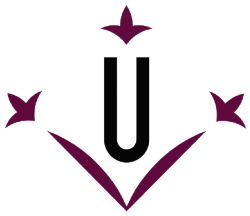
Precision Medicine in Obstructive Sleep Apnea: Deciphering Novel Biomarkers and Biometrics

Lucía Pinilla Latorre



Universitat de Lleida

DOCTORAL THESIS

Precision Medicine in Obstructive Sleep Apnea: Deciphering Novel Biomarkers and Biometrics

Lucía Pinilla Latorre

Doctoral Dissertation submitted to fulfill the requirements for the Doctor of Philosophy degree from the University of Lleida

Health Doctoral Program

Directors:

Dr. Manuel Sánchez de la Torre
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Tutor:

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Universitat de Lleida
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Lleida, September 20, 2023

We, Dr. Ferran Barbé Illa, Chair of Pneumology at the University of Lleida, and Dr. Manuel Sánchez de la Torre, Associate Professor at the University of Lleida, as directors of the Doctoral Thesis entitled “Precision Medicine in Obstructive Sleep Apnea: Deciphering Novel Biomarkers and Biometrics”, conducted by Lucía Pinilla Latorre,

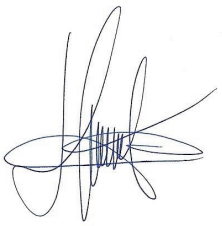
ASSERT:

That the present Doctoral Dissertation, submitted to fulfill the requirements for the Doctor of Philosophy degree in the Department of Medicine at the University of Lleida, has successfully achieved the objectives established at the onset of the Doctoral Thesis. This Dissertation constitutes a comprehensive work that addresses a series of questions related to the identification of biomarkers and biometrics for the characterization and clinical management of obstructive sleep apnea, focusing on their utility for diagnosis, risk stratification, and response to treatment. We deem this Doctoral Dissertation suitable for proceeding to the reading and defense in view of the Health Doctoral Commission.

This certification is hereby signed and documented for official record-keeping purposes, in Lleida, September 20, 2023.

Dr. Ferran Barbé Illa

Dr. Manuel Sánchez de la Torre



Si el futuro no te emociona,

estás en el presente equivocado.

ACKNOWLEDGMENTS

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PRESENTATION

This Doctoral Dissertation has been structured in accordance with the academic regulations for the presentation of Doctoral Theses as a compendium of articles, approved by the Steering Committee of the Doctoral School of the University of Lleida on July 5, 2022, and subsequently by the Governing Council on July 21, 2022.

The studies presented in this Doctoral Dissertation belong to the same research line initiated in 2019, aimed at deciphering novel biomarkers and biometrics for the characterization and clinical management of obstructive sleep apnea, focusing on their utility for diagnosis, risk stratification, and response to treatment. The findings have made significant contributions to the field, resulting in five original research articles that are published or currently under review in indexed international journals.

1. Lucía Pinilla, Iván D Benítez, Fernando Santamaria-Martos, Adriano Targa, Anna Moncusí-Moix, Mireia Dalmases, Olga Mínguez, Maria Aguilà, Mariona Jové, Joaquim Sol, Reinald Pamplona, Ferran Barbé, Manuel Sánchez-de-la-Torre. **Plasma Profiling Reveals a Blood-Based Metabolic Fingerprint of Obstructive Sleep Apnea**. *Biomedicine & Pharmacotherapy*. 2022. Vol. 145, No. 112425. DOI: 10.1016/j.biopha.2021.112425.

2. Lucía Pinilla, Fernando Santamaria-Martos, Iván D Benítez, Andrea Zapater, Adriano Targa, Olga Mediano, Juan F Masa, Maria J Masdeu, Olga Mínguez, Maria Aguilà, Ferran Barbé, Manuel Sánchez-de-la-Torre. **Association of Obstructive Sleep Apnea with the Aging Process.** *Annals of the American Thoracic Society*. 2021. Vol. 18, No. 9, pp. 1540 - 1547. DOI: 10.1513/AnnalsATS.202007-771OC.

3. Lucía Pinilla, Iván D Benítez, Esther Gracia-Lavedan, Gerard Torres, Olga Mínguez, Maria Aguilà, Adriano Targa, Mireia Dalmases, Olga Mediano, Juan F Masa, Maria J Masdeu, Ferran Barbé, Manuel Sánchez-de-la-Torre. **Polysomnographic Characterization of Circadian Blood Pressure Patterns in Patients with Obstructive Sleep Apnea**. *Sleep*. 2023. Vol. 46, No. 4, pp. zsad031. DOI: 10.1093/sleep/zsad031.

4. Lucía Pinilla, Iván D. Benítez, Esther Gracia-Lavedan, Gerard Torres, Olga Mínguez, Rafaela Vaca, Mariona Jové, Joaquim Sol, Reinald Pamplona, Ferran Barbé, Manuel Sánchez-de-la-Torre. **Metabolipidomic Analysis in Patients with Obstructive Sleep Apnea Discloses a Circulating Metabotype of Non-Dipping Blood Pressure**. Second revision in *Antioxidants*.

5. Lucía Pinilla, Neda Esmaeili, Gonzalo Labarca, Miguel A Martinez-Garcia, Gerard Torres, Esther Gracia-Lavedan, Olga Mínguez, Dolores Martínez, Jorge Abad, Maria José Masdeu, Olga Mediano, Carmen Muñoz, Valentín Cabriada, Joaquín Duran-Cantolla, Mercè Mayos, Ramón Coloma, Josep María Montserrat, Mónica de la Peña, Wen-Hsin Hu, Ludovico Messineo, Sehhati MohammadReza, Andrew Wellman, Susan Redline, Scott Sands, Ferran Barbé, Manuel Sánchez-de-la-Torre, Ali Azarbarzin. **Hypoxic Burden to Guide CPAP Treatment Allocation in Patients with Obstructive Sleep Apnoea: A *post-hoc* Study of the ISAACC Trial**. *European Respiratory Journal*. 2023. In press. DOI: 10.1183/13993003.00828-2023.

Furthermore, during the completion of the present Doctoral Thesis, the PhD candidate has actively participated in other research projects related to the respiratory and sleep medicine field, resulting in additional scientific publications, listed in the Appendix of this Dissertation.

CONTENT

SUMMARIES

ABSTRACT

Obstructive sleep apnea (OSA) is a chronic disorder characterized by recurrent episodes of upper airway collapse during sleep, affecting nearly one billion individuals worldwide. The cardinal features of OSA, which are intermittent hypoxia, sleep fragmentation, and large intrathoracic pressure swings, initiate a cascade of mechanisms that lead to wide-ranging adverse health consequences, especially in the cardiovascular and hemodynamic sphere. The timely diagnosis of OSA faces challenges. The gold-standard diagnostic sleep test, polysomnography (PSG), is labor-intensive, time-consuming, and inconvenient for patients. The first-line treatment for OSA is the use of continuous positive airway pressure (CPAP). Although OSA is widely recognized as an independent risk factor for cardiac and vascular disease, the beneficial effects of CPAP therapy on cardiovascular outcomes remain controversial. Embracing a precision medicine perspective, this Doctoral Thesis is directed towards: (i) identifying biomarker candidates to facilitate a more accessible and streamlined OSA diagnosis; (ii) enhancing prognostic insights into the aging-related, hemodynamic, and cardiovascular implications of OSA; and (iii) providing a more tailored approach to identify the patients who are more likely to exhibit a clinical benefit from CPAP treatment. First, a complete analysis of the circulating metabolipidome in patients with suspected OSA revealed diagnostic biomarker candidates and suggested the activation of adaptive mechanisms in response to OSA-derived injury. Second, the evaluation of cellular and molecular hallmarks of aging in patients with suspected OSA showed that OSA severity was associated with a dose-response increase in specific aging markers, specifically in younger patients. Third, a comprehensive characterization of the PSG parameters associated with alterations in the circadian blood pressure (BP) pattern in patients with OSA, revealed that the respiratory arousal index was the most relevant predictor of non-dipping, highlighting the role of sleep fragmentation on the loss of circadian BP rhythmicity. Fourth, a complete analysis of the blood metabolipidome in patients with OSA depicted a specific metabotype of non-dipping BP, suggesting systemic metabolic implications for the circadian control of BP in OSA. Fifth, among non-sleepy patients with acute coronary syndrome, there was a differential impact of CPAP treatment on cardiovascular prognosis according to the baseline hypoxic burden, and only patients with high baseline hypoxic burden levels displayed a long-term protective effect of the treatment. Within this Doctoral Thesis, we leverage advanced technologies and comprehensive analyses from different sources of data to improve the biological characterization and clinical management of OSA. Based on the exposed findings, the combination of molecular information provided by -omics and physiological information derived from sleep data constitutes a promising approach to assist in medical decision-making in OSA.

RESUMEN

La apnea obstructiva del sueño (AOS) es un trastorno crónico caracterizado por episodios recurrentes de colapso de la vía respiratoria superior durante el sueño, que afecta a casi mil millones de personas en todo el mundo. Las características principales de la AOS, que son hipoxia intermitente, fragmentación del sueño y grandes oscilaciones en la presión intratorácica, inician una cascada de mecanismos que conducen a una plétora de consecuencias adversas para la salud, especialmente en la esfera cardiovascular y hemodinámica. El diagnóstico de la AOS enfrenta diversos desafíos. La prueba diagnóstica de referencia, la polisomnografía (PSG), es laboriosa y requiere mucho tiempo, además de ser costosa e incómoda para los pacientes. El tratamiento de primera línea para la AOS es el uso de presión positiva continua en la vía aérea (CPAP). Aunque la AOS está ampliamente reconocida como un factor de riesgo independiente para el desarrollo de enfermedades cardíacas y vasculares, el posible beneficio de la terapia con CPAP sobre la recurrencia de eventos cardiovasculares es un tema controvertido. Adoptando una perspectiva de medicina de precisión, la presente Tesis Doctoral está dirigida a: (i) identificar biomarcadores que faciliten un diagnóstico de la AOS más accesible y sencillo; (ii) ampliar el conocimiento sobre el pronóstico de las implicaciones cardiovasculares, hemodinámicas y las relacionadas con el envejecimiento, propiciadas por la AOS; y (iii) aportar un enfoque individualizado para identificar aquellos pacientes que mostrarán un beneficio clínico del tratamiento con CPAP. En primer lugar, un análisis completo del metabolipidoma circulante en pacientes con sospecha de AOS, puso de manifiesto la existencia de biomarcadores con potencial diagnóstico y sugirió la activación de mecanismos de adaptación en respuesta al daño inducido por la AOS. En segundo lugar, la evaluación de marcadores celulares y moleculares de envejecimiento en pacientes con sospecha de AOS, demostró que la gravedad de la enfermedad se asoció con un aumento del efecto dosis-respuesta en marcadores específicos de envejecimiento, una asociación especialmente significativa en los pacientes más jóvenes. En tercer lugar, una caracterización minuciosa de los parámetros de la PSG asociados a las alteraciones del patrón circadiano de la presión arterial (PA) en pacientes con AOS, reveló que el índice de arousals respiratorios era el factor predictivo más relevante del patrón de PA non-dipping, lo que resalta el papel de la fragmentación del sueño en la pérdida del ritmo circadiano de la PA. En cuarto lugar, un análisis exhaustivo del metabolipidoma circulante en pacientes con AOS, reveló un metabotipo específico del patrón de PA non-dipping, lo que sugería implicaciones metabólicas sistémicas para el control circadiano de la PA en la AOS. En quinto lugar, en pacientes con síndrome coronario agudo que no presentaban somnolencia, se observó un efecto diferencial del tratamiento con CPAP sobre el pronóstico cardiovascular en función de la carga hipóxica inicial. Así, se observó que únicamente aquellos pacientes con

niveles iniciales elevados de carga hipóxica mostraron un efecto protector del tratamiento a largo plazo. En el presente trabajo de Tesis Doctoral, aprovechamos tecnologías avanzadas y análisis exhaustivos de diferentes fuentes de datos para mejorar la caracterización biológica y el manejo médico de la AOS. En base a los hallazgos expuestos, la combinación de información molecular proporcionada por las tecnologías -ómicas, y la información fisiológica derivada de los estudios de sueño, constituye un enfoque prometedor para ayudar en la toma de decisiones clínicas para un manejo integral de la AOS.

RESUM

L'apnea obstructiva del son (AOS) és un trastorn crònic caracteritzat per episodis recurrents de col·lapse de la via respiratòria superior durant el son, que afecta gairebé mil milions de persones a tot el món. Les característiques principals de l'AOS, com son l'hipòxia intermitent, la fragmentació del son i grans oscil·lacions en la pressió intratoràcica, inicien una cascada de mecanismes que condueixen a una plètora de conseqüències adverses per a la salut. El diagnòstic de l'AOS planteja diversos reptes. La prova diagnòstica de referència, la polisomnografia (PSG), és laboriosa i requereix molt de temps, a més de ser costosa i incòmoda per als pacients. El tractament de primera línia per a l'AOS és l'ús de pressió positiva contínua a la via aèria (CPAP). Tot i que l'AOS està àmpliament reconeguda com a factor de risc independent per al desenvolupament de malalties cardíaques i vasculars, el possible benefici de la teràpia amb CPAP sobre la recurrència d'esdeveniments cardiovasculars és un tema controvertit. El marc actual per al maneig de l'AOS segueix un enfocament uniforme per a tots els pacients. Adoptant una perspectiva de medicina de precisió, aquesta Tesi Doctoral està dirigida a: (i) identificar biomarcadors que facilitin un diagnòstic de l'AOS més accessible i senzill; (ii) ampliar el coneixement sobre el pronòstic de les implicacions cardiovasculars, hemodinàmiques i les relacionades amb l'envelliment, propiciades per l'AOS; i (iii) aportar un enfocament individualitzat per identificar aquells pacients que mostraran un benefici clínic del tractament amb CPAP. En primer lloc, una anàlisi completa del metabolipidoma circulant en pacients amb sospita d'AOS va posar de manifest l'existència de biomarcadors amb potencial diagnòstic i va suggerir l'activació de mecanismes d'adaptació en resposta al dany induït per l'AOS. En segon lloc, l'avaluació de marcadors cel·lulars i moleculars d'envelliment en pacients amb sospita d'AOS, va demostrar que la gravetat de la malaltia es va associar amb un augment de l'efecte dosi-resposta en marcadors específics d'envelliment, essent l'associació especialment significativa en els pacients més joves. En tercer lloc, una minuciosa caracterització dels paràmetres de la PSG associats a les alteracions del patró circadià de la pressió arterial (PA) en pacients amb AOS, va revelar que l'índex d'arousals respiratoris era el factor predictiu més rellevant del patró de PA non-dipping, cosa que ressalta el paper de la fragmentació del son en la pèrdua del ritme circadià de la PA. En quart lloc, una anàlisi exhaustiva del metabolipidoma circulant en pacients amb AOS, va revelar un metabotip específic del patró de PA non-dipping, cosa que suggeria implicacions metabòliques sistèmiques per al control circadià de la PA a l'AOS. En cinquè lloc, en pacients amb síndrome coronària aguda que no presentaven somnolència, es va observar un efecte diferencial del tractament amb CPAP sobre el pronòstic cardiovascular en funció de la càrrega hipòxica inicial. Així, es va observar que només aquells pacients amb nivells inicials elevats de càrrega hipòxica van mostrar un efecte protector del tractament a llarg termini. En aquest

treball de Tesi Doctoral, aprofitem tecnologies avançades i anàlisis exhaustives de diferents fonts de dades per millorar la caracterització biològica i el maneig mèdic de l'AOS. En base a les troballes exposades, la combinació d'informació molecular proporcionada per les tecnologies -òmiques i la informació fisiològica derivada dels estudis de son constitueix un enfocament prometedor per ajudar en la presa de decisions clíniques per a un maneig integral de l'AOS.

ABBREVIATION INDEX

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AASM	American Academy of Sleep Medicine
ABPM	Ambulatory blood pressure monitoring
ACS	Acute coronary syndrome
AF	Atrial fibrillation
AHI	Apnea-hypopnea index
AutoPAP	Autotitrating positive airway pressure
BiPAP	Bilevel positive airway pressure
BMI	Body mass index
BP	Blood pressure
CI	Confidence interval
CNS	Central nervous system
CPAP	Continuous positive airway pressure
CRP	C-reactive protein
CSA	Central sleep apnea
CVD	Cardiovascular disease
DR	Dipping ratio
ECG	Electrocardiography
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalography
EMG	Electromyography

EOG	Electrooculography
ESI-Q-TOF	Electrospray-ionization quadrupole time of flight
ESS	Epworth sleepiness scale
FC	Fold change
GAM	Generalized additive model
HB	Hypoxic burden
HF	Heart failure
HIF	Hypoxia-inducible factor
HMDB	Human Metabolome Database
HR	Hazard ratio
HSAT	Home sleep apnea testing
ICAM	Intercellular adhesion molecule
ICSD	International classification of sleep disorders
IQR	Interquartile range
LASSO	Least absolute shrinkage and selection operator
LAUP	Laser-assisted uvulopalatoplasty
LC-MS/MS	Liquid chromatography/tandem mass spectrometry
MAD	Mandibular advancement devices
MI	Myocardial infarction
MMA	Maxilla-mandibular advancement
MS	Mass spectrometry

mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
NF-κB	Nuclear factor kappa B
NREM	Non-rapid eye movement
ODI	Oxygen desaturation index
OR	Odds ratio
OSA	Obstructive sleep apnea
PAP	Positive airway pressure
PAT	Peripheral arterial tonometry
PCA	Principal component analysis
PLS-DA	Partial least squares-discriminant analysis
PSG	Polysomnography
qPCR	Quantitative polymerase chain reaction
RCT	Randomized controlled trial
REM	Rapid eye movement
RF	Random forest
RFA	Radiofrequency ablation
ROS	Reactive oxygen species
RP	Respiratory polygraphy
RT	Retention time
SaO_2	Oxygen saturation

SCOPER	Sleep, cardiovascular, oximetry, position, effort, and respiratory
SD	Standard deviation
TIA	Transient ischemic attack
TRD	Tongue-retaining devices
TSat90	Percent of total sleep time spent with SaO_2 <90%
UHPLC	Ultra-high-performance liquid chromatography
UPPP	Uvulopalatopharyngoplasty
VEGF	Vascular endothelial growth factor
WASO	Wake after sleep onset
WHO	World health organization
8-OHdG	8-hydroxy-2'-deoxyguanosine

INTRODUCTION

1. OBSTRUCTIVE SLEEP APNEA

1.1. Definition

Sleep is a universal function of living species, comprising one-third of the human lifespan [1]. Sleep health represents a multidimensional concept. It can be defined as a pattern of sleep-wakefulness adapted to individual, social, and environmental demands, that promotes physical and mental well-being [2]. Good sleep health is characterized by subjective satisfaction, appropriate timing, adequate duration, high efficiency, and sustained alertness during waking hours.

Sleep health can be compromised by what is referred to as sleep disorders, which encompass a variety of conditions. The International Classification of Sleep Disorders (ICSD) [3], published by the American Academy of Sleep Medicine (AASM), provides a standardized manual for sleep disorders nosology and definition. Accordingly, sleep disorders can be classified into seven categories, which are, insomnia, sleep-related breathing disorders, central disorders of hypersomnolence, circadian rhythm sleep-wake disorders, parasomnias, sleep-related movement disorders, and other sleep disorders. Sleep-related breathing disorders can be subsequently divided into four subcategories, including obstructive sleep apnea (OSA), central sleep apnea (CSA), sleep-related hypoventilation, and sleep-related hypoxemia.

OSA is a chronic respiratory disorder characterized by recurrent episodes of upper airway collapse during sleep. The degree of airflow obstruction can range from partial narrowing, defined as hypopnea, to complete pharyngeal occlusion with cessation of airflow, defined as apnea (**Figure 1**). The number of apneas plus hypopneas per hour of sleep is termed the apnea-hypopnea index (AHI). The International Consensus Document on Obstructive Sleep Apnea [4] defines the presence of OSA as an AHI of 5 or more events per hour accompanied by clinical symptomatology of non-restorative sleep, or alternatively, as an AHI of 15 or more events per hour *per se*.

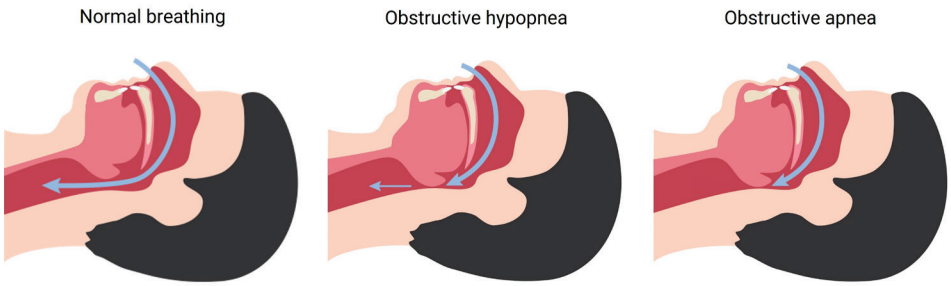

Figure 1. Maintenance of airway patency during normal breathing (left). Partial upper airway narrowing during sleep, defined as hypopnea (center). Complete pharyngeal collapse with cessation of airflow during sleep, defined as apnea (right). Adapted from [5].

1.2. Epidemiology

OSA is the most common sleep-related breathing disorder, globally affecting more than 20% of the adult population [6] and up to 5% of children [7,8]. The high prevalence of OSA has been well-documented by several population-based cohort studies during the past decades [9–11]. In 2007, the World Health Organization (WHO) estimated that more than 100 million individuals were affected by this condition worldwide [12]. Recent global estimates raise this rate to almost 1 billion people between the ages of 30 and 69 years [13]. OSA affects all continents, with a calculated prevalence of 35.2% in Spain and exceeding 50% in certain countries, such as Switzerland and Germany (**Figure 2**) [13]. OSA has been recognized as a major health concern worldwide. However, despite growing awareness in the last decades, it remains a significantly underdiagnosed and untreated condition, especially in minorities [14].

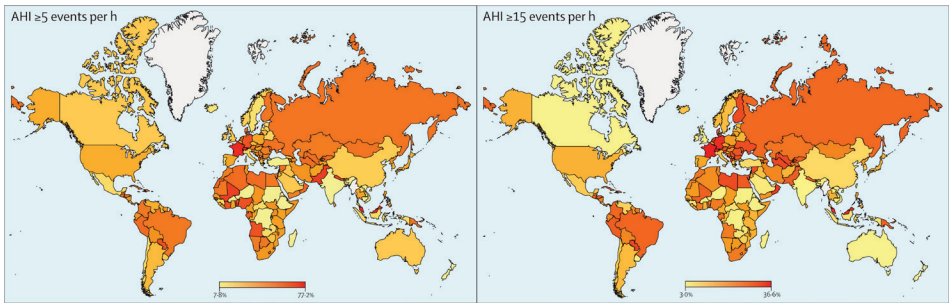

Figure 2. Global heat maps of the estimated prevalence of OSA for each country, considering an AHI of 5 or more events per hour (left), and an AHI of 15 or more events per hour (right). Adapted from Benjafield *et al.* 2019 *Lancet Respir Med* [15].

1.3. Etiology and risk factors

The etiology of OSA is multifactorial and heterogeneous [16]. Upper airway patency depends on a balance between dilatating and collapsing forces. Contraction of the dilator muscles is necessary to maintain airway patency during inspiration. The largest and most important upper airway dilator muscle is the genioglossus, which contracts with each inspiration to prevent the posterior collapse of the tongue. A variety of factors influence the stability of the pharynx and contribute to its closure during sleep. Current evidence indicates that at least four key pathophysiological phenotypes, or more recently termed "endotypic traits", contribute to OSA development (**Figure 3**) [17].

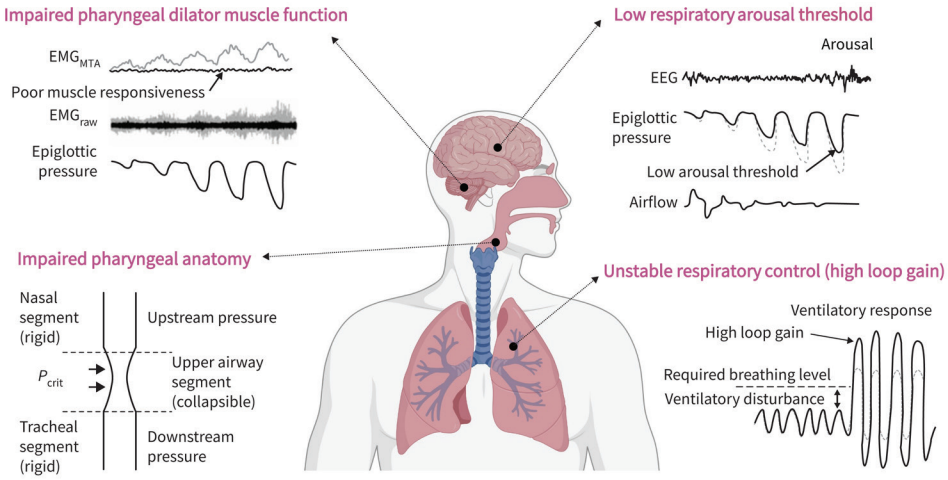

Figure 3. Key endotypic traits that contribute to OSA development: impaired pharyngeal dilator muscle function, unfavorable upper airway anatomy, decreased respiratory arousal threshold, and an unstable/over-sensitive ventilatory control system (increased loop gain). Adapted from Pépin *et al.* 2021 *Eur Respir J* [18].

Unfavorable pharyngeal anatomy is the most common factor contributing to upper airway obstruction [19]. Abnormalities in skeletal and soft tissue structure can alter the mechanical properties of the upper airway and increase its vulnerability to collapse during sleep [20]. Morphological conditions that compromise upper airway dimensions include micrognathia, retrognathia, enlarged tonsils and adenoids, and increased soft tissue in the neck, typically caused by fat deposition in the pharyngeal fat pads and pharyngeal muscles [21].

Non-anatomical features also influence respiratory mechanics and play an important role in upper airway collapsibility [22]. Sleep-related alterations in airway function comprise impaired dilator muscle function (reduced neuromuscular responsiveness to airway narrowing) [23], premature awakening to mild airway narrowing (decreased respiratory arousal threshold) [24], and an unstable/over-sensitive ventilatory control system (increased loop gain) [25]. These mechanisms lead to compromised airway patency resulting in pharynx collapse, although the relative contribution of each varies markedly among and within individuals [18].

Susceptibility to OSA reflects multiple factors that reduce the size of the pharynx or increase airway collapsibility [26]. The following section briefly describes the key risk factors for OSA. However, several other conditions have also been associated with an increased prevalence of OSA, such as pregnancy [27], polycystic ovary syndrome [28], hypothyroidism [29], and allergic rhinitis [30].

- Obesity. Excess weight is a major cause of a narrow pharyngeal airway, representing one of the major modifiable risk factors for OSA. Deposition of adipose tissue in regions surrounding the airway reduces the pharyngeal airspace, contributing to airway compression. The risk for OSA progressively rises with increases in body mass index (BMI) [31] and neck circumference [32]. Weight gain and loss have been consistently associated with increasing and decreasing OSA severity, in observational [33] and intervention studies [34].
- Age. OSA can occur throughout the entire lifespan, from neonates [35] to the very elderly [36], although the mean age at diagnosis is usually between 40 and 50 years [37]. The incidence of OSA increases with age, with a 2- to 3-fold higher prevalence in older adults (over 65 years) compared with those in middle age (from 30 to 65 years) [38,39].
- Sex. It has long been recognized that men have greater vulnerability than women to developing OSA. Population-based studies have estimated a 2 to 3 times greater prevalence of OSA in men than in women [40]. The male predisposition for the disorder has been attributed to sex differences in

anatomical and functional properties of the upper airway. However, hormonal influences are also likely to play an important role in the pathogenesis of OSA, as postmenopausal women have 3 times the odds of having OSA compared with premenopausal women [41].

- Airway morphology. Craniofacial dysmorphisms and upper airway structural changes are typically observed in patients with OSA [21]. Common anatomical abnormalities that increase the likelihood of OSA include retrognathia, micrognathia, and enlarged upper airway soft tissues, such as tonsillar hypertrophy, adenoid hypertrophy, and macroglossia [42].
- Genetics. The prevalence of OSA in first-degree relatives of patients suffering from the disease is 2-fold higher compared with first-degree relatives of healthy individuals [43]. Furthermore, susceptibility to OSA increases directly with the number of affected relatives [44]. Although this association may reflect shared risk factors related to lifestyle, it is estimated that 40% of AHI variance is explained by genetic factors [45,46], suggesting that there are strong genetic underpinnings that predispose to OSA development.
- Race. Available evidence indicates an elevated prevalence and severity of OSA among African Americans [47], Hispanic/Latinos [48], and Asians [49] as compared to Caucasians. However, race and ethnicity may be surrogates for other predisposing features, such as craniofacial differences [50], higher comorbid medical conditions [48], disparities in sleep patterns [51], or lower socioeconomic status [52].
- Lifestyle health behaviors. Several epidemiologic studies have associated cigarette smoking with OSA [53]. Data indicates that current smokers are 3 times more likely to have OSA than never-smokers [54]. Alcohol is also a well-known aggravator factor for OSA, as it compromises airway patency during sleep [55]. Experimental studies have shown that alcohol intake before sleep acutely affects OSA severity [56,57]. Additionally, the consumption of sedative medications and other substances such as benzodiazepines and narcotics, promotes relaxation of the upper airway and exacerbates OSA [58].

1.4. Clinical manifestations

Patients with OSA have different patterns of signs and symptoms (**Figure 4**), which can be clustered into distinct clinical phenotypes [59–61]. The most common clinical manifestation of OSA is unrefreshing sleep, leading to excessive daytime sleepiness, which is reported by up to 90% of patients with OSA referred to sleep clinics [62]. Patients may also experience fatigue, tiredness, and lack of energy [63], ultimately hindering daily functioning [64]. Individuals with OSA are twice as likely as the general population to have chronic morning headaches [65], and habitual dry mouth upon awakening [66]. Other commonly reported daytime symptoms include irritability, memory loss, difficulties concentrating, and cognitive decline [67]. Typical nighttime signs of OSA involve habitual snoring, which is present in 50% to 60% of individuals with OSA [68], and witnessed pauses in breathing, found in 10% to 15% of the cases [69]. However, while snoring and witnessed apneas are considered the hallmark symptoms of OSA, a recent systematic review concluded that nocturnal gasping or choking was the most reliable indicator of the disease [70]. Some patients also report nocturnal gastroesophageal reflux [71] and nocturia [72]. Nonetheless, a significant proportion of patients with OSA are either asymptomatic or minimally symptomatic [73].

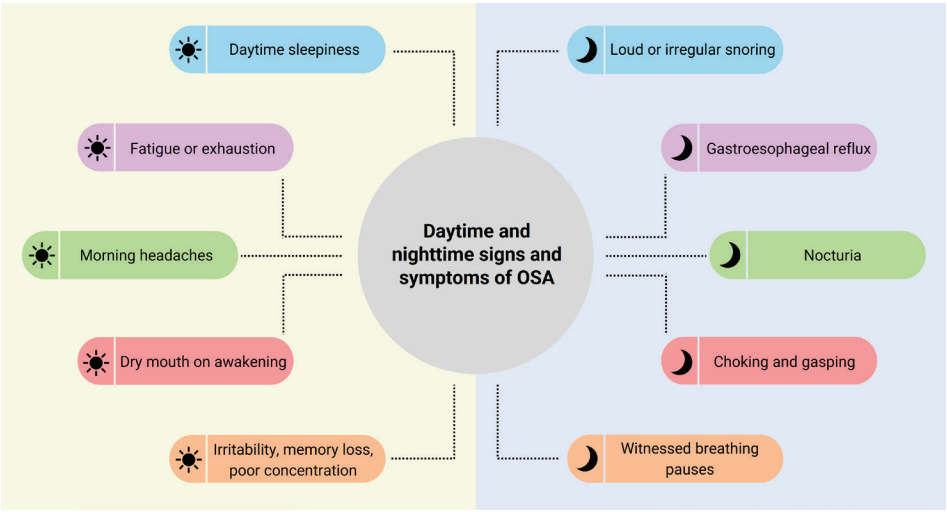

Figure 4. Daytime (left) and nighttime (right) signs and symptoms that should trigger the suspicion of OSA. Own work.

1.5. Diagnosis

Clinical evaluation

A comprehensive medical history and clinical examination are essential steps in the evaluation of patients with suspicion of having OSA. This assessment should include a detailed review of signs and symptoms, along with a physical examination of the facial and upper airway morphology, which may help identify any anatomical abnormalities.

Questionnaires

Self-reported questionnaires can provide valuable predictive information and serve as screening tools to simplify the assessment of OSA. Available questionnaires include the Berlin [74] and the STOP-Bang questionnaires [75], among others [76]. The most widely used questionnaire to assess the subjective degree of sleepiness is the Epworth sleepiness scale (ESS) [77], which asks the patients to rate their tendency to fall asleep in eight different situations. However, although questionnaires can be used to prioritize patients for a sleep study, they cannot replace a formal diagnosis. If the clinical evaluation is suggestive of OSA, overnight testing is required to confirm diagnosis.

Diagnostic procedures

The diagnosis of OSA involves the direct measurement of breathing patterns during sleep. In 1994, the AASM categorized sleep studies into four types based on the number and class of physiological variables recorded [78]. Type I corresponds to attended polysomnography (PSG), which is the most exhaustive sleep study conducted in a sleep laboratory. Home sleep apnea testing (HSAT) englobes unattended sleep studies performed with portable devices (Types II to IV) [79]. Ambulatory PSG falls under Type II, while home respiratory polygraphy (RP) is classified as Type III. Type IV refers to simplified sleep testing devices based on single- or dual-bioparameter recording.

The traditional classification of sleep studies fails to consider newly emerging technologies beyond this schema, such as under-the-mattress sleep trackers [80], or peripheral arterial tonometry (PAT) devices [81]. In 2011, the AASM proposed a new classification system specifically for out-of-center devices [78]. This alternative categorization was based on the type of sensors used to measure Sleep, Cardiovascular, Oximetry, Position, Effort, and Respiratory (SCOPER) parameters. However, due to the complexity and limited familiarity with the SCOPER classification, the sleep medicine community commonly refers to HSAT devices using the traditional classification system [82].

