ Western blot detection using the ECL system and imaging by X-ray processor. Samples were separated on SDS-PAGE gels before transfer to PVDF membranes. Detection of proteins was via using the Ab-PRDX $SO_{2/3}$ (dilution 1:2000 – methods, see page 29). The samples were grouped into 18 different groups for ease of analysis. The order of samples in each group is always the same and is indicated in samples 1, 2, 3, 10, 11 and 12 – Control (C), non-treated OSA (OSA) and post CPAP treatment (CP) samples, n=54. One monomeric and one higher-order PRDX $SO_{2/3}$ forms can be observed.

This study revealed two distinct hyperoxidized bands, one with a molecular weight of ≈25 kDa (also previously identified with the specific antibody against PRDX2), and another of about 50 kDa (Figure 4.18).

In what concerns the presence of the hyperoxidized PRDX $SO_{2/3}$ forms in plasma, it is important to point out that the ≈25 kDa band's protein abundance is similar among control and OSA groups, but there is, however, a significant decrease after six months of CPAP treatment. On the other hand, the results concerning the 50 kDa band reveal a significant increase of this hyperoxidized form in OSA group, when compared to the control and a significant decrease after treatment (Figure 4.19.)

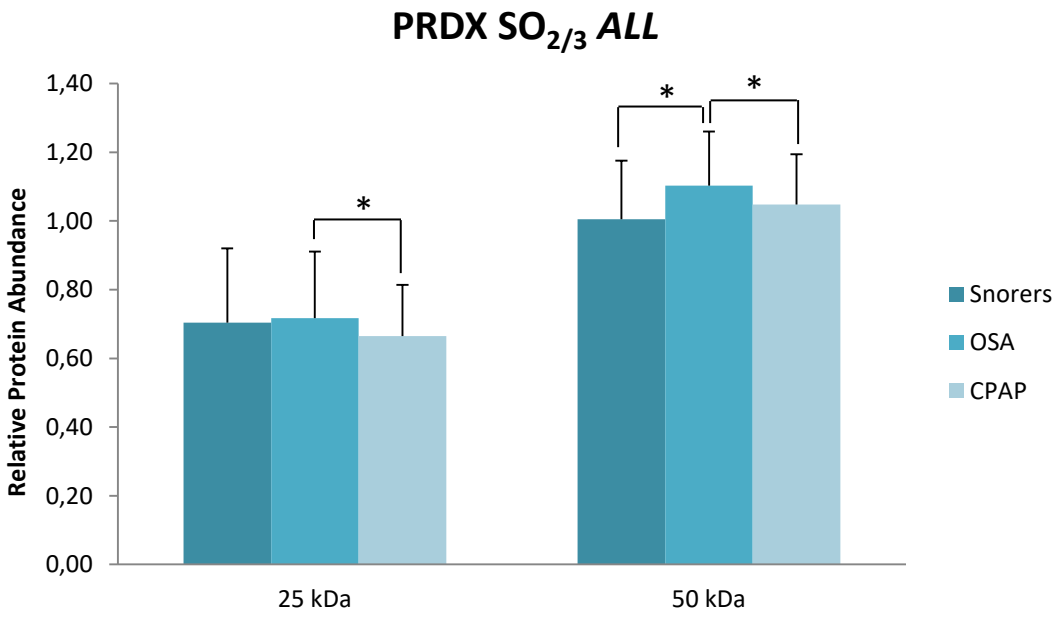

Figure 4.19: Plasma relative protein abundance of PRDX $SO_{2/3}$ 25 kDa and 50 kDa hyperoxidized forms in samples, in Snorers (n = 18) and OSA (n = 18) groups, and after CPAP (n = 18) treatment. Significant differences between groups are denoted by "*" p<0.05.

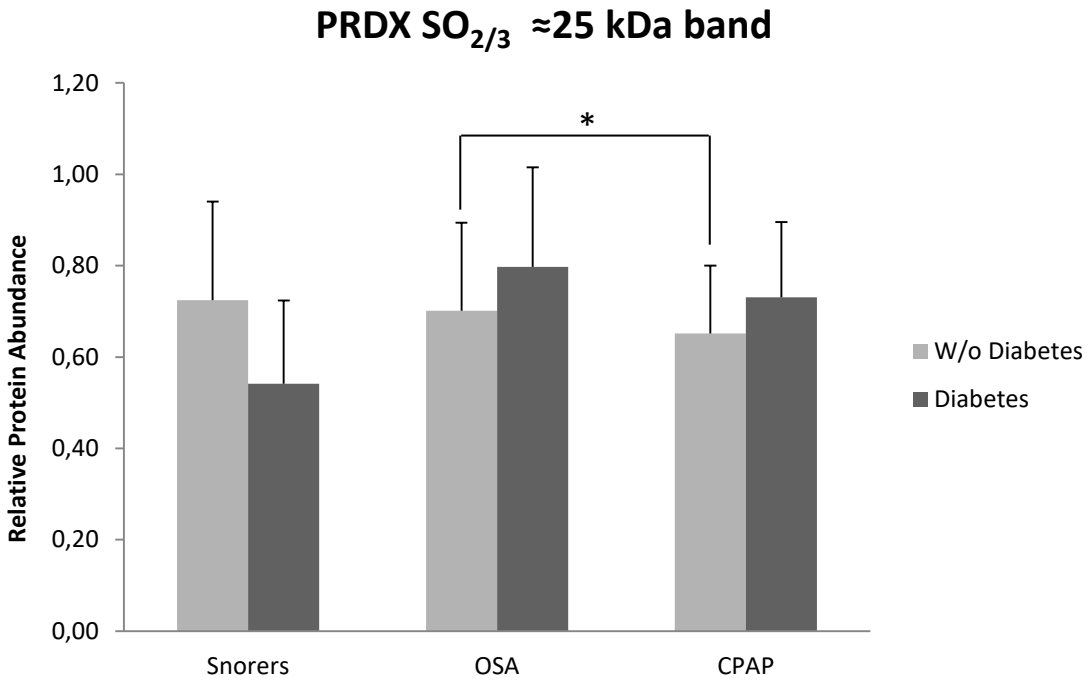

Figure 4.20: Plasma relative protein abundance of PRDX $SO_{2/3}$ 25 kDa hyperoxidized monomeric form in samples, in Snorers (n = 18; 16 samples from non-diabetic snorers and 2 samples from diabetic snorers) and OSA group (n = 18; 15 samples from non-diabetic OSA patients and 3 from diabetic OSA patients, and after CPAP treatment (n=18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients). Significant differences between groups are denoted by "*" $p < 0.05$.

Analyzing more carefully the ≈25 kDa form, no significant alterations have been found, unless in the non-diabetic group, where there is a significant decrease of protein abundance after CPAP treatment, compared with the OSA group (Figure 4.20). Nevertheless, analyzing the hyperoxidized form of 50 kDa, it is possible to verify a significant increase of protein abundance in the non-treated OSA group, compared with control, and a significant decrease after treatment, in the non-diabetic group (Figure 4.21). Diabetic patients show a slight higher abundance of this protein form than non-diabetics, maintaining the same tendency described before, i.e. an increase in non-treated OSA compared with the control group and a decrease after CPAP treatment. These differences are, however, not significant (Figure 4.21).