- Polysomnography. The gold-standard diagnostic sleep test, known as attended PSG, is conducted in a laboratory setting and involves the continuous monitoring of several neurophysiologic and cardiorespiratory parameters (**Figure 5**). A typical polysomnogram includes measures of (1) airflow through the nose using a nasal cannula connected to a pressure transducer or through the nose and mouth using a thermal sensor or thermistor; (2) sleep stage and arousals using electroencephalography (EEG), electrooculography (EOG), and chin electromyography (EMG); (3) cardiac function using electrocardiography (ECG); (4) respiratory effort using thoracic and abdominal inductance plethysmography; (5) snoring using a microphone affixed over the trachea; (6) body position; (7) oxygen hemoglobin saturation (SaO_2) using finger pulse-oximetry; and (8) leg movement using leg EMG.
- Respiratory polygraphy. RP is the most common HSAT for OSA, but it is only recommended when there is a high clinical suspicion of the disease and no conditions that predispose to non-obstructive sleep-disordered breathing. This study involves devices that measure limited cardiopulmonary parameters, recording at least two respiratory variables (e.g., airflow and respiratory effort), SaO_2, and cardiac function (heart rate or ECG), but not measures of sleep or leg movements.

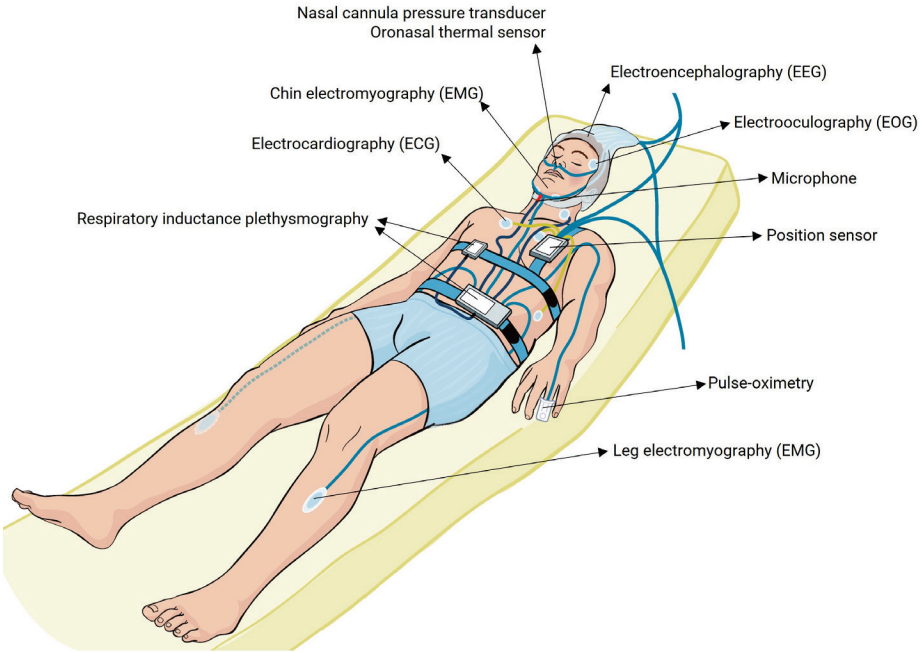

Figure 5. Illustration of all the sensors and measures recorded during a complete overnight PSG, the gold-standard diagnostic sleep test. Adapted from Siyanbade *et al.* 2022 *BioMedInformatics* [83].

Diagnostic criteria

The initial effort to establish standardized scoring definitions for assessing OSA took place in 2007 with the publication of the first edition of the Manual for the Scoring of Sleep and Associated Events by the AASM [84]. This comprehensive and continuously updated resource provides a consensus on rules, terminology, and technical specifications for scoring PSG and HSATs. An apnea is defined as an interruption or nearly complete cessation (≥90%) in airflow for at least 10 seconds. A hypopnea is defined as a partial reduction (30% to 90%) in airflow for at least 10 seconds, accompanied by a concurrent oxygen desaturation of at least 3% or evidence of cortical arousal from sleep. The AHI, calculated as the average number of apnea plus hypopnea events per hour of sleep (or per hour of recording for HSATs), is used for diagnosing the disease and categorizing its severity. The presence of OSA is defined as an AHI of 5 or more events per hour. An AHI of 5 to 15 events per hour indicates mild OSA, of 15 to 30 indicates moderate OSA, and greater than 30 indicates severe OSA.

1.6. Treatment

Therapeutic options for OSA span from behavioral modifications to medical devices and surgery. Recommended lifestyle changes include incorporating regular aerobic exercise, losing weight, and avoiding medications and substances that promote relaxation of the upper airway muscles or suppress respiratory drive, such as alcohol, benzodiazepines, and opioids [85]. Weight loss has been proven to effectively improve the severity of OSA [33] and it is strongly recommended for all patients with overweight or obesity, in conjunction with other therapies if necessary [86]. In asymptomatic or minimally symptomatic patients, it can be considered as the initial standalone treatment [87]. For adult obese patients, bariatric surgery may be suggested if weight has not improved despite the participation in a comprehensive weight reduction program [88]. Behavioral interventions for OSA treatment can also include positional therapy, which is aimed to prevent the supine sleep position [89].

The fist-line treatment for OSA is the use of continuous positive airway pressure (CPAP). CPAP was designed by Colin Sullivan in 1984 [90] to address upper airway collapsibility by delivering a constant positive inspiratory and expiratory pressure to the nose and/or mouth. CPAP devices act as a mechanical splint to maintain the upper airway open during sleep (**Figure 6**).

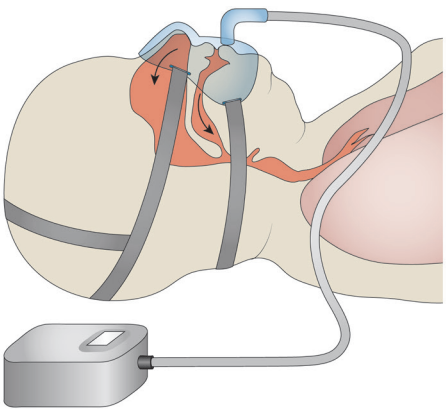

Figure 6. Representation of a CPAP device composed of three main parts: a mask that fits over the nose and/or mouth, with straps that maintain the mask in its position; a tube that connects the mask to the machine's motor; and a motor that injects air into the tube. Adapted from Lévy *et al.* 2015 *Nat Rev Dis Primers* [91].

There are various variable-pressure approaches to positive airway pressure (PAP), including higher pressure during inspiration than during expiration (bilevel PAP or BiPAP), and autoadjusting pressure in response to airflow changes throughout the night (autotitrating PAP or autoPAP). Alternative treatments to PAP include the use of oral appliances, such as tongue-retaining devices (TRD) [92] and mandibular advancement devices (MAD) [93]. Surgical interventions are also available for OSA [94], including maxilla-mandibular advancement (MMA), procedures targeting the pharyngeal region such as uvulopalatopharyngoplasty (UPPP), laser-assisted uvulopalatoplasty (LAUP), and upper airway radiofrequency ablation (RFA). Another potential treatment option for OSA is the stimulation of the hypoglossal nerve [95]. This approach involves the use of a device that delivers electrical stimulation to the hypoglossal nerve, which controls the movement of the tongue and helps to maintain airway patency open during sleep.

2. PHYSIOPATHOLOGY OF OSA

OSA acutely induces several physiological disturbances (**Figure 7**) [92]. Each apnea and hypopnea triggers an episodic cessation or reduction of airflow, respectively. Respiratory events be classified as obstructive, when accompanied by paradoxical movement of the abdomen and thorax against the closed upper airway, or central, when there is a transient reduction of central respiratory motor output to the respiratory pump muscles. Reduced ventilation during apneas and hypopneas leads to a decrease in blood SaO_2 (hypoxemia), and a concomitant increase in carbon dioxide concentration (hypercapnia). During obstructive episodes, the inspiratory effort against the occluded airway leads to exaggerated negative intrathoracic pressures. Respiratory events typically conclude with an arousal of the central nervous system (CNS). Cortical arousals activate the pharyngeal muscles to restore upper airway patency. The resumption of normal breathing is accompanied by the restoration circulating gases back to pre-event baseline levels.

Transitioning back to sleep is associated with the reoccurrence of upper airway collapse, creating a cycle that continues repetitively during sleep. Over time, individuals with OSA are exposed to repetitive episodes of nocturnal hypoxemia-reoxygenation cycles (intermittent hypoxia), abrupt intrathoracic pressure swings, and recurrent arousals from sleep, which lead to sleep fragmentation and affect sleep quantity and quality.

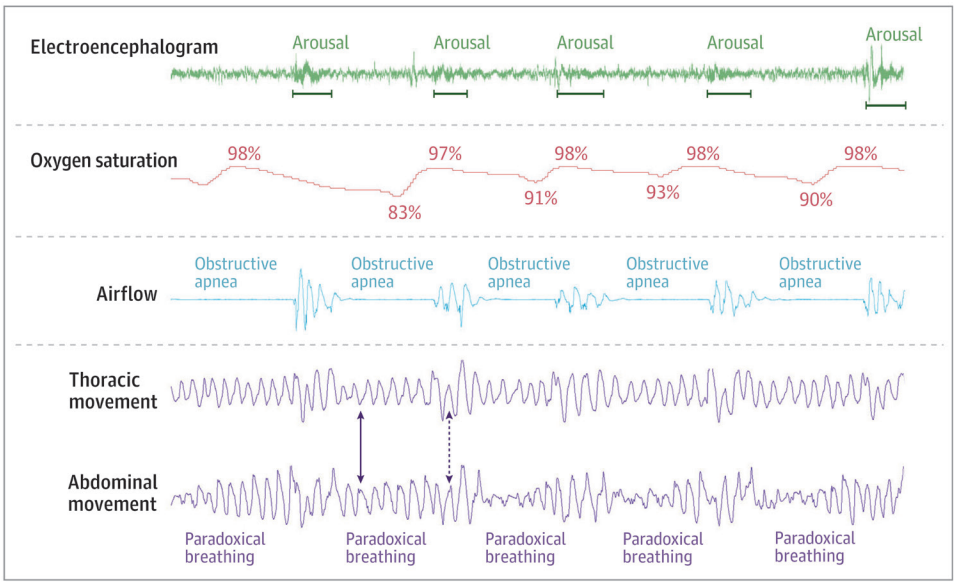

Figure 7. Polysomnographic tracing of OSA events. With each obstructive episode, the absence of airflow is accompanied by a decrease in SaO_2, with movement of the thorax and abdomen (solid arrow), known as paradoxical breathing. EEG arousals terminate the obstructive events with resumption of normal breathing (dotted arrow) and restoration of SaO_2 to normal levels. Extracted from Gottlieb *et al.* 2020 *JAMA* [96].

2.1. Disease mechanisms

The physiopathology of OSA is complex and not entirely understood [97]. The cardinal features of OSA, i.e., intermittent hypoxia, large intrathoracic pressure swings, and sleep fragmentation, initiate the activation of a cascade of physiopathological pathways. The following section briefly describes the intermediate disease mechanisms that are thought to underpin the link between OSA and its associated adverse health consequences (**Figure 8**).

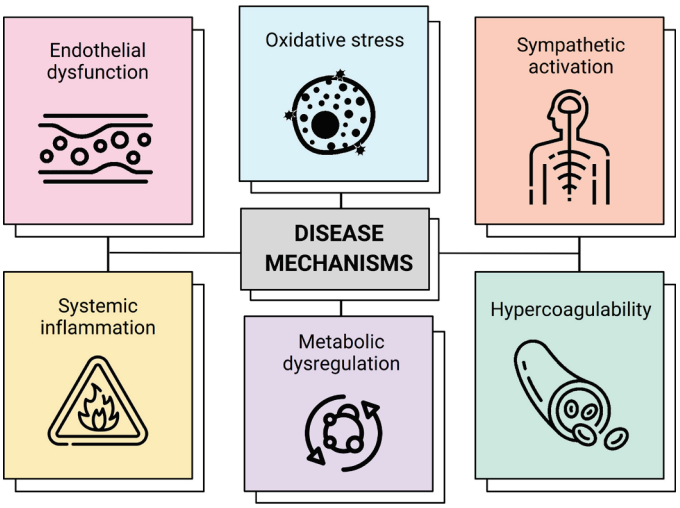

Figure 8. Representation of the intermediate disease mechanisms that are thought to underly the adverse health consequences of OSA. Although illustrated separately, these mechanisms are intertwined and can manifest simultaneously. Own work.

Endothelial dysfunction

Evidence linking OSA to impaired endothelial function and reduced endothelial repair capacity is accumulating [98]. Endothelial dysfunction, characterized by impaired endothelium-dependent vasodilatation, is an early marker of vascular damage. Hypoxia, hypercapnia, and pressor surges accompanying obstructive events are believed to act as potent stimuli for the release of vasoconstrictive molecules that negatively affect endothelial function [99]. OSA patients have exhibited elevated concentrations of plasma markers associated with endothelial dysfunction, such as endothelin-1 [100], intercellular adhesion molecule (ICAM)-1 [101], and vascular endothelial growth factor (VEGF) [102], which were normalized after effective OSA treatment. Furthermore, randomized controlled trials (RCTs) have provided evidence of a functional improvement in endothelial-dependent vascular relaxation following CPAP therapy, leading support to a causal relationship between OSA and endothelial dysfunction [103].

Oxidative stress

OSA has consistently been associated with elevated oxidative stress, as evidenced by animal models of intermittent hypoxia [104] and experimental studies

involving humans [105]. Oxidative stress arises from an imbalance between prooxidant and antioxidant systems. The repetitive cycles of hypoxia and reoxygenation experienced in OSA are believed to be analogous to ischemia-reperfusion injury. The rapid reoxygenation that occurs after apneas and hypopneas is linked to the production and release of reactive oxygen species (ROS), thereby triggering mechanisms of oxidative stress [106]. Oxidative stress markers have long been observed in OSA patients, including increased free radical production in leukocytes, compromised cellular antioxidant capacity, activation of redox-sensitive gene expression, enhanced plasma-lipid peroxidation, and nitric oxide deficiency [107,108]. Moreover, randomized intervention studies have demonstrated the attenuation of intracellular ROS production and oxidative stress mediators by CPAP therapy [109].

Sympathetic activation

There is compelling evidence linking OSA with sympathetic activation. Episodic apneas and hypopneas elicit an increase in sympathetic activity that occurs upon event termination[110]. This heightened sympathetic drive eventually persists during normoxic daytime wakefulness [111]. Patients with OSA are exposed to elevated sympathetic nerve traffic to peripheral blood vessels, with consequent vasoconstriction, changes in cardiac output, surges in systemic and pulmonary blood pressures, and acceleration of heart rate [112]. RCTs have demonstrated that CPAP treatment reduces sympathetic nervous system activity and mitigates the increased sympathetic tone observed in OSA patients [113,114].

Systemic inflammation

OSA has been associated with a chronic state of low-grade systemic inflammation [115]. Hypoxia is implicated in initiating and propagating inflammatory responses through the activation of inflammatory signaling pathways [116]. Sleep deprivation has also been linked to the production of inflammatory cytokines [117]. Multiple studies have reported activation of the proinflammatory nuclear

transcription factor kappa B (NF-κB) in OSA [118], with its levels correlating with disease severity and decreasing after CPAP treatment [119]. Indeed, evidence from cell culture models has shown that intermittent hypoxia selectively activates NF-κB-mediated inflammatory pathways, in contrast to sustained hypoxia, which preferentially activates hypoxia-inducible factor (HIF)-1-dependent pathways [120]. NF-κB serves as a crucial regulator of inflammation, immune response, and cell survival, orchestrating the expression of proinflammatory mediators [121]. The combination of intermittent hypoxia and sleep deprivation may explain the increased levels of circulating inflammatory molecules observed in OSA, including cytokines [122], interleukins [123], and C-reactive protein (CRP) [124].

Metabolic dysregulation

Findings from numerous studies provide solid evidence that OSA is independently associated with metabolic alterations [125], including metabolic syndrome [126], insulin resistance [127], and type 2 diabetes [128]. OSA has been shown to alter fasting levels of insulin, glycemia, and free fatty acids [129,130], independently of body weight and adiposity. Additionally, OSA has been associated with impairments in insulin sensitivity, glucose effectiveness, and pancreatic beta-cell function [131]. RCTs have provided contradictory results of OSA treatment on metabolic outcomes, rendering uncertain whether they are a consequence of OSA or primarily reflect the effects of coexisting obesity [132].

Hypercoagulability

There is evidence supporting a hypercoagulable state in OSA. Patients with OSA have exhibited elevated levels of plasma fibrinogen, enhanced platelet activity, reduced fibrinolytic capacity, and other markers of thrombotic risk [133]. Abolition of OSA by CPAP therapy has demonstrated to reduce platelet aggregability [134]. The heightened coagulation state observed in OSA is thought to be related to elevated levels of catecholamines [135,136]. However, further studies are needed to understand the role of hemostatic mechanisms and coagulability in OSA.

3. CONSEQUENCES OF OSA

OSA is associated with a broad spectrum of health consequences (**Figure 9**). It has a detrimental impact on the quality of life, which is the most essential patient-reported outcome [137]. Excessive daytime sleepiness and reduced vigilance are the cardinal effects of sleep deprivation and sleep fragmentation caused by recurrent arousals throughout the night. These features of OSA are believed to explain the increased rates of depression, anxiety, and mood changes observed among OSA patients [138,139]. Inattention and fatigue resulting from hypersomnolence hinder academic performance [140] and contribute to work disability [141] and absenteeism[142]. Even individuals with mild OSA encounter difficulties in carrying out their daily activities [143]. In this context, there is substantial evidence reporting that OSA treatment improves subjective sleepiness [144], quality of life [145], and symptoms of anxiety and depression [146,147].

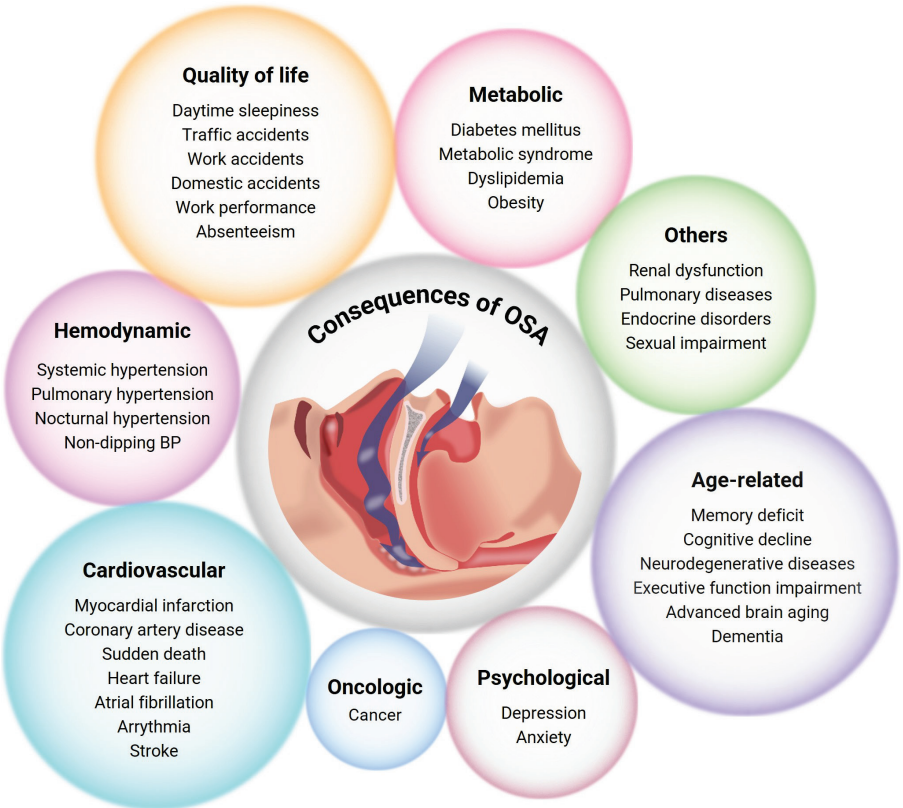

Figure 9. Overall overview of the adverse health consequences associated with OSA. Own work.

Patients with OSA are at a 5 to 7 times greater risk of being involved in motor vehicle crashes [148], as well as domestic [149] or workplace accidents [150]. Indeed, drivers who are not adherent to OSA treatment with CPAP are 5 times more likely to experience a serious preventable road traffic accident compared to those who use the treatment effectively [151].

Additionally, OSA is associated with a plethora of adverse health consequences, including metabolic disorders [152], cancer [153], renal dysfunction [154], pulmonary diseases [155], sexual impairment [156], and endocrine alterations [157]. Nonetheless, the primary focus of this Doctoral Thesis revolves around three major consequences of OSA: aging- and age-related consequences, hemodynamic consequences, and cardiovascular consequences. Subsequent sections will delve into these specific areas in detail.

3.1. Aging- and age-related consequences

OSA has traditionally been considered an age-related disorder. Aging is known to be an important factor contributing to the risk of OSA, with a rising disease prevalence as age increases [158]. Patients with OSA demonstrate significant impairment in vigilance, coordination, executive function, and memory, in contrast to healthy subjects [159–161]. Although these cognitive impairments are primarily observed in the elderly population, OSA leads to impairments in performance and brain function in adults who are younger than expected[162]. Furthermore, recent findings have related untreated OSA with aging-related cognitive deficit [163], as well as an increased vulnerability to early stages of dementia and the development of neurodegenerative diseases [164,165]. Literature suggests that patients with OSA are susceptible to developing cerebral and cognitive impairments at an earlier age than would normally be expected [166].

Evidence from clinical and experimental studies supports that the physiologic changes induced by OSA resemble those induced by aging [167–169]. Additionally, OSA and aging have risk factors in common, suggesting shared disease

mechanisms. Therefore, OSA and aging could be regarded as two different manifestations of the same underlying process, namely, the accumulation of cellular and molecular alterations that promote time-dependent functional decline. However, the precise mechanisms by which OSA could induce or accelerate aging have not been characterized extensively[170].

In the past decade, López-Otín *et al.* [171] identified the main factors that contribute to the aging phenotype and proposed a classification into nine categories, named "The Hallmarks of Aging" (**Figure 10**). The proposed hallmarks of aging are as follows: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. A comprehensive study of the hallmarks of aging in OSA may contribute to elucidate the potential contribution of the disease to the aging process.

Figure 10. Enumeration of "The Hallmarks of Aging" proposed by López-Otín *et al.* 2013 *Cell* [171]. According to authors, each hallmark fulfills the following criteria: (1) it manifests during normal aging; (2) its aggravation accelerates aging; and (3) its amelioration retards the normal aging process. Extracted from López-Otín *et al.* 2013 *Cell* [171].

Several preclinical studies have provided robust evidence supporting the assumption that the pathological mechanisms underlying OSA are influenced by age, with more pronounced effects observed in younger populations [172,173]. In this regard, available evidence from clinical settings also indicates that the impact of OSA on various outcomes is more marked in young patients than in elderly individuals. This age-dependent differential effect of OSA has been described on vascular function [174], daytime sleepiness [175], likelihood of obesity [176], risk of incident hypertension [177], cancer incidence [178], and all-cause mortality [179], among others. Relatedly, our group recently demonstrated that the exposure of mice to chronic intermittent hypoxia induced deleterious cardiovascular effects resembling chronological age-related decline [180]. These effects were more prominent in young mice, supporting the hypothesis that OSA may lead to age-dependent premature aging. Nevertheless, this association of OSA with age-dependent premature aging observed in an experimental murine model of OSA is yet to be illuminated in humans.

3.2. Hemodynamic consequences

Patients with OSA are exposed to a wide range of acute hemodynamic consequences during each respiratory event, including sympathetic-mediated vasoconstriction, increased left ventricular afterload, and changes in cardiac output [181]. These hemodynamic derangements lead to marked fluctuations in blood pressure (BP) that occur repetitively throughout the night. Impairments in BP have been largely documented in OSA. Up to 70% of OSA patients are hypertensive [182,183]. Large-scale cross-sectional and longitudinal studies have established a strong correlation between OSA and systemic hypertension [184,185]. Prospective studies have further demonstrated that OSA predisposes normotensive individuals to the development of hypertension [186,187]. Furthermore, RCTs and subsequent meta-analyses have provided evidence that CPAP treatment significantly reduces BP levels in OSA patients [188,189].

In addition to systemic hypertension, OSA has been linked to other BP-related complications, such as pulmonary hypertension [190], masked hypertension [191], resistant hypertension [192], nocturnal hypertension [193], and impaired BP dipping. This section will focus on the impact of OSA on the circadian regulation of BP.

Healthy individuals exhibit BP variations over a 24-hour period. BP typically declines by 10% to 20% at nighttime during sleep, compared with BP values during daytime wakefulness. The concept of circadian patterns of BP was first introduced by O'Brien *et al.* in 1988 [194] (**Figure 11**). When BP during sleep follows a physiological decrease, this is called a dipping pattern, designating individuals with this pattern as dippers. Conversely, a blunted or absent nocturnal BP decrease is termed a non-dipping pattern, and these individuals are classified as non-dippers.

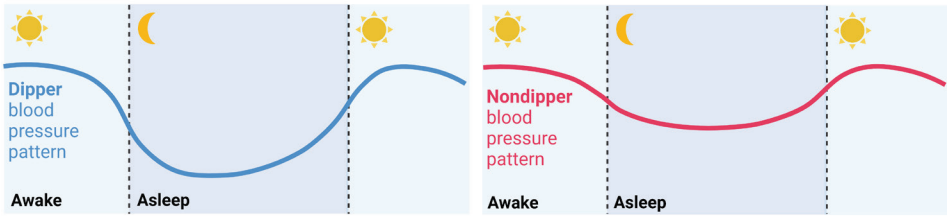

Figure 11. Illustration of the circadian patterns of BP. According to the European Society of Hypertension practice guidelines for office and out-of-office BP measurement [195], dipping is defined as a nocturnal BP decrease ≥10% of daytime levels (left), and non-dipping is defined as a nocturnal BP decrease <10% of daytime levels (right). Adapted from Pinilla *et al.* 2023 *Sleep* [196].

Patients who exhibit a non-dipping BP pattern are at an increased risk of suffering adverse cardiovascular events and developing hypertensive end-organ damage [197–200]. Despite the accumulating body of evidence highlighting the clinical significance and prognostic value of non-dipping BP in normotensive [201], hypertensive [202–205], and population-based cohorts [206], the pathophysiology underlying this phenomenon remains not fully understood [207]. The etiology of impaired nocturnal BP dipping is complex and may be influenced by various factors, among which the disruption of biological circadian rhythms during sleep appears to play a pivotal role [208].

The evidence linking OSA to impaired nocturnal BP dipping extends beyond cross-sectional studies [209]. Longitudinal studies have demonstrated that OSA is a risk factor for developing a non-dipping BP profile [210]. In terms of treatment, our group has observed a differential BP response to CPAP depending on the circadian pattern of BP, both in normotensive [211] and hypertensive [212] patients with OSA. Patients with a non-dipper BP profile exhibited a higher benefit from CPAP treatment in terms of BP reduction, remarkably in nocturnal BP. However, the precise mechanisms that explain the close association between OSA and the development of BP abnormalities are not completely depicted [213]. Several major features of OSA have been proposed as potential triggers for nocturnal non-dipping [214], but the relative importance of each OSA-related factor is unclear. A comprehensive characterization of the sleep parameters and molecular pathways underlying the dysregulation of circadian BP rhythmicity in OSA may shed light on the physiopathological connection between these two conditions.

3.3. Cardiovascular consequences

OSA activates a series of mechanisms known to elicit cardiac and vascular damage, potentially contributing to the initiation and progression of cardiovascular disease (CVD) (**Figure 12**) [215–217]. In the cardiovascular sphere, untreated OSA has been associated with wide-ranging complications, including myocardial infarction (MI) [218], heart failure (HF) [219], atrial fibrillation (AF) [220] and other cardiac arrhythmias [221], stroke [222], transient ischemic attack (TIA) [223], and cardiovascular mortality [224]. OSA frequently co-exists in patients with CVD. It is estimated that 40-80% of patients with established CVD have undiagnosed OSA [225–227]. Furthermore, the presence of comorbid OSA in patients with pre-existing CVD has been associated with a poorer long-term prognosis [228–230]. Prospective studies indicate that among patients who have experienced a MI, those with OSA are more likely to have suffered their cardiac event during the nighttime period, presumably due to the acute nocturnal hypoxic, adrenergic, and hemodynamic stress induced by obstructive respiratory events [231].

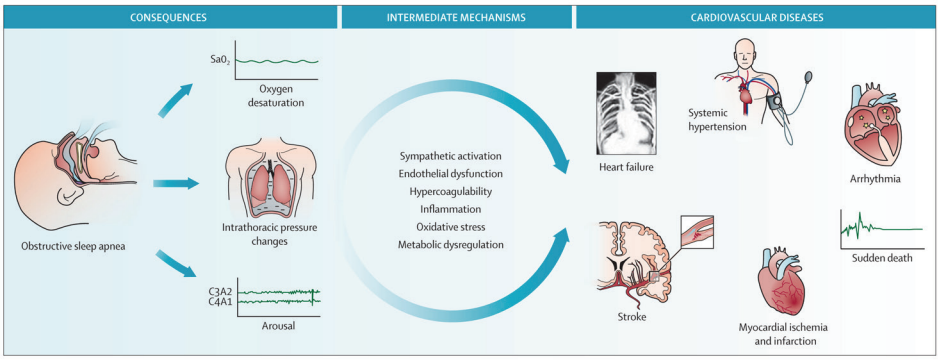

Figure 12. Illustration of the physiopathological events and intermediate disease mechanisms induced by OSA that potentially contribute to the initiation and progression of CVD. Adapted from Sánchez-de-la-Torre *et al.* 2012 *Lancet Respir Med* [217].

Cumulative clinical and experimental data points to OSA as a modifiable risk factor for CVD [232–234]. A seminal observational study by Marin et al. [235] showed that patients with untreated severe OSA were at an increased risk of incident fatal and non-fatal cardiovascular events compared to healthy participants, while those who received CPAP treatment exhibited a similar risk profile to that of healthy individuals. Additionally, previous evidence from RCTs had shown a favorable impact of CPAP treatment on a variety of CVD drivers [236–239]. However, the effect of CPAP treatment on cardiovascular outcomes remains controversial.

The CERCAS trial [240] (Effect of Continuous Positive Airway Pressure on Hypertension and Cardiovascular Morbidity-Mortality in Patients with Sleep Apnea and no Daytime Sleepiness; NCT00127348) involved 725 non-sleepy patients with moderate-to-severe OSA and no prior history of CVD. Patients were randomized to CPAP or no active intervention other than dietary and sleep hygiene advice. In this trial, CPAP did not reduce the incidence of hypertension or cardiovascular events over a median follow-up of 4 years. Since then, three major secondary prevention RCTs have been conducted to address the cardiovascular benefits of CPAP treatment in patients with moderate-to-severe non-sleepy OSA. First, the RICCADSA trial (Continuous Positive Airway Pressure Treatment in Coronary Artery Disease and Sleep Apnea; NCT00519597) [241] included 244 patients with newly revascularized coronary artery disease who were randomly

assigned to receive CPAP or no-CPAP. The treatment had no effect on the incidence of adverse cardiovascular outcomes during a median follow-up of 4.75 years. In the SAVE trial (Continuous Positive Airway Pressure Treatment of Obstructive Sleep Apnea to Prevent Cardiovascular Disease; NCT00738179) [242], 2,717 adults with established coronary or cerebrovascular disease were randomized to CPAP plus usual care, or usual care alone. After a median follow-up period of 3.7 years, the use of CPAP did not reduce the incidence of major adverse cardiovascular endpoints. More recently, the ISAACC trial (Continuous Positive Airway Pressure in Patients with Acute Coronary Syndrome and Obstructive Sleep Apnea; NCT01335087) [243] enrolled 1,264 patients with acute coronary syndrome (ACS) who were randomized to CPAP or usual care. Once again, the use of CPAP treatment over a median follow-up of 3.35 years did not reduce the prevalence of cardiovascular events.

The reasons for RCTs failing to demonstrate a positive effect of CPAP treatment on cardiovascular outcomes have been extensively debated. Together with the modest adherence to treatment reported in these trials, one key aspect of this discussion is based on questioning the omnibus use of the AHI to assess OSA [244]. Despite its standard use as the primary diagnostic, prognostic, and treatment-guiding criterion [245,246], available literature suggests that the AHI alone is an oversimplification of a complex and diverse disease phenomenon [247]. The substantial variability of response observed across studies underscores the existence of high heterogeneity in the impact of OSA and its treatment with CPAP on the cardiovascular system. This clinical scenario prompts us to question whether specific patient subgroups may be more susceptible to the detrimental effects of OSA and thus derive greater benefits from its treatment. Aspirational goals of the sleep medicine community include adopting a more tailored approach to optimize the management of this heterogeneous disease. The development of novel metrics that accurately foresee cardiovascular risk in OSA and predict response to treatment represents a hot topic in the field.

4. PRECISION MEDICINE IN OSA

The current framework for the management of OSA follows a one-size-fits-all approach. This generalist way for addressing the multifactorial, heterogeneous, and complex nature of OSA can have important repercussions for patients and healthcare systems. Rather than considering OSA as a single, universally applicable disease model, a more comprehensive characterization of the disease is crucial to gain understanding of the impact of OSA on each individual [248]. It is relevant to consider that distinct phenotypes of OSA may be driven by different physiological etiologies, may result in different clinical manifestations, may be linked to different health complications, and may display divergent responses to therapy. Under this paradigm, there is a growing emphasis on transitioning towards more targeted and personalized approach to addressing the disease [249].

Precision medicine represents an innovative approach to healthcare that takes into account individual variability to provide personalized care for patients. This emerging medical paradigm aims to align preventive and therapeutic interventions to each individual's needs [250]. The implementation of precision medicine in OSA involves the integration of different sources of data, including molecular data derived from omics analysis and physiological data derived from sleep tests [251]. These two aspects will be described in the following subsections.

4.1. Biomarkers

In the recent decades, technological and methodological advances have propelled the rapid screening of thousands of molecules in complex biological samples with high reproducibility, giving rise to the field known as "omics". Omics encompasses various branches of biology that involve the study of different molecular domains, including genomics, epigenomics, transcriptomics, proteomics, and metabolomics (**Figure 13**). Over the past few years, omics technologies have yielded important new insights into biological systems, playing a pivotal role in advancing our understanding of human biology and diseases [252].