Results regarding the relative quantification of PRDX $SO_{2/3}$ bands (25 and 50 kDa) in plasma are summed up and properly marked in figure 4.22.

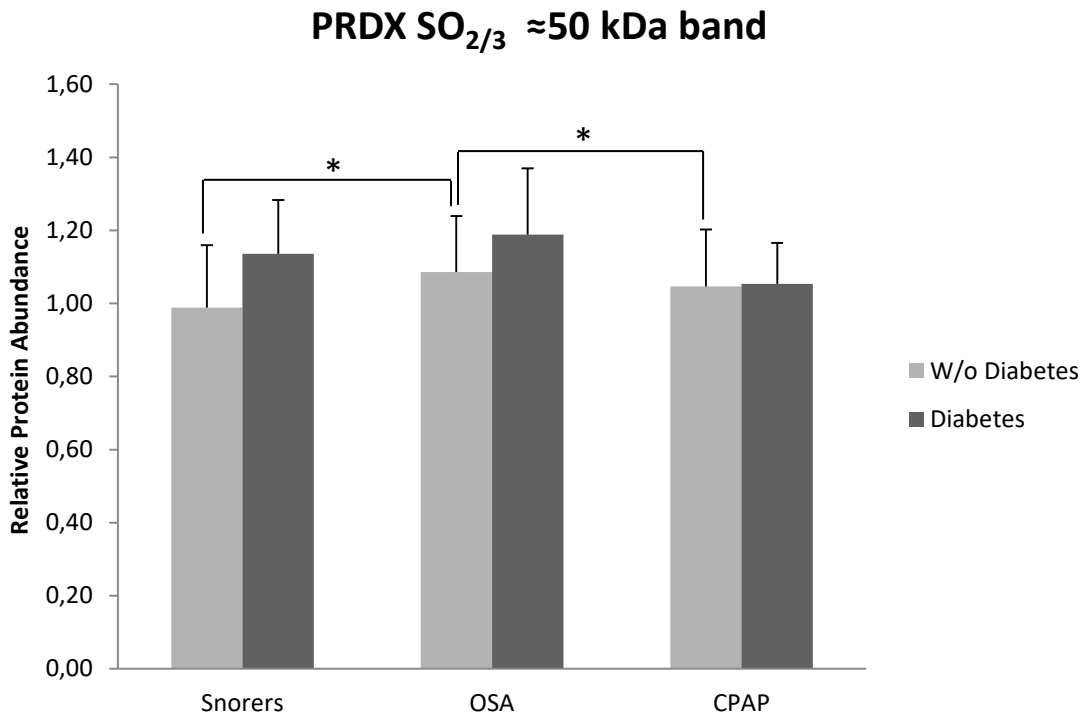

Figure 4.21: Plasma relative protein abundance of PRDX $SO_{2/3}$ 50 kDa hyperoxidized form in samples, in Snorers (n = 18; 16 samples from non-diabetic snorers and 2 samples from diabetic snorers) and OSA group (n = 18; 15 samples from non-diabetic OSA patients and 3 from diabetic OSA patients, and after CPAP treatment (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients). Significant differences between groups are denoted by "*" $p < 0.05$.

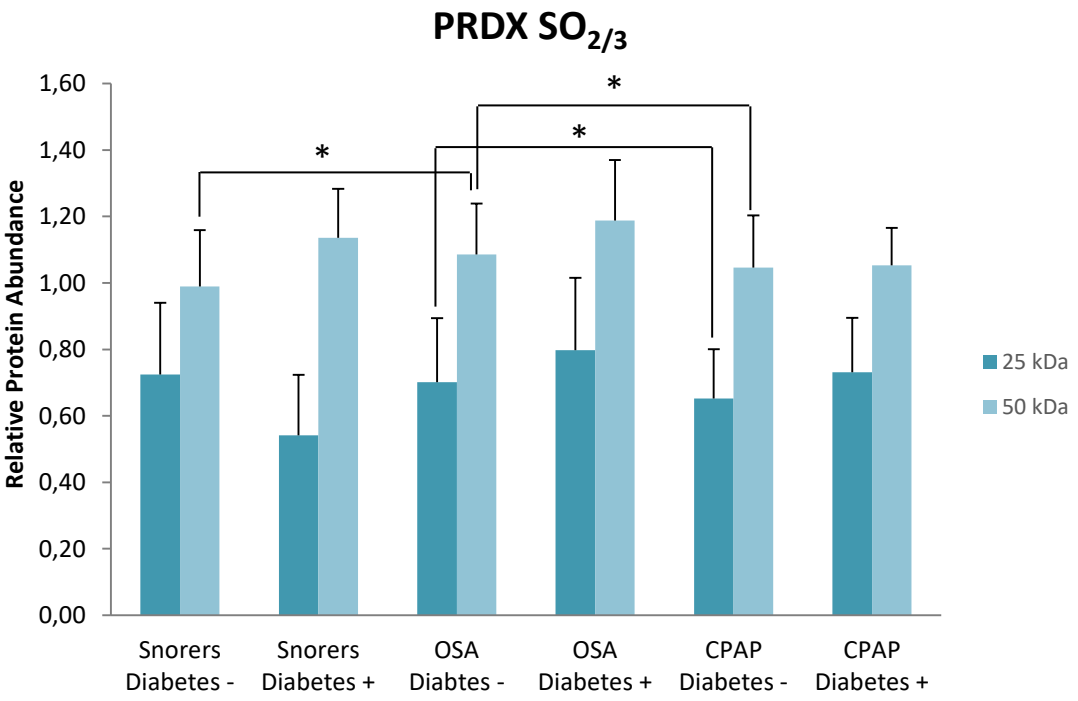

Figure 4.22: Plasma relative protein abundance of PRDX $SO_{2/3}$ 25kDa and 50 kDa hyperoxidized forms for diabetic (Diabetes+) and non-diabetic (Diabetes-) patients, in Snorers (n = 18; 16 samples from non-diabetic snorers and 2 samples from diabetic snorers) and OSA (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients) groups, and after CPAP treatment (n = 18; 15 samples from non-diabetic OSA patients and 3 samples from diabetic OSA patients). Significant differences between groups are denoted by "*" $p < 0.05$.

4.3 RBCs results for PRDX2 and PRDX $SO_{2/3}$ Correlation with PSG and Clinical Parameters

Correlations between RBC PRDX2 redox oligomeric status and polysomnographic/clinical parameters for OSA patients are shown in table 4.1. Monomeric and dimeric forms positively correlate to PSG parameters and negatively correlate to CBC results, while multimeric forms positively correlate with Hb A1C.

Table 4.1: Correlations between RBC PRDX2 and PSG/Clinical parameters concerning non-treated patients

PRDX2	Snorers + OSA (N=36)		
	Correlation	Pearson r Value	p Value
Monomer	EPW	-,334(*)	0,046
	RDI	,368(*)	0,027
	T90	,386(*)	0,020
	Min sat (%)	-,366(*)	0,028
	ODI	,421(*)	0,010
	Hemoglobin	-,376(*)	0,024
	Hematocrit	-,395(*)	0,017
	WBC count x 10^9/L	-,411(*)	0,013
S-S Dimer	no significant correlation observed		
S-S/S-S Dimer	Triglycerides	,406(*)	0,014
	RDW	-,367(*)	0,028
$SO_{2/3}$ Monomer	EPW	-.334(*)	0,046
	RDI	.368(*)	0,027
	T90	.386(*)	0,020
	Min sat (%)	-.366(*)	0,028
	ODI	.421(*)	0,010
	Hemoglobin	-,376(*)	0,024
	Hematocrit	-,395(*)	0,017
	WBC count x 10^9/L	-,411(*)	0,013
S-S/$SO_{2/3}$ Dimer	Triglycerides	.458(**)	0,005
	Platelets, count x 10^9/L	.399(*)	0,016
$SO_{2/3}$ Multimer	Hb A1C	.783(*)	0,037

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.2: Correlations between RBC PRDX2 and PSG/Clinical parameters after CPAP treatment

PRDX2	CPAP ALL (N=18)		
	Correlation	Pearson r Value	p Value
Monomer	no significant correlation observed		
S-S Dimer	WBC count x 10^9/L	,585(*)	0.011
S-S/S-S Dimer	Triglycerides	,585(*)	0.011
$SO_{2/3}$ Monomer	no significant correlation observed		
S-S/$SO_{2/3}$ Dimer	MCHC	,660(**)	0.003
$SO_{2/3}$ Multimer	no significant correlation observed		

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

After CPAP treatment, different correlations are shown – table 4.2. Dimeric forms positively correlate with CBC results, and no correlations were observed neither in monomeric nor multimeric forms. Remaining detailed tables concerning correlation concerning specific groups are shown in supplemental data (annex IV).

4.4 Plasma results for PRDX2 and PRDX $SO_{2/3}$ Correlation with PSG and Clinical Parameters

Correlations between Plasma PRDX2 redox oligomeric status and polysomnographic/clinical parameters for OSA patients are shown in table 4.3. PRDX 2 monomeric forms positively correlate mainly with glucose metabolism related parameters, as well as hyperoxidized forms. Also, PDRX2 monomeric form revealed a positive correlation with Hb and a negative correlation with homocysteine.

Table 4.3: Correlations between plasma PRDX2 and PSG/Clinical parameters concerning non-treated patients

PRDX2	Snorers + OSA ALL (N=36)		
	Correlation	Pearson r Value	p Value
PRDX2 25 kDa	Oximetry	,402(*)	0,017
PRDX2 20 kDa Monomer	Fasting glucose	,491(**)	0,002
	Hb A1C	,441(**)	0,007
	HOMA-IR	,457(**)	0,005
	Homocysteine	-,427(**)	0,009
	Hemoglobin	,332(*)	0,048
$SO_{2/3}$ 25 kDa	Oximetry	,376(*)	0,026
$SO_{2/3}$ 50 kDa	Cervical perimeter	,346(*)	0,048
	Min sat (%)	-,338(*)	0,044
	Fasting glucose	,342(*)	0,042
	Hb A1C	,339(*)	0,043

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.4: Correlations between plasma PRDX2 and PSG/Clinical parameters concerning non-treated patients

PRDX2	CPAP ALL (N=18)		
	Correlation	Pearson r Value	p Value
PRDX2 25 kDa	Days w/o use	-,600(**)	0,008
	MCHC	-,495(*)	0,037
PRDX2 20 kDa Monomer	Hematocrit	-,477(*)	0,046
	RDW	,587(*)	0,010
$SO_{2/3}$ 25 kDa	no significant correlation observed		
$SO_{2/3}$ 50 kDa	no significant correlation observed		

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

After CPAP treatment, different correlations are shown – table 4.4. Despite no major correlation was observed, plasma PRDX2 monomeric forms reveled a negative correlation with some CBC results. No glucose metabolism related correlations were observed after six months of CPAP therapy, in none of the oligomeric plasma PRDX2 forms. Remaining detailed tables concerning PRDX2 correlation with PSG/Clinical parameters of specific groups are shown in supplemental data (annex VI).

5 Discussion

Previous studies developed by INSA Proteomics Laboratory have showed that monomeric and dimeric forms of a RBC antioxidant enzyme, PRDX2, were significantly hyperoxidized in OSA RBCs, mainly during the morning, suggesting severe oxidative stress and altered antioxidant homeostasis in OSA RBC. Six months of CPAP treatment decreased this hyperoxidation and generated multimeric hyperoxidized forms associated with chaperone function of PRDX2, described to enhance cell stress-resistance [46]. Levels of oxidized PRDX2 correlated to PSG and metabolic parameters in these patients. Therefore, the redox/oligomeric state of RBC PRDX2 has been proposed by these authors as a promising candidate biomarker for OSA severity and treatment monitoring [39].

In this work, we intended to go further with this investigation by studying in a larger cohort of patients the impact of comorbidities, namely diabetes, on the morning redox/oligomeric state of RBC PRDX2 before and after CPAP therapy. Overall, the results corroborated the previous data by showing a significant increase in the morning level of hyperoxidized monomeric/dimeric PRDX2 forms in OSA patients with/without diabetes compared with snorer ones. The hyperoxidized multimeric form – associated with chaperone protective function of PRDX2 – was observed in some subjects, mainly in snorer patients (six of 18, one was diabetic) compared with OSA patients (one of 18, none were diabetics).

No significant differences were observed between diabetic and non-diabetic OSA patients in regards to the level of hyperoxidation in the monomeric and dimeric forms of PRDX2, although dimeric forms showed a tendency, still not significant, to be less hyperoxidized in diabetic OSA patients when compared with OSA non-diabetic ones. Interestingly, as mentioned above, none of the OSA diabetic patients showed the presence of hyperoxidized PRDX2 multimers. These data suggest that the putative stress-resistance effect conferred by these hyperoxidized PRDX2 multimers seemed to be severely compromised in OSA patients with diabetes comorbidity.

Six months of CPAP therapy decreased the hyperoxidation level of monomeric and dimeric forms of OSA RBC PRDX2, as previously shown [39]. However, this effect was significantly evident in dimeric PRDX2 forms in non-diabetic patients when compared

with diabetic ones. In diabetic OSA patients, hyperoxidation seemed to remain higher in PRDX2 monomers and dimers after treatment.

However, CPAP treatment showed to increase the level of PRDX2 multimeric protective structures in OSA patients, with higher evidence in OSA patients without diabetes, in response to treatment. Further studies will be necessary to better elucidate the therapeutic effect of CPAP-induced hyperoxidized PRDX2 multimers in OSA patients and its higher modulation under diabetes comorbidity.