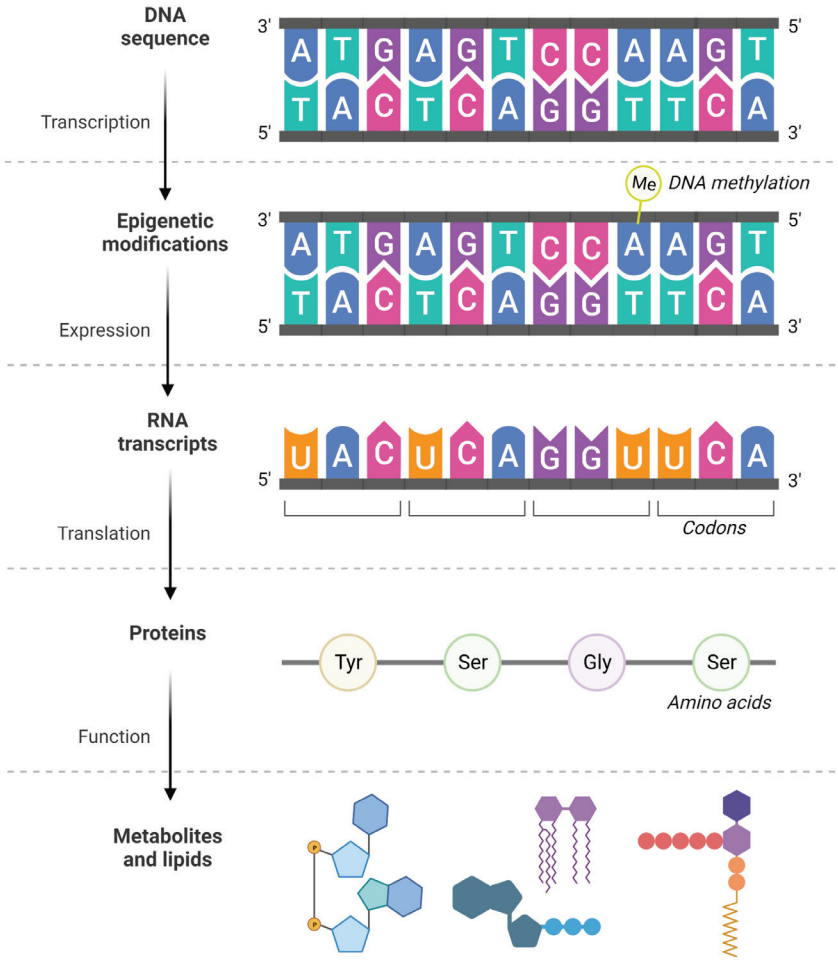

Figure 13. Schematic diagram of the omics cascade, including genomics (study of DNA sequence), epigenomics (study of mechanisms influencing gene expression that do not rely on differences in DNA sequence), transcriptomics (study of RNA transcripts), proteomics (study of proteins), and metabolomics (study of small molecules comprising the substrates and products of metabolism). Own work.

Metabolomics, the focus of this subsection, represents one of the newest disciplines within the omics field. The concept was first introduced by Nicholson et al. in 1999 [253], involving the comprehensive study the entire metabolome expressed in a given biological sample or organism. The metabolome is defined as the complete collection of metabolites, i.e., small molecules (molecular mass <1,5000 Da) that are chemically transformed during metabolism. Metabolites can be categorized as endogenous or exogenous [254]. Endogenous metabolites are routinely produced by the host catabolism or anabolism, including lipids, amino acids, short peptides, nucleic acids, sugars, alcohols, and organic acids, among

others. The synthesis of endogenous metabolites is encoded by the genome and is highly conserved across species [255]. Endogenous metabolites play essential roles in growth, development, and other key physiological functions in all living species. Exogenous metabolites, collectively forming the exposome, are xenobiotic compounds that are extrinsic to the genetically encoded metabolic pathways of an organism [256]. These compounds can originate from various sources, such as diet, environment, and lifestyle habits, and include plant phytochemicals, food additives, drugs, microbial products, pollutants, etc.

Collectively, metabolites represent the ultimate downstream product of multiple intracellular actors, including genes, transcriptional activators, RNA transcripts, protein transporters, enzymes, and other cellular components. The metabolome provides a quantifiable readout of the biochemical state of cells and tissues, resulting from the interplay between intrinsic and extrinsic factors. Metabolomics is therefore regarded as the final layer of the omics cascade [257].

In chronic diseases like OSA, the phenotype is complex and dynamic due to the occurrence of multiple interactions among genetic and environmental factors. As a complementary technique to other omics, metabolomics reflects the upstream input from the environment. Therefore, the study of metabolites offers a plethora of information that can contribute to the understanding of disease pathogenesis, helping to bridge the genotype-to-phenotype gap [258].

The term “biomarker”, a portmanteau of “biological marker”, refers to an objective and quantifiable indicator of a biological state, process, or condition [259]. Metabolites, used as single targets or in combination within a molecular signature, have long served as biomarkers for a variety of conditions, with cholesterol emerging as a prominent example. In recent times, cutting-edge high-throughput profiling strategies have revolutionized biomarker analysis [260,261], holding promising potential in the era of personalized medicine, not only for candidate biomarker discovery [262–264] (**Figure 14**), but also for the pathophysiological exploration of underlying disease mechanisms [265–267].

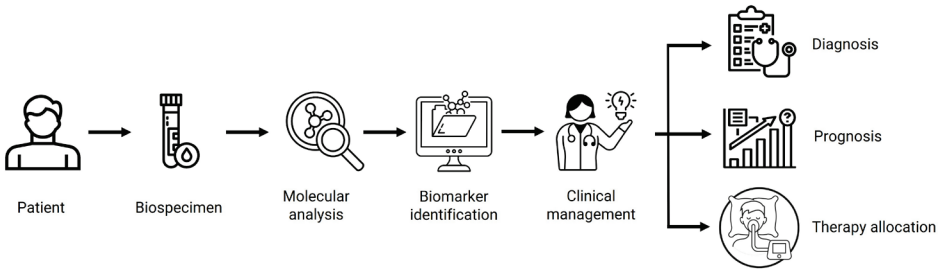

Figure 14. Diagram representing the potential translational applicability of molecular-based biomarkers from biospecimens to guide clinical decision-making. Adapted from Pinilla *et al*. 2021 *Sleep Med Rev*[268].

4.2. Biometrics

The current framework for the management of OSA reflects a "one size fits all" approach, where a single parameter, the AHI, is used to define the presence, categorize the severity, and guide the physician prescription for therapy. However, the AHI entails several inherent limitations [269,270]. Firstly, it assumes that apneas and hypopneas are fundamentally equal in their biologic effects. Moreover, the computation of the AHI neglects the temporal distribution of the events, fails to consider event duration, and does not differentiate between obstructive and central events. The definition of hypopnea is also a matter of controversy [271], as it depends on the guidelines or manual followed (European *vs.* American), the type of sleep test conducted (PSG *vs.* Types II to IV), and an arbitrary threshold for desaturation scoring (3% *vs.* 4%) [272]. Furthermore, all events are equally quantified independently of whether they are associated with an arousal, an increase in heart rate, or a drop in oxygenation. Finally, the AHI is prone to interscorer and individual night-to-night variability.

Overall, the AHI fails to capture all the key physiopathological aspects of OSA. Therefore, substantial efforts are currently underway to discover additional metrics that can effectively characterize OSA and predict adverse outcomes [273]. Major advances have been achieved by applying computational methods to derive new biometrics from raw sleep data [274]. In recent years, a growing list of candidate metrics has been proposed [275]. Examples of novel physiologically-informed indices include sleep depth [276], sleep drive [277], arousal intensity [278],

arousal threshold [279], pulse-rate response respiratory events [279], and the focus of this subsection, the hypoxic burden (HB).

The HB was developed by Ali Azarbarzin and colleagues [280] as a single measure that encapsulates the frequency of nighttime respiratory events with the depth and duration of event-related hypoxemia. This metric can be easily derived from overnight sleep studies and is calculated as the total area under the oxygen desaturation curve in association with each respiratory event, normalized by the sleep duration, with the units of HB being (%min)/h (**Figure 15**). For instance, a HB of 20 (%min)/h is equivalent to experiencing 20 min of 1% desaturation per hour or 5 min of 4% desaturation per hour. Since its introduction to the sleep community in 2018, a growing number of studies have demonstrated the capacity of HB for predicting CVD-related outcomes, including morbidity [280–284] and mortality [285]. This biometric could become a target to assist therapeutic decision-making in OSA. However, to date, no study has evaluated the potential of HB for predicting the therapeutic benefit of CPAP treatment.

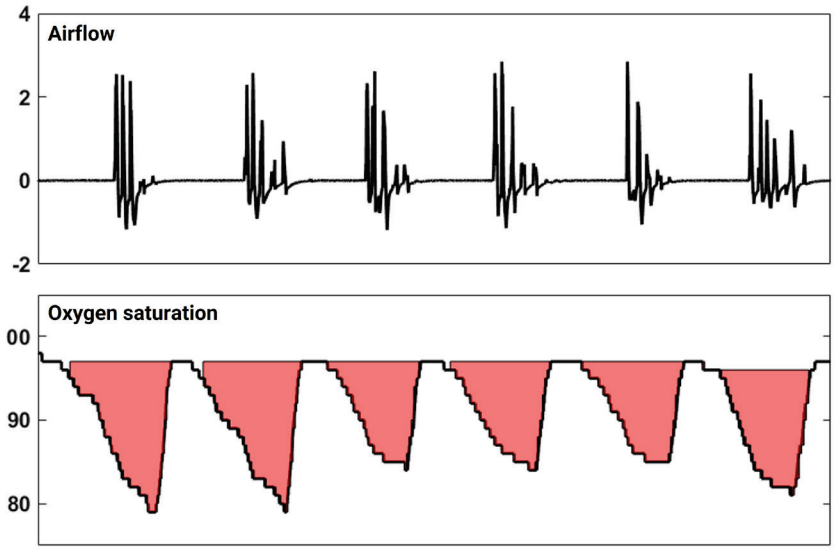

Figure 15. Illustration of the HB calculation. For each individual apnea and hypopnea, airflow and SaO_2 signals are synchronized at the termination of the respiratory event (time zero). Overlaid SaO_2 signals associated with all respiratory events are ensemble-averaged to obtain a patient-specific search window. This search window is used to quantify the individual area under the desaturation curve for each respiratory event. Total HB is defined as the sum of all individual areas (%min) divided by total sleep time (h). Adapted from Martinez-Garcia *et al.* 2022 *Arch Bronconeumol* [286].

5. CONTEXTUAL FRAMEWORK OF THE DOCTORAL THESIS

OSA is a prevalent chronic disorder with potentially devastating consequences. Its economic impact is far-reaching [287]. The latest reports estimate the cost of diagnosing and treating OSA in the USA to surpass 12 billion US dollars annually [288]. However, a significant proportion of the patients remain undiagnosed, up to 80% in the case of moderate-to-severe OSA [289,290]. The indirect costs of undiagnosed OSA are estimated at nearly 150 billion US dollars per year, in terms of loss of productivity, public safety, and consumption of health resources due to comorbidities. Moreover, it has been observed that patients with undiagnosed OSA use, on average, twice as many healthcare services in the decade before diagnosis compared to matched controls [291,292].

The timely diagnosis of OSA faces challenges, with delays of up to 10 years from the onset of initial symptoms to the proper disease diagnosis [293]. The gold-standard diagnostic test for OSA, in-laboratory PSG, is labor-intensive, time-consuming, and inconvenient for the patient. Moreover, it requires specialized facilities and trained technicians, generating long waiting lists [90,294]. Limited access to PSG and associated costs renders high rates of undiagnosed and undertreated OSA, especially in low-medium income settings [295–297]. HSAT constitutes a cheaper alternative for ambulatory OSA diagnosis [298]. However, polygraphy lacks recording of neurophysiological signals, which may result in an underestimation of the AHI and inaccurate interpretations of the sleep test that would require further confirmation by PSG [299]. Given the multiple pathophysiological risks and consequences of OSA, coupled with the inconvenience, laboriousness, and economic impact derived from its diagnosis, there is a compelling need to discover and implement alternatives for early and effective OSA detection [271]. The identification of biomarkers in readily accessible biospecimens holds great promise in bridging the current diagnostic gap. Despite research efforts in recent years, there is a shortage of simple, reliable, and non-invasive biomarkers to accurately detect the disease.

Embracing a precision medicine perspective, this Doctoral Thesis is focused on the study of biomarkers and biometrics for the characterization and clinical management of OSA, and addresses three major areas of investigation: diagnosis, risk stratification, and response to treatment (**Figure 16**).

Figure 16. Major target areas for the application of precision medicine-based approaches to improve the clinical management of OSA, from disease diagnosis to risk assessment and prediction of treatment response. Adapted from Pinilla *et al*. 2021 *Sleep Med Rev* [268].

HYPOTHESIS AND OBJECTIVES

STUDY 1

Hypothesis

OSA induces a unique metabolomic and lipidomic profile that is detectable in plasma. This blood-based fingerprint of OSA can contribute to the understanding of disease pathogenesis. A circulating metabolipidomic signature is useful to accurately detect OSA among individuals with suspicion of the disease. OSA treatment with CPAP triggers changes in the plasma metabolomic/lipidomic profile.

Objectives

We analyzed the complete circulating metabolome and lipidome of patients with suspected OSA, aiming to explore diagnostic biomarker candidates, and to unravel potential pathophysiological mechanisms underlying the disease. Additionally, we aimed to evaluate changes in the plasma metabolomic/lipidomic profile following CPAP treatment.

STUDY 2

Hypothesis

OSA promotes cellular and molecular alterations that accelerate the aging process. This premature aging induced by OSA is age-dependent, expecting to be more prominent in younger populations than in older individuals.

Objectives

We evaluated specific cellular and molecular hallmarks of aging in patients with OSA, aiming to elucidate the contribution of OSA to the aging process. Additionally, we aimed to assess whether this association is influenced by the age of the patient.

STUDY 3

Hypothesis

OSA promotes the alteration of the circadian BP pattern through specific physiopathological mechanisms. The characterization of the PSG parameters associated with impaired BP dipping in OSA can enlighten the physiological connection between these two conditions.

Objectives

We characterized the PSG parameters that are associated with alterations in the circadian BP pattern in patients with OSA, aiming to identify the main sleep features that explain hemodynamic non-dipping in OSA.

STUDY 4

Hypothesis

OSA promotes systemic metabolomic and lipidomic changes that are characteristic of the non-dipping BP pattern. The study of the metabolipidomic pathways that characterize the disruption of BP dipping in OSA can shed light on the molecular link between sleep-disordered breathing and the dysregulation of circadian BP rhythmicity.

Objectives

We comprehensively analyzed the blood metabolipidome of patients with OSA aiming to depict a specific metabotype of non-dipping which can provide new insights into the molecular mechanisms that mediate the circadian BP variations in OSA.

STUDY 5

Hypothesis

Among non-sleepy ACS patients with OSA, there is a differential pattern of cardiovascular response to CPAP treatment depending on the baseline HB level. This biometric is useful to identify a specific subgroup of OSA patients who exhibit long-term cardiovascular benefits from CPAP therapy.

Objectives

In non-sleepy ACS patients, we evaluated whether the effect of CPAP treatment on the long-term incidence of cardiovascular events was dependent on the baseline HB level, aiming to assess the potential of this biometric to predict the cardiovascular benefit of treating OSA with CPAP.

METHODOLOGY

1. STUDY DESIGNS

1.1. Sleep unit cohort (Studies 1 to 4)

Population

Multicenter, observational, prospective, and longitudinal study including consecutive adult participants who were referred to the sleep unit due to suspected OSA (ClinicalTrials.gov: NCT03513926). The exclusion criteria included the following: existence of a previously diagnosed sleep disorder, history of CPAP treatment, psychophysical inability to complete the questionnaires, or any medical, social, or geographical circumstance that, in the opinion of the responsible investigator, affected the eligibility of the subject (e.g., pregnancy, drug or alcohol consumption, or less than 1 year of life expectancy).

Recruiting centers

Patient enrollment took place in four hospitals in Spain: University Hospital Arnau de Vilanova and Santa Maria of Lleida, San Pedro de Alcántara Hospital, Guadalajara University Hospital, and Parc Taulí University Hospital.

Ethical aspects

The Clinical Research Ethics Committee of the coordinating center (University Hospital Arnau de Vilanova and Santa Maria of Lleida) approved the study (No. 1153/1411), and every enrolled patient provided informed written consent to participate in the study.

1.2. Cardiology unit cohort (Study 5)

Population

Post-hoc analysis of the ISAACC study (ClinicalTrials.gov: NCT01335087), a multicenter, open-label, parallel, prospective, and randomized controlled trial [300]. Consecutive adult subjects admitted for ACS to the coronary care unit or cardiology hospitalization ward were eligible to participate in the ISAACC study if

they did not exhibit excessive daytime sleepiness, defined as ESS ≤10. ACS was defined as the acute presentation of coronary disease with or without ST-segment elevation infarction, unstable angina, or type 1 MI. The exclusion criteria included the following: previous treatment with CPAP, psychophysical inability to complete questionnaires, presence of any previously diagnosed sleep disorder, more than 50% central apneas or the presence of Cheyne–Stokes respiration, presence of severe chronic diseases, a medical history that could interfere with the study objectives or compromise conclusions, any medical, social or geographical factor that might jeopardize patient compliance, and any processes that reduce life expectancy to less than 1 year.

Recruiting centers

Patient enrollment took place throughout 15 hospitals in Spain: University Hospital Arnau de Vilanova and Santa María of Lleida, University and Polytechnic Hospital La Fe of Valencia, University Hospital Germans Trias i Pujol, University Hospital Parc Taulí, Guadalajara University Hospital, Burgos University Hospital, Cruces University Hospital, Araba University Hospital, Santa Creu i Sant Pau Hospital, Albacete University Hospital, Clinic Hospital of Barcelona, Son Espases University Hospital, San Pedro de Alcántara Hospital, Sant Joan d'Alacant University Hospital, Sant Joan XXIII Hospital of Tarragona, and Central University Hospital of Asturias.

Ethical aspects

The Clinical Research Ethics Committee of each participating center approved the trial protocol (approval number in the coordinating center: 2010/852), and every enrolled patient provided informed written consent to participate in the study.

2. CLINICAL PROCEDURES AND MEASUREMENTS

2.1. Sleep unit cohort (Studies 1 to 4)

Baseline procedures and measurements

- Clinical evaluation: Detailed information about sociodemographic characteristics, unhealthy lifestyle habits, and medical history, including comorbidities and prescribed medications, were collected from the patients by trained clinicians. General physical and anthropometric parameters were documented, and the degree of self-reported daytime somnolence was evaluated using the ESS. Patient symptomatology of OSA was collected and the STOP-Bang questionnaire was assessed.
- Sleep evaluation: All patients who satisfied the selection criteria underwent an overnight in-lab PSG sleep study (Philips Sleepware G3, Amsterdam, Netherlands). All methods were performed according to national clinical practice guidelines and regulations [4,301]. The results from the sleep studies were analyzed by trained personnel using standard criteria [302]. The AHI and other conventional OSA severity paraments were calculated according to international scoring guidelines. Treatment recommendations in those patients diagnosed with OSA were based on the national clinical guidelines, according to usual clinical practice [4,301].
- BP evaluation: In the morning immediately after the sleep study, the patients were self-reported to 24-hour ambulatory BP monitoring (ABPM) (Mortara Ambulo 2400; Milwaukee, USA), according to internationally recommended procedures [303]. Awake and asleep periods were defined using the sleeping time reported by each participant. The dipping ratio (DR) was calculated as the ratio between average nighttime and daytime BP values. A dipper circadian BP pattern was defined as nocturnal BP decrease of >10% relative to daytime values (DR ≤0.9), and

a non-dipper circadian BP pattern was defined as a nocturnal BP decrease of ≤10% relative to daytime values (DR >0.9).

- Sample collection: Overnight fasting urine and venous blood samples were obtained from each patient in the morning immediately after the sleep study, between 08:00 and 09:00 a.m. Urine samples were centrifuged to separate the urine fraction from the sediment. Blood samples collected in ethylenediaminetetraacetic acid (EDTA) tubes were centrifuged to separate the plasma fraction from the serum and buffy coat. All specimens were immediately aliquoted, frozen, and stored in a dedicated -80ºC freezer.

Follow-up procedures and measurements

Patients treated with CPAP were evaluated after 6 months of follow-up.

- Clinical evaluation: The follow-up clinic visit included a physical examination, record of current medication use, unhealthy life habits, and changes in anthropometric measurements.
- OSA treatment: The level of adherence to the treatment with CPAP was recorded in the 6-months visit by means of the machines' internal clocks. Good compliance was defined as the use of a CPAP device for an average of at least 4 hours per night.
- BP evaluation: In the 6-month follow-up visit, patients underwent a 24-hour ABPM using the same device and following the same internationally recommended guidelines as employed during the baseline visit.
- Sample collection: In the 6-month follow-up visit, overnight fasting urine and venous blood samples were obtained from each patient between 08:00 and 09:00 a.m. The collected samples were processed, aliquoted, frozen, and stored identically as in the baseline visit.

Study groups

Based on the data obtained from the PSG and ABPM procedures, the subjects were classified into different groups to address the specific objectives of each study (**Figure 17**).

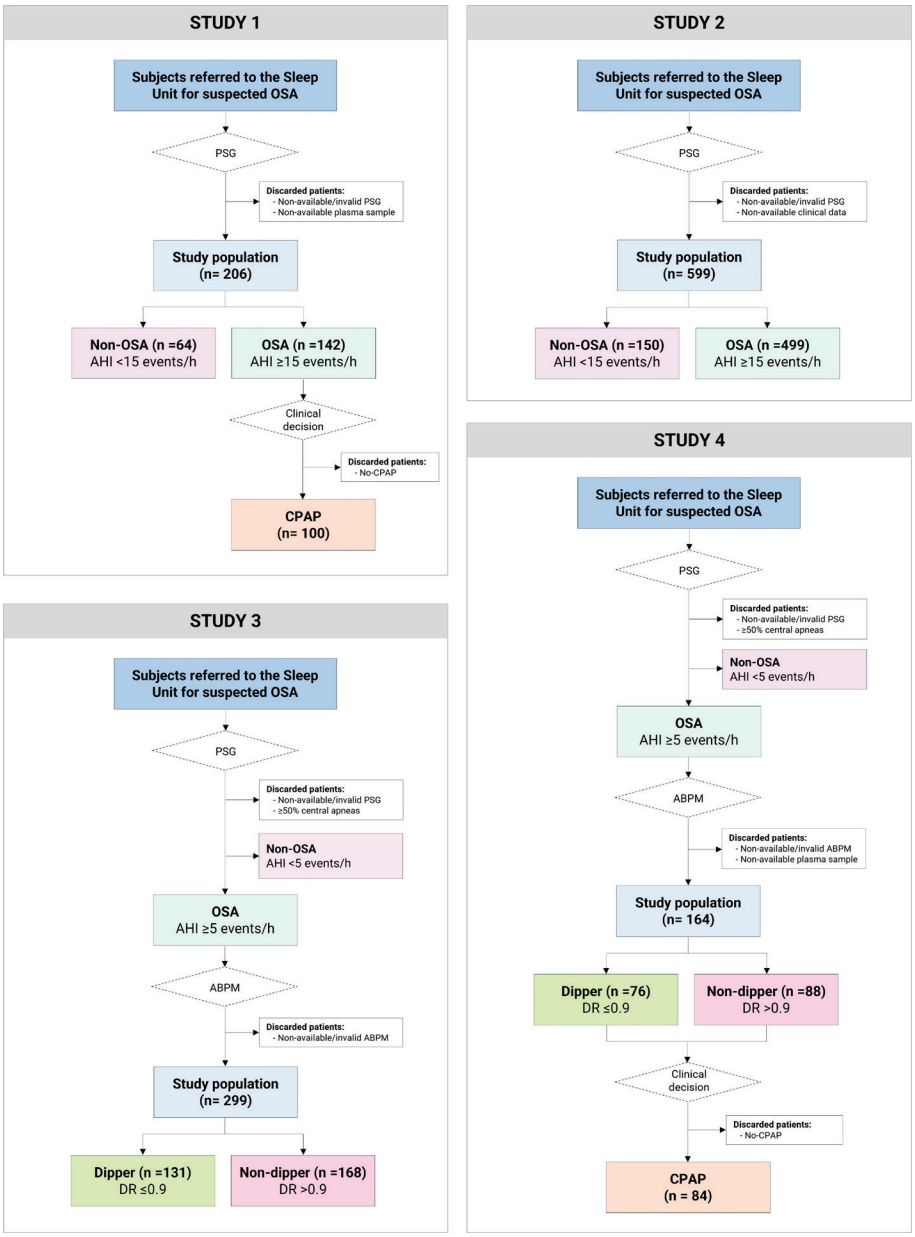

Figure 17. Flowcharts of the Studies 1 to 4. Consecutive adult subjects who were referred to the Sleep unit for suspected OSA were eligible to participate. Participants who met the inclusion and exclusion criteria underwent a PSG sleep study and ambulatory BP evaluation. Patients diagnosed with OSA were treated following usual clinical practice and were followed for 6 months. To address the specific objectives of each study, the subjects were classified into different groups. Own work.

2.2. Cardiology unit cohort (Study 5)

Baseline procedures and measurements

- Clinical evaluation: Detailed information regarding sociodemographic characteristics, health behaviors, and medical history, including comorbidities and prescribed medications, was collected from all patients by trained clinicians. General physical and anthropometric parameters were recorded, and the degree of self-reported daytime sleepiness was assessed with the validated Spanish version of the ESS.
- Sleep evaluation: All patients who satisfied the selection criteria underwent a RP (Embletta, ResMed, Bella Vista, Australia) during the first 24-72 h after the time of hospitalization for ACS. All methods were performed according to the national clinical practice guidelines and regulations [304]. The AHI, oxygen desaturation index (ODI), and other conventional OSA severity paraments were calculated according to international scoring manuals and guidelines.
- Randomization: Patients diagnosed with OSA (AHI ≥15 events/h) were randomly assigned (1:1) to the CPAP group or the Usual Care group. Usual are instructions comprised education on lifestyle and behavioral modifications.

Follow-up procedures and measurements

Follow-up visits were scheduled for all patients at 1, 3, 6, 12, 18, 24, 30, and 36 months and annually thereafter. All patients were monitored and followed-up for a minimum of 1 year.

- Clinical evaluation: Each follow-up clinic visit included a physical examination, record of current medication use, unhealthy life habits, changes in anthropometric measurements, and the assessment of the primary endpoint. The primary endpoint of the ISAACC trial was a composite of death from any cardiovascular cause, or non-fatal events,

including acute myocardial infarction, non-fatal stroke, hospital admission for heart failure and new hospitalizations for unstable angina or transient ischemic attack. A blinded committee adjudicated both fatal and non-fatal cardiovascular outcomes specified in the protocol. The follow-up time was defined as the time between the baseline visit and the end of the study or the occurrence of a cardiovascular event, whichever occurred first.

– OSA treatment: In each follow-up visit, the patient's level of adherence to CPAP treatment was measured by means of the machines' internal clocks, dividing the hours of CPAP use by the days of treatment. Good compliance was defined as a mean use of the CPAP device ≥4 h per day.

Study groups

All patients with available RP data were included in this *post-hoc* analysis of the ISAACC trial (**Figure 18**).

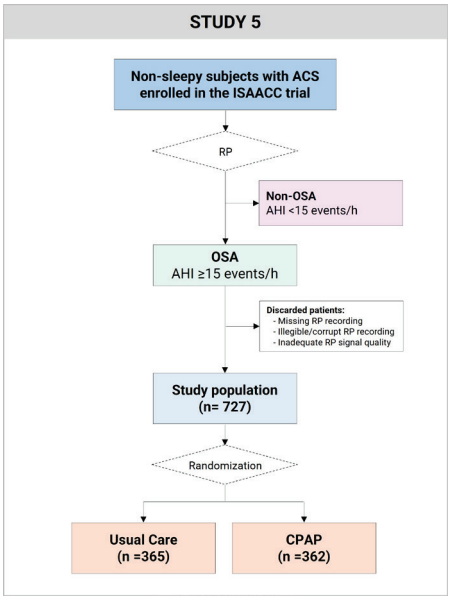

Figure 18. Flowcharts of the Study 5. Consecutive adult non-sleepy subjects admitted for ACS to the coronary care unit or cardiology hospitalization ward were eligible to participate in the ISAACC study. Participants who met the inclusion and exclusion criteria underwent a RP sleep study. Patients diagnosed with OSA were randomized to receive CPAP treatment or Usual care measures and were followed for a minimum of 1 year. Own work.

3. BIOMARKER AND BIOMETRIC DETERMINATIONS

The methodological approach applied to analyze the biomarkers and biometrics among the different studies is briefly described below. Detailed information for each determination can be found in the *Articles* section.

3.1. Plasma metabolomic and lipidomic profiling (Studies 1 & 4)

In Studies 1 and 4, metabolites [305] and lipids [306] were isolated from the same patient-derived plasma sample. The metabolic and lipidic extracts were analyzed via liquid chromatography coupled to tandem mass spectrometry [307]. Ultra-high-performance liquid chromatography (UHPLC) was performed using an Agilent 1290 series system, and mass spectrometry analyses through electrospray ionization quadrupole time of flight (ESI-Q-TOF), with an Agilent 6520 instrument (**Figure 19**). Data were acquired in both positive and negative ionization modes using the MassHunter Data Acquisition software [308,309]. To identify the compounds, annotated features, defined by exact mass and retention time (RT), were compared against the Human Metabolome Database (HMDB) [310]. Potential identities were confirmed by comparison of the exact mass, RT, and MS/MS spectra fragmentation pattern of the class representative internal standards, when available, with data from public databases [311]. Bioinformatic pathway enrichment analyses were conducted using the MetaboAnalyst web service [312].

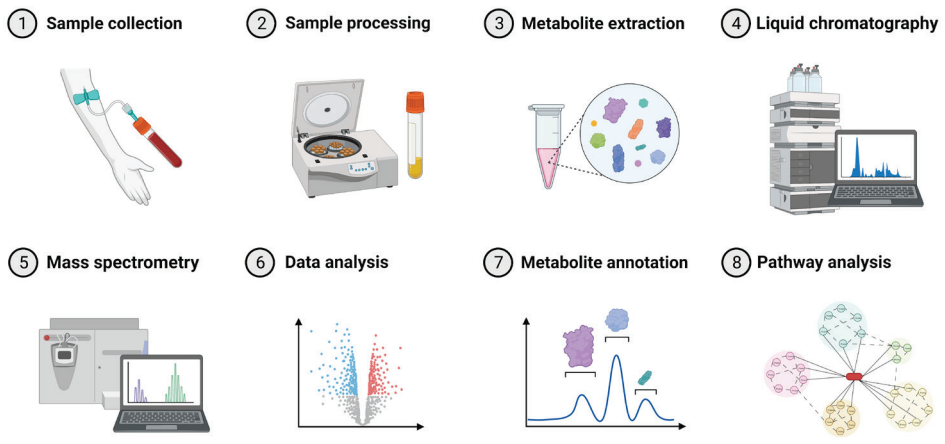

Figure 19. Untargeted metabolipidomic profiling workflow, based on LC-MS/MS. Own work.

3.2. Molecular determination of the hallmarks of aging (Study 2)

In Study 2, a total of five hallmarks of aging were assessed. The alteration of intercellular communication was estimated through the serum CRP concentration, measured using an immunoturbidimetric assay [313]. To evaluate the deregulation of nutrient sensing, we used the homeostasis model assessment index to assess the degree of insulin resistance [314]. Telomere attrition was evaluated by measuring the leukocyte telomeric length. Leukocytic DNA was extracted from the buffy coat, and the telomeric length was quantified using a quantitative polymerase chain reaction (qPCR) with QuantStudio 7 Flex (Applied Biosystems) [315]. To estimate the degree of mitochondrial dysfunction, we measured the leukocytic mitochondrial DNA (mtDNA) copy number with qPCR and normalized it to the nuclear DNA (nDNA) copy number [316]. Finally, as a marker of genomic instability, we measured the urinary concentration of 8-hydroxy-2-deoxyguanosine (8-OHdG) [317].

3.3. Calculation of the PSG parameters (Study 3)

In Study 3, measures of OSA severity derived from PSG were categorized into four groups. Respiratory disturbances included the AHI, obstructive apnea index and hypopnea index. Nocturnal hypoxemia parameters recorded by pulse oximetry included the mean SaO_2, minimum SaO_2, percent of time spent with SaO_2 <90% (TSat90), and desaturation index (number of SaO_2 <90% events per hour of sleep). Measures of sleep fragmentation included arousals from sleep, which were further classified into three categories: arousals associated with a respiratory event, arousals associated with limb movement, and non-specific/spontaneous arousals. To evaluate sleep architecture, the entire sleep period was divided into four stages: nonrapid eye movement (NREM) stage 1 (N1), non-REM stage 2 (N2), non-REM stage 3 (N3), and rapid eye movement (REM) stage (R). The sleep macrostructure was calculated as the proportions of time spent in the N1, N2, N3, and R stages. Additionally, conventional sleep

quality parameters were determined, including total sleep time, sleep latency (transition time from wakefulness to non-REM sleep onset), sleep efficiency (proportion of total time in bed spent asleep), total wake time, and wake after sleep onset (WASO; total time of wakefulness occurring after the sleep onset).

3.4. Calculation of the oximetry-based HB (Study 5)

In Study 5, the HB was calculated for all OSA patients with available RP data in the ISAACC cohort. The original HB calculation algorithm [280] has been modified to calculate the HB using automatically-identified desaturations from pulse oximetry, regardless of the scored respiratory events [318]. All oxygen desaturation/recovery episodes with at least a 2% drop and a 2% rise in magnitude were automatically detected from the SaO_2 signal. The identified desaturation episodes were then synchronized with respect to the minimum saturation points. Desaturation events were ensemble-averaged to obtain the individual-specific search window, defined as the time interval between the two peaks around the minimum value of the ensemble average signal (**Figure 20**). HB is determined by summing all the individual areas under the desaturation curve restricted by the search window (%/min), normalized by the total sleep time (h).

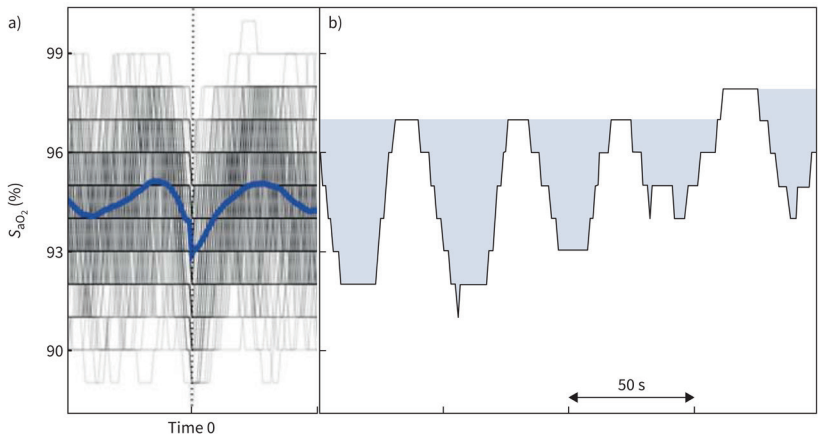

Figure 20. Calculation of the oximetry-based HB. (a) All automatically-detected desaturations are synchronized at their minimum saturation levels (time zero) and ensemble averaged to obtain the search window (duration between two maximum peaks around time zero). (b) HB is calculated as the summation of the colored SaO_2 areas within the search window for all desaturation episodes divided by total sleep time. Extracted from Pinilla *et al.* 2023 *Eur Respir J* [319].