In general, the correlation study between redox oligoforms of PRDX2 and patients clinical parameters showed mainly that the morning level of hyperoxidized monomeric forms of PRDX2 positively correlated to PSG parameters such as RDI, ODI, T90%, while the level of S-S/$SO_{2/3}$ dimer and $SO_{2/3}$ multimers showed a positive correlation with metabolic parameters, triglycerides and HbA1C, respectively.

A previous study by the laboratory performed with a smaller cohort of patients (n=20), which included mild OSA patients, also showed a positive correlation between the morning level of hyperoxidized PRDX2 monomers and PSG parameters, but only with PSG-arousal index, while the morning level of S-S/$SO_{2/3}$ dimer (but not multimers) showed a larger positive correlation with metabolic parameters such as HbA1C, insulin and HOMA-IR [39]. These data prompt the authors to suggest that during the morning, the non-peroxidase (hyperoxidation) cycle of PRDX2 predominates in OSA and is mainly associated with the metabolic status of the patients.

Here the study was performed with a larger cohort of subjects (n=36), and included only moderate/severe OSA patients with/without diabetes, which may explain some observed differences between this study and the previous one. Although herein the data confirmed that morning increased the predomination of hyperoxidation PRDX2 cycle in OSA, it also seemed to be associated, not only with the metabolic status, but also with the OSA severity PSG parameters. Interestingly, however, is that the level of protective hyperoxidized multimers, which was mainly observed in Snorer patients, strongly positively correlated with several glycemic parameters in this group of patients. In patients with moderate/severe OSA with/without diabetics there was no such correlation. This data suggested that in the presence of moderate/severe OSA-induced hypoxia/sleep disruptions, patients may

have a lower capacity to produce PRDX2 multimeric protective forms in response to increased plasma glucose and/or RBC Hb1AC.

The correlation study, which studied OSA patients before and after CPAP, showed that before treatment, the level of PRDX2 dimers (oxidized/hyperoxidized) positively correlated with PSG-minimum oxygen saturation parameter, which is known to associated with OSA severity [47]. After treatment, this correlation was not observed, i.e., had ceased to exist. Other (positive/negative) correlations between clinical parameters and the different redox/oligomeric states of PRDX2 before and after CPAP, were obtained (see annex IV), the interpretation of which needs further investigation.

After studying the PRDX2 in RBCs, we decided it would be interesting to recreate the same experience, but this time testing plasma samples, in order to understand the state of PRDX2 oligomeric redox-status in plasma. We used the same cohort, in order to further compare and possibly relate plasma to RBC results.

Upon analyzing plasma proteins separated by reduced SDS-PAGE, we detected two immune-reactive bands (20 kDa and 25 kDa) using the Ab-PRDX2 and two immune-reactive bands (25 KDa a 50 kDa) using the Ab-PRDXSO$_{2/3}$ that is specific for hyperoxidized forms of PRDX1-4. From this data, we assumed 20 kDa and 25kDa bands to be PRDX2 forms, namely non-oxidized and hyperoxidized forms, respectively. Since the 50 kDa band did not react with the Ab-PRDX2, we considered this reactive band may correspond to PRDXs 1 or 4, excluding PRDX2 or PRDX3 (the latter is mostly found in mitochondria) [44].

Overall, the results showed that the OSA patients with/without diabetes had higher blood circulating levels of PRDX2, especially hyperoxidized forms, and hyperoxidized PRDXs 1/4. Six months of CPAP treatment reduced significantly the plasma levels of hyperoxidized PRDXs but only in OSA patients without diabetes. In OSA patients with diabetes only circulating levels of non-oxidized PRDX2 were reduced after treatment.

This study also brought interesting results concerning correlations between PRDXs redox state in plasma and PSG/clinical parameters. The plasma level of non-oxidized PRDX2 and hyperoxidized PRDXs 1/4 positively correlated with several glucose-metabolism parameters, such as fasting glucose, Hb A1C and/or HOMA-IR. A positive correlation between non-oxidized PRDX2 and glycemic parameters was also

observed when the OSA diabetic group is separately analyzed. After CPAP, those correlations were no longer observed. However, in the diabetic OSA group, a positive correlation was observed between non-oxidized forms of PRDX2 and metabolic parameters, namely insulin and Hb A1C, possibly meaning that the glycemic status of these patients unchanged after treatment.

These results confirm previous studies reporting higher levels of plasma PRDXs (PRDX1,2,4 and 6) in the diabetic patients than in the healthy ones [48,49]. However, differential associations of circulating PRDXs levels with indicators of cardiovascular risk factors in type 2 diabetes mellitus were observed [48,49]. For example, PRDX2 and PRDX6 were found to be negatively correlated with fasting blood glucose and Hb A1C. In contrast, PRDX1 levels were positively correlated with low-density lipoprotein and C-reactive protein levels, and PRDX4 levels were negatively correlated with triglycerides. Differences in the type of cohort studied and the quantification method used in the analysis may explain some of the contradictions this study showed concerning the positive correlation between PRDXs and glycemic profile that these authors found. This result should encourage further research in order to better clarify the impact of these PRDXs in diabetic patients with/without OSA syndrome and the benefic effect of CPAP treatment in glycemic control in these patients.

Intracellular PRDX2 is a peroxidase and chaperone protein [43], whereas extracellular PDRX2 , which is stress-induced and secreted from different cells such as activated macrophages, presents chemokine-like activity, enabling induction of inflammatory responses [50]. Herein, no correlation was found between the level of the different redox/oligomeric states of RBC PRDX2 and those PRDXs from plasma, PRDX2 (non-oxidized/oxidized) or PRX1/4 $SO_{2/3}$. More studies will be needed to better define the role(s) of different PRDXs proteoforms in different cells and subcellular location in diseases associated with redox imbalance oxidative conditions such as OSA and OSA-associated metabolic disorders.

There are some limitations of this study, such as reduced sample size and female gender exclusion. Also, the control group was constituted by non-completely healthy snorer subjects, the snorer diabetics group was constituted by only two individuals and the non-diabetic OSA group included patients with higher levels of blood insulin and HOMA-IR. Taken all together, this is limiting for data generalization. Although patients

were instructed to follow a restricted diet for three days before urine/blood collection to minimize the impact on antioxidant status, their dietary habits were not fully controlled. Validation analyses were performed on -80 ºC stored samples, which could introduce some bias in the molecular events of both control and disease samples.

6 Conclusion

In summary, the present findings confirmed previous laboratory data, showing that the antioxidant defense and/or redox-signaling involving RBC PRDX2 seemed highly modulated and correlated with OSA severity and/or metabolic status of the patients. Six months of CPAP significantly increased the chaperone/transduction signaling function of RBC PRDX2 that might be linked with beneficial effects of CPAP in improving oxidative-stress and the metabolic status of the patients.

However, diabetes comorbidity in these patients appears to compromise these modulations in PRDX2 redox/oligomeric status. Overall, diabetes seems to worsen OSA's implications, as the presence of oxidative stress caused by hypoxia, along with glucose metabolism-induced problems, seems to restrain the formation of higher order oligomers in RBCs. Nevertheless, we noted a decrease of plasma PRDXs stress-induced inflammation pathways after CPAP treatment. Also, interesting correlations were noted between plasma PRDX2 and PSG/clinical parameters, especially those related to glucose-metabolism parameters, thus deserving special attention in further studies.

PRDX2's oligomeric redox-state relation to hemoglobin autoxidation and PRDX's excretion from blood cells to plasma are critical points which must be carefully analyzed in order to further validate PRDX2's promising potential as a biomarker candidate for OSA severity and metabolic status.

Future studies encompass different proteomic approaches.

2D-DIGE individual studies are currently in progress. Further LC-MS/MS studies might be needed in order to further investigate PTM involved in PRDX2 redox and signaling pathways.

7 Bibliography

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8 Annexes

Annex I

Solutions Composition

Incomplete Transfer Buffer (10xs)

30,2g Tris-HCL 0,025 M

144g Glycine 0,192 M

Add type II water for a final volume of 1L

Complete Transfer Buffer

100 mL Incomplete Transfer Buffer

200 mL Methanol

Add ultrapure water for a final volume of 1L

***Ponceau* Solution at 0,1% in 5% de Acetic Acid**

50 mL *Ponceau S* at 0,1%

450 mL acetic acid at 5%

PBS 1x (Saline Phosphate Buffer)

100 mL mineral medium 10xs

900 mL ultrapure water

PBS-T (Saline Phosphate Buffer w/ Tween 20 at 0,1%)

100 mL PBS 1x

900 mL ultrapure water

1 mL *Tween 20* at 0,1%

Annex II

Total protein quantification for RBCs samples (1:200) of Snorer, OSA and OSA – CPAP individuals (N=54)

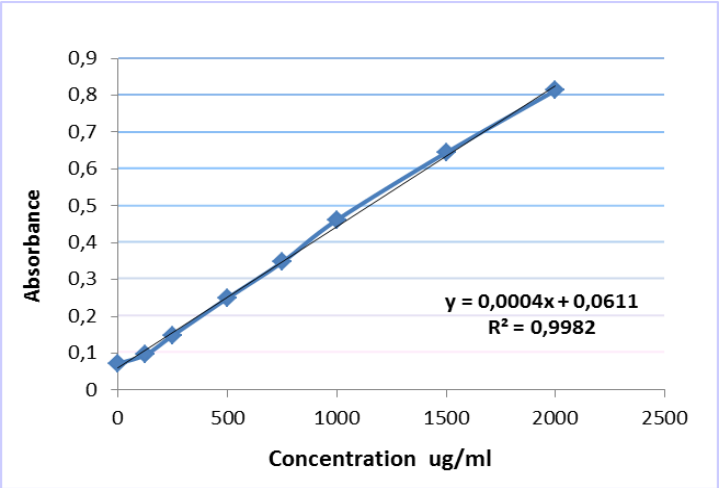

Figure Annex II. 1: Calibration line used to calculate the volume of RBC sample needed to adjust to 70 µG

Table Annex II. 1: Absorbance values for standards.

Standards	Absorbance (λ= 660 nm)		Mean
0	0,072	0,072	0,072
125	0,098	0,094	0,096
250	0,144	0,153	0,1485
500	0,248	0,251	0,2495
750	0,351	0,343	0,347
1000	0,463	0,46	0,4615
1500	0,61	0,679	0,6445
2000	0,809	0,818	0,8135

Table Annex II. 2: Volume needed to adjust each RBC sample to 70 µG of total protein

Samples 1:200	Volume for 70 µG protein (µL)	Samples 1:200	Volume for 70 µG protein (µL)	Samples 1:200	Volume for 70 µG protein (µL)
C1	47,6	OSA1	42,0	CP1	50,0
C2	42,7	OSA2	46,0	CP2	49,3
C3	84,7	OSA3	42,2	CP3	47,4
C4	50,7	OSA4	46,4	CP4	47,8
C5	50,0	OSA5	42,7	CP5	48,6
C6	50,8	OSA6	42,3	CP6	53,2
C7	47,8	OSA7	48,3	CP7	50,8
C8	48,5	OSA8	46,4	CP8	48,9
C9	52,8	OSA9	51,6	CP9	45,8
C10	51,4	OSA10	45,6	CP10	47,1
C11	43,9	OSA11	42,0	CP11	46,5
C12	52,5	OSA12	47,0	CP12	32,6
C13	54,0	OSA13	46,4	CP13	40,6
C14	49,3	OSA14	41,9	CP14	48,3
C15	53,8	OSA15	46,9	CP15	51,5
C16	51,1	OSA16	44,6	CP16	46,3
C17	43,8	OSA17	45,1	CP17	48,5
C18	52,6	OSA18	49,1	CP18	46,2

Total protein quantification on Plasma samples (1:10) of Snorer, OSA and OSA – CPAP individuals (N=54)

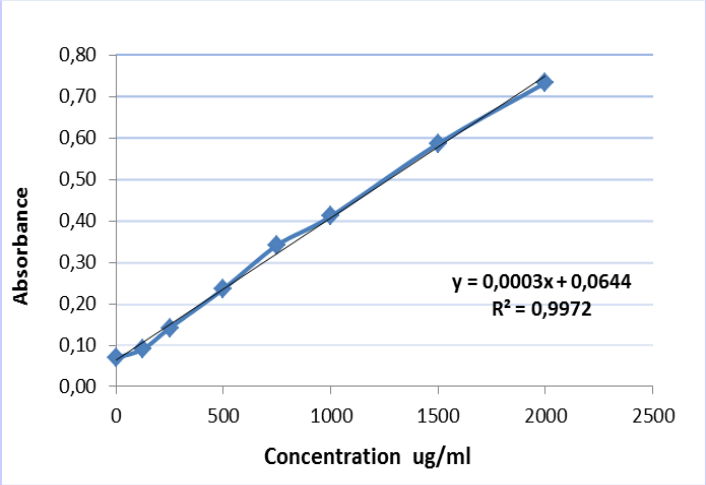

Figure Annex II. 2: Calibration line used to calculate the volume of Plasma sample needed to adjust to 40 µG

Table Annex II. 3: Absorbance values for standards.