4. DATA ANALYSIS

Descriptive statistics were used to provide an overview of the patient characteristics within each study population. For quantitative data, the mean (standard deviation; SD) was calculated to summarize normally distributed variables. The median [p_{25} to p_{75}] or median [interquartile range; IQR] was calculated for non-normally distributed variables. The normality assumption of each distribution was analyzed using the Shapiro-Wilk test. Qualitative data were summarized using absolute and relative frequencies. Descriptive characteristics were compared between the different study groups using a chi-squared test for qualitative variables, and a t-test or equivalent non-parametric test for quantitative variables.

For Studies 1 and 4, metabolite and lipid levels were log-transformed for statistical purposes. Differences in metabolite and lipid levels between groups were evaluated using adjusted linear models for arrays and empirical Bayes statistics, controlling for confounders. Molecules with a significant difference between groups and a fold change (FC) >1.25 (or <0.8 for downregulated molecules) were considered as differentially expressed. Differential expression between study groups were depicted through volcano plots. Correlations between the differentially expressed molecules and clinical parameters were determined using Spearman's rank correlation coefficient. Principal component analysis (PCA) and hierarchical clustering were conducted on the differentially expressed metabolites and lipids. In Study 1, a stepwise feature selection process based on random forest (RF) was performed to construct a metabolic signature to predict the OSA status. The accuracy [95% confidence interval (CI)] of the model was estimated and compared to that of a reference questionnaire. Paired t-tests were applied to assess the pre-post change of the differentially expressed metabolites and lipids after CPAP treatment. In study 4, a partial least squares-discriminant analysis (PLS-DA) was performed to identify a metbolipidomic signature of non-dipping. Generalized additive models (GAM)

with penalized thin plate regression splines were used to evaluate the type of associations between the selected components of PLS-DA and the clinical variables (DR, OSA metrics, and change in the DR after CPAP treatment).

For Study 2, the relationship between the OSA parameters and the hallmarks of aging was determined through linear models. GAM models with a penalized cubic regression spline were used to evaluate the type and nature of association between each OSA parameter and each measurement of the hallmarks of aging. The relationship between OSA parameters and the hallmarks of aging by age group was evaluated by using adjusted linear models.

For Study 3, individual associations between the PSG parameters and the dipping status were assessed using adjusted logistic regression models, and displayed as odds ratios (OR) (95% CI). Multivariate GAM models with a penalized thin plate regression spline were fitted to evaluate the dose-response relationships between the PSG parameters and the DR. Multivariate models for the prediction of non-dipping status were constructed using a relaxed least absolute shrinkage and selection operator (LASSO) and a RF method.

For Study 5, patients were categorized according to the median HB value. Unadjusted and adjusted Cox regression models were used to assess the interaction between treatment arm and HB category, and displayed as hazard ratios (HR) (95% CI). Kaplan-Meier survival curves were plotted to illustrate incident cardiovascular events per treatment arm in the overall sample and in the subgroups with low or high HB. Log-rank tests were used to assess differences between survival curves. These analyses were repeated for conventional OSA severity parameters. The dose-response relationship between HB and incident CVD in each treatment arm was evaluated.

The p-value threshold defining statistical significance in all analyses was set at <0.05. All statistical analyses were performed using R software (R Foundation for Statistical Computing, Vienna, Austria).

ARTICLES

STUDY 1

Plasma Profiling Reveals a Blood-Based Metabolic Fingerprint of Obstructive Sleep Apnea

Lucía Pinilla, Iván D Benítez, Fernando Santamaria-Martos, Adriano Targa, Anna Moncusí-Moix, Mireia Dalmases, Olga Mínguez, Maria Aguilà, Mariona Jové, Joaquim Sol, Reinald Pamplona, Ferran Barbé, Manuel Sánchez-de-la-Torre.

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Plasma profiling reveals a blood-based metabolic fingerprint of obstructive sleep apnea

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ABSTRACT

Introduction: Obstructive sleep apnea (OSA) is a chronic, heterogeneous and multicomponent disorder with associated cardiovascular and metabolic alterations. Despite being the most common sleep-disordered breathing, it remains a significantly undiagnosed condition.
Objective: We examined the plasma metabolome and lipidome of patients with suspected OSA, aiming to identify potential diagnosis biomarkers and to provide insights into the pathophysiological mechanisms underlying the disease. Additionally, we evaluated the impact of continuous positive airway pressure (CPAP) treatment on the circulating metabolomic and lipidomic profile.
Material and methods: Observational-prospective-longitudinal study including 206 consecutive subjects referred to the sleep unit. OSA was defined as an apnea-hypopnoea index $\geq$ 15 events/h after polysomnography (PSG). Patients treated with CPAP were followed-up for 6 months. Untargeted plasma metabolomic and lipidomic profiling was performed using liquid chromatography coulpled to massspectrometry.
Results: A plasma profile composed of 33 metabolites (mainly glycerophospholipids and bile acids) was identified in OSA *vs.* non-OSA patients. This profile correlated with specific PSG measures of OSA severity related to sleep fragmentation and hypoxemia. Machine learning analyses disclosed a 4-metabolites-signature that provided an accuracy (95% CI) of 0.98 (0.95–0.99) for OSA detection. CPAP treatment was associated with changes in 5 plasma metabolites previously altered by OSA.
Conclusions: This analysis of the circulating metabolome and lipidome reveals a molecular fingerprint of OSA, which was modulated after effective CPAP treatment. Our results suggest blood-based biomarker candidates with potential application in the personalized management of OSA and suggest the activation of adaptive mechanisms in response to OSA-derived hypoxia.

List of abbreviations: AHI, apnea-hypopnea index; BMI, body mass index; CI, confidence interval; CL, cardiolipin; CPAP, continuous positive airway pressure; ESI-Q-TOF, electrospray-ionization quadrupole time of flight; ESS, Epworth sleepiness scale; FC, fold change; HIF-1-alpha, hypoxia inducible factor-1-alpha; HMDB, human metabolome database; LC-MS/MS, liquid chromatography/tandem mass spectrometry; MS/MS, tandem mass spectrometry; OSA, obstructive sleep apnea; PC, phosphatidylcholine; PCA, principal component analysis; PE, phosphatidylethanolamine; PSG, polysomnography; SaO_2, oxygen saturation; TSat90, time with SaO_2 < 90%; UHPLC, ultra-high-performance liquid chromatography.

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1. Introduction

Obstructive sleep apnea (OSA) is characterized by complete cessation or a partial reduction in the airflow during sleep caused by obstruction of the upper airway. The immediate effects caused by apnoeic and hypopnoeic episodes include shifts in the intrathoracic pressure, intermittent hypoxia, recurrent arousals and sleep fragmentation [1]. As the most common sleep-disordered breathing, it is estimated to affect more than 20% of the adult population [2], exceeding 50% in some countries [3]. Despite its high incidence, it remains a significantly undiagnosed and untreated condition in the majority of individuals [4,5].

There is extensive evidence linking this sleep disorder to a broad spectrum of complications, from behavioral and cognitive deficits [6] to major cardiovascular sequelae [7]. Given its increasing prevalence [8], its high burden of associated morbidity [9], and its subsequent economic impact on healthcare systems [10], OSA has been recognized as a major public health concern worldwide. However, its diagnosis is complex, laborious, slow and expensive. Overnight polysomnography (PSG) is the gold-standard procedure for this purpose [11]. However, this sleep study entails several relevant limitations that constrain its widespread use, as it requires to be conducted by trained personnel in specialized facilities. Although domiciliary respiratory polygraphy has contributed to OSA diagnosis as a simplified method, it remains a time and resources consuming procedure. Currently, questionnaires are one of the best validated strategies available for screening at-risk populations, where the STOP-Bang questionnaire [12] stands out as the most accurate [13].

In the last decade, there have been ongoing efforts to discover and implement alternative approaches to enable early and effective OSA detection. The identification of biological markers in easily accessible biospecimens would be extensively useful for clinicians in this clinical arena [14]. Nevertheless, to date, there is a shortage of simple, reliable and inexpensive indicators with sufficient precision to accurately detect patients with the disease.

Metabolomics and lipidomics, aiming to comprehensively study the entire metabolome and lipidome expressed in a given biological sample, are newly emerged analytic disciplines with promising potential in the area of personalized medicine [15,16]. Metabolites reflect the underlying biochemical activity and state of cells and tissues and are therefore directly connected to pathological conditions [17]. As molecular fingerprints of disease progression, alterations in these features can aid not only in the identification of novel biomarkers [18] but can also offer a plethora of information contributing to the understanding of disease pathogenesis [19]. In the context of such a heterogeneous disorder as OSA [20], the combination of metabolomic and lipidomic high-throughput technologies can enable the obtainment of a more complete view of its physiopathology.

Through an untargeted profiling strategy, in the present study, we analyzed the complete plasma metabolome and lipidome of patients with suspected OSA, aiming to explore diagnosis biomarker candidates and to elucidate potential pathophysiological mechanisms underlying the disease. Additionally, we evaluated the changes in the circulating metabolomic/lipidomic profile after continuous positive airway pressure (CPAP), the gold-standard treatment for OSA.

2. Materials and methods

2.1. Study design and population

This is an observational prospective and longitudinal study that included consecutive subjects who were referred to the sleep unit for suspected OSA (ClinicalTrials.gov identifier: NCT03513926). Recruitment took place at the University Hospital Arnau de Vilanova-Santa María of Lleida in Spain. Current evidence indicates that physiopathological consequences and clinical manifestations of OSA vary with age [21]. For the current study we enrolled patients that were aged between 18 and 60 years, contributing to the homogenization of the age of the subjects included. Patients were excluded based on the following criteria: presence of a previously diagnosed sleep disorder, history of CPAP treatment, pregnancy, and/or any medical, social or geographic factor that could jeopardize patient compliance.

2.2. Ethics statement

Enrolled patients provided informed written consent to participate in the study, and the ethics committee of the center approved the study (Clinical Research Ethics Committee of the University Hospital Arnau de Vilanova-Santa María of Lleida no. 1153/1411).

2.3. Clinical procedures and measurements

All patients who satisfied the selection criteria underwent a full polysomnographic sleep study (Philips Sleepware G3, Amsterdam, Netherlands). The results from all sleep studies were analyzed by trained personnel using standard criteria [22]. Apnea was defined as an interruption or reduction in oronasal airflow by ≥90% for at least 10 s. Hypopnea was defined as a 30–90% reduction in oronasal airflow for at least 10 s associated with oxygen desaturation by at least 3% or an arousal on the electroencephalogram. The apnea-hypopnea index (AHI) that estimates the severity of OSA, was calculated based on the average number of apnea plus hypopnea events per hour of sleep. Following the PSG study, the subjects were divided according to the current International Consensus Document on Obstructive Sleep Apnea [23] into the

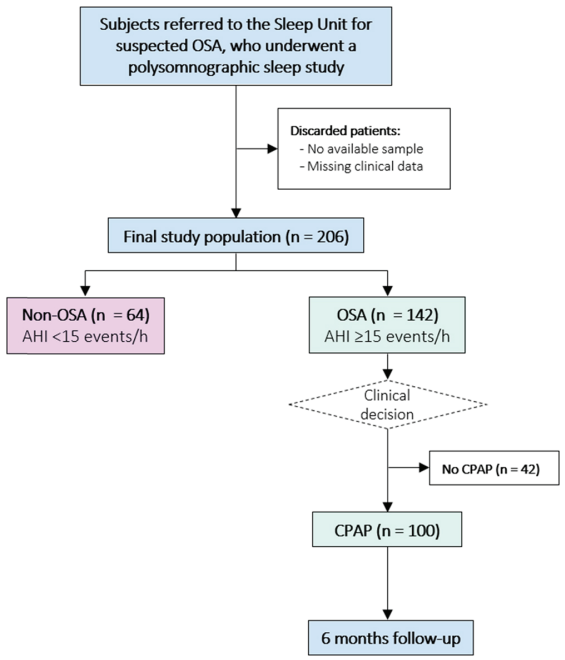

Fig. 1. Flow chart of the study. Consecutive subjects with suspected OSA who fulfilled the inclusion and exclusion criteria and completed a polysomnographic sleep study were assessed for eligibility. Patients were removed from the analysis when plasma samples were not available or there were insufficient clinical data to perform the analysis. The final study population included in the metabolomic and lipidomic analyses comprised 206 individuals: 64 without OSA and 142 with OSA, from which 100 were treated with CPAP. Definition of abbreviations: AHI = apnea-hypopnea index; CPAP = continuous positive airway pressure; OSA = obstru ctive sleep apnea.

Table 1
Clinical and polysomnographic characteristics of the study population at baseline.

	All (n = 206)	Non-OSA (n = 64) AHI< 15 events/h	OSA (n = 142) AHI≥ 15 events/h	p-value
Demographic variables				
Age, years	50.0 [44.0;55.0]	47.0 [40.0;54.0]	51.0 [45.0;56.0]	**0.016**
Sex, male	139 (67.5%)	39 (60.9%)	100 (70.4%)	**0.236**
Anthropometric measurements				
BMI, kg/m^2	29.3 [26.5;33.3]	28.0 [25.5;32.2]	29.8 [27.2;33.7]	**0.017**
Smoking status				0.323
Never	80 (38.8%)	20 (31.2%)	60 (42.3%)	
Former	61 (29.6%)	21 (32.8%)	40 (28.2%)	
Current	65 (31.6%)	23 (35.9%)	42 (29.6%)	
Polysomnography parameters				
AHI, events/h	27.6 [12.8;50.2]	9.26 [5.18;11.9]	41.0 [26.8;62.4]	**<0.001**
TSat90, %	1.76 [0.18;8.86]	0.08 [0.00;0.67]	3.65 [0.74;14.6]	**<0.001**
Mean SaO_2, %	94.0 [92.0;95.0]	94.0 [93.0;95.0]	93.0 [91.0;94.0]	**<0.001**
Minimum SaO_2, %	83.5 [76.0;88.0]	88.5 [85.0;90.2]	81.5 [72.8;86.0]	**<0.001**
Arousal index, events/h	33.7 [21.2;53.6]	20.7 [14.1;25.2]	43.3 [32.1;62.3]	**<0.001**
Epworth Sleepiness Scale	11.0 [7.00;14.2]	10.0 [6.50;13.5]	11.0 [7.00;15.0]	0.442
Medical history				
Hypertension	72 (35.1%)	15 (23.8%)	57 (40.1%)	**0.036**
Diabetes	21 (10.2%)	3 (4.69%)	18 (12.7%)	0.132
Medications				
ACE inhibitors	50 (24.3%)	8 (12.5%)	42 (29.6%)	**0.014**
Beta-blockers	36 (17.5%)	7 (10.9%)	29 (20.4%)	0.144
Diuretic agents	28 (13.7%)	7 (11.1%)	21 (14.9%)	0.613
Calcium-channel blockers	17 (8.29%)	3 (4.69%)	14 (9.93%)	0.323
Angiotensin II receptor blockers	15 (7.35%)	4 (6.25%)	11 (7.86%)	0.780
Lipid-lowering agents	43 (21.0%)	8 (12.5%)	35 (24.8%)	0.068
Anticoagulants	5 (2.43%)	0 (0.00%)	5 (3.52%)	0.327
Insulin	10 (4.85%)	1 (1.56%)	9 (6.34%)	0.178

Data are presented as the median [p25;p75] for quantitative variables and n (%) for qualitative variables. Definition of abbreviations: ACE = angiotensin-converting enzyme; AHI = apnea-hypopnea index; BMI = body mass index; IQR = interquartile range; OSA = obstructive sleep apnea; SaO2 = oxygen saturation; TSat90 = time with SaO2 < 90%.

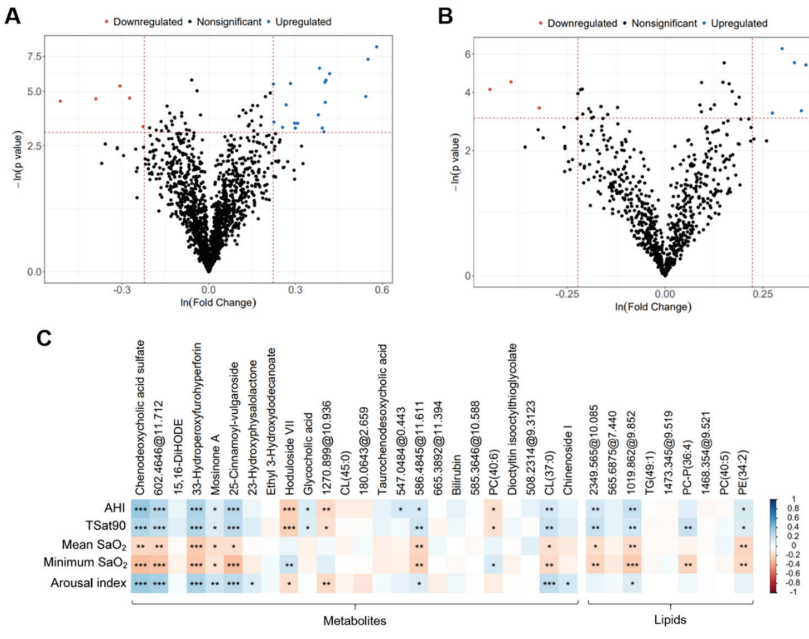

Fig. 2. Untargeted metabolomic and lipidomic profiling in patients with suspected OSA. (A & B) Volcano plots of the FC (x-axis) and p value (y-axis) for each detected metabolite (A) and lipid (B) in the comparison of OSA *vs.* non-OSA subjects. Red dots represent significantly downregulated (FC < 0.8) molecules, and blue dots represent significantly upregulated (FC > 1.25) molecules in OSA patients. The results are adjusted by confounding factors (age, sex and BMI). The p value threshold defining statistical significance was <0.05. (C) Correlations between PSG parameters of OSA severity and the differentially expressed metabolites and lipids. Unknown features are presented as exact mass@retention time. The color scale illustrates the degree of correlation and ranges from red to blue, indicating negative and positive correlations, respectively. Significance is illustrated by *: p < 0.05, **: p < 0.01, ***: p < 0.001. Definition of abbreviations: AHI = apnea-hypopnea index; BMI = body mass index; CL = cardiolipin; FC = fold change; OSA = obstructive sleep apnea; PC = phosphatidylcholine; PE = phosphatidyle thanolamine; SaO2 = oxygen saturation; TG = triglyceride; TSat90 = time with SaO2 < 90%. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

Table 2
Potential identities of the significantly differentially expressed metabolites and lipids between OSA and non-OSA patients.

Mass	RT (min)	*m/z*	Methodology	Regulation (OSA *vs.* non-OSA)	Putative identification	Family	Ion species	Reliability level
472.2503	10.17247	471.2503	M	Up	Chenodeoxycholic acid sulfate	ST	$[M + H]^-$	a
602.4646	11.71269	601.4646	M	Up	Unknown			
312.2267	10.35376	311.2267	M	Up	15,16-DiHODE	FA	$[M-H]^-$	a
628.3827	9.728171	627.3827	M	Up	33-Hydroperoxyfurohyperforin	PR	$[M+Hac-H]^-$	b
602.4654	11.61746	603.4654	M	Up	Mosinone A	FA	$[M+H-H2O]^+$	b
566.3101	9.029786	565.3101	M	Up	25-Cinnamoyl-vulgaroside	PR	$[M-H]^-$	a
536.2064	11.3893	535.2064	M	Up	23-Hydroxyphysalolactone	ST	$[M-H2O-H]^-$	b
244.2047	9.438738	243.2047	M	Up	Ethyl 3-Hydroxydodecanoate	FA		a
930.5211	12.7908	929.5211	M	Down	Hoduloside VII	PR	$[M-H]^-$	a
465.3101	8.988933	464.3101	M	Up	Glycocholic acid	ST	$[M-H]^-$	a
1270.899	10.93675	1269.899	M	Down	Unknown			
1064.646	10.94879	1063.646	M	Down	CL(45:0)	GP	$[M-H2O-H]^-$	b
180.0643	2.659617	181.0643	M	Down	Unknown			
499.2975	10.32784	498.2975	M	Up	Taurochenodesoxycholic acid	ST	$[M-H]^-$	a
547.0484	0.4439751	548.0484	M	Up	Unknown			
586.4845	11.61156	587.4845	M	Up	Unknown			
665.3892	11.39443	664.3892	M	Up	Unknown			
584.26285	7.601593	585.26285	M	Up	Bilirubin	TP	$[M+H]^+$	a
585.3646	10.58821	584.3646	M	Up	Unknown			
833.5856	12.39143	832.5856	M	Down	PC(40:6)	GP	$[M-H]^-$	a
812.3854	7.521849	811.3854	M	Up	Dioctyltin isooctylthioglycolate	CA	$[M+Hac-H]^-$	b
508.2314	9.312367	507.2314	M	Up	Unknown			
956.5291	11.57792	955.5291	M	Up	CL(37:0)	GP	$[M-H2O-H]^-$	b
1036.506	11.37844	103.5506	M	Up	Chinenoside I	ST	$[M-H]^-$	a
2349.565	10.08554	2348.565	L	Up	Unknown			
565.6875	7.440328	564.6875	L	Up	Unknown			
1019.862	9.852908	1020.862	L	Up	Unknown			
835.7769	10.12173	836.7769	L	Down	TG(49:1)	GL	$[M+NH4]^+$	a
1473.345	9.519504	1472.345	L	Down	Unknown			
765.567	7.535114	766.567	L	Up	PC-P(36:4)	GP	$[M+Na]^+$	a
1468.354	9.521306	1467.354	L	Down	Unknown			
895.6334	7.75234	894.6334	L	Up	PC(40:5)	GP	$[M+Hac-H]^-$	a
715.5194	7.073215	716.5194	L	Up	PE(34:2)	GP	$[M+NH4]^+$	a

The methodological approach used for the detection of the molecules is represented as M for metabolomics and L for lipidomics. All compounds are putatively annotated based on physicochemical properties and/or spectral similarity with public/commercial spectral libraries: (a) ID based on exact mass, RT, and MS/MS spectrum; (b) ID based on exact mass and RT. Definition of abbreviations: CA = carboxylic acid; CL = cardiolipin; FA = fatty acyl; GL = glycerolipid; GP = glycerophospholipid; OSA = obstructive sleep apnea; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PR = prenol; RT = retention time; ST = steroid; TP = tetrapyrrol; TG = triglyceride.

following (Fig. 1): the non-OSA group, including patients with an AHI less than 15 events/h; and the OSA group, including patients with an AHI equal to or more than 15 events/h [24]. Treatment recommendations in those patients diagnosed with OSA were based on the National Clinical Guidelines [25], in accordance with usual clinical practice. Patients treated with CPAP were evaluated after 6 months of follow-up. The level of adherence to the treatment with CPAP was recorded in each patient by means of the machines' internal clocks. Good compliance was defined as the use of a CPAP device for an average of at least 4 h per night [24].

At the initial visit, detailed information pertaining to the socio-demographic characteristics, medical history, medication use, and toxic habits was collected from all patients by trained clinicians. General physical and anthropometric parameters were recorded. Self-reported sleepiness status was measured by the Epworth Sleepiness Scale (ESS) [26]. The STOP-Bang questionnaire [12] was assessed at baseline and included 4 questions (STOP) related to symptomatology and comorbidities and 4 questions (BANG) related to anthropometric and demographic variables.

Overnight fasting venous blood samples were obtained at the same time of day (between 08:00 and 09:00 a.m.) at baseline (in the morning immediately after the sleep study) and after 6 months of follow-up. Whole blood samples collected in EDTA (ethylene diamine tetra acetic acid) anticoagulant tubes (Vacuette, Greiner Bio-One, Kremsmünster, Austria) were centrifuged at 1500 *g* for 10 min at 4 °C to separate the plasma fraction. All specimens were immediately aliquoted, frozen, and stored in a dedicated −80 °C freezer. No freeze-thaw cycles were performed during the experiments.

2.4. Metabolomic and lipidomic profiling

Metabolomic and lipidomic profiling analyses were performed from the same patient-derived plasma sample. For untargeted metabolomic analysis, metabolites were isolated from plasma samples using liquid-liquid extraction with methanol, as previously described [27]. Metabolic extracts were analyzed via ultra-high-performance liquid chromatography (UHPLC) coupled to electrospray ionization quadrupole time of flight (ESI-Q-TOF) tandem mass spectrometry (MS/MS) following a previously published method [28]. An Agilent 1290 liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA) coupled to an ESI-Q-TOF mass spectrometer 6520 instrument (Agilent Technologies, Santa Clara, CA, USA) was used. Data were acquired in both positive (+) and negative (-) polarity. Detailed information regarding metabolite extraction and analysis can be found in the Supplementary Material.

Lipid extraction from plasma samples was based on a previously validated method [29]. For untargeted lipidomics analysis, lipid extracts were subjected to mass spectrometry using an UHPLC 1290 series coupled to an ESI-Q-TOF MS/MS 6520 unit, operating in both the positive and negative ion modes, as previously described [30,31]. Further information about lipid extraction and profiling appears in the online Supplementary Material.

Data collected from the untargeted metabolomic and lipidomic profiling were acquired and analyzed using different software programs, as detailed in the Supplementary Material.

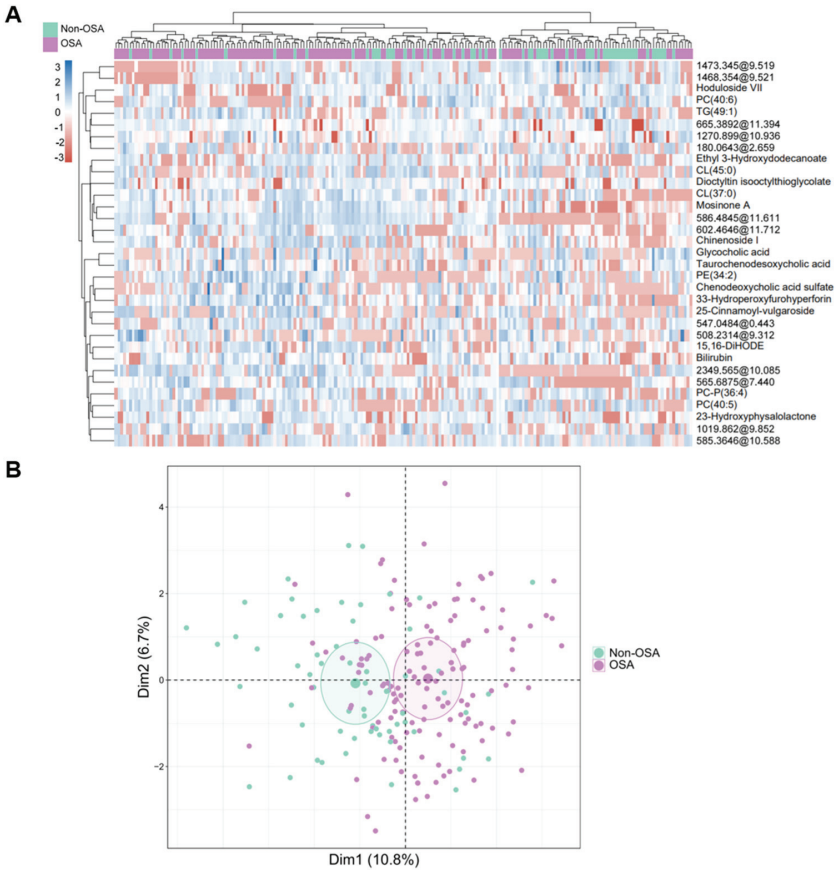

Fig. 3. Unsupervised clustering and PCA of plasma metabolites and lipids that are significantly relevant in OSA. (A) Heat map representing the hierarchical clustering of dysregulated features in OSA. Each column represents a patient. Non-OSA patients appear in green, and OSA patients appear in pink. Each row represents a metabolite or lipid. Unknown features are presented as exact mass@retention time. The color scale illustrates the relative expression level of each molecule in each patient and ranges from red to blue, indicating relatively low to high expression, respectively. (B) PCA using the dysregulated features in OSA. Each dot represents a patient. Green dots represent the non-OSA subjects, and pink dots represent the OSA patients. Definition of abbreviations: CL = cardiolipin; OSA = obstructive sleep apnea; PC = phosphatidylcholine; PCA = principal component analysis; PE = phosphatidylethanolamine; TG = triglyceride. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

2.5. Metabolite identification

According to previously published work, differentially expressed features, defined by exact mass and retention time, were searched against the Human Metabolome Database (HMDB) [32] (accuracy <30 ppm). Potential identities were confirmed by comparison of the exact mass, retention time and MS/MS spectra fragmentation pattern of the class representative internal standards, when available, with public databases [33].

2.6. Statistical analyses

Descriptive statistics were used to summarize the characteristics of the study population. Continuous variables were summarized using mean (standard deviation) for normally distributed data and median (25th percentile; 75th percentile) for nonnormally distributed data. Normal distributions were assessed by the Shapiro–Wilk test. Categorical data were summarized using frequency (percentage). Clinical and sociodemographic characteristics were compared between the study groups (OSA *vs.* non-OSA) using a *t*-test (or an equivalent nonparametric test) or chi-squared test depending on whether the variables were quantitative or categorical, respectively. Metabolite/lipid levels were log-transformed for statistical purposes. Differences in metabolite or lipid levels between groups were evaluated using linear models for arrays [34], adjusted for age, sex and body mass index (BMI). Molecules with a significant difference (p value < 0.05) between groups and a fold change (FC) higher than 1.25 (or lower than 0.8 for downregulated molecules) were considered differentially expressed. Differential expression between study groups is displayed in volcano plots. Correlations between differentially expressed metabolites/lipids and PSG parameters were evaluated using Spearman's rank correlation coefficient.

2.7. Machine learning and unsupervised analysis

Principal component analysis (PCA) and hierarchical clustering were performed on the differentially expressed metabolites and lipids. Metabolite/lipids levels were scaled to unit variance. In PCA, the first two components were selected and represented graphically. The coordinates of the top 5 metabolites with the highest contribution to the first component were represented (Fig. S1). In hierarchical clustering, distance matrix was calculated with Manhattan distance and Ward's minimum variance algorithm was used to clustering.

A stepwise feature selection process based on random forest [35] (RF) was performed to construct a metabolic signature that predicted the OSA status. The process was based on three steps [36]: First, the variables with low importance were eliminated by ranking the average of the variable importance measure on 50 runs of RF; Second, the Out-of-bag (OOB) error rates of 50 RF runs were calculated for each nested model (from the most important variable to the model with all previously selected variables). The variables included in the model with the lowest OOB error were selected; Third, the final model was selected by performing an ascending sequence of RF that tests the inclusion of each variable selected in the second step. The accuracy (95% confidence

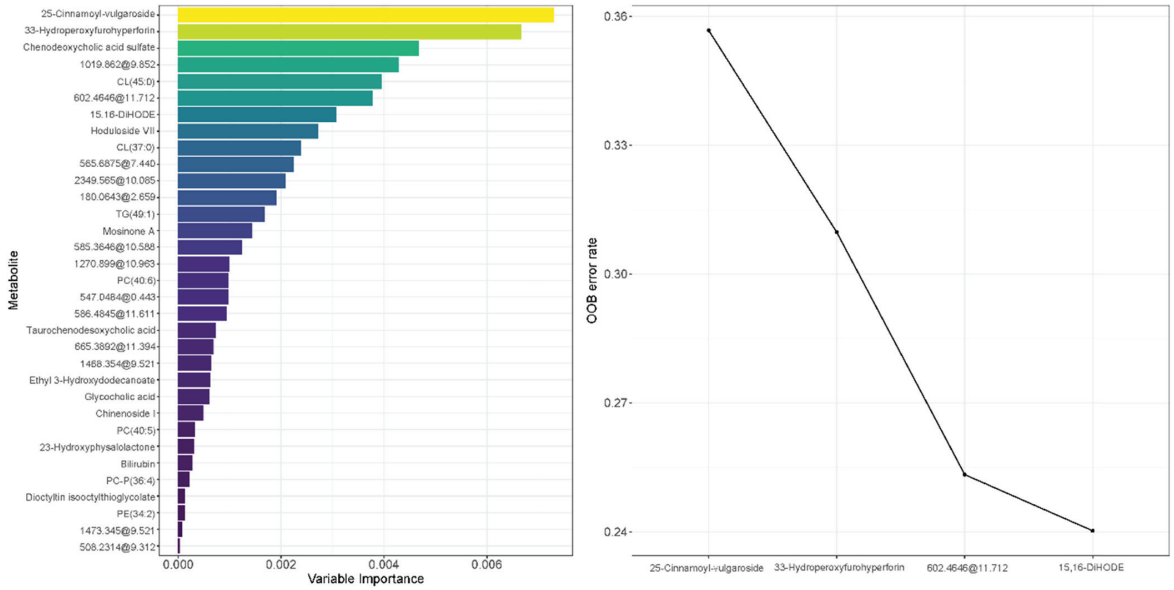

Fig. 4. Construction of the metabolic prediction model for OSA detection based on random forest. (A) Importance of each metabolite and lipid in the classification of the study groups (OSA *vs.* non-OSA). Unknown features are presented as exact mass@retention time. (B) Selection of the best combination of molecules that provides the lowest error rate for the construction of the metabolic signature. Definition of abbreviations: CL = cardiolipin; OOB = out-of-bag; OSA = obstructive sleep apnea; PC = phosphatidylcholine; PE = phosphatidylethanolamine; TG = triglyceride.

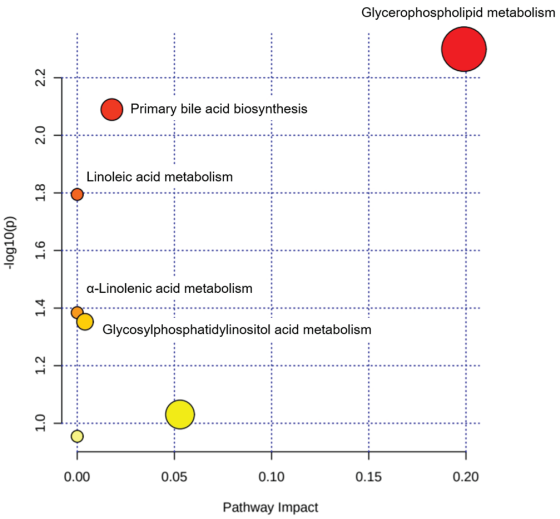

Fig. 5. Cross-omic pathway enrichment analysis of annotated features relevant in OSA. Scatter plot presenting the enriched metabolic pathways in which the 20 identified metabolites and lipids are involved. Each circle represents a pathway. The color gradient indicates the significance of the pathway ranked by p value, with yellow indicating higher p values and red indicating lower p values (y-axis). The size of the circles represents the impact score of the pathway based on the number of molecules contained in the pathway (x-axis). Significantly affected pathways with a p value < 0.05 appear with their name., Definition of abbreviations: OSA = obstructive sleep apnea. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

interval (CI)) of the model was estimated and compared to a reference questionnaire (STOP-Bang).

2.8. Pathway enrichment analysis

The differential metabolites and lipids that were annotated were searched against the KEGG library of *H. sapiens*. Pathway enrichment analysis was conducted through the MetaboAnalyst web service (https://www.metaboanalyst.ca/) [37]. A hypergeometric test was performed for overrepresentation analysis. Paired *t*-tests were used to evaluate the metabolite/lipid pre-post change after CPAP treatment. The p value threshold defining statistical significance in all analyses was set at < 0.05. All statistical analyses were performed using R software, version 4.0.2.

2.9. Role of Funders

The funding sources had no role in the writing, data collection, analysis or interpretation of the study.

3. Results

3.1. Description of the study population

A total of 206 patients with available clinical data and plasma sample were included in the metabolomic and lipidomic profiling analyses, of which 142 were OSA and 64 were non-OSA subjects (Fig. 1). The study population was mainly middle-aged, male and overweight-obese. Patients with OSA were older and presented a higher BMI and a higher prevalence of hypertension than patients without OSA (Table 1).

3.2. Untargeted metabolomic and lipidomic analyses reveal a plasma profile of OSA

The first aim of this study was to analyze global metabolomic and lipidomic differences between patients with and without OSA.

Table 3
Changes in the circulating metabolomic/lipidomic profile after CPAP treatment.

Metabolite/Lipid	Mean change after CPAP treatment
Chenodeoxycholic acid sulfate	-0.04 [−0.26;0.17]
602.4646@11.712	-0.13 [−0.47;0.22]
15,16-DiHODE	0.00 [−0.16;0.17]
33-Hydroperoxyfurohyperforin	-0.06 [−0.21;0.10]
Mosinone A	-0.03 [−0.23;0.17]
25-Cinnamoyl-vulgaroside	**0.18 [0.01;0.34]**
23-Hydroxyphysalolactone	-0.08 [−0.25;0.09]
Ethyl 3-Hydroxydodecanoate	-0.04 [−0.19;0.11]
Hoduloside VII	0.01 [−0.17;0.20]
Glycocholic acid	**0.38 [0.11;0.65]**
1270.899@10.936	0.05 [−0.15;0.26]
CL(45:0)	-0.03 [−0.27;0.21]
180.0643@2.659	0.23 [−0.05;0.51]
Taurochenodesoxycholic acid	0.17 [−0.07;0.41]
547.0484@0.443	**-0.23 [−0.42; −0.05]**
586.4845@11.611	-0.05 [−0.26;0.16]
665.3892@11.394	-0.08 [−0.22;0.07]
Bilirubin	**-0.24 [−0.45; −0.02]**
585.3646@10.588	-0.06 [−0.30;0.17]
PC(40:6)	-0.14 [−0.30;0.01]
Dioctyltin isooctylthioglycolate	0.00 [−0.18;0.19]
585.3646@10.588	0.02 [−0.29;0.34]
CL(37:0)	-0.14 [−0.40;0.12]
Chinenoside I	0.02 [−0.34;0.38]
2349.565@10.085	0.05 [−0.15;0.25]
565.6875@7.440	-0.11 [−0.32;0.10]
1019.862@9.852	**-0.35 [−0.60; −0.11]**
TG(49:1)	-0.08 [−0.35;0.19]
1473.345@9.519	0.13 [−0.19;0.44]
PC-P(36:4)	-0.18 [−0.45;0.09]
1468.354@9.521	-0.01 [−0.29;0.27]
PC(40:5)	0.01 [−0.23;0.26]
PE(34:2)	0.11 [−0.09;0.31]

Post-CPAP treatment indicates changes in the differentially expressed metabolites and lipids. Unknown features are represented as exact mass@retention time. Significant differences (p value < 0.05) are presented in bold. Definition of abbreviations: CL = cardiolipin; CPAP = continuous positive airway pressure; OSA = obstructive sleep apnea; PC = phosphatidylcholine; PE = phosphatidylethanolamine; RT = retention time; TG = triglyceride.

Following the gold-standard procedure for untargeted metabolomic and lipidomic profiling, an LC-MS-based strategy was applied. After quality control (see the Supplementary Material for detailed information), 1506 metabolites and 748 lipids were detected and included in the subsequent analyses. After adjustment for confounding factors (age, sex and BMI), 33 molecules were found to be differentially expressed between the groups: 22 compounds from the metabolomics analysis (Fig. 2A) and 9 lipidic species from the lipidomic analysis (Fig. 2B). Specifically, decreased levels of 5 metabolites and 3 lipids were observed in OSA patients (FC < 0.8), whereas 19 metabolites and 6 lipids were upregulated (FC > 1.25). The complete list of the significantly dysregulated metabolites and lipids between the study groups appears in Tables S1 and S2, respectively. Based on the exact experimental mass, retention time, isotopic distribution and/or MS/MS spectrum of the dysregulated molecules between the groups, identification was achieved for 20 entities, and the remaining 13 were unidentified. A full list of the candidates with putative identification is listed in Table 2. The identified features included different classes of molecules: 6 glycerophospholipids, 5 steroids, 3 fatty acyls, 3 prenols, 1 tetrapyrrol, 1 carboxylic acid and 1 glycerolipid.

Correlation analysis between the deregulated molecules and specific PSG parameters related to sleep fragmentation (arousal index) and different hypoxemia measurements (AHI, mean oxygen saturation (SaO_2), minimum SaO_2, and time with $SaO_2 < 90\%$ (TSat90)) revealed that the circulating levels of 18 molecules were significantly associated with one or more parameters of OSA severity (Fig. 2C).

To obtain a global overview of the metabolomic and lipidomic differences between OSA and non-OSA subjects, unsupervised multivariate statistics were applied to the differentially expressed molecules. Hierarchical clustering, represented by a heat map, generated two main clusters of patients, separating the population according to the presence of OSA (Fig. 3A). The first cluster was mostly represented by OSA patients, exhibiting, in general terms, higher expression levels of the metabolites that compose the differential metabolic profile. The second cluster, which was represented by a high presence of non-OSA patients, denoted a general downregulation of the metabolic expression. The PCA showed that the differentially detected metabolites were able to separate patients with OSA from those without OSA, depicting a differential metabolic plasma pattern of the OSA patient (Fig. 3B). The first and second component represented the 10.8% and 6.7% of the total variance, respectively. Given that the first component was the highest contributor to the separation of the study groups, we performed deeper a study of the top 5 metabolites with the greater contribution to this component. This plot is presented in Fig. S1. Together, these analyses suggested the existence of a specific metabolic signature for the OSA condition.

3.3. Metabolomic prediction model for OSA detection

The next objective of this study was to identify a molecular signature based on the plasma metabolic content, which could identify OSA among individuals with suspicion of this disease. The 33 differentially abundant plasma metabolites identified earlier were included in the construction of the prediction models by using machine learning approaches. Multivariate analysis of variable selection based on random forest revealed a specific fingerprint of OSA composed of 4 metabolites (Fig. 4): 25-cinnamoyl-vulgaroside; 33-hydroxyfurohyperforin; 15,16-DiHODE and 602.4646@11.712 (exact mass@retention time). The predictive performance of this fingerprint showed an accuracy (95% CI) of 0.98 (0.95–0.99) for OSA detection. The predictive potential of the derived signature was compared to the commonly used STOP-Bang questionnaire (accuracy (95% CI) = 0.65 (0.59–0.72)), and it was found that it was significantly higher than that observed for the questionnaire ($p < 0.01$).

3.4. Integrated enrichment analysis of the metabolic pathways relevant in OSA

We next performed a bioinformatic analysis integrating the metabolomic with the lipidomic results, with the aim of uncovering molecular clues that could be of relevance for disease pathogenesis. Pathway enrichment analysis, using all the dysregulated molecules with a putative identity as inputs, was carried out using the MetaboAnalyst platform (see the Methods section). As shown in Fig. 5, this integrated analysis approach yielded 5 significantly enriched pathways, including glycerophospholipid metabolism, primary bile acid biosynthesis, linoleic acid metabolism, α-linolenic acid metabolism and glycosylphosphatidylinositol acid metabolism.

3.5. Changes in the circulating metabolome and lipidome after CPAP treatment

Once we observed that OSA was associated with a specific molecular profile, we then explored the effect of CPAP treatment on this circulating profile. OSA patients treated with CPAP showed a mean (95% CI) CPAP use of 5.21 (2.84) h/night, i.e., 69% of treated patients were good compliers (CPAP use $\geq$4 h/night). In general terms, the metabolic plasma content of CPAP-treated patients changed after 6 months of therapy (Table 3). Despite this observation, only changes in 5 of the 33 differentially abundant molecules at baseline reached statistical significance in the pre-post comparison. Stratified analysis including adherent patients showed similar results as to the set of molecules previously observed, plus one additional molecule that was added (Table S3). It should be noted that no significant changes were found after follow-up

in the variables that were considered potential confounders.

4. Discussion

In this study, we identified a circulating metabolic profile associated with the presence of OSA, which strongly correlated with different polysomnographic measures of OSA severity related to sleep fragmentation and hypoxemia. Indeed, we provide here the cross-sectional identification of 33 plasma molecules that were differentially abundant in OSA *vs.* non-OSA patients, independently of several known confounding factors. The annotated features were mainly glycerophospholipids and bile acids, suggesting that the metabolic impact of OSA at the circulating level is mainly mediated by a dysregulation in glycerophospholipid metabolism and the biosynthesis of primary bile acids. Notably, multivariate machine learning analyses revealed that the circulating levels of 4 of these molecules provided a strong discriminative power for OSA detection, that was higher than that of the STOP-Bang questionnaire. Finally, the longitudinal evaluation of the plasma metabolic content indicated that CPAP treatment was associated with a significant modulation of 5 molecules that were previously altered by OSA.

OSA is a common and heterogeneous chronic disorder with a growing global prevalence that remains undiagnosed in a high proportion of patients [4] – up to 80% in the case of moderate-to-severe OSA [38]. Underdiagnosis of chronic disorders represents an additional cost for healthcare systems compared to the proper diagnosis and treatment of these diseases [39]. The multiple pathophysiological risks and consequences of OSA, as well as the inconvenience, laboriousness and economic impact derived from PSGs, justify the effort to explore new biomarkers for its early detection. Over the last decade, improvements in liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based technologies have powered the rapid screening of thousands of molecules in complex biological samples with high reproducibility, rendering this technology a promising tool for the discovery of sensitive and robust biomarkers [40,41]. Especially when considering an untargeted approach, metabolomic/lipidomic profiling constitutes an expanding research concept that is currently being applied for biomarker discovery in a variety of diseases [42–45]. Here, we found a signature composed of 4 metabolites, which provided powerful biomarker performance that was significantly higher than that of the STOP-Bang questionnaire, a currently available OSA screening tool for clinicians. This result suggests the potential of these molecules for the personalized management of OSA in the clinical setting of patients with suspicion of the disease.

Although metabolomic and lipidomic procedures have been applied to study a range of respiratory diseases [46–49], most of the research on OSA had been focused on the search for biomarkers in exhaled breath condensate or urine [50–54]. A limited number of studies have explored blood-based biofluids to address this purpose. Ferrarini et al. [55] identified 14 significant metabolites that distinguished severe from mild OSA patients but did not evaluate biomarker performance for OSA prediction. Lebkuchen et al. [56] showed that the combination of the NoSAS (Neck circumference, Obesity, Snoring, Age, Sex) score with a selection of metabolites or lipids raised the detection of OSA up to an AUC = 0.911 and 0.951, respectively. However, as was pointed out by Zhang et al. [57], the reliability of these studies is limited due to the small sample sizes, cross-sectional designs and lack of adjustment for confounding variables. Here, we sought to overcome these limitations, providing a more realistic setting with a larger sample size, longitudinal design and consideration of variables that could confound the associations. This is, to the best of our knowledge, the most comprehensive concurrent metabolomic and lipidomic study to date, aiming to identify circulating profiles associated with OSA, its severity, and its treatment.

As complementary techniques to other omics, metabolomics and lipidomics are influenced by both genetic and environmental factors, providing a more accurate image of the real physiological status of a biological system [58]. Metabolites represent the ultimate downstream product of the whole omics cascade, directly reflecting the molecular phenotype of an organism at a specific moment [59]. Our omics approaches revealed that despite the corrections introduced to eliminate the effect of confounding variables, there were still significant changes induced by OSA conditions, although it was only a minor fraction, representing approximately 1.5% (33 out of 2254 molecular species) of the detected metabolome and lipidome. The majority of these metabolites (lipids included) that were found to be differentially abundant in the plasma of OSA *vs.* non-OSA patients, were also strongly correlated with different polysomnographic measurements of OSA severity. Specifically, remarkable correlations were found with diverse well-established measurements of sleep fragmentation (arousal index) and/or hypoxemia (AHI, mean SaO_2, minimum SaO_2, and TSat90).

Collectively, the vast majority of the molecules with putative identification (20 out of 33) were lipids. The most affected lipid classes were glycerophospholipids (cardiolipin (CL), phosphatidylcholine (PC), and phosphatidylethanolamine (PE)), sterols (bile acids), and fatty acid derivatives (oxylipids). Notably, 4 functional categories associated with the different lipid classes were identified: membrane structural components, cellular protective antioxidants, lipids involved in mitochondrial bioenergetics, and bioactive lipids. Thus, PC and PE are essential and represent the major components of both cellular and subcellular membranes, and it is particularly remarkable that PE(34:2) is a primary molecular species involved in the *de novo* synthesis of PC and PE [60], from which other glycerophospholipid species can be generated by remodeling. PC-P(36:4) is a plasmalogen, a major component of cell membranes that plays a structural role in maintaining membrane stability and has antioxidant properties by acting as a reactive oxygen species (ROS) scavenger, thus helping to reduce hypoxia-derived oxidative stress [61]. Cardiolipin is a glycerophospholipid specifically located at the inner mitochondrial membrane, where it plays a key role in the integrity and activity of the mitochondrial electron transport chain complex I and, consequently, in cell bioenergetic processes [62]. Bile acids not only serve as detergents for lipid absorption but also function as bioactive lipids that activate different nuclear receptors [63]. Chenodeoxycholic acid, a major component of bile acids that we found to be markedly associated with OSA, has been shown to act as a signaling molecule, regulating hypoxia inducible factor-1-alpha (HIF-1-alpha) under hypoxic conditions [64]. Taking into account the strong correlation that we found between the plasma levels of these molecules and the nocturnal hypoxemia parameters, such as TSat90 and the minimum SaO_2, our results would suggest the activation of adaptive mechanisms directed to counter hypoxia-derived injury. Our findings support prior observations, as hypoxia-induced changes had been previously associated with a dysregulation of bile acid metabolism and ultimately bile acid synthesis [65]. Furthermore, sleep disturbances have been shown to significantly suppress genes involved in bile acid synthesis, such as Cyp7a1 [66]. Together, these results are aligned with ours and support that the observed changes could indicate an impairment in bile acid homeostasis.

Finally, regarding CPAP treatment, we found that after 6 months of therapy, there was a significant modulation of 5 plasma metabolites previously altered by OSA. This result suggests that CPAP seems to have an effect on the metabolic plasma content but might not be enough to reverse all the alterations induced by OSA, highlighting the importance of furthering the research in this regard.

The present study has some limitations worth noting. Due to the incipient state of this research field, an exploratory approach was applied, aiming to gain new insights from which new hypotheses might be developed. Therefore, further development of a confirmatory targeted methodology is needed to determine the validity of our findings in another independent group of individuals, especially in those of older ages. The strengths of this study include the evaluation of a relatively large number of consecutively recruited patients, which would contribute to the widespread use of the findings. Considering the study

population, instead of using an artificial design including extreme cases *vs.* healthy controls, which could have overestimated biomarker performance, we included a cohort of subjects referred to the sleep unit, which provides a realistic level of heterogeneity in the population with suspicion of OSA. In addition, all patients underwent a complete PSG study, which is the gold-standard technique for the diagnosis of OSA, and it also enabled the performance of correlation analyses with different PSG variables related to OSA severity. Finally, the longitudinal design of the study and the consequent evaluation of the effect of CPAP treatment on the plasma metabolic content would help to reinforce the etiological role of OSA in the observed associations.

5. Conclusions

In this study, an untargeted metabolomic and lipidomic profiling strategy was used to identify a circulating molecular fingerprint of OSA, which markedly correlated with different polysomnographic measurements of disease severity. This fingerprint was mainly composed of glycerophospholipids and bile acids, suggesting the activation of adaptive mechanisms in response to OSA-derived hypoxia. Additionally, we identified a metabolic signature that accurately detected OSA among individuals with suspicion of the disease, suggesting blood-based biomarker candidates with potential for the personalized management of OSA. Finally, we found that CPAP treatment was associated with significant changes in the metabolic profile previously altered by OSA.

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CRediT authorship contribution statement

MSdT is the guarantor of the study. LP, IDB, FSM, FB and MSdT contributed to the study design. LP, FSM, MD, OM, MA and MSdT contributed to data acquisition. LP, IDB, FSM, AT, AMM, MJ, JS, RP, FB and MSdT contributed to data analysis, interpretation and drafting of the manuscript. All authors contributed to the revision of the manuscript for intellectual content and approved of the final version.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112425.

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SUPPLEMENTARY INFORMATION

Plasma Profiling Reveals a Blood-Based Metabolic Fingerprint of Obstructive Sleep Apnoea

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METHODOLOGY SECTION

Samples were randomized before metabolite and lipid extraction.

Metabolomic profiling

Metabolite extraction

Metabolites were isolated from plasma samples using liquid-liquid extraction with methanol according to previously described methods [1]. Briefly, 30 µl of cold methanol containing 1 µg/mL Phe-13C as an internal standard was added to 10 µl of plasma, vortexed for 1 min and incubated for 1 h at -20°C to precipitate proteins. Samples were centrifuged for 3 min at 12,000g, and the supernatant was filtered through a 0.22-µm pore size nylon filter (Sigma-Aldrich, St. Louis, MO, USA). Metabolic extracts were collected, and quality control samples were prepared by pooling equal volumes of all metabolite extracts [2].

Liquid chromatography

Ultra-high-performance liquid chromatography (UHPLC) was performed using an Agilent 1290 series system (Agilent Technologies, Santa Clara, CA, USA) [3]. An injection volume of 2 µl of the metabolomic extract was applied to a 1.8-µm reversed-phase column, 2.1 mm × 50 mm (Zorbax SB-Aq 2.1 mm x 50 mm; Agilent Technologies, Santa Clara, CA, USA) equipped with a 3.5-µm precolumn (Zorbax SB-C8 Rapid Resolution Cartridge 2.1 mm × 30 mm; Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed by gradient elution of mobile phase A composed of water containing 0.2% acetic acid (v/v) and mobile phase B composed of methanol with 0.2% acetic acid (v/v). The gradient started with solvent B from 2% to 98% over 13 min, held at 98% solvent B for 6 min, and re-equilibrated at 2% solvent B for 5 min. The column temperature was maintained at 60°C, and the flow rate was set to 600 µL/min.

Mass spectrometry

Mass spectrometry (MS) metabolite detection was carried out with an ESI-Q-TOF mass spectrometer 6520 instrument (Agilent Technologies, Santa Clara, CA, USA) [3]. Duplicate runs of the samples were performed separately to collect positive and negative electrospray ionized metabolite species. The instrument was operated in full-scan mode

at 100 to 3000 m/z in an extended dynamic range (2 GHz) using N2 as the nebulizer gas (5 L/min, 350°C). The capillary voltage was set at 3500 V with a scan rate of 1 scan/s. Continuous infusion using a double spray with masses 121.050873 and 922.009798 (positive ion mode) and 119.036320 and 966.000725 (negative ion mode) was used for in-run calibration of the mass spectrometer [4]. Samples were processed in batches with consistent quality control samples included in each batch. Potential batch effects for each metabolite were adjusted by dividing by the corresponding average value identified in the quality control samples from the same batch.

Lipidomic profiling

Lipid extraction

Lipid extraction was based on a previously validated method [5]. First, to precipitate the protein fraction, 5 µL of Milli-Q water and 20 µL of cold methanol were added to 5 µL of plasma sample and vigorously shaken by vortexing for 2 min. For lipid extraction, 250 µL of methyl tert-butyl ether (MTBE) (Sigma-Aldrich, St. Louis, MO, USA), containing internal isotopically labelled lipid standards (Avanti Polar Lipids, Alabaster, AL, USA), was added [6]. Stock solutions were prepared by dissolving lipid standards in MTBE (1 mg/mL), and working solutions were diluted to 2.5 µg/mL in MTBE. Samples were then immersed in a water bath (ATU Ultrasonidos, Valencia, Spain) with an ultrasound frequency and power of 40 kHz and 100 W, respectively, at 10°C for 30 min. After the addition of 25 µL of Milli-Q water to the mixture, the organic phase was separated by centrifugation (1400g) at 10°C for 10 min. Lipid extracts contained in the upper phase were collected, and a pool of all lipid extracts was prepared and used as quality control samples.

Liquid chromatography

Chromatographic lipid separation was performed with the Agilent 1290 system by injection of 10 µl of lipid extracts onto an XBridge BEH C18 shield column, 100 mm × 2.1 mm (Waters, Milford, MA, USA) [7]. Two solvents were used for gradient elution, which consisted of solvent A composed of 10 mM ammonium acetate in acetonitrile-water (40:60, v/v) and solvent B composed of 10 mM ammonium acetate in acetonitrile-isopropanol (10:90, v/v). The chromatographic conditions were as follows: the gradient started at 40% mobile phase B, reached 100% B in 10 min and was held for 2 min. Finally,

the system was switched back to 60% mobile phase B and was equilibrated for 3 min. The column temperature was maintained at 55°C, and the flow rate was 400 µL/min.

Mass spectrometry

For MS analysis, data were collected with an ESI-Q-TOF mass spectrometer 6520 instrument (Agilent Technologies, Santa Clara, CA, USA) in full-scan mode at 100 to 3000 m/z in an extended dynamic range (2 GHz) [8]. Duplicate runs of the samples were performed to collect positive and negative electrospray ionized lipid species. The nebulizer gas, N2, was set to 5 L/min at a temperature of 350°C. The capillary voltage was set at 3500 V with a scan rate of 1 scan/s. Continuous infusion using a double spray with masses 121.050873 and 922.009798 (positive ion mode) and 119.036320 and 966.000725 (negative ion mode) was used for in-run calibration of the mass spectrometer [4]. All batches contained quality control samples to avoid interbatch confounding effects and to ensure the reliability of the data.

Metabolomic and lipidomic data analysis

Data collected from the untargeted metabolomic and lipidomic analyses were acquired using MassHunter Data Analysis Software (Agilent Technologies, Santa Clara, CA, USA). MassHunter Qualitative Analysis Software (Agilent Technologies, Santa Clara, CA, USA) was employed to obtain the molecular features of the samples, representing different comigrating ionic species of a given molecular entity using the molecular feature extractor (MFE) algorithm. This algorithm uses the accuracy of the mass measurements to group related ions (based on charge-state envelope, isotopic distribution, and/or the presence of different adducts and dimers/trimers), assigning multiple ionic species to a single compound referred to as a feature. Finally, MassHunter Mass Profiler Professional Software (Agilent Technologies, Santa Clara, CA, USA) and MetaboAnalyst Software [9,10] were used to perform nontargeted metabolomic and lipidomic analyses of the extracted features. Only those features with a minimum of 2 ions were selected. Compounds from different samples were aligned using retention time windows of 0.1% ± 0.25 min and 30.0 ppm ± 2.0 mDa. Only common features (found in at least 70% of the quality control samples) were taken into account to correct for individual bias [11]. The signal was corrected using a LOESS approach [12]. Features with a standard

deviation of the signal higher than 20% in the quality control samples after normalization were excluded [11].

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Figure S1. Coordinates of the top 5 metabolites with the highest contribution to the first component of the PCA. Unknown features are presented as exact mass@retention time. Definitions of abbreviations: CL = cardiolipin; PCA = principal component analysis.

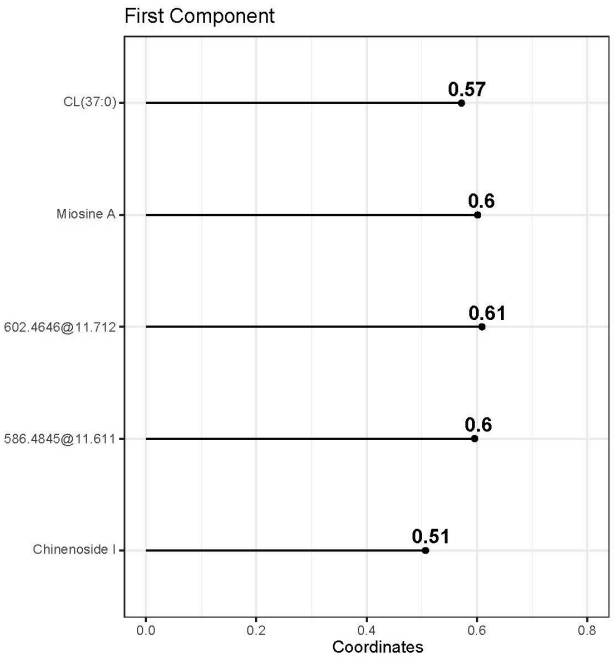

Table S1. Differentially expressed metabolites between non-OSA and OSA patients. Data are adjusted for age, sex, and BMI. Metabolites with FC <0.8 (downregulated) or >1.25 (upregulated) and p value <0.05 were considered. Features are represented as exact mass and retention time. Definition of abbreviations: BMI = body mass index; FC = fold change; OSA = obstructive sleep apnoea; RT = retention time.

Metabolite		FC	p value
Mass	RT (min)		
472.2503	10.17247	1.788	<0.001
602.4646	11.71269	1.735	0.001
312.2267	10.35376	1.467	0.001
628.3827	9.728171	1.519	0.002
602.4654	11.61746	1.500	0.003
566.3101	9.029786	1.494	0.004
536.2064	11.3893	1.327	0.004
244.2047	9.438738	1.251	0.004
930.5211	12.7908	0.735	0.005
465.3101	8.988933	1.721	0.009
1270.899	10.93675	0.760	0.01
1064.646	10.94879	0.676	0.01
180.0643	2.659617	0.598	0.012
499.2975	10.32784	1.496	0.012
547.0484	0.4439751	1.307	0.014
586.4845	11.61156	1.461	0.023
665.3892	11.39443	1.253	0.033
584.26285	7.601593	1.346	0.034
585.3646	10.58821	1.360	0.034
833.5856	12.39143	0.797	0.04
812.3854	7.521849	1.291	0.041
508.2314	9.312367	1.481	0.042
956.5291	11.57792	1.349	0.042
1036.506	11.37844	1.489	0.049

Table S2. Differentially expressed lipids between non-OSA and OSA patients. Data are adjusted for age, sex, and BMI. Lipids with FC <0.8 (downregulated) or >1.25 (upregulated) and p value <0.05 were considered. Features are represented as exact mass and retention time. Definition of abbreviations: BMI = body mass index; FC = fold change; OSA = obstructive sleep apnoea; RT = retention time.

Lipid		FC	p value
Mass	RT (min)		
2349.565	10.08554	1.349	0.002
565.6875	7.440328	1.392	0.004
1019.862	9.852908	1.434	0.005
835.7769	10.12173	0.674	0.011
1473.345	9.519504	0.639	0.016
765.567	7.535114	1.476	0.019
1468.354	9.521306	0.725	0.035
895.6334	7.75234	1.417	0.039
715.5194	7.073215	1.316	0.042

Table S3. Changes in the circulating metabolomic/lipidomic profile after CPAP treatment in patients with good compliance. Post-CPAP treatment indicates changes in the differentially expressed metabolites and lipids, considering patients with good adherence to the treatment (CPAP use ≥4 h/night). Unknown features are represented as exact mass and retention time. Significant differences (p value <0.05) are presented in bold. Definition of abbreviations: CL = cardiolipin; CPAP = continuous positive airway pressure; OSA = obstructive sleep apnoea; PC = phosphatidylcholine; PE = phosphatidylethanolamine; RT = retention time; TG = triglyceride.

Metabolite/Lipid	Mean change after CPAP treatment
Chenodeoxycholic acid sulfate	0.10 [-0.17;0.38]
602.4646@11.712	-0.06 [-0.51;0.38]
15,16-DiHODE	-0.09 [-0.29;0.11]
33-Hydroperoxyfurohyperforin	-0.04 [-0.22;0.15]
Mosinone A	-0.03 [-0.27;0.22]
25-Cinnamoyl-vulgaroside	**0.31 [0.11;0.51]**
23-Hydroxyphysalolactone	0.01 [-0.17;0.18]
Ethyl 3-Hydroxydodecanoate	-0.08 [-0.27;0.11]
Hoduloside VII	0.05 [-0.17;0.27]
Glycocholic acid	**0.46 [0.15;0.76]**
1270.899@10.936	-0.07 [-0.32;0.19]
CL(45:0)	-0.08 [-0.33;0.17]
180.0643@2.659	**0.39 [0.04;0.73]**
Taurochenodesoxycholic acid	0.28 [-0.02;0.59]
547.0484@0.443	**-0.30 [-0.50;-0.09]**
586.4845@11.611	0.03 [-0.22;0.27]
665.3892@11.394	-0.09 [-0.27;0.09]
Bilirubin	-0.20 [-0.47;0.07]
585.3646@10.588	-0.05 [-0.33;0.24]
PC(40:6)	-0.15 [-0.33;0.03]
Dioctyltin isooctylthioglycolate	0.10 [-0.12;0.31]
585.3646@10.588	0.09 [-0.30;0.47]
CL(37:0)	-0.11 [-0.44;0.22]
Chinenoside I	0.09 [-0.38;0.56]
2349.565@10.085	0.19 [-0.07;0.46]
565.6875@7.440	-0.20 [-0.45;0.06]
1019.862@9.852	**-0.41 [-0.69;-0.13]**
TG(49:1)	-0.10 [-0.46;0.25]
1473.345@9.519	0.19 [-0.19;0.57]
PC-P(36:4)	-0.16 [-0.49;0.17]
1468.354@9.521	-0.12 [-0.46;0.22]
PC(40:5)	-0.03 [-0.34;0.27]
PE(34:2)	0.12 [-0.13;0.37]

STUDY 2

Association of Obstructive Sleep Apnea with the Aging Process

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Association of Obstructive Sleep Apnea with the Aging Process

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Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LP, FS, AZ, FB, AT and MSdT conceived and designed the study; LP, FS, AZ, OM, JFM, MJM, OM, and AM acquired the data; LP, FS, IB, AZ, AT, FB and MSdT analyzed and interpreted the data; and LP, FS, IB, AZ, AT, OM, JM, FB and MSdT drafted the manuscript, critically revised the manuscript for important intellectual content and approved the final version. MSdT is the guarantor of the paper.

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Online supplement

This article has an online supplement, which is accessible from this issue's table of contents online at www.atsjournals.org

LIST OF ABBREVIATIONS

OSA: obstructive sleep apnea

AHI: apnea-hypopnea index

TSat90: night time with oxygen saturation less than 90%

CRP: C-reactive protein

HOMA-IR: homeostasis model assessment index for insulin resistance

qPCR: quantitative PCR

T: telomere

S: single copy gene

mtDNA: mitochondrial DNA

nDNA: nuclear DNA

8-OHdG: 8-hydroxy-2′-deoxyguanosine

ELISA: enzyme-linked immunosorbent assay

GAM: generalized additive model

BMI: body mass index

CI: confidence interval

ABSTRACT

Rationale

Evidence suggests that the physiopathological consequences of obstructive sleep apnea (OSA) resemble those induced by aging. Some studies report that the deleterious effects associated with OSA might be age dependent. The objectives of the study were to evaluate the association of OSA with the aging process and to determine whether this association is maintained across different age-groups.

Methods

Observational-prospective study including 599 patients with suspected OSA. Five hallmarks of aging were evaluated: alteration of cellular communication (serum C-reactive protein concentration), deregulation of nutrient sensing (insulin resistance), telomere attrition (leukocyte telomeric length), mitochondrial dysfunction (leukocyte mitochondrial mtDNA copy number) and genomic instability (urinary 8-hydroxy-2-deoxyguanosine concentration). For age-stratified analyses, subjects were divided into four groups according to the apnea-hypopnea index (AHI) and the median age (50 years): young non-OSA patients (age <50 years old, AHI <15 events/h), young OSA patients (age <50 years old, AHI ≥15 events/h), elderly non-OSA patients (age ≥50 years old, AHI <15 events/h) and elderly OSA patients (age ≥50 years old, AHI ≥15 events/h).

Results

A dose-response relationship was found between AHI, arousal index and night time with oxygen saturation less than 90%, and the following hallmarks: alteration of cellular communication, deregulation of nutrient sensing, mitochondrial dysfunction and genomic instability. Considering age-stratified analyses, OSA was associated with an

increase in several hallmarks of aging in young patients, but no significant association of OSA was identified in elderly patients.

Conclusions

In subjects under 50 years of age, OSA is associated with an increase in specific hallmarks of aging, independent of several known confounding factors.

INTRODUCTION

Obstructive sleep apnea (OSA) is a common sleep disorder characterized by repetitive episodes of complete (apnea) or incomplete (hypopnea) upper airway collapse during sleep (1). OSA is a major health concern affecting more than 20% of the adult population (2). The main physiopathological features of OSA include episodes of hypoxemia and recurrent arousals from sleep, which lead to oxidative stress (3), inflammation (4) and endothelial dysfunction (5). These events link OSA to an increased incidence and the accelerated progression of several diseases, including cardiovascular diseases (6), metabolic disorders (7), cancer (8) and neurodegenerative diseases (9).