Standards	Absorbance (λ= 660 nm)		Mean
0	0,067	0,072	0,070
125	0,093	0,088	0,091
250	0,145	0,138	0,142
500	0,243	0,229	0,236
750	0,353	0,330	0,342
1000	0,432	0,391	0,412
1500	0,616	0,558	0,587
2000	0,748	0,720	0,734

Table Annex II. 4: Volumes needed to adjust each Plasma sample to 40 µG of total protein

Samples 1:10	Volume for 40 µG protein (µL)	Volume of Ultra-pure Water	Samples 1:10	Volume for 40 µG protein (µL)	Volume of Ultra-pure Water	Samples 1:10	Volume for 40 µG protein (µL)	Volume of Ultra-pure Water
C1	5,4	1,1	OSA1	4,7	1,8	CP1	4,8	1,7
C2	5,5	1,0	OSA2	5,0	1,5	CP2	5,4	1,1
C3	5,3	1,2	OSA3	4,7	1,8	CP3	5,0	1,5
C4	5,3	1,2	OSA4	5,4	1,1	CP4	5,1	1,4
C5	5,1	1,4	OSA5	5,1	1,4	CP5	5,1	1,4
C6	4,8	1,7	OSA6	5,1	1,4	CP6	5,1	1,4
C7	5,2	1,3	OSA7	5,2	1,3	CP7	5,2	1,3
C8	4,8	1,7	OSA8	5,0	1,5	CP8	5,2	1,3
C9	4,7	1,8	OSA9	4,7	1,8	CP9	4,9	1,6
C10	4,8	1,7	OSA10	4,8	1,7	CP10	4,6	1,9
C11	5,0	1,5	OSA11	4,4	2,1	CP11	4,7	1,8
C12	4,5	2,0	OSA12	4,9	1,6	CP12	4,9	1,6
C13	5,2	1,3	OSA13	4,9	1,6	CP13	4,7	1,8
C14	5,5	1,0	OSA14	5,0	1,5	CP14	5,3	1,2
C15	5,1	1,4	OSA15	5,4	1,1	CP15	5,4	1,1
C16	4,7	1,8	OSA16	5,5	1,0	CP16	5,2	1,3
C17	5,5	1,0	OSA17	4,9	1,6	CP17	5,0	1,5
C18	4,9	1,6	OSA18	5,2	1,3	CP18	5,0	1,5

Annex III

Table Annex III. 1: Demographic, Polysomnographic and Analytical Characterization

Demographic, Polysomnographic and Analytical Characterization											
Demographic and PSG parameters	**Screened Subjects**										
	Mean (St Dev)			***p value***							
				ALL		***OSA***	***CPAP***	***W/o diabetes***		***Diabetes***	
	Snorers (n=18)	**OSA (n=18)**	**CPAP (n=18)**	**Snorers vs. OSA**	***OSA vs. CPAP***	***Diabetes vs. W/o diabetes***	***Diabetes vs. W/o diabetes***	**Snorers vs. OSA**	***OSA vs. CPAP***	**Snorers vs. OSA**	***OSA vs. CPAP***
Age (years)	45,8	47,4	-	NS	n/a	NS	-	NS	n/a	0,028	n/a
Habits											
Current Smoking (n)	5	3	-	-	-	-	-	-	-	-	-
Current Drinking (n)	10	14	-	-	-	-	-	-	-	-	-
EPW Score	10,4 (5,4)	8,8 (3,6)	5,8 (4,2)	NS	0,026	0,024	NS	NS	NS	NS	NS
Observational features											
Morning arterial pressure (mmHg) *	135,5 (21,0)/ 83,3 (12,3)	142,2 (22,8)/ 90,2 (14,7)	-	NS	n/a	NS	-	NS	n/a	NS	n/a
BMI (kg/m2)	28,1 (3,4)	31,3 (3,3)	-	0,0014	n/a	NS	-	0,008	n/a	NS	n/a
Abdominal perimeter (cm)	100,1 (8,4)	109,7 (10,0)	-	0,0009	n/a	NS	-	0,004	n/a	NS	n/a
Comorbidities											
Hypertension (n)	9	13	-	-	-	-	-	-	-	-	-
Respiratory diseases (n)	0	3	-	-	-	-	-	-	-	-	-
Dyslipidemia (n)	10	14	-	-	-	-	-	-	-	-	-
Diabetes (n)	2	6	-	-	-	-	-	-	-	-	-
Polysomnographic parameteres											
RDI (events/h)	2,8 (1,2)	50,2 (21,6)	-	7,01E-11	n/a	NS	-	2,12E-10	n/a	NS	n/a
Arousal Index (events/h)	13,8 (6,3)	31,5 (17,3)	-	0,0003	n/a	NS	-	2,05E-04	n/a	0,023	n/a
Minimum arterial saturation (%)	88,1 (4,3)	76,1 (6,6)	-	2,41E-07	n/a	NS	-	1,61E-07	n/a	NS	n/a
ODI (events/h)	2,7 (3,5)	42,2 (22,4)	-	1,45E-08	n/a	NS	-	4,38E-07	n/a	0,011	n/a
T90 (%)	0,1 (0,1)	12,7 (14,6)	-	0,0008	n/a	NS	-	0,004	n/a	NS	n/a
Sleep efficiency (%)	79,7 (11,7)	77,1 (13,6)	-	NS	n/a	NS	-	NS	n/a	NS	n/a

CPAP Record											
Number of days without use	–	–	55,2 (69,3)	-	n/a	-	NS	-	n/a	-	n/a
Total of recording days	–	–	192,3 (32,3)	-	n/a	-	NS	-	n/a	-	n/a
Use per night (minutes)	–	–	266,7 (146,0)	-	n/a	-	NS	-	n/a	-	n/a
Residual AHI (events/hour)	–	–	2,0 (1,4)	-	n/a	-	NS	-	n/a	-	n/a
Analytical parameteres											
Glycemic profile											
Glucose (70-110 mg/dl)	101,9 (27,2)	115,4 (35,3)	106,4 (26,5)	NS	NS	5,98E-06	0,002	NS	NS	NS	NS
Hb A1C (4-6%)	5,8 (0,8)	6,4 (1,1)	6,3 (1,2)	NS	NS	5,05E-06	0,003	0,037	NS	NS	NS
Insulin (3-25 mU/L)	14,7 (7,5)	21,4 (11,0)	31,6 (33,9)	0,0401	NS	NS	NS	0,023	NS	NS	NS
HOMA-IR (≥ 3,8)	3,9 (3,4)	6,1 (3,0)	8,5 (8,9)	0,0522	NS	NS	NS	0,008	NS	NS	NS
Lipid profile											
Cholesterol (< 190 mg/dl)	189,3 (39,6)	187,4 (30,7)	180,1 (32,9)	NS	NS	NS	NS	NS	NS	NS	NS
Triglycerides (< 150 mg/dl)	131,6 (77,6)	142,8 (82,4)	143,7 (100,8)	NS	NS	NS	NS	NS	NS	NS	NS
Cardiovascular marker											
Homocysteine (3,7-13,9 µmol/L)	14,3 (3,1)	15,5 (2,8)	16,4 (2,8)	NS	NS	NS	NS	NS	NS	NS	NS
Urinary catecholamines											
Adrenalin (1,7-22,4 µg/24h)	22,8 (18,6)	17,4 (12,2)	21,3 (15,1)	NS	NS	NS	NS	NS	NS	NS	NS
Nor-adrenalin (12,1-85,5 µg/24h)	60,4 (25,6)	81,9 (45,8)	58,8 (28,4)	NS	NS	NS	NS	NS	NS	NS	NS
Complete Hemogram											
RBC (4.5-5.9x10^12/L)	5,2 (0,3)	5,1 (0,4)	5,1 (0,3)	NS	NS	0,046	NS	NS	NS	NS	NS
Hemoglobin (13-17.5 g/dl)	15,5 (0,7)	15,7 (1,4)	15,1 (1,2)	NS	NS	NS	NS	NS	NS	NS	NS
Hematocrit (40-50%)	45,7 (2,3)	46,3 (4,1)	45,1 (3,2)	NS	NS	NS	NS	NS	NS	NS	NS
MCV (80-97 fl)	88,8 (4,6)	89,9 (4,5)	89,4 (4,6)	NS	NS	NS	NS	NS	NS	NS	NS
MCH (27-33 pg)	30,2 (1,5)	30,4 (1,6)	30,0 (1,7)	NS	NS	0,036	NS	NS	NS	NS	NS
MCHC (31.5-35.5 g/dl)	34,0 (1,0)	33,9 (1,0)	33,6 (0,7)	NS	NS	NS	NS	NS	NS	NS	NS
RDW (11.5-14.5)	13,8 (0,5)	13,7 (0,7)	13,7 (0,8)	NS	NS	NS	NS	NS	NS	NS	NS
Leukocytes (4.0-11x10^9/L)	7,5 (2,2)	6,9 (1,9)7	6,3 (1,3)	NS	NS	NS	NS	NS	NS	NS	NS
Platelets (150-450 10^3µL)	229,2 (41,3)	217,1 (49,0)	196,7 (47,2)	NS	NS	NS	NS	NS	NS	NS	NS

NS: non-statistical meaning ; n/a: not-applicable

* PA max / PA min

Annex IV

RBCs results for PRDX2 and PRDX $SO_{2/3}$ Correlation with PSG and Clinical Parameters

Table Annex IV. 1: RBC PRDX2 correlations with PSG/Clinical parameters for snorer group

PRDX2	Snorers ALL (N=18)		
	Correlation	Pearson r Value	p Value
Monomer	EPW	-,497(*)	0,036
S-S Dimer	no significant correlation observed		
S-S/S-S Dimer	no significant correlation observed		
$SO_{2/3}$ Monomer	EPW	-.505(*)	0,032
	Noradrenalin U24	-.474(*)	0,047
S-S/$SO_{2/3}$ Dimer	no significant correlation observed		
$SO_{2/3}$ Multimer	T90	,483(*)	0,042
	Fasting glucose	,776(**)	0,000
	Hb A1C	,642(**)	0,004
	HOMA-IR	,737(**)	0,000
	Noradrenalin U24	,565(*)	0,015

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table Annex IV. 2: RBC PRDX2 correlation with PSG/Clinical parameters for snorer group without diabetes

PRDX2	Snorers W/o diabetes (N=16)		
	Correlation	Pearson r Value	p Value
Monomer	Sat min (%)	,516(*)	0,041
S-S Dimer	Oximetry	,631(*)	0,012
S-S/S-S Dimer	Fasting glucose	,526(*)	0,037
$SO_{2/3}$ Monomer	Insulin	,560(*)	0,024
	HOMA-IR	,559(*)	0,024
S-S/$SO_{2/3}$ Dimer	Fasting glucose	,691(**)	0,003
	Cholesterol	,672(**)	0,004
$SO_{2/3}$ Multimer	Height	-,963(*)	0,037
	Cholesterol	,950(*)	0,050

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table Annex IV. 3: RBC PRDX2 correlations with PSG/Clinical parameters for OSA group

PRDX2	OSA ALL (N=18)		
	Correlation	Pearson r Value	p Value
Monomer	no significant correlation observed		
S-S Dimer	Min sat (%)	,477(*)	0,045
S-S/S-S Dimer	Min sat (%)	,615(**)	0,007
	MCHC	,489(*)	0,039
$SO_{2/3}$ Monomer	RBC count	-.624(**)	0,006
	Hemoglobin	-.613(**)	0,007
	Hematocrit	-.624(**)	0,006
	WBC count x 10^9/L	-.484(*)	0,042
S-S/$SO_{2/3}$ Dimer	Age	-,502(*)	0,034
	Systolic BP	-,500(*)	0,035
	Triglycerides	,469(*)	0,050
	Platelets, count x 10^9/L	,521(*)	0,026
$SO_{2/3}$ Multimer	MCHC	,473(*)	0,047

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table Annex IV. 4: RBC PRDX2 correlations with PSG/Clinical parameters for non-treated OSA group without diabetes

PRDX2	OSA W/o diabetes (N=15)		
	Correlation	Pearson r Value	p Value
Monomer	Triglycerides	-,515(*)	0,050
	Homocysteine	,518(*)	0,048
S-S Dimer	Min sat (%)	,532(*)	0,041
S-S/S-S Dimer	Min sat (%)	,624(*)	0,013
$SO_{2/3}$ Monomer	RBC count	-,669(**)	0,006
	Hemoglobin	-,654(**)	0,008
	WBC count x 10^9/L	-,540(*)	0,038
	Hematocrit	-,682(**)	0,005
S-S/$SO_{2/3}$ Dimer	Age	-,573(*)	0,026
	Diastolic BP	-,642(**)	0,010
	Homocysteine	-,585(*)	0,022
	RDW	-,576(*)	0,025
$SO_{2/3}$ Multimer	Systolic BP	,565(*)	0,028

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table Annex IV. 5: RBC PRDX2 correlations with PSG/Clinical parameters for diabetic non-treated OSA group

PRDX2	OSA diabetes (N=3)		
	Correlation	Pearson r Value	p Value
Monomer	MCV	,999(*)	0,025
S-S Dimer	MCHC	,999(*)	0,034
	Weight	,998(*)	0,039
	Abdominal perimeter	,998(*)	0,042
S-S/S-S Dimer	Adrenalin	1,000(*)	0,016
$SO_{2/3}$ Monomer	no significant correlation observed		
S-S/$SO_{2/3}$ Dimer	Weight	,998(*)	0,040
	Abdominal perimeter	,998(*)	0,037
	MCHC	,997(*)	0,045
$SO_{2/3}$ Multimer	.(a)		

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

a. Cannot be computed because at least one of the variables is constant.