With the actual increase in life expectancy, delaying the aging process and age-related diseases becomes a crucial aspect of interest to society. In the past decade, the relationship between OSA and aging has been widely studied, suggesting that the physiological changes induced by OSA resemble those induced by aging (10). OSA has been traditionally considered a sleep disorder related to age, as its prevalence is 2-3 times higher in elderly adults (> 65 years) than in younger adults (30-65 years) (11). However, some studies suggest that the deleterious consequences associated with OSA could be more pronounced in younger populations. Relatedly, a recent experimental

study showed that in a murine model of OSA, the detrimental cardiovascular changes induced by chronic intermittent hypoxia were modulated by age, suggesting that intermittent hypoxia may induce age-dependent premature cardiovascular aging (12). Moreover, the evidence available from clinical settings indicates that OSA is associated with an increased incidence of cancer mortality only in patients younger than 65 years old but not in older ones (13).

Evidence from clinical and experimental studies suggest that OSA could induce cellular and molecular alterations that promote the aging process (10, 14). Nevertheless, the mechanisms by which OSA might induce or accelerate aging have not been characterized extensively. Additionally, according to previous studies, these mechanisms may be more prominent or more severe in younger populations than in older ones. Therefore, in the present study, we aimed to evaluate the association of OSA with the aging process and to determine whether this association is maintained across different age ranges. To that end, we evaluated specific hallmarks clearly identified as contributors to the aging process (15), including alteration of cellular communication, deregulation of nutrient sensing, telomere attrition, mitochondrial dysfunction and genomic instability.

METHODOLOGY

Study cohort and sample collection

This was a multicenter, observational and prospective study that included a total of 599 subjects referred for suspected OSA to the sleep units in the University Hospital Arnau de Vilanova-Santa Maria of Lleida, Hospital San Pedro Alcántara of Cáceres, University Hospital of Guadalajara, and Hospital Parc Taulí (Spain). The exclusion criteria included

patients with a history of continuous positive airway pressure (CPAP) use or any condition that, in the opinion of the responsible investigator, affected the eligibility of the patient for inclusion in the study (e.g., pregnancy, drug or alcohol consumption, or less than one year of life expectancy). Patients with missing or unavailable data were excluded from the corresponding analysis, as displayed in Figure S1. Additionally, molecular determinations with discrepant values among replicas were eliminated. All recruited patients signed an informed consent form, and the ethics committee of the center (Clinical Research Ethics Committee of the University Hospital Arnau de Vilanova-Santa Maria of Lleida no. 1153/1411) approved the study. All methods were performed in accordance with current clinical practice guidelines (16) and regulations.

All patients underwent a full polysomnographic sleep study. Apnea was defined as an interruption or reduction in oronasal airflow by ≥90% for at least 10 s. Hypopnea was defined as a 30% to 90% reduction in oronasal airflow for at least 10 s that was associated with oxygen desaturation by at least 3% or an arousal on the electroencephalogram. The apnea-hypopnea index (AHI) was defined as the number of apnea and hypopnea events per hour of sleep.

To investigate the association of OSA with the aging process, we evaluated the dose-response relationship between different OSA parameters: AHI, arousal index and night time with oxygen saturation less than 90% (TSat90,) and the hallmarks of aging. To evaluate the association of OSA with the hallmarks of aging in different age ranges, age groups were determined by the median of the population. Thus, the subjects were divided into four groups according to their AHI (17, 18) and age: young non-OSA patients (age < 50 years old, AHI < 15 events/h), young OSA patients (age < 50 years old, AHI ≥ 15

events/h), elderly non-OSA patients (age ≥ 50 years old, AHI < 15 events/h) and elderly OSA patients (age ≥ 50 years old, AHI ≥ 15 events/h).

Fasting urine and venous blood samples were obtained from each patient in the morning immediately after the sleep study between 08:00 and 09:00 a.m. The urine samples were centrifuged to separate the urine from the sediment. The blood samples were centrifuged to separate the plasma, serum and buffy coat. Leukocytic DNA was extracted from the buffy coat through the salt precipitation method with the MasterPure DNA Extraction kit (Ecogen, Spain). All specimens were immediately aliquoted, frozen, and stored in a dedicated −80 °C freezer. No freeze-thaw cycles were performed during the experiment.

Molecular measurements

Alteration of cellular communication

To study this hallmark, we evaluated the inflammation process, as "inflammaging" is a prominent aging-associated alteration in intercellular communication. As it is a well-established marker of inflammation, we assessed the serum concentration of high-sensitivity C-reactive protein (CRP) (19). This determination was measured by immunoturbidimetric assays in accordance with the standard routine method used in the hospital and presented in mg/L.

Deregulation of nutrient sensing

To assess the deregulation of nutrient sensing, we estimated the insulin resistance of the patients (20). The degree of insulin resistance was estimated by the Homeostasis Model Assessment Index for Insulin Resistance (HOMA-IR), as described by Matthews et al. (21). This index is calculated as the product of fasting insulin (mIU/mL) and fasting glucose (mmol/L) divided by 22.5. The serum glucose and insulin concentrations were

measured by enzymatic and chemiluminescence assays, respectively, in accordance with the standard routine methods used in the hospital.

Telomere attrition

Regarding telomere attrition, we estimated the telomeric length by quantitative PCR (qPCR) using QuantStudio 7 Flex (Applied Biosystems, USA), as described previously (22). In brief, the relative ratio T/S of ng of telomeres (T) to ng of a single copy gene (S) in experimental samples is determined using a standard curve generated by serial dilutions from standard DNA. The T/S ratio is proportional to the average telomere length. The assessments of samples and standard curves were all run in triplicates. The sequences of the primers and TaqMan probes are listed in Table S1. The PCR mix was 5 µL 2x PowerUp SYBR Green Master Mix (Applied Biosystems, USA), 0.857 µL of each primer (100 µM) and 20 ng of genomic DNA extract in a 10 µL PCR. The qPCR conditions were as follows: 2 min at 50°C, 15 min at 95°C, 2 cycles of 15 s at 94°C and 15 s at 49°C, and 32 cycles of 15 s at 94°C, 10 s at 62°C, 15 s at 74°C (with signal acquisition for telomere amplification), 10 s at 84°C, and 15 s at 88°C (with signal acquisition for single copy gene amplification).

Mitochondrial dysfunction

To estimate the degree of mitochondrial dysfunction of the patients, we measured their leukocytic mitochondrial DNA (mtDNA) content (23). This was measured by qPCR using QuantStudio 7 Flex (Applied Biosystems, USA), as described previously (24). Briefly, the leukocytic mtDNA copy number was measured by the quantification of a unique mitochondrial fragment relative to a single copy region of the nuclear beta-2-microglobulin gene. The sequences of primers and TaqMan probes are listed in

Supplementary Table S2. The mitochondrial and nuclear probes were labeled at the 5' end with FAM and VIC fluorochromes, respectively, and both probes were labeled at the 3' end with nonfluorescent quencher QSY. The PCR mix was 5 µL 2x TaqMan Fast Advanced Master Mix (Applied Biosystems, USA), 0.5 µL 20x of each TaqMan assay and 20 ng of genomic DNA extract in a 10 µL PCR. The qPCR conditions were as follows: 2 min at 50°C, 2 min at 95°C, and 40 cycles of 1 s at 95°C and 20 s at 60°C. Standard curves obtained from serial dilutions of PCR-amplified target sequences were used for the quantification of mtDNA and nuclear DNA (nDNA), and then the mt/n DNA ratio was calculated. The assessments of samples and standard curves were all run in duplicates.

Genomic instability

As it is a marker of genomic instability, we assessed the urinary concentration of 8-hydroxy-2′-deoxyguanosine (8-OHdG) (25), as previously described(26). 8-OHdG is a molecule develops as a result of DNA damage due to the accumulation of oxidative stress in organisms. The 8-OHdG concentration was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Elabscience, USA). The urinary 8-OHdG excretion value was normalized to the urinary creatinine level, which was determined by a standard automated colorimetric assay (Arbor Assays, USA). Each determination was made in duplicates, yielding the urinary 8-OHdG [nanograms/milliliter]/ creatinine [milligrams/milliliter] ratio.

Statistical analysis

Descriptive statistics of the mean (standard deviation) and median (interquartile range) were estimated for quantitative variables with normal and nonnormal distributions, respectively. The absolute and relative frequencies were calculated for qualitative

variables. The normality of each distribution was analyzed using the Shapiro–Wilk test. The relationship between OSA severity (AHI, arousal index and TSat90) and the hallmarks of aging was determined using linear models. A generalized additive model (GAM) with penalized cubic regression spline was used to evaluate the type of association between each OSA parameter and measurements of the hallmarks of aging. The relationship of OSA and the hallmarks of aging by age groups was evaluated using linear models that included confounder factors, OSA factor (AHI≥ 15 events/h), Age factor (age≥ 50 years) and Age-OSA interaction. The p-value threshold defining statistical significance in all analyses was set at <0.05. Data management and statistical analyses were performed using R (version 3.4.2) (27).

RESULTS

A total of 150 subjects were included in the study as non-OSA patients, and 449 were included as OSA patients. The patients were mainly middle-aged, overweight-obese and male. The characteristics of the study population are shown in Table 1. Significant differences were identified between the non-OSA and OSA groups in physiological characteristics (BMI, age) sex, presence of diabetes, medications (angiotensin-converting enzyme inhibitors, beta-blockers, diuretic agents) and the expected polysomnographic parameters (AHI, arousal index and TSat90).

The linear relationship between the parameters of OSA severity (AHI, arousal index and TSat90), and the hallmarks of aging is reported in Table 2. Regarding the alteration of cellular communication, AHI, arousal index and TSat90 were associated with the serum CRP concentration. In relation to the deregulation of nutrient sensing, AHI, arousal index

and TSat90 were associated with insulin resistance. As regards to telomere attrition, no association was found between AHI, arousal index and TSat90, and leukocyte telomeric length. With regard to mitochondrial dysfunction, AHI, arousal index and TSat90 were associated with mitochondrial mtDNA copy number. As for genomic instability, AHI and arousal index were associated with urinary 8-OHdG levels only when the model was adjusted for confounding factors.

The non-linearity of the associations was evaluated using GAM models. In general, all the associations showed linearity except for the relationships of TSat90 with the hallmarks of aging and the relationships of genomic instability with the parameters of OSA severity (Figure 1).

The adjusted association of OSA with the hallmarks of aging in the young and elderly groups, and the interaction between them, is reported in Table 3. The number of subjects per group of OSA and age is specified in Table S3. In patients younger than 50 years, we found that OSA was associated with an increase in the following hallmarks of aging after adjustment for cofounding factors: alteration of intercellular communication, deregulation of nutrient sensing and genomic instability. However, no significant association of OSA was identified in elderly patients for any of the evaluated hallmarks.

DISCUSSION

The present study shows that OSA is independently associated with an increase in specific hallmarks of aging, in a dose-response manner. When the cohort was stratified by age groups, we observe that OSA is associated with an increase in specific hallmarks

of aging in patients under 50 years of age, independently of several known confounding factors.

The relationship between OSA and aging has been widely investigated. According to previous literature, in comparison to healthy subjects, patients with OSA show significant impairment in vigilance, coordination, executive function, and verbal and visuospatial memory (28). Even though these cognitive impairments are often observed in older people, OSA leads to impairments in performance and brain function in individuals younger than expected compared with age-matched controls (29). Recent findings have associated untreated OSA with an aging-related cognitive deficit (30), as well as an increased vulnerability for early stages of dementia and the development of neurodegenerative diseases (28). The previous findings propose that OSA patients are especially susceptible to developing cerebral and cognitive impairments at an earlier age than would be expected. To the best of our knowledge, this is the first study to address the contribution of OSA to the aging process in an overall manner, and the results show that there is a dose-response relationship between AHI, arousal index and TSat90, and four of the studied hallmarks: alteration of cellular communication, deregulation of nutrient sensing, mitochondrial dysfunction and genomic instability.

Prior investigations have showed results similar to those shown in the present study in terms of insulin resistance (31), CRP concentration (32), the mtDNA copy number (33) and the 8-OHdG levels (26). However, different results have been previously published regarding telomere shortening; while we did not observe any association of OSA with telomere length, other researchers have reported this association (34–37). Our results suggest that OSA could have an association in early stages of the aging process, leading to inflammation, metabolic impairments, mitochondrial alterations or oxidative damage,

but this association is not displayed in an ultimate structural damage as is telomere shortening. Collectively, the assumptions and our current findings led us to agree with the preceding proposal that OSA accelerates or potentiates aging mechanisms. However, studies elucidating the causative and mechanistic roles of OSA in the aging process are lacking. Additional studies are needed to clearly distinguish between correlative and causal observations in the proposed link between OSA and aging.

Regarding the role of age in the aforementioned association of OSA with the aging process, our results suggest that the detrimental mechanisms associated with OSA have a greater impact in young patients. Nevertheless, it is important to note that whereas for some specific hallmarks of aging the magnitude of the effect of OSA was greater in younger than elderly patients, the sample size does not allow us to conclude that these differences are statistically significant. Our results would suggest a potential differential effect depending on the age of the patient that should be explored and demonstrated in future studies. These findings are consistent with those proposed in earlier studies. Castro-Grattoni et al (12) observed an accelerated decline in cardiovascular structural integrity with characteristics strikingly similar to those observed during the natural aging process in young mice exposed to chronic intermittent hypoxia. In the clinical context, Lavie et al (38) reported an increased mortality in only patients with severe OSA who were younger than 50 years, and Campos-Rodriguez et al (13) described an increased incidence of cancer in patients with OSA, but only those younger than 65 years. Our data could partly contribute to explain some of the causes related to the lack of cardiovascular consequences found in elderly OSA patients, the higher cancer incidence in younger OSA patients, and the results extracted from animal models, suggesting that the detrimental effects induced by OSA are modulated by age, with younger patients exhibiting higher

susceptibility. Nevertheless, in the analysis of the relationship between OSA and aging, it is also worth considering the possibility that an accelerated aging process is favoring the development of episodes of OSA, as it is largely described that the prevalence of this disease increases with age, independently of other risk factors including obesity (39).

The socioeconomic impact of OSA together with its implication in life quality and health status of the patients make this disease to emerge as a major public health problem (40, 41). Considering the high rates of undiagnosed cases, it is imperative to develop early, personalized, and robust diagnosis strategies. In this context, stratifying OSA patients by age may lead to considerably better diagnoses and treatments in terms of personalized medicine. Considering the observed association of OSA with the hallmarks of aging in young subjects, an early OSA diagnosis and treatment early might slow the progression of cellular and molecular changes that may become irreversible, thus delaying the progression of aging and maybe even age-related disorders. Understanding the molecular biology of aging has several potential clinical implications that may give rise to new ways of managing OSA. In this way, additional knowledge of how aging affects organ systems and induces dysfunction can lead to the development of methods for revising or limiting these effects in specific populations who may be more vulnerable to the pathological consequences of this disease. Relatedly, our results highlight the importance of raising awareness about the potential detrimental effects of OSA in young population and would suggest the need for specific efforts to evaluate the impact of this disease in younger patients. Our study presents several limitations that need to be commented upon. First, since the patients were consecutively recruited on the basis of suspicions of OSA, all age ranges are not equally represented, hindering the establishment of study subgroups with considerably distant ages. Second, as this is a cross-sectional study, no data are

available corresponding to the onset of OSA, preventing us from inferring the causality of the results. Third, the number of patients included in each determination was not homogeneous due to the different reasons for exclusion detailed in the methods section. However, in every experiment, the number of patients considered was suitable in terms of the representability of the target population and provided sufficient statistical power for each determination. Fourth, the potential role of OSA and its association with the aging process has been explored in a population of patients referred to the sleep unit for evaluation of suspected OSA. In this way, the results may not be generalizable to the global population. However, this design of the present study has allowed to limit the possible selection biases of patients in the group without OSA as a control group. Finally, for the present study we explored specific mechanisms widely described and linked to the aging process. We evaluated extensively established marker as a subrogated indicator of each specific hallmark. However, not all mechanisms related to the aging process have been explored in the present work. The evaluation of these other mechanisms should be explored in future studies. The main strength of the study is related to its multicenter design, which facilitates the recruitment of a large cohort of patients and allows us to appropriately address the main association of OSA with the hallmarks of aging. Moreover, patients were recruited consecutively from sleep units, which leads to a realistic level of heterogeneity in the population with suspected OSA. In addition, all patients underwent complete polysomnography, a gold standard technique for the diagnosis of OSA. Furthermore, we used robust and widely described techniques that had been previously used in the literature, allowing the accurate measurement of the determinations.

CONCLUSIONS

In conclusion, the results of the present study indicate that OSA is associated with an increase in specific hallmarks of aging in people under 50 years of age, independent of several known confounding factors. Our results would suggest the need of specific efforts for early diagnosis and evaluation of the impact of OSA in younger patients.

Acknowledgements

The authors thank Maricel Abornés, Silvia Ortega, Alicia Martín and Cristina Giron for their technical support. We thank the individuals who participated in this trial, their families, and the clinical and research teams in the various sleep departments.

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FIGURES:

Figure 1: Dose-response relationship between OSA parameters and hallmarks of aging.

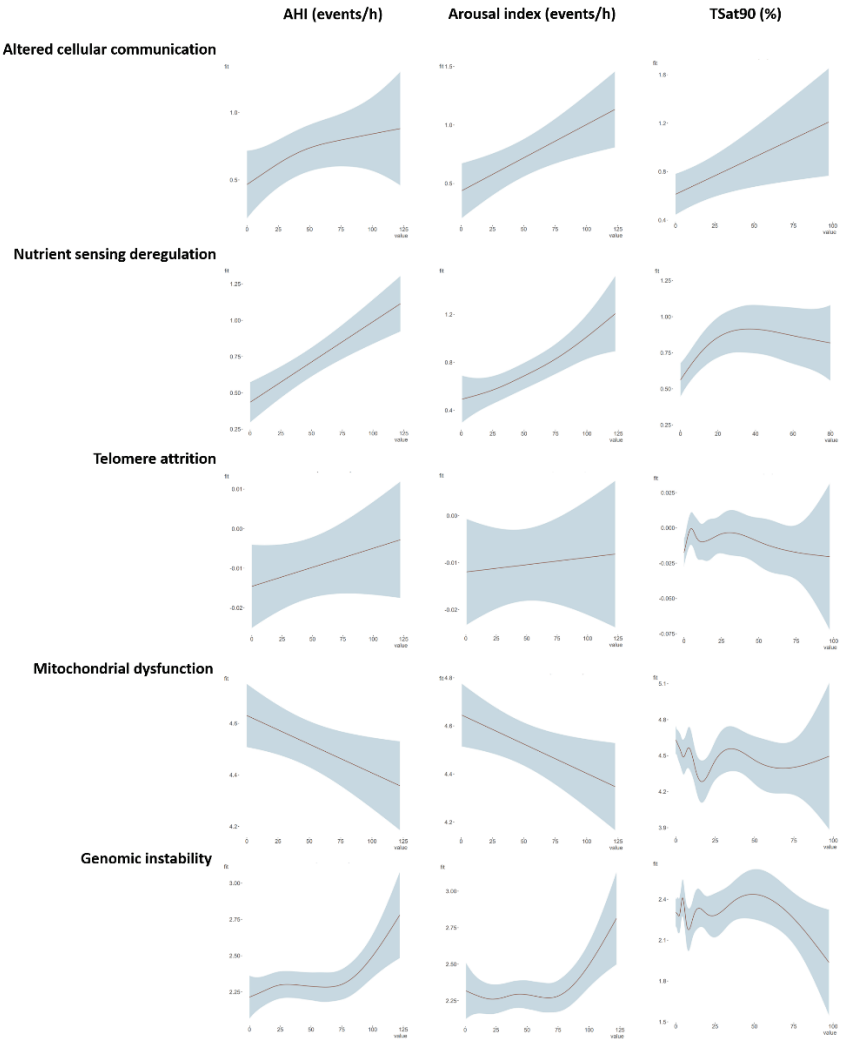

Figure caption: The line represents the estimate of the change in each hallmark of aging, according to the value of each polysomnographic parameter, estimated by generalized additive model. The gray areas correspond to the 95% confidence intervals. The hallmarks of aging were log-transformed. AHI: apnea-hypopnea index; OSA: obstructive sleep apnea; TSat90: night time spent with oxygen saturation less than 90%.

TABLES

Table 1. Characteristics of the study population and division by age.

	Non-OSA (AHI <15 events/h)			OSA AHI (AHI ≥15 events/h)		
	All n = 150	Young Age <50 years	Elderly Age ≥50 years	All n = 449	Young Age <50 years	Elderly Age ≥50 years
Physiological characteristics						
BMI, kg/m^2	27.8 [25.0;32.1]	27.6 [24.7;31.5]	32.0 [28.8;36.1]	31.8 [28.4;36.1]	28.5 [25.5;32.9]	31.3 [28.3;36.1]
Age, years	48.0 [40.0;55.0]	42.0 [37.0;45.5]	58.0 [54.0;65.0]	53.0 [46.0;59.0]	56.0 [54.0;59.0]	45.0 [40.0;47.0]
Sex						
Male, n	81 (54.0%)	52 (59.8%)	192 (69.1%)	331 (73.7%)	29 (46.0%)	139 (81.3%)
Female, n	69 (46.0%)	35 (40.2%)	86 (30.9%)	118 (26.3%)	34 (54.0%)	32 (18.7%)
Smoking habits						
Non-smoker, n	57 (38.0%)	34 (39.1%)	113 (41.2%)	178 (40.1%)	23 (36.5%)	65 (38.2%)
Smoker, n	44 (29.3%)	29 (33.3%)	51 (18.6%)	112 (25.2%)	15 (23.8%)	61 (35.9%)
Ex-smoker, n	49 (32.7%)	24 (27.6%)	110 (40.1%)	154 (34.7%)	25 (39.7%	44 (25.9%)
Diabetes						
Non-diabetic, n	142 (95.3%)	85 (97.7%)	213 (77.7%)	367 (82.7%)	57 (91.9%)	154 (90.6%)
Diabetic, n	7 (4.70%)	2 (2.30%)	61 (22.3%)	77 (17.3%)	5 (8.06%)	16 (9.41%)
Sleep parameters						
AHI, events/h	8.48 [4.25;11.6]	7.80 [3.99;11.4]	43.5 [28.9;63.3]	43.0 [28.6;63.9]	9.40 [4.90;12.3]	41.0 [27.7;70.2]
ESS	11.0 [7.00;14.0]	11.0 [8.00;14.8]	10.0 [6.00;14.0]	10.0 [6.75;14.0]	9.00 [5.00;14.0]	11.0 [7.00;15.0]
Arousal index, events/h	19.1 [14.4;25.3]	18.2 [12.2;25.1]	48.8 [33.2;64.8]	46.5 [32.3;65.4]	0.35 [0.00;1.49]	42.3 [32.0;67.8]
TSat90, %	0.10 [0.00;0.90]	0.06 [0.00;0.60]	7.04 [2.20;21.9]	5.48 [1.40;21.2]	21.2 [16.5;25.8]	3.80 [0.64;16.8]
Medications						
ACE inhibitors, n	19 (12.7%)	6 (6.90%)	13 (20.6%)	106 (23.7%)	28 (16.4%)	78 (28.2%)
Beta-blockers, n	10 (6.67%)	3 (3.45%)	7 (11.1%)	71 (15.8%)	14 (8.19%)	57 (20.6%)
Diuretic agents, n	14 (9.40%)	6 (6.90%)	8 (12.9%)	76 (17.0%)	17 (10.0%)	59 (21.3%)
Calcium-channel blockers, n	12 (8.00%)	8 (9.20%)	4 (6.35%)	46 (10.3%)	10 (5.85%)	36 (13.0%)
Angiotensin II receptor blockers, n	10 (6.67%)	6 (6.90%)	4 (6.35%)	51 (11.4%)	9 (5.26%)	42 (15.3%)
Anticoagulants, n	3 (2.00%)	1 (1.15%)	2 (3.17%)	3 (5.13%)	1 (0.58%)	22 (7.94%)
Insulin, n	2 (1.33%)	0 (0.00%)	2 (3.17%)	25 (5.58%)	7 (4.09%)	18 (6.50%)

Data are presented as median [p25;p75] for quantitative variables and n(%) for qualitative variables. P-values <0.05 are presented in bold. ACE: angiotensin-converting enzyme; AHI: Apnea-Hypopnea Index; BMI: Body Mass Index; ESS: Epworth sleepiness scale. OSA: Obstructive Sleep Apnea; TSat90: night time with oxygen saturation less than 90%.

Table 2. Linear dose-response relationship between OSA parameters and the hallmarks of aging.

	AHI (events/h)		Arousal index (events/h)		TSat90 (%)	
	Rate ratio (95% CI)	*p value*	*Rate ratio (95% CI)*	*p value*	*Rate ratio (95% CI)*	*p value*
Alteration of cellular communication						
Unadjusted	7,81 (4,26 to 11,48)	**<0.001**	9,36 (5,31 to 13,56)	**<0.001**	13,81 (8,46 to 19,42)	**<0.001**
Adjusted*	3,84 (0,39 to 7,41)	**0,029**	5,85 (2,06 to 9,78)	**0,002**	6,31 (1,4 to 11,45)	**0,011**
Deregulation of nutrient sensing						
Unadjusted	11,43 (9,08 to 13,83)	**<0.001**	11,73 (9,02 to 14,5)	**<0.001**	13,74 (10,35 to 17,23)	**<0.001**
Adjusted#	5,71 (3,53 to 7,94)	**<0.001**	5,51 (3,11 to 7,97)	**<0.001**	4,49 (1,54 to 7,53)	**0,003**
Telomere attrition						
Unadjusted	0,05 (-0,09 to 0,19)	0,469	-0,02 (-0,18 to 0,14)	0,836	-0,06 (-0,27 to 0,15)	0,578
Adjusted*	0,1 (-0,06 to 0,26)	0,237	0,03 (-0,14 to 0,21)	0,729	-0,04 (-0,26 to 0,19)	0,199
Mitochondrial dysfunction						
Unadjusted	-2,57 (-4,15 to -0,97)	**0,002**	-2,92 (-4,7 to -1,11)	**0,002**	-3,15 (-5,47 to -0,76)	**0,010**
Adjusted*	-2,2 (-4,01 to -0,36)	**0,020**	-2,41 (-4,37 to -0,4)	**0,019**	-2,4 (-4,99 to 0,26)	**0,038**
Genomic instability						
Unadjusted	0,36 (-1,12 to 1,86)	0,636	0,52 (-1,12 to 2,19)	0,537	-0,43 (-2,62 to 1,81)	0,704
Adjusted#	1,88 (0,22 to 3,57)	**0,014**	1,53 (-0,21 to 3,31)	**0,026**	-0,34 (-2,65 to 2,02)	0,218

* Adjusted for age, BMI, sex and smoking habits.

Adjusted for age, BMI, sex, smoking habits and the presence of diabetes.

Results for each hallmark of aging are expressed as the rate ratio for a 10-unit increase in each polysomnographic parameter. P-values <0.05 are presented in bold. AHI: apnea-hypopnea index; BMI: body mass index. CI: confidence interval; OSA: obstructive sleep apnea; TSat90: night time with oxygen saturation less than 90%.

Table 3. Adjusted association of OSA with the hallmarks of aging by age groups.

			Adjusted rate ratio or mean difference (95% CI)	*p-value*
Altered cellular communication *				
	Young	n= 188	1.37 (1.05 to 1.79)	**0.019**
	Elderly	n= 233	1.32 (0.99 to 1.77)	0.059
	Interaction		0.96 (0.66 to 1.41)	0.852
Deregulation of nutrient sensing #				
	Young	n= 253	1.25 (1.05 to 1.48)	**0.011**
	Elderly	n= 327	1.06 (0.88 to 1.27)	0.552
	Interaction		0.85 (0.66 to 1.08)	0.177
Telomere attrition *				
	Young	n= 191	<0.01 (-0.01 to 0.01)	0.610
	Elderly	n= 226	<0.01 (-0.01 to 0.01)	0.880
	Interaction		<0.01 (-0.02 to 0.01)	0.819
Mitochondrial dysfunction *				
	Young	n= 195	-6.62 (-20.89 to 7.66)	0.363
	Elderly	n= 235	-13.43 (-29.38 to 2.53)	0.099
	Interaction		-6.81 (-27.49 to 13.87)	0.518
Genomic instability #				
	Young	n= 116	1.47 (0.12 to 2.81)	**0.033**
	Elderly	n= 113	0.68 (-0.84 to 2.21)	0.377
	Interaction		-0.78 (-2.74 to 1.17)	0.431

* Adjusted for age, BMI, sex and smoking habits.

Adjusted for age, BMI, sex, smoking habits and the presence of diabetes.

Alteration in cellular communication and deregulation of nutrient sensing are compared by the rate ratio, while telomere attrition, mitochondrial dysfunction and genomic instability are compared by the mean difference. P-values <0.05 are presented in bold.

AHI: apnea-hypopnea index; BMI: body mass index; CI: confidence interval; OSA: obstructive sleep apnea.

SUPPLEMENTARY INFORMATION

Association of Obstructive Sleep Apnea with the Aging Process

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Figure E1. Flowchart showing the patients included in each molecular determination. Patients with missing or unavailable data were excluded from the corresponding analysis. Molecular determinations with discrepant values among replicas were considered not valid and eliminated. Definition of abbreviations: OSA = obstructive sleep apnea.

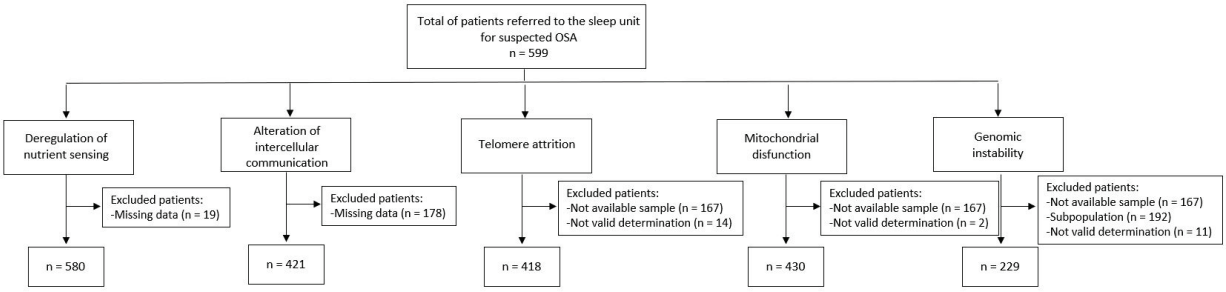

Table E1. Sequence of the primers used to evaluate leukocyte telomeric length.

Definition of abbreviations: T: telomere; S: single-copy gene.

Primer	Sequence
T forward primer	ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT
T reverse primer	TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA
S forward primer	cggcggcgggcggcgcgggctgggcggAAATGCTGCACAGAATCCTTG
S reverse primer	gcccggcccgccgcgcccgtcccgccgGAAAAGCATGGTCGCCTGTT

Table E2. Sequence of the primers used to evaluate leukocyte mtDNA copy number.

Definition of abbreviations: Mt. mitochondrial; N: nuclear.

Primer / Probe	Sequence
Mt forward primer	TTAAACACATCTCTGCCAAACC
Mt reverse primer	AGATTAGTAGTATGGGAGTGGGA
Mt probe	AACCCTAACACCAGCCTAACCAGA
N forward primer	CTTTCTGGCTGGATTGGTATCT
N reverse primer	CAGAATAGGCTGCTGTTCCTAC
N probe	AGTAGGAAGGGCTTGTTCCTGCTG

Table E3. Number of subjects per group of OSA and age: young non-OSA patients, young OSA patients, elderly non-OSA patients and elderly OSA patients. Definition of abbreviations: AHI: apnea-hypopnea index; OSA = obstructive sleep apnea.

	Non-OSA (AHI <15 events/h)		OSA (AHI ≥15 events/h)	
	Young Age < 50 years	Elderly Age 50 ≥ years	Young Age < 50 years	Elderly Age ≥ 50 years
Alteration of cellular communication	67	43	121	190
Deregulation of nutrient sensing	86	62	167	265
Telomere attrition	72	41	120	185
Mitochondrial dysfunction	72	44	123	191
Genomic instability	38	27	78	86

STUDY 3

Polysomnographic Characterization of Circadian Blood Pressure Patterns in Patients with Obstructive Sleep Apnea

Lucía Pinilla, Iván D Benítez, Esther Gracia-Lavedan, Gerard Torres, Olga Mínguez, Maria Aguilà, Adriano Targa, Mireia Dalmases, Olga Mediano, Juan F Masa, Maria J Masdeu, Ferran Barbé, Manuel Sánchez-de-la-Torre.

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Polysomnographic Characterization of Circadian Blood Pressure Patterns in Patients with Obstructive Sleep Apnea

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ABSTRACT

We characterized the polysomnography (PSG) parameters associated with alterations in the circadian blood pressure (BP) pattern aiming to identify the main contributors to explain the nondipper profile in obstructive sleep apnea (OSA).

Observational-prospective-multicenter study that included subjects referred to the sleep unit for suspected OSA. Following a PSG study, subjects with an apnea-hypopnea index (AHI) ≥5 events/h were included. Two groups were established based on the 24-h ambulatory blood pressure monitoring (ABPM) dipping ratio (DR; night/day BP ratio): dippers (DR ≤0.9) and nondippers (DR >0.9).

The cohort consisted of 299 patients: 131 (43.8%) dippers and 168 (56.2%) nondippers. A significant increase in the risk of presenting a nondipper BP pattern was found along with AHI gain [odds ratio (OR) (95% (CI) = 1.71 (1.28 to 2.28)]. The best AHI cut-off for predicting nondipper status was 25.2 events/h, increasing the OR (95% CI) to 3.50 (2.02 to 6.07). The hypopnea index [OR (95% CI) = 1.70 (1.27 to 2.26), TSat90 [OR (95% CI) = 1.41 (1.06 to 1.87)] and respiratory arousal index [OR (95% CI) = 1.74 (1.30 to 2.34)] were individually associated with the risk of a nondipping pattern. Multivariate variable selection processes identified the respiratory arousal index as the most relevant risk factor for the nondipper profile, beyond classical clinical risk factors and usual PSG metrics.

KEYWORDS

Obstructive sleep apnea; Nondipping; Circadian rhythm; Blood pressure; Polysomnography.

STATEMENT OF SIGNIFICANCE

A close relationship between obstructive sleep apnea (OSA) and blood pressure (BP) impairments, including an increased risk for nocturnal nondipping, has been consistently observed. However, the precise mechanisms of this interaction are incompletely understood. We characterized the polysomnography parameters associated with alterations in the circadian BP pattern, aiming to identify the main contributors to explain the nondipper profile in OSA. We found that the respiratory arousal index was a key OSA-related parameter associated with the loss of nocturnal BP dipping. The results suggest that sleep fragmentation is an important mechanistic pathway underlying the relationship between OSA and circadian BP abnormalities.