Table Annex IV. 6: RBC PRDX2 correlations with PSG/Clinical parameters for non-diabetic OSA patients after CPAP treatment

PRDX2	CPAP W/o diabetes (N=15)		
	Correlation	Pearson r Value	p Value
Monomer	no significant correlation observed		
S-S Dimer	Hb A1C	,545(*)	0,036
	Adrenalin	,558(*)	0,031
	WBC count x 10^9/L	,623(*)	0,013
S-S/S-S Dimer	Triglycerides	,596(*)	0,019
	MCHC	,518(*)	0,048
$SO_{2/3}$ Monomer	no significant correlation observed		
S-S/$SO_{2/3}$ Dimer	MCHC	,705(**)	0,003
$SO_{2/3}$ Multimer	Hb A1C	,559(*)	0,030

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table Annex IV. 7: RBC PRDX2 correlations with PSG/Clinical parameters for diabetic OSA patients after CPAP treatment

PRDX2	CPAP diabetes (N=3)		
	Correlation	Pearson r Value	p Value
Monomer	Days w/o use	-,998(*)	0,042
S-S Dimer	Hb A1C	1,000(*)	0,012
	Hemoglobin	,998(*)	0,042
S-S/S-S Dimer	no significant correlation observed		
$SO_{2/3}$ Monomer	Insulin	1,000(*)	0,008
S-S/$SO_{2/3}$ Dimer	P95	-,998(*)	0,039
	MCH	-1,000(**)	0,010
$SO_{2/3}$ Multimer	no significant correlation observed		

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Plasma results for PRDX2 and PRDX $SO_{2/3}$ Correlation with PSG and Clinical Parameters

Table Annex IV. 8: Plasma PRDX2 correlations with PSG/Clinical parameters for snorer group

PRDX2	Snorers ALL (N=18)		
	Correlation	Pearson r Value	p Value
PRDX2 25 kDa	Height	,496(*)	0,036
	Dopamine U24	,522(*)	0,026
	Oximetry	,532(*)	0,028
PRDX2 20 kDa Monomer	RDI	-,540(*)	0,021
	Arousal Index	-,498(*)	0,035
	Hb A1C	,500(*)	0,034
$SO_{2/3}$ 25 kDa	Oximetry	,586(*)	0,014
$SO_{2/3}$ 50 kDa	Adrenaline U24	-,522(*)	0,026
	Noradrenalin U24	,501(*)	0,034

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table Annex IV. 9: Plasma PRDX2 correlations with PSG/Clinical parameters for non-treated OSA group

PRDX2	OSA ALL (N=18)		
	Correlation	Pearson r Value	p Value
PRDX2 25 kDa	Age	,616(**)	0,007
PRDX2 20 kDa Monomer	ODI	-,668(**)	0,002
	Fasting glucose	,505(*)	0,033
	Homocysteine	-,709(**)	0,001
	RDW	-,659(**)	0,003
$SO_{2/3}$ 25 kDa	no significant correlation observed		
$SO_{2/3}$ 50 kDa	RDW	-,526(*)	0,025

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table Annex IV. 10: Plasma PRDX2 correlations with PSG/Clinical parameters for non-treated OSA group without diabetes

PRDX2	OSA W/o DIABETES (N=15)		
	Correlation	Pearson r Value	p Value
PRDX2 25 kDa	Age	,628(*)	0,012
	Cholesterol	-,516(*)	0,049
PRDX2 20 kDa Monomer	Homocysteine	-,775(**)	0,001
	RDI	-,531(*)	0,042
	ODI	-,748(**)	0,001
	RDW	-,596(*)	0,019
$SO_{2/3}$ 25 kDa	Age	,529(*)	0,043
$SO_{2/3}$ 50 kDa	ODI	-,526(*)	0,044
	Platelets, count x 10^9/L	,598(*)	0,019

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table Annex IV. 11: Plasma PRDX2 correlations with PSG/Clinical parameters for non-treated diabetic OSA group

PRDX2	OSA diabetes (N=3)		
	Correlation	Pearson r Value	p Value
PRDX2 25 kDa	Systolic BP	1,000(**)	0,003
	Diastolic BP	,998(*)	0,036
	Triglycerides	-,999(*)	0,021
	Dopamine	-,999(*)	0,021
PRDX2 20 kDa Monomer	Fasting glucose	,999(*)	0,032
$SO_{2/3}$ 25 kDa	Height	-,999(*)	0,029
	Arousal Index	1,000(**)	0,002
	Hemoglobin	,997(*)	0,047
	Hematocrit	1,000(*)	0,011
	RDW	-1,000(**)	0,001
$SO_{2/3}$ 50 kDa	WBC count x 10^9/L	-,999(*)	0,029
	Platelets, count x 10^9/L	-1,000(**)	0,010

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table Annex IV. 12: Plasma PRDX2 correlations with PSG/Clinical parameters for non-diabetic OSA patients after CPAP treatment

PRDX2	CPAP W/o DIABETES (N=15)		
	Correlation	Pearson r Value	p Value
PRDX2 25 kDa	Days w/o use	-,586(*)	0,022
	MCHC	-,590(*)	0,021
	RDW	,517	0,048
PRDX2 20 kDa Monomer	RDW	,581(*)	0,023
	P95	,619(*)	0,014
$SO_{2/3}$ 25 kDa	no significant correlation observed		
$SO_{2/3}$ 50 kDa	no significant correlation observed		

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table Annex IV. 13: Plasma PRDX2 correlations with PSG/Clinical parameters for diabetic OSA patients after CPAP treatment

PRDX2	CPAP DIABETES (N= 3)		
	Correlation	Pearson r Value	p Value
PRDX2 25 kDa	RDW	,999(*)	0,026
	Hb A1C	-,998(*)	0,041
PRDX2 20 kDa Monomer	Insulin	,999(*)	0,030
$SO_{2/3}$ 25 kDa	Dopamine	-,999(*)	0,024
	MCHC	-1,000(**)	0,009
$SO_{2/3}$ 50 kDa	Platelets, count x 10^9/L	-1,000(**)	0,006

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Annex V

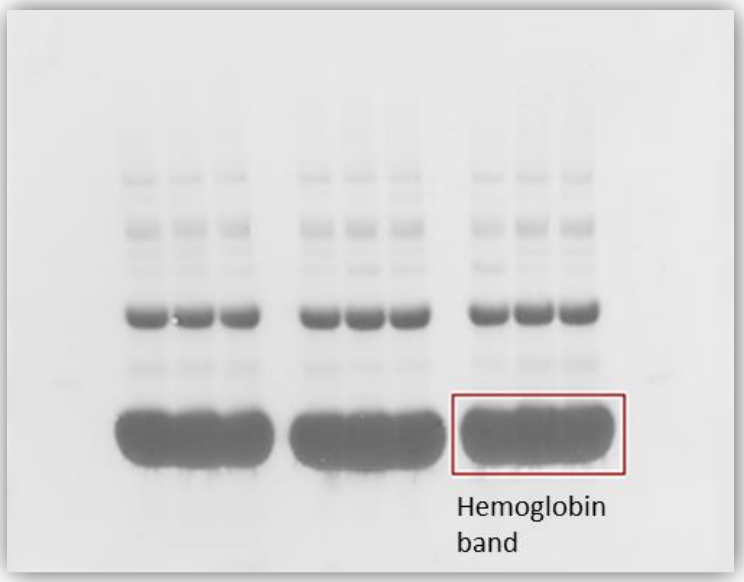

Figure Annex V. 1: Example of a membrane colored with Ponceau solution after transference. Hemoglobin band was used to normalize areas obtained from final WB results of RBC PRDX2 and PRDX $SO_{2/3}$ quantification using ImageJ software.

Figure Annex V. 2: Example of a membrane colored with Ponceau solution after transference. Albumin band was used to normalize areas obtained from final WB results of Plasma PRDX2 and PRDX $SO_{2/3}$ quantification using ImageJ software.