LIST OF ABBREVIATIONS

ABPM = ambulatory blood pressure monitoring

AHI = apnea-hypopnea index

BMI = body mass index

BP = blood pressure

CI = confidence interval

COPD = chronic obstructive pulmonary disease

DR = dipping ratio

EEG = electroencephalogram

ESS = Epworth sleepiness scale

GAM = generalized additive model

LASSO = least absolute shrinkage and selection operator

MSE = mean square error

NREM = non-rapid eye movement

OR = odds ratio

OSA = obstructive sleep apnea

PLMS = periodic limb movements in sleep

REM = rapid eye movement

RLS = restless legs syndrome

SaO_2 = oxygen saturation

SD = standard deviation

TSat90 = percent of total sleep time spent with SaO2 <90%

WASO = wake after sleep onset

INTRODUCTION

Blood pressure (BP) fluctuates daily during different physiologic states, declining by 10% to 20% at nighttime during sleep, compared with BP values at daytime during wakefulness. Nocturnal BP decline is related to the physiological lessening of the sympathetic nervous system during the nighttime period. The lack of BP fall at night (nocturnal BP decrease of less than 10% of daytime BP), defined as nondipping, is an independent risk factor for the development of adverse cardiovascular events and hypertensive end-organ damage [1–4]. Several prospective studies have substantiated the prognostic value of nocturnal BP fall in normotensive [5] and hypertensive [6–9] population-based cohorts. Despite the increasing body of evidence supporting the prognostic value and clinical significance of impaired circadian BP variation, the pathophysiology underlying this phenomenon remains to be fully understood. The etiology of nocturnal nondipping is complex, and may co-occur with several clinical factors; among these, the disruption of biological circadian rhythms during sleep seems to play a pivotal role [10].

Obstructive sleep apnea (OSA), the most common sleep disorder, is caused by repetitive episodes of upper airway collapse and has been associated with marked dysregulation of circadian BP control as well as wide-ranging cardiovascular consequences [11]. Within the OSA population, a high proportion of patients exhibit abnormal nocturnal BP dipping, the frequency of which increases with OSA severity. A recent meta-analysis revealed a nondipping prevalence of 59% among patients with OSA, indicating that individuals with OSA have a 1.5 times higher risk of presenting this abnormal circadian pattern than individuals without OSA [12]. Data on the association of OSA with nocturnal nondipping are not limited to cross-sectional studies; longitudinal studies have demonstrated that OSA is a risk factor for the development of a nondipping BP profile [13]. Furthermore, it has been

shown that the presence of nondipping is independently associated with a poor cardiovascular prognosis, specifically among OSA patients [14].

The physiopathological mechanisms underlying the close association between OSA and the development of abnormalities in 24-h circadian BP patterns are not completely understood [15]. Emerging evidence has proposed several major features of OSA as potential triggers for nocturnal nondipping [16], including intermittent hypoxemia and hypercapnia, sympathetic hyperactivation, intrathoracic pressure fluctuations, awakening or arousal, and ultimately the disruption of the structure and quality of sleep [17]. However, the respective importance of these OSA-related pathogenic mechanisms in the development of impaired BP dipping is unclear. Furthermore, to the best of our knowledge, the relationships of OSA severity metrics with the night-to-day variation of BP have not been studied extensively. In this study, we characterized the polysomnography (PSG) parameters associated with alterations in the circadian BP pattern aiming to identify the main contributors to explain the nondipper profile in OSA.

METHODOLOGY

Study design

This was a multicenter, observational, prospective, cross-sectional study including consecutive subjects ≥18 years old who were referred to the Sleep Unit due to suspected OSA (ClinicalTrials.gov: NCT03513926). The exclusion criteria included the following: existence of a previously diagnosed sleep disorder, a history of continuous positive airway pressure (CPAP) treatment, diagnosis of chronic obstructive pulmonary disease (COPD), pregnancy, a psychophysical inability to complete the questionnaires, or any medical

social or geographical circumstance that may jeopardize patient compliance. Recruitment took place in 4 hospitals in Spain: 439 (73.3%) University Hospital Arnau de Vilanova and Santa Maria of Lleida, 132 (22.0%) San Pedro de Alcántara Hospital, 22 (3.67%) Guadalajara University Hospital, and 6 (1.03%) Parc Taulí University Hospital). The Clinical Research Ethics Committee of the coordinating center approved the study (No. 1153/1411), and every enrolled patient provided informed written consent to participate in the study.

Clinical evaluation

Detailed information about sociodemographic characteristics, unhealthy life habits and medical history, including comorbidities and prescribed medications, was collected from the patients by trained clinicians. General physical and anthropometric parameters were recorded, and self-reported daytime somnolence was assessed with the Epworth sleepiness scale (ESS) [18].

Sleep evaluation

All patients underwent an overnight in-laboratory polysomnographic sleep study (Philips Sleepware G3, Amsterdam, Netherlands). All methods were performed according to current clinical practice guidelines and regulations [19]. The results from the sleep studies were analyzed by trained personnel using standard criteria [20,21]. Apnea was defined as an interruption or reduction in oronasal airflow by ≥90% for at least 10 s. Hypopnea was defined as a 30–90% reduction in oronasal airflow for at least 10 s associated with oxygen desaturation ≥3% or evidence of arousal on the electroencephalogram (EEG). The apnea-hypopnea index (AHI) was calculated based on the average number of apnea plus hypopnea events per hour of sleep. To ensure coverage of multiple OSA severity ranges,

the analysis included patients with an AHI equal to or more than 5 events/h. Mild OSA was defined as 5 ≤AHI <15 events/h, moderate OSA as 15≤AHI <30 events/h and severe OSA as an AHI ≥30 events/h. To focus on the obstructive component of sleep apnea, patients with ≥50% central apnea were excluded from the analysis [22]. OSA severity parameters derived from PSG were grouped into 4 categories according to the physiopathology of the disease: respiratory disturbances, nocturnal hypoxemia, sleep fragmentation and sleep architecture. Respiratory disturbances included the obstructive apnea index and the hypopnea index. Nocturnal hypoxemia parameters recorded by pulse oximetry included the mean overnight oxygen saturation (SaO2), minimum overnight SaO2, percent of total sleep time spent with SaO2 <90% (TSat90), and desaturation index (number of SaO2 <90% events per hour of sleep). Measures of sleep fragmentation included arousal from sleep, which was classified into 3 categories, namely, arousal associated with a respiratory event, arousal associated with limb movement and nonspecific/spontaneous arousal. To study sleep architecture, the entire nighttime sleep period, excluding wakefulness periods, was divided into four stages: nonrapid eye movement (NREM) stage 1 (N1), non-REM stage 2 (N2), non-REM stage 3 (N3; also known as slow-wave sleep [SWS]), and rapid eye movement (REM) stage (R). The sleep macrostructure was calculated as the proportions of time spent in the N1, N2, N3, and R stages. Additional conventional sleep quality parameters were determined, including total sleep time, sleep latency (transition time from wakefulness to non-REM sleep onset), sleep efficiency (proportion of total time in bed spent asleep), total wake time, and wake after sleep onset (WASO; total time of wakefulness occurring after sleep onset).

Ambulatory BP evaluation

In the morning immediately after the PSG sleep study, the patients were subjected to 24-h ambulatory blood pressure monitoring (ABPM) (Mortara Ambulo 2400; Milwaukee, WI, USA), according to internationally recommended procedures [23]. During ABPM, BP measurements were obtained every 15 minutes during the daytime interval and every 30 minutes during the nighttime interval. Awake and asleep periods were defined using the sleeping time reported by each subject. ABPM recordings were considered optimal when the percentage of the measurements was >70%, with at least one measurement every hour, or else the monitoring was repeated. Following the BP evaluation, the subjects were classified based on the dipping ratio (DR), which was calculated as the ratio between average nighttime and daytime BP values. A dipper circadian BP pattern was defined as nocturnal BP decrease of >10% relative to daytime values (DR ≤0.9), and a nondipper circadian BP pattern was defined as a nocturnal BP decrease of ≤10% relative to daytime values (DR >0.9). For detailed information regarding the study cohort, see Figure 1.

Statistical analysis

Descriptive statistics were used to summarize the characteristics of the study population. Absolute and relative frequencies were used for qualitative data. Medians (25th–75th percentiles) were estimated for quantitative variables. Sociodemographic and clinical data of dipper and nondipper patients were compared using the Wilcoxon signed-rank test for continuous variables and the chi-square test (or Fisher's exact test when the expected frequencies were less than 5 in some cells) for qualitative variables. Individual associations between the PSG parameters and dipping status were assessed using logistic regression models adjusted for the following confounding factors: age, sex, body mass index (BMI), mean daytime BP value, and antihypertensive drug use. Multivariate generalized additive models (GAMs) with a penalized thin plate regression spline were

fitted to evaluate the dose–response relationships between the PSG parameters and the DR. A multivariate model for the prediction of nondipping status was constructed using a relaxed least absolute shrinkage and selection operator (LASSO) model [24,25]. The variable selection process included sociodemographic characteristics (age, sex and BMI), pharmacological data (antihypertensive drug use) and office BP measurements (systolic and diastolic BP) as well as the PSG data. Tenfold cross-validation was performed to determine the lambda and gamma parameters of the LASSO model. The lambda and gamma parameters were selected as the values associated with one standard error greater than the minimum mean square error (MSE). To perform the LASSO analysis, missing values were replaced with the means of the nonmissing values, due to low percentage of missing data on the input variables (Table S1). Furthermore, an ensemble learning process based on the random forest method [26] was applied to evaluate potential complex interactions and nonlinear relationships between the predictive factors and nondipping status. The p value threshold defining statistical significance was set at <0.05. All analyses were performed using R statistical software, version 4.0.1 (R Project for Statistical Computing; Vienna, Austria).

RESULTS

Characteristics of the study population

The final study cohort consisted of 299 individuals, mainly middle-aged, men, and overweighted/obese. Considering the OSA severity ranges, 62 (20.7%) of the patients presented mild OSA, 67 (22.4%) moderate OSA and 170 (56.9%) severe OSA. A total of 119 (40.8%) subjects had a positive diagnosis of hypertension, of whom 114 (96.6%)

used BP-lowering drugs. One hundred thirty-one (43.8%) patients presented a dipping BP pattern, and 168 (56.2%) presented a nondipping BP pattern. Descriptive characteristics of the study population by DR category are presented in Table 1. The median [p25;p75] DRs in the dipper and nondipper groups were 0.85 [0.81;0.87] and 0.96 [0.93;1.01], respectively. Differences in the DR were mainly due to nighttime BP values, with no significant differences in daytime and mean BP levels between the groups observed. Compared to participants with a dipper profile, those with a nondipper profile had a significantly larger number of comorbid conditions and thus had a higher number of prescribed drugs (antidiabetic, antihypertensive and lipid-lowering medications). Nondipper subjects had more severe OSA (median [p25;p75] AHIdippers = 25.4 [13.4;48.6] vs. AHInondippers = 41.4 [24.5;63.5], $p<0.001$), with significant differences in disease-related sleep characteristics and sleep quality parameters (see Table 1 for detailed information).

Individual associations of OSA parameters with the risk of impaired nocturnal BP dipping

W e first focused on the AHI, which is a ubiquitous disease-defining metric of OSA. After adjustment for various confounding factors, a significant increase in the risk of a nondipping BP pattern was observed along with AHI gain [odds ratio (OR) (95% confidence interval (CI)) = 1.71 (1.28 to 2.28) per 1-standard deviation (SD) AHI change]. When classifying the AHI by OSA severity categories, with mild OSA as the reference group, patients with severe OSA were 3.42 times more likely to present a nondipper profile [OR (95% CI) = 3.42 (1.76 to 6.64)]. Additionally, as an exploratory objective, we investigated the AHI cutoff value that best predicted the nondipping circadian pattern. The optimal cutoff value for the differentiation of nondipper and dipper patients in our cohort

was 25.2 events/h, increasing the OR (95% CI) of nondipping to 3.50 (2.02 to 6.07) (Figure 2A-1 and Table S2A). Finally, we aimed to deepen our understanding of the type and nature of the association between the continuous AHI and nocturnal BP fall. By using a GAM model, a linear dose–response relationship between these two variables was observed (Figure 2A-2).

To further assess the associations of OSA with the circadian variation of BP, we analyzed a set of OSA severity metrics beyond the AHI. Adjusted logistic regression models were used to examine the individual associations of PSG-derived parameters with the risk of a nondipping pattern (Figure 2B-1 and Table S2B). Regarding respiratory disturbances, the hypopnea index was associated with an increased risk of a nondipper profile [OR (95% CI) = 1.70 (1.27 to 2.26)]. In relation to nocturnal hypoxemia, only the association of the TSat90 with an increased risk of nondipping reached statistical significance, with an OR (95% CI) of 1.41 (1.06 to 1.87). For sleep fragmentation, the respiratory arousal index was associated with an increased risk of a nondipper profile, with an OR (95% CI) of 1.74 (1.30 to 2.34). No significant associations were found for sleep architecture, although a tendency toward an increased percentage of sleep time spent in stage N1 was detected in nondippers. Additionally, we selected the parameter in each category with the highest effect size to evaluate the nature of these associations with nocturnal BP fall. The GAM models indicated that the selected OSA parameters showed dose–response relationships with the DR, though the association with stage N1 sleep did not reach statistical significance (Figure 2B-2).

Multivariate analyses for identification of risk factors for a nondipping BP pattern

Aiming to identify the most relevant factors of impaired dipping in OSA patients, we performed a multivariate analysis, combining the PSG data with a set of clinical variables

previously described as physiologically related to BP control, i.e., age, sex, BMI, office systolic and diastolic BP values, and antihypertensive drug use. The LASSO regression analysis identified a combination of 5 variables as independent predictors of nondipper status: the respiratory arousal index, the proportion of sleep time spent in stage N2, the hypopnea index, antihypertensive drug use and female sex. However, the last two factors did not achieve statistical significance in the multivariable model (Figure 3A). Multicollinearity was tested for the constructed LASSO model by assessing the variance-inflation factors (VIF). All VIF values were <2, showing no collinearity between the predictor variables. Furthermore, a random forest method was applied to evaluate potential complex interactions and nonlinear relationships between the factors and nondipping status. The ranking of variable importance revealed that the respiratory arousal index was the principal risk factor for the nondipper BP profile (Figure 3B). Moreover, the top 5 predictive factors identified by the random forest method were related to both the macro- and microstructure of sleep.

DISCUSSION

Scientific evidence indicates relevant impacts of sleep disturbances on various components of human homeostasis, including cardiac and vascular system homeostasis [27,28]. A close relationship between OSA and BP impairment, including an increased risk for nocturnal hypertension and abnormal BP dipping, has been consistently observed 29. However, the precise mechanisms of this interaction are incompletely understood. Here, we aimed to characterize the PSG parameters associated with circadian BP pattern alteration in OSA. The present findings confirm those of previous investigations, showing that OSA is independently associated with impaired BP dipping. Furthermore, while the

AHI, hypopnea index, TSat90 and respiratory arousal index were individually associated with the risk of a nondipping BP pattern, the multivariate analyses identified the respiratory arousal index as the most relevant predictor of nondipping, beyond classical clinical risk factors and usual PSG metrics.

Consistent with previous reports indicating that the risk of nondipping is linked to OSA severity [12], we found that patients with severe OSA were the most likely to present a nondipper profile. However, the AHI cutoff value that best predicted the nondipping status in our population was 25 events/h, increasing the OR of this BP pattern to OR (95% CI) = 3.50 (2.02 to 6.07). Our results are in line with previous investigations. Staats et al. [30] investigated the beat-to-beat impact of OSA on the evolution of hemodynamic parameters during the wake-sleep transition. The authors found that sleep fragmentation due to arousals correlated better with systolic BP evolution and the development of a nondipping systolic BP pattern than the AHI.

Several pathological mechanisms have been proposed to be responsible for the BP changes associated with OSA, although the relative contribution of each factor to the integrated BP response has not been fully elucidated. Repeated oxygen desaturations have traditionally been considered one of the main contributors to the development of BP abnormalities among patients with OSA [31–33]. However, it has been reported that hypoxemia alone does not explain BP elevations after obstructive apnea events. Ringler et al. [34] showed that hypoxic apnea events were associated with equivalent BP elevations compared to apnea events without desaturation due to oxygen supplementation. In addition, in the absence of respiratory and sleep disruptions, chronic hypoxemia at 80% oxygen saturation was not associated with BP elevations. Indeed, available evidence indicates that other sleep disorders without effects on arterial oxygen saturation, such as

chronic insomnia and restless legs syndrome (RLS), also increase BP values during sleep, diminishing the BP dipping pattern [35]. A number of studies have observed transient increases in BP and heart rate values after each episode of periodic limb movements in sleep (PLMS), which acts as a trigger for sympathetic activation during sleep [36–38]. Krzyzaniak et al. [39] recently described a direct relationship between the number of PLMS during the night and the value of nocturnal BP. Other forms of arousal from sleep without airway obstruction or airflow limitation have also been shown to induce transient BP fluctuations. In a seminal study, Morgan et al. [40] applied auditory stimuli during non-REM sleep in healthy subjects to assess the neurocirculatory effects of arousal alone. The authors found that auditory arousals evoked abrupt increases in BP, the heart rate and sympathetic outflow to skeletal muscle. Consistent results were obtained by Davies et al. [41], who showed that the application of variable-length transient stimuli induced cortical arousals and led to a substantial nighttime BP increase in healthy volunteers.

Given the high prevalence of abnormal dipping in OSA patients and the prognostic implications of this BP pattern, defining the factors that affect nocturnal BP patterns and applying appropriate therapeutic interventions could prevent subsequent cardiovascular and organ damage in patients with OSA. In addition, risk stratification using metrics beyond the AHI is a new paradigm in OSA management [42]. Our results suggest that measures of sleep fragmentation should be considered when diagnosing OSA, as they could better predict OSA-related BP abnormalities and hence potential future cardiovascular consequences, highlighting the importance of performing complete PSG as opposed to respiratory polygraphy. Additionally, this study provides insights into the potential physiopathological link between OSA and BP hemodynamics, emphasizing the role of recurrent arousals, as triggers of sympathetic overactivity, which is a plausible

mechanism underlying this relationship, although this should be confirmed in further additional investigations. Moreover, our results suggest that future studies addressing nocturnal hypertension, circadian abnormalities, or other hemodynamic implications of OSA should not focus on only the severity of hypoxemia but also consider OSA-induced sleep fragmentation. Nevertheless, although the literature encourages the ability of sleep disruption alone to elevate BP, it should be noted that other factors associated with OSA could be, at least partially, responsible for BP elevations during sleep.

Our study has certain limitations that should be noted. First, no data on the onset of OSA were available, preventing inference of temporal causality in the results. Second, due to the observational design of the study, cause-effect relationships between the sleep parameters and the nocturnal nondipping pattern remain unproven, although it is biologically plausible that OSA physiology may contribute to nondipping hemodynamics. Third, as OSA was diagnosed by in-laboratory PSG, sleep quality parameters were not included in the analyses because measurements were not taken in the participants' usual sleep environments, and actigraphy, the reference method to assess sleep quality, was not available. Fourth, although we considered various confounding factors all the analyses, individual biologic conditions and/or lifestyle behaviors, such as shift work routines, caffeine intake and psychological stress, may attenuate or potentiate the effects of OSA on BP, and thus may limit the generalizability of the results. Fifth, no information regarding antihypertensive drug ingestion timing was collected. Therefore, a potential chronotherapeutic effect of evening vs. morning antihypertensive medication intake may exist. Finally, the classification of dippers and nondippers was based on a single 24-h ABPM. The strengths of this study include its multicenter design, which enabled the evaluation of a relatively large number of patients to appropriately address the

associations of sleep characteristics with circadian BP patterns. Moreover, we included a cohort of consecutive subjects who were referred to the sleep unit, resulting in a realistic level of heterogeneity among the population with OSA and enabling the evaluation of the complete spectrum of disease severity. Other strengths include the use of 24-h ABPM and PSG, which are gold-standard techniques for the identification of circadian patterns of BP and the diagnosis of OSA, respectively. Standardized methods were applied for the collection of sleep and BP measurements, which were scored by certified technicians. Finally, disposing of data on nocturnal BP fall as a continuous variable (the night-to-day BP ratio) helped to delve into the associations of OSA severity markers with the nocturnal variation of BP, to avoid the possibility of misleading or nonrepresentative associations based solely on the circadian categories of BP.

In conclusion, the present study suggests that the respiratory arousal index is a key OSA-related parameter associated with the loss of nocturnal BP dipping. Although the intermediate mechanisms of the development of BP abnormalities in OSA patients are likely multifactorial, the results suggest that sleep fragmentation is an important mechanistic pathway underlying this relationship.

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FIGURES

Figure 1. Flowchart of the study cohort.

Consecutive subjects with suspected OSA who fulfilled the inclusion and exclusion criteria were subjected to in-laboratory PSG. Patients with missing or invalid sleep study data, ≥50% central apnea, or an AHI <5 events/h were not considered for the analysis. Following ABPM, the patients were classified into two groups: dippers (DR ≤0.9) and nondippers (DR >0.9). Definitions of abbreviations: ABPM = ambulatory blood pressure monitoring; AHI = apnea-hypopnea index; DR = dipping ratio; OSA = obstructive sleep apnea; PSG = polysomnography.

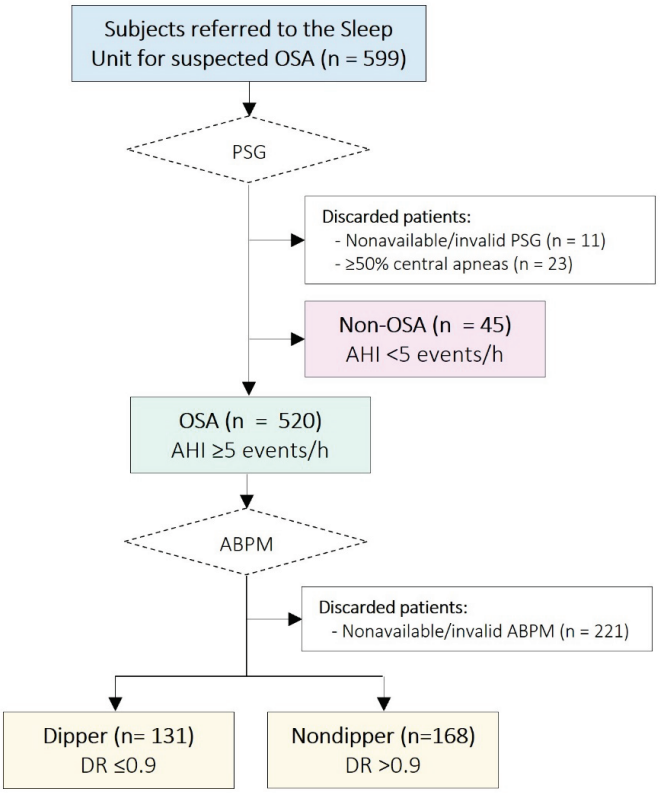
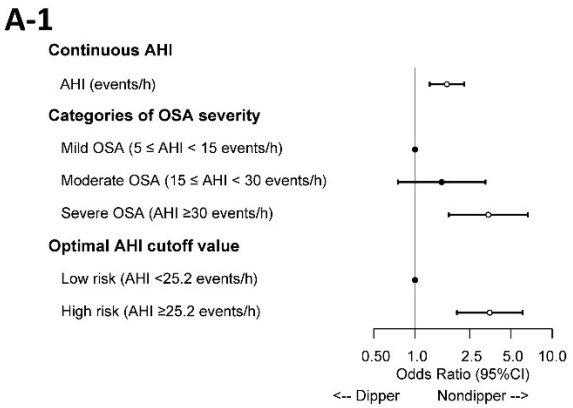
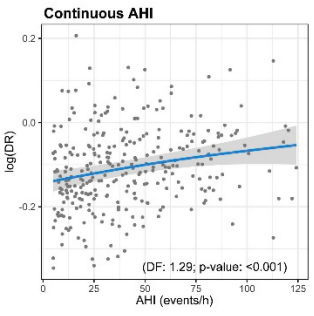
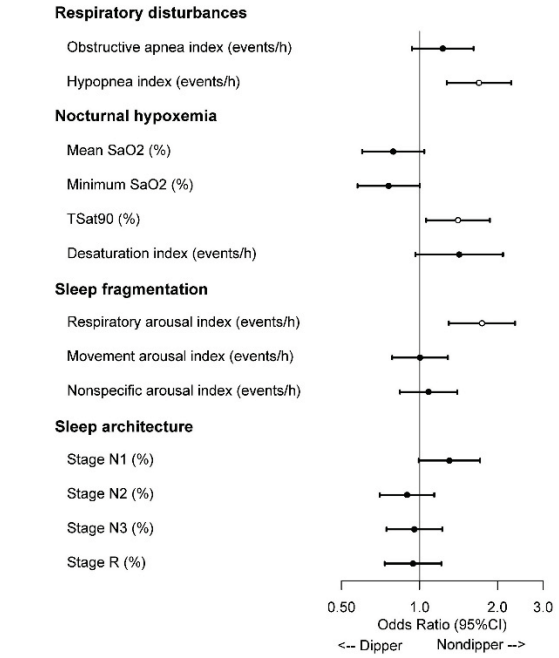


Figure 2. Individual associations of OSA parameters with the risk of impaired nocturnal BP dipping.

(A) Apnea-hypopnea index. A-1: Logistic regression model for evaluating the individual association between the AHI and the risk of a nondipper BP profile. Data are presented as the OR (95% CI), which represents the risk of nondipping per 1-SD increase in the AHI, the AHI categorized by OSA severity, or the AHI categorized by the optimal cutoff value. **A-2**: GAM models was used to estimate the dose–response relationship between the AHI and the log(DR) as continuous variables. The AHI is plotted along the x-axis, and the log(DR) is plotted along the y-axis. **(B) OSA parameters. B-1**: Logistic regression model for evaluating the individual associations between the OSA metrics and the risk of a nondipper BP profile. Data are presented as ORs (95% CIs), which represent the risk of nondipping per 1-SD increases in the OSA parameters. **B-2**: GAM models were used to estimate the dose–response relationships between the selected OSA parameters and the log(DR) as continuous variables. The OSA parameters are plotted along the x-axis and the log(DR) along the y-axis. All logistic regression models and GAM models were adjusted for age, sex, BMI, mean daytime BP value, and use of antihypertensive drug treatment. The blue lines in the GAM plots represent the estimate of the change in the log(DR) according to the value of the AHI or OSA parameter, and the gray area corresponds to the 95% CI. Definitions of abbreviations: AHI = apnea-hypopnea index; BMI = body mass index; BP = blood pressure; CI = confidence interval; DR = dipping ratio; GAM = generalized additive model; OR = odds ratio; OSA = obstructive sleep apnea; REM = rapid eye movement; SaO_2 = oxygen saturation; SD = standard deviation; TSat90 = time with SaO_2 <90%.


A-1
Continuous AHI
AHI (events/h)
Categories of OSA severity
Mild OSA (5 ≤ AHI < 15 events/h)
Moderate OSA (15 ≤ AHI < 30 events/h)
Severe OSA (AHI ≥30 events/h)
Optimal AHI cutoff value
Low risk (AHI <25.2 events/h)
High risk (AHI ≥25.2 events/h)
0.50 1.0 2.5 5.0 10.0
Odds Ratio (95%CI)
<-- Dipper Nondipper -->


A-2
Continuous AHI
log(DR)
(DF: 1.29; p-value: <0.001)
AHI (events/h)


B-1
Respiratory disturbances
Obstructive apnea index (events/h)
Hypopnea index (events/h)
Nocturnal hypoxemia
Mean SaO2 (%)
Minimum SaO2 (%)
TSat90 (%)
Desaturation index (events/h)
Sleep fragmentation
Respiratory arousal index (events/h)
Movement arousal index (events/h)
Nonspecific arousal index (events/h)
Sleep architecture
Stage N1 (%)
Stage N2 (%)
Stage N3 (%)
Stage R (%)
0.50 1.0 2.0 3.0
Odds Ratio (95%CI)
<-- Dipper Nondipper -->

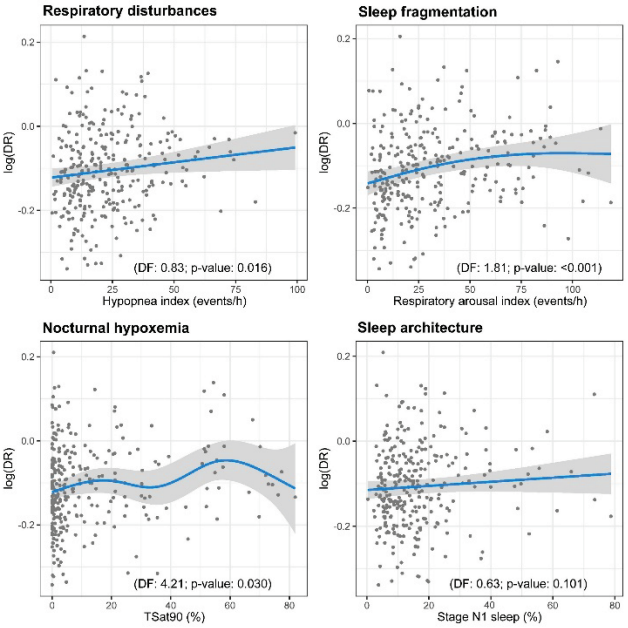
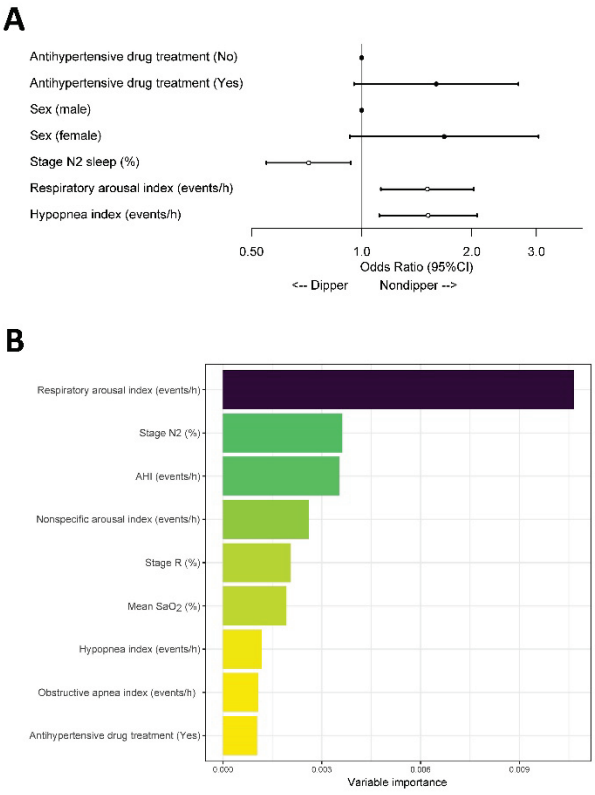

B-2
Respiratory disturbances
log(DR)
(DF: 0.83; p-value: 0.016)
Hypopnea index (events/h)
Sleep fragmentation
(DF: 1.81; p-value: <0.001)
Respiratory arousal index (events/h)
Nocturnal hypoxemia
(DF: 4.21; p-value: 0.030)
TSat90 (%)
Sleep architecture
(DF: 0.63; p-value: 0.101)
Stage N1 sleep (%)

Figure 3. Multivariate analyses for identification of the key factors of nondipping BP.

(A) LASSO: Predictive model for the detection of nondipper status constructed using a variable selection process based on LASSO regression. Data are presented as the OR (95% CI), which represents the risk of nondipping BP per 1-SD increase in the predictor variables. **(B) RF:** Ranking of the importance of each variable in the classification of the study groups (nondippers vs. dippers), based on RF analysis. Only variables displaying a minimum discrimination are shown in the graphic. Definitions of abbreviations: AHI = apnea-hypopnea index; BMI = body mass index; CI = confidence interval; LASSO = least absolute shrinkage and selection operator; OR = odds ratio; R = rapid eye movement; RF = random forest; SaO_2 = oxygen saturation; TSat90 = time with SaO_2 <90%.



TABLES

Table 1. Characteristics of the study cohort by categories of dipping ratio. Data are presented as the median [p25;p75] for quantitative variables and n (%) for qualitative variables. BP measurements are expressed in mmHg. P values <0.05 are presented in bold. Definitions of abbreviations: ABPM = ambulatory blood pressure monitoring; ACE = angiotensin-converting enzyme; AHI = apnea-hypopnea index; BMI = body mass index; BP = blood pressure; DR = dipping ratio; ESS = Epworth sleepiness scale; OSA = obstructive sleep apnea; PSG = polysomnography; R = rapid eye movement; SaO_2 = oxygen saturation; TSat90 = time with SaO_2 <90%; WASO = wake after sleep onset.

	All **n = 299**	***Dipper*** **n = 131**	***Nondipper*** **n = 168**	**p value**
Clinical data				
Demographic/anthropometric				
Age (years)	52.0 [45.0;56.5]	50.0 [44.0;56.0]	52.0 [46.0;57.0]	0.149
Sex				0.177
Male	220 (73.6%)	102 (77.9%)	118 (70.2%)	
Female	79 (26.4%)	29 (22.1%)	50 (29.8%)	
BMI (kg/m^2)	30.8 [27.4;34.9]	29.8 [26.9;34.0]	31.6 [27.9;35.7]	0.060
Smoking status				0.646
Never	110 (37.2%)	45 (34.9%)	65 (38.9%)	
Former	104 (35.1%)	49 (38.0%)	55 (32.9%)	
Current	82 (27.7%)	35 (27.1%)	47 (28.1%)	
Comorbidities				
Diabetes	43 (14.5%)	11 (8.46%)	32 (19.2%)	**0.015**
Hypertension	119 (40.8%)	40 (31.7%)	79 (47.6%)	**0.009**
Dyslipidemia	91 (31.2%)	29 (22.7%)	62 (37.8%)	**0.008**
Cardiovascular disease	50 (16.9%)	16 (12.4%)	34 (20.4%)	0.098
Medication use				
Insulin	14 (4.70%)	2 (1.53%)	12 (7.19%)	**0.044**
Any antihypertensive drug	114 (38.3%)	39 (29.8%)	75 (44.9%)	**0.011**
ACE inhibitors	69 (23.2%)	23 (17.6%)	46 (27.5%)	0.059
Beta-blockers	44 (14.8%)	16 (12.2%)	28 (16.8%)	0.350
Diuretic agents	45 (15.2%)	13 (10.0%)	32 (19.2%)	**0.043**
Calcium-channel blockers	30 (10.1%)	8 (6.11%)	22 (13.3%)	0.066
Angiotensin II receptor blockers	28 (9.46%)	11 (8.40%)	17 (10.3%)	0.721
Lipid-lowering drugs	67 (22.6%)	17 (13.0%)	50 (30.1%)	**0.001**
Hemodynamic data				
Dipping ratio	0.91 [0.85;0.97]	0.85 [0.81;0.87]	0.96 [0.93;1.01]	

24 h				
Mean BP	97.1 [90.2;105]	95.4 [90.0;102]	98.2 [90.6;108]	0.098
Systolic BP	128 [119;142]	127 [118;138]	130 [120;145]	0.055
Diastolic BP	79.9 [75.3;84.6]	79.5 [75.2;83.8]	80.3 [75.5;84.7]	0.424
24 h pulse frequency	70.8 [63.5;76.5]	69.5 [63.5;76.1]	71.2 [63.8;77.2]	0.468
Daytime				
Mean BP	99.0 [92.8;107]	99.8 [94.0;107]	98.9 [92.0;107]	0.187
Systolic BP	131 [122;144]	131 [123;144]	131 [121;145]	0.842
Diastolic BP	81.7 [76.9;86.5]	82.1 [78.1;87.5]	81.3 [76.2;85.3]	0.086
Daytime pulse frequency	71.1 [64.7;78.8]	70.3 [65.4;78.2]	72.2 [64.3;79.2]	0.681
Nighttime				
Mean BP	90.1 [82.1;99.0]	83.2 [78.2;90.8]	96.2 [86.5;107]	**<0.001**
Systolic BP	120 [109;138]	112 [104;125]	129 [114;146]	**<0.001**
Diastolic BP	74.4 [69.2;79.3]	70.4 [66.8;74.3]	77.5 [73.2;85.0]	**<0.001**
Nighttime pulse frequency	65.4 [59.6;72.5]	64.9 [57.8;71.0]	66.0 [60.8;74.2]	0.069
Polysomnographic data				
Respiratory disturbances				
AHI (events/h)	34.7 [18.8;58.3]	25.4 [13.4;48.6]	41.4 [24.5;63.5]	**<0.001**
Obstructive apnea index (events/h)	7.36 [2.15;23.4]	5.93 [1.57;16.3]	8.46 [2.54;28.3]	**0.047**
Hypopnea index (events/h)	18.5 [11.0;30.6]	15.3 [9.32;25.4]	21.9 [12.6;35.7]	**<0.001**
Nocturnal hypoxemia				
Mean SaO_2 (%)	93.0 [91.0;94.0]	93.0 [92.0;94.0]	93.0 [91.0;94.0]	**0.022**
Minimum SaO_2 (%)	82.0 [73.0;86.0]	82.0 [75.0;87.0]	80.0 [72.0;85.0]	**0.033**
TSat90 (%)	3.23 [0.54;14.8]	2.40 [0.32;9.63]	4.50 [0.76;20.9]	**0.010**
Desaturation index (events/h)	8.02 [1.78;24.0]	4.86 [1.34;14.4]	9.75 [2.29;32.3]	0.057
Sleep fragmentation				
Respiratory arousal index (events/h)	23.4 [11.0;45.7]	18.5 [7.96;34.4]	27.7 [16.4;50.8]	**<0.001**
Movement arousal index (events/h)	3.18 [1.30;6.60]	3.06 [1.58;6.48]	3.18 [1.14;7.02]	0.844
Nonspecific arousal index (events/h)	6.68 [3.14;11.5]	6.92 [3.01;9.96]	6.56 [3.28;13.1]	0.734
Sleep architecture				
Stage N1 (%)	12.4 [7.41;18.9]	11.7 [6.70;17.0]	13.4 [7.58;21.5]	0.121
Stage N2 (%)	43.3 [36.5;53.2]	44.6 [37.5;53.1]	42.4 [35.4;53.4]	0.191
Stage N3 (%)	26.6 [15.7;36.0]	26.6 [15.6;35.4]	26.6 [16.7;36.3]	0.787
Stage R (%)	13.8 [9.09;18.1]	14.1 [10.4;17.7]	13.0 [8.16;18.3]	0.351
Sleep quality				
Total sleep time (min)	340 [306;374]	344 [318;375]	336 [300;374]	0.252
Sleep latency (min)	14.8 [7.30;29.0]	12.5 [7.15;24.0]	17.0 [8.00;31.6]	0.082
Sleep efficiency (%)	84.8 [75.7;90.4]	86.5 [77.4;91.7]	82.8 [74.8;88.9]	**0.014**
Total wake time (min)	64.0 [40.4;103]	56.0 [34.0;92.0]	70.0 [48.9;108]	**0.018**
WASO (min)	45.5 [27.2;77.0]	40.0 [23.0;71.0]	50.0 [29.6;80.3]	0.127
Somnolence (ESS)	11.0 [7.00;14.0]	11.0 [6.00;13.5]	10.5 [7.00;15.0]	0.209

SUPPLEMENTARY INFORMATION

Polysomnographic Characterization of Circadian Blood Pressure Patterns in Patients with Obstructive Sleep Apnea

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TABLE OF CONTENTS

Table S1. Missing data on the input variables for the LASSO analysis. Data are presented as n (%), representing the proportion of missing information on the variables used as input for the construction of the LASSO regression model for the prediction of nondipping. Definitions of abbreviations: AHI = apnea-hypopnea index; BMI = body mass index; BP = blood pressure; LASSO = least absolute shrinkage and selection operator; R = rapid eye movement; SaO_2 = oxygen saturation; TSat90 = time with SaO_2 <90%.

Input variables for the LASSO analysis	Missings (%)
Dipping ratio	0 (0.00%)
Age	0 (0.00%)
Sex	0 (0.00%)
BMI	2 (0.67%)
Antihypertensive drug treatment	1 (0.33%)
Office systolic BP	7 (2.34%)
Office diastolic BP	7 (2.34%)
AHI	0 (0.00%)
Obstructive apnea index	5 (1.67%)
Hypopnea index	5 (1.67%)
Stage N1	5 (1.67%)
Stage N2	5 (1.67%)
Stage N3	5 (1.67%)
Stage R	5 (1.67%)
Mean SaO_2	3 (1.00%)
Minimum SaO_2	9 (3.01%)
TSat90	2 (0.67%)
Respiratory arousal index	11 (3.68%)
Movement arousal index	6 (2.01%)
Unspecific arousal index	25 (8.36%)

Table S2. Individual associations of OSA parameters with the risk of impaired nocturnal BP dipping. (A) Apnea-hypopnea index. Data are presented as the OR (95% CI), which represents the risk of nondipping per 1-SD increase in the AHI, treated as a continuous variable, the OSA severity category considering mild OSA as the reference group, or the AHI categorized by the optimal cut-off value (25.24 events/h). **(B) OSA parameters.** Data are presented as the OR (95% CI), which represents the risk of nondipping per 1-SD increases in the OSA parameters. P values <0.05 are presented in bold. All logistic regression models were adjusted for age, sex, BMI, mean daytime BP value, and antihypertensive drug use. Definitions of abbreviations: AHI = apnea-hypopnea index; BMI = body mass index; BP = blood pressure; CI = confidence interval; OR = odds ratio; OSA = obstructive sleep apnea; R = rapid eye movement; SaO_2 = oxygen saturation; SD = standard deviation; TSat90 = time with SaO_2 <90%.

A. APNOEA-HYPOPNOEA INDEX	OR (95% CI)	p value
Continuous AHI		
AHI (events/h)	1.71 (1.28 to 2.28)	**<0.001**
Categories of OSA severity		
Mild OSA (5 ≤ AHI < 15 events/h)	1	
Moderate OSA (15 ≤ AHI < 30 events/h)	1.56 (0.75 to 3.26)	0.232
Severe OSA (AHI ≥30 events/h)	3.42 (1.76 to 6.64)	**<0.001**
Optimal AHI cut-off value		
Low risk (AHI <25.2 events/h)	1	
High risk (AHI ≥25.2 events/h)	3.50 (2.02 to 6.07)	**<0.001**
B. OSA PARAMETERS	**OR (95% CI)**	**p value**
Respiratory disturbances		
Obstructive apnea index (events/h)	1.23 (0.94 to 1.62)	0.138
Hypopnea index (events/h)	1.70 (1.27 to 2.26)	**<0.001**
Nocturnal hypoxemia		
Mean SaO_2 (%)	0.79 (0.60 to 1.04)	0.100
Minimum SaO_2 (%)	0.76 (0.58 to 1.00)	0.052
TSat90 (%)	1.41 (1.06 to 1.87)	**0.018**
Desaturation index (events/h)	1.42 (0.96 to 2.10)	0.075
Sleep fragmentation		
Respiratory arousal index (events/h)	1.74 (1.30 to 2.34)	**<0.001**
Movement arousal index (events/h)	1.00 (0.78 to 1.29)	0.971
Nonspecific arousal index (events/h)	1.08 (0.84 to 1.40)	0.532
Sleep architecture		
Stage N1 (%)	1.69 (1.27 to 2.25)	0.056
Stage N2 (%)	1.00 (0.78 to 1.29)	0.369
Stage N3 (%)	1.74 (1.30 to 2.34)	0.718
Stage R (%)	0.94 (0.73 to 1.21)	0.658

STUDY 4

Metabolipidomic Analysis in Patients with Obstructive Sleep Apnea Discloses a Circulating Metabotype of Non-Dipping Blood Pressure

Lucía Pinilla, Iván D. Benítez, Esther Gracia-Lavedan, Gerard Torres, Olga Mínguez, Rafaela Vaca, Mariona Jové, Joaquim Sol, Reinald Pamplona, Ferran Barbé, Manuel Sánchez-de-la-Torre.

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Metabolipidomic Analysis in Patients with Obstructive Sleep Apnea Discloses a Circulating Metabotype of Non-Dipping Blood Pressure

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Abstract:

A non-dipping blood pressure (BP) pattern, which is frequently present in patients with obstructive sleep apnea (OSA), confers high cardiovascular risk. The mechanisms connecting these two conditions remain unclear. In the present study we performed a comprehensive anal-ysis of the blood metabolipidome that aims to provide new insights into the molecular link be-tween OSA and the dysregulation of circadian BP rhythmicity. This was an observational pro-spective longitudinal study involving adults with suspected OSA who were subjected to full polysomnography (PSG). Patients with an apnea–hypopnea index ≥5 events/h were included. Fasting plasma samples were obtained the morning after PSG. Based on the dipping ratio (DR; ratio of night/day BP values) measured via 24 h ambulatory BP monitoring, two groups were established: dippers (DR ≤ 0.9) and non-dippers (DR > 0.9). Treatment recommendations for OSA followed the clinical guidelines. Untargeted metabolomic and lipidomic analyses were performed in plasma samples via liquid chromatography-tandem mass spectrometry. Non-dipper patients represented 53.7% of the cohort (88/164 patients). A set of 31 metabolic species and 13 lipidic species were differentially detected between OSA patients who present a physiologic nocturnal BP decrease and those with abnormal BP dipping. Among the 44 differentially abundant plasma compounds, 25 were putatively identified, notably glycerophospholipids, glycolipids, sterols, and fatty acid derivates. Multivariate analysis defined a specific metabotype of non-dipping BP, which showed a significant dose-response relationship with PSG parameters of OSA severity, and with BP dipping changes after 6 months of OSA treatment with continuous positive airway pressure (CPAP). Bioinformatic analyses revealed that the identified metabolipidomic profile was found to be implicated in multiple systemic biological pathways, with potential physiopathologic implica-tions for the circadian control of BP among individuals with OSA.

Keywords: obstructive sleep apnea; blood pressure; non-dipping; metabolomic; lipidomic; met-abolic pathways; CPAP.

1. Introduction

Healthy sleep is closely linked to the physiological reduction of the sympathetic nervous system activity during the nighttime period, leading to a nocturnal decline in blood pressure (BP). Obstructive sleep apnea (OSA), the most prevalent sleep-disordered breathing, is a chronic disorder that stems from repetitive obstruction of the upper airway during sleep and affects nearly 1 billion people globally [1]. The cardinal features of OSA include intermittent hypoxia, sleep fragmentation, and swings in intrathoracic pressure [2]. Oxidative stress is regarded as a fundamental component of OSA pathophysiology, arising from an imbalance between the prooxidant and anti-oxidant systems [3]. The repetitive cycles of hypoxia and reoxygenation experienced in OSA are considered to be analogous to repeated ischemia-reperfusion injury [4], re-sulting in an increased production of reactive oxygen species (ROS) that exceeds the antioxidant supply [5,6]. Apneic episodes trigger hyperactivation of sympathetic nerve drive, resulting in transient vasoconstriction, accelerated heart rate, increased cardiac output, and surges in BP [7]. These acute hemodynamic disruptions, combined with the stress induced by intermittent hypoxia and frequent arousals from sleep, cause signif-icant fluctuations in BP that manifest in a recurrent manner throughout the night. This situation can ultimately disrupt the circadian rhythmicity of arterial BP [8].

In the healthy state, a physiological decrease in BP occurs during sleep; this is re-ferred to as a dipping pattern, and individuals with this pattern are designated dippers. Conversely, a reduction in or absence of this nocturnal BP decrease is termed a non-dipping pattern, and these individuals are classified as non-dippers [9]. A non-dipping BP pattern is widely recognized as a primary contributory risk factor for cardiovascular disease and organ damage [10–18]. Indeed, epidemiological investiga-tions have reported that nighttime BP values are the strongest predictor of cardiovas-cular mortality [19]. Approximately 60% of individuals with OSA exhibit a non-dipping BP pattern [20]. The evidence linking OSA to impaired nocturnal dipping extends be-yond cross-sectional data. Longitudinal studies have demonstrated that OSA serves as a risk factor for the development of this pathological BP pattern [21]. Moreover, the cooccurrence of OSA and a non-dipping BP pattern has been proposed to exert a mul-tiplicative effect on adverse cardiovascular outcomes [22]. Unfortunately, neither the pathogenesis of OSA and the non-dipping pattern nor the interactions of these condi-tions have been

completely elucidated [23]. A comprehensive understanding of the molecular mechanisms underpinning a non-dipping BP pattern in OSA may shed light on the physiopathological connection between these two prevalent conditions, and could help to design therapeutic interventions to prevent subsequent cardiometabolic damage.

Metabolomics, which is dedicated to the global study of all metabolites found in a given biospecimen, is one of the newest disciplines within the omics field [24]. The metabolome is defined as the complete collection of metabolites, i.e., small molecules (molecular mass <1,5000 Da), that are chemically transformed during cellular metabolism. Metabolites are the ultimate downstream products of multiple intracellular elements, including genes, transcriptional activators, RNA transcripts, protein transport-ers, enzymes, and other cellular components. Metabolomics is regarded as the final layer of the omics cascade, providing a direct functional readout of the biochemical ac-tivity of cells and tissues [25].

In chronic diseases such as OSA, the phenotype is complex and dynamic due to the interplay of multiple intrinsic and extrinsic factors. By incorporating the down-stream input from the genome and the upstream input from environmental exposures, metabolomics can help to bridge the genotype-to-phenotype gap [26–28]. Global profiling of metabolites in accessible biosamples is currently being applied to investigate disease mechanisms and to identify new therapeutic targets in a variety of conditions [29–31]. This study presents a comprehensive analysis of the blood metabolipidome that aims to enhance our understanding of the molecular mechanisms underlying the alteration of nocturnal hemodynamic dipping in OSA.

2. Materials and Methods

2.1. Study cohort

2.1.1. Study design

This was an observational, prospective, and longitudinal study involving consecu-tive adult participants referred to the sleep unit due to suspected OSA (ClinicalTri-als.gov: NCT03513926). The exclusion criteria included the following situations: exist-ence of a previously diagnosed sleep disorder, a history of OSA treatment, a psycho-physical inability to complete the questionnaires, age >60 years, or any medical, social, or

geographical circumstance that, as determined by the responsible investigator, could affect the eligibility of the subject (e.g., pregnancy, drug or alcohol consumption, or life expectancy below 1 year). The Clinical Research Ethics Committee approved the study (University Hospital Arnau de Vilanova and Santa Maria of Lleida, No. 1153/1411), and all enrolled patients provided informed written consent for participation in the study.

2.1.2. Baseline clinical evaluation

During the baseline visit, detailed information about sociodemographic charac-teristics, unhealthy lifestyle habits, and medical history, including comorbidities and prescribed medications, was gathered from the patients by trained clinicians. General physical and anthropometric parameters were documented, and the degree of self-reported daytime somnolence was assessed with the Epworth sleepiness scale (ESS) [32].

2.1.3. Polysomnography for OSA diagnosis

All patients who met the established selection criteria underwent an overnight in-lab polysomnography (PSG) sleep study using the Philips Sleepware G3 system (Amsterdam, Netherlands). All procedures were performed according to national guidelines and regulations for clinical practice [33]. The results from the sleep studies were analyzed by trained personnel using standard criteria [34]. Apnea was defined as a cessation or reduction in oronasal airflow of at least 90% for a minimum duration of 10 sec. Hypopnea was defined as a 30%–90% reduction in oronasal airflow for a mini-mum duration of 10 sec, associated with oxygen desaturation ≥3% or evidence of arousal on the electroencephalogram. The apnea–hypopnea index (AHI) was computed based on the average number of apnea and hypopnea events/h of sleep. OSA severity param-eters derived from PSG were evaluated according to international scoring guidelines [34], as previously published [35]. To ensure coverage of multiple OSA severity ranges, the analysis included patients with an AHI ≤5 events/h. To focus on the obstructive component of sleep apnea, patients with central apnea ≥50% were excluded from the analysis [36]. Treatment recommendations for the patients diagnosed with OSA were based on national clinical guidelines, according to usual clinical practice [37].

2.1.4. Ambulatory BP assessment

In the morning immediately after the sleep study, the patients were subjected to 24 h ambulatory BP monitoring (ABPM) (Mortara Ambulo 2400; Milwaukee, USA), ac-cording to internationally recommended procedures [38]. Awake and asleep periods were defined using the sleeping time reported by each participant. BP measurements were obtained every 20 min during the daytime interval and every 30 min during the nighttime interval. ABPM recordings were considered optimal when >70% of the measurements were adequately recorded, with at least one measurement every hour; otherwise, the monitoring was repeated. The dipping ratios (DRs) for mean, systolic, and diastolic BPs were calculated as the ratios between the average nighttime and day-time BP values. Based on the DR of mean BP, the subjects were classified into two groups (Figure 1). A dipper pattern was defined as a nocturnal BP decrease of >10% relative to daytime values (DR ≤0.9), and a non-dipper pattern was defined as a noc-turnal BP decrease of ≤10% relative to daytime values (DR >0.9).

2.1.5. Sample collection

Overnight fasting venous blood samples were obtained at the same time of day (be-tween 08:00 and 09:00 a.m.) the morning immediately after the sleep study. Whole-blood samples collected in ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes (Vacuette, Greiner Bio-One, Kremsmünster, Austria) were centrifuged at 1500 × g for 10 min at 4 °C to separate the plasma fraction. All specimens were immediately aliquoted, frozen, and stored in a dedicated − 80 °C freezer. No freeze–thaw cycles were performed during the experiments.

2.1.6. Post-treatment evaluation

Continuous positive airway pressure (CPAP) constitutes the first-line treatment for OSA. Patients undergoing CPAP treatment (according to national clinical guidelines [37]) were scheduled for a 6-month follow-up clinic visit. The follow-up visit included a physical examination and collection of information regarding current medication use, unhealthy life habits, and changes in anthropometric measurements. The level of ad-herence to the treatment protocol was recorded from the CPAP device. Good compli-ance was defined as the use of the CPAP device for an average of at least 4 hours per night.

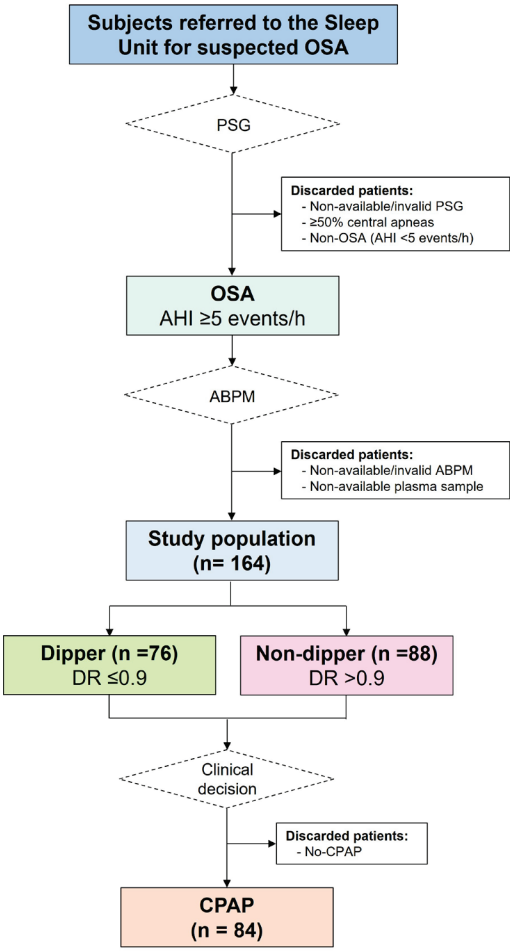

Figure 1. Flowchart of the study. Consecutive participants with suspected OSA who fulfilled the inclusion and exclusion criteria were subjected to in-laboratory PSG. Following ABPM, the pa-tients were classified into two groups: dippers (DR ≤0.9) and non-dippers (DR > 0.9). Treatment recommendations for patients diagnosed with OSA were based on usual clinical practice, and patients treated with CPAP were evaluated after 6 months of follow-up. Abbreviations: ABPM: ambulatory blood pressure monitoring; AHI: apnea-hypopnea index; CPAP: continuous positive airway pressure; DR: dipping ratio; OSA: obstructive sleep apnea; PSG: polysomnography.

2.2. Metabolomic and lipidomic profiling

2.2.1. Metabolite/lipid isolation and untargeted -omic analysis

Metabolites and lipids were isolated from the same patient-derived plasma sample following previously validated extraction methods [39,40]. The metabolic and lipidic extracts were analyzed via liquid chromatography coupled to tandem mass spectrom-etry (LC–MS/MS), as previously described [41–44]. Ultra-high-performance liquid

chromatography (UHPLC) was performed using an Agilent 1290 series system (Agilent Technologies, Santa Clara, CA, USA). Mass spectrometry analyses were performed via electrospray ionization quadrupole time of flight (ESI-Q-TOF) with an Agilent 6520 instrument (Agilent Technologies, Santa Clara, CA, USA). The order used to inject the samples was randomized, and quality control samples (pools of all the samples distributed in different aliquots and inserted after every five real samples) were used to control instrumental drift [45]. Data were acquired in both positive (+) and negative (-) ionization modes using MassHunter Data Acquisition software (Agilent Technologies, Barcelona, Spain) and were preprocessed using MassHunter Mass Profiler Professional software (Agilent Technologies, Barcelona, Spain), as previously described. Data were normalized using a LOESS (LOcally WEighted Scatter-plot Smoother) signal correction approach [46]. To correct individual bias, only stable features (found in at least 70% of the quality control samples) were considered for the analyses [47].

2.2.2. Feature identification and pathway enrichment analysis

According to previously published work, the potential identities of the features of interest, defined by exact molecular mass and retention time (RT), were compared against the Human Metabolome Database (HMDB) [48]. Potential identities were con-firmed via comparison to the exact mass, RT, and MS/MS spectra fragmentation pattern of class-representative internal standards, when available, using data from public da-tabases [49]. Bioinformatic pathway enrichment analyses were conducted using the MetaboAnalyst web service (https://www.metaboanalyst.ca/) [50]. Detailed information regarding the untargeted metabolomic and lipidomic analysis, feature identification, and pathway enrichment analysis can be found elsewhere [51].

2.3. Statistical analysis

Descriptive statistics were used to determine the characteristics of the study population. The normality of the distributions was assessed by the Shapiro–Wilk test. The median [25th percentile; 75th percentile] and frequency (percentage) were used to summarize continuous and categorical data, respectively. The clinical and sociodemographic characteristics of the patients were compared between the dipper and non-dipper groups using the Mann–Whitney-Wilcoxon test for quantitative variables and the chi-squared test for categorical variables. Plasma metabolite and lipid levels were log-

transformed for statistical purposes. Missing data were not imputed because there were no missing data for the variables used for the differential expression analy-sis. Linear models and empirical Bayes statistics were used to evaluate the differences in metabolite and lipid levels between the study groups [52], controlling for age, sex, body mass index (BMI), and antihypertensive medication use. Differential expression of metabolite/lipid species was defined as a significant difference (p value <0.05) and a fold change (FC) >1.15 (or <0.83 for downregulation) between the groups. Due to the exploratory nature of the study, p values were not adjusted for multiple comparisons. Linear dose–response relationships were assessed between the identified metabo-lite/lipid levels and the ABPM parameters using linear models adjusted for confounding factors. To evaluate the magnitudes of the identified associations, metabolite/lipid levels were standardized in this analysis.

A partial least squares-discriminant analysis (PLS-DA) was performed integrating the identified metabolite/lipid patterns previously associated with the baseline dipping status. Ten 3-fold cross-validations were performed to select the optimal number of components. The types of associations between the selected components of PLS-DA with the DR as a continuous variable and the PSG parameters of OSA severity (AHI, TSat90 (time with oxygen saturation <90%), and respiratory arousal index) were explored using generalized additive models (GAMs) with penalized thin plate regression splines. Similarly, we assessed the association of the first component of PLS-DA with the change in the DR after 6 months of treatment with CPAP. The threshold for statis-tical significance was set at a p value <0.05 for all analyses. All statistical analyses were performed using R software, version 4.0.2 [53].

3. Results

3.1. Characteristics of the study groups at baseline

A total of 164 OSA patients with available PSG data, ABPM data, and plasma sample were analyzed in this study. The population was middle-aged (median age 51 years) and overweight-obese (median BMI 29.3 kg/m3), and most participants were males (69.5%). The baseline characteristics of the study cohort according to nocturnal BP dipping category are outlined in Table 1. Non-dippers represented 53.7% of the cohort. As expected, marked differences were observed between the study groups in relation to

ambulatory BP measurements. Differences in the DRs mainly stemmed from higher nighttime BP values within the non-dipper group. Non-dippers were more obese, presented a higher number of comorbidities, and therefore were more medicated. Compared to dippers, non-dippers exhibited increased values for the AHI and other OSA severity markers. Accordingly, individuals with a non-dipping profile exhibited lower sleep quality.

Table 1. Clinical, ABPM, and PSG characteristics of the study population at baseline. Data are presented as the median [p25;p75] for quantitative variables and n (%) for qualitative variables. P values <0.05 are presented in bold. Abbrevia-tions: ABPM: ambulatory blood pressure monitoring; ACE: angiotensin-converting enzyme; AHI: apnea-hypopnea index; BMI: body mass index; BP: blood pressure; DR: dipping ratio; ESS: Epworth sleepiness scale; OSA: obstructive sleep apnea; R: rapid eye movement; SaO2: oxygen saturation; TSat90: time with SaO2 <90%.

	All N = 164	Dippers N = 76	Non-dippers N = 88	p value
Clinical data				
Demographic/anthropometric				
Age (years)	51.0 [44.8;55.0]	50.0 [44.8;56.0]	52.0 [44.8;55.0]	0.504
Sex				0.112
Male	114 (69.5%)	58 (76.3%)	56 (63.6%)	
Female	50 (30.5%)	18 (23.7%)	32 (36.4%)	
BMI (kg/m^2)	29.3 [26.7;33.4]	28.5 [26.1;31.8]	30.2 [27.1;34.6]	**0.029**
Smoking status				
Never	67 (40.9%)	27 (35.5%)	40 (45.5%)	0.433
Former	52 (31.7%)	26 (34.2%)	26 (29.5%)	
Current	45 (27.4%)	23 (30.3%)	22 (25.0%)	
Comorbidities				
Diabetes	17 (10.4%)	4 (5.26%)	13 (14.8%)	0.083
Hypertension	56 (34.1%)	21 (27.6%)	35 (39.8%)	0.142
Dyslipidemia	38 (23.5%)	12 (16.2%)	26 (29.5%)	0.071
Cardiovascular disease	29 (17.7%)	9 (11.8%)	20 (22.7%)	0.106
Medication use				
Insulin	7 (4.27%)	1 (1.32%)	6 (6.82%)	0.124
Any antihypertensive drug	62 (37.8%)	21 (27.6%)	41 (46.6%)	**0.020**
ACE inhibitors	41 (25.0%)	13 (17.1%)	28 (31.8%)	**0.047**
Beta-blockers	29 (17.7%)	12 (15.8%)	17 (19.3%)	0.700
Diuretic agents	23 (14.1%)	6 (8.00%)	17 (19.3%)	0.065
Calcium-channel blockers	14 (8.59%)	4 (5.26%)	10 (11.5%)	0.256
Angiotensin II receptor blockers	13 (8.02%)	3 (3.95%)	10 (11.6%)	0.132
Lipid-lowering drugs	32 (19.6%)	8 (10.5%)	24 (27.6%)	**0.011**
ABPM data				
Dipping ratios				

24 h DR	0.91 [0.85;0.97]	0.85 [0.82;0.87]	0.97 [0.93;1.00]	**<0.001**
Systolic DR	0.92 [0.86;0.98]	0.86 [0.83;0.89]	0.96 [0.93;1.02]	**<0.001**
Diastolic DR	0.91 [0.87;0.97]	0.86 [0.84;0.89]	0.96 [0.92;1.00]	**<0.001**
Nighttime BP				
Mean (mmHg)	88.8 [80.3;97.5]	83.2 [77.4;91.3]	94.0 [85.3;106]	**<0.001**
Systolic (mmHg)	118 [106;136]	110 [103;127]	122 [112;142]	**<0.001**
Diastolic (mmHg)	74.6 [68.9;79.0]	70.4 [67.0;75.4]	76.6 [72.8;84.4]	**<0.001**
Daytime BP				
Mean (mmHg)	97.1 [90.5;106]	99.0 [92.3;108]	96.0 [89.0;103]	**0.034**
Systolic (mmHg)	128 [120;143]	128 [121;145]	127 [119;141]	0.292
Diastolic (mmHg)	81.3 [76.2;85.4]	82.1 [77.1;87.2]	79.6 [74.7;84.8]	**0.029**
24 h BP				
Mean (mmHg)	95.0 [88.5;105]	94.6 [89.1;105]	95.8 [88.1;104]	0.924
Systolic (mmHg)	125 [117;140]	123 [117;139]	128 [117;142]	0.498
Diastolic (mmHg)	79.4 [74.0;84.2]	79.4 [74.8;84.4]	79.4 [73.5;84.2]	0.568
Polysomnography data				
Respiratory disturbances				
AHI (events/h)	28.6 [14.0;50.4]	22.4 [11.8;41.8]	34.9 [21.4;60.5]	**0.001**
Obstructive apnea index (events/h)	5.23 [1.59;14.5]	3.26 [1.30;12.4]	6.46 [1.92;26.2]	**0.043**
Hypopnea index (events/h)	18.0 [9.89;27.2]	13.0 [8.50;25.1]	20.8 [12.0;30.4]	**0.011**
Nocturnal hypoxemia				
Mean SaO_2 (%)	94.0 [92.0;95.0]	94.0 [93.0;95.0]	93.0 [91.0;95.0]	0.052
Minimum SaO_2 (%)	83.0 [76.0;88.0]	85.0 [78.2;89.0]	82.0 [73.5;87.0]	**0.027**
TSat90 (%)	1.97 [0.22;8.30]	1.33 [0.16;4.50]	2.40 [0.29;15.9]	**0.028**
Desaturation index (events/h)	4.47 [1.27;19.4]	4.34 [1.19;10.4]	7.01 [1.46;23.8]	0.268
Sleep fragmentation				
Respiratory arousal index (events/h)	20.1 [8.92;37.2]	15.0 [7.14;24.2]	25.8 [14.5;46.8]	**<0.001**
Movement arousal index (events/h)	3.49 [1.33;6.59]	3.67 [1.71;5.50]	3.49 [1.31;7.42]	0.564
Unspecific arousal index (events/h)	6.32 [3.04;10.6]	6.46 [2.61;9.90]	6.21 [3.19;11.9]	0.927
Sleep architecture				
Stage N1 (%)	11.4 [7.09;16.9]	11.3 [6.50;16.8]	11.8 [7.44;17.2]	0.737
Stage N2 (%)	43.7 [37.6;53.2]	47.0 [40.5;53.8]	40.4 [34.2;52.1]	**0.014**
Stage N3 (%)	26.3 [17.6;37.2]	24.2 [15.9;35.2]	27.8 [19.1;38.9]	0.130
Stage R (%)	14.1 [9.59;18.3]	14.1 [11.0;17.2]	14.1 [9.00;19.3]	0.992
Sleep quality				
Total sleep time (min)	346 [318;375]	344 [322;372]	347 [311;376]	0.786
Sleep latency (min)	14.6 [7.80;29.0]	12.5 [7.03;20.1]	18.0 [8.00;32.4]	0.085
Sleep efficiency (%)	86.0 [77.9;90.6]	87.2 [79.9;91.7]	83.2 [76.9;89.4]	**0.025**
Total wake time (min)	58.0 [36.2;89.7]	51.4 [30.2;84.0]	68.1 [48.0;96.0]	**0.021**
WASO (min)	40.0 [24.5;68.7]	35.7 [21.3;52.0]	47.8 [29.3;72.5]	0.062
Somnolence (ESS)	11.0 [7.00;14.5]	11.0 [7.00;14.0]	11.0 [7.00;15.0]	0.736

3.2. Untargeted analysis of the circulating metabolipidome

The first objective of the study was to assess the differences in the blood metabo-lome and lipidome between OSA patients who present a physiologic nocturnal BP de-crease and those with abnormal BP dipping. An untargeted metabolomic and lipidomic approach was applied via LC–MS/MS, the gold-standard technique for this purpose. After quality control and signal correction, 1,506 metabolites and 748 lipids were de-tected in the plasma and included in the unsupervised analysis. After adjustment for confounding factors, 31 metabolic species and 13 lipidic species were found to be dif-ferentially expressed between dipper and non-dipper OSA patients (Figure 2 and Sup-plementary Tables 1 and 2). Out of the 44 differentially expressed metabolites and lipids, a total of 25 compounds were putatively annotated based on the physicochemical properties and/or spectral similarity with public/commercial spectral libraries (Table 2). Collectively, the identified features of interest were mainly lipidic species, with a smaller set of lysine derivatives. The most affected lipid classes were glycerophospho-lipids (lysophospholipids, phosphatidic acid, cardiolipin, phosphatidyl-choline, and serine), glycerolipids (diacylglycerols), sterols (bile acids and steroids), and fatty acid derivatives. These 25 putative features of interest were included in the subsequent analyses.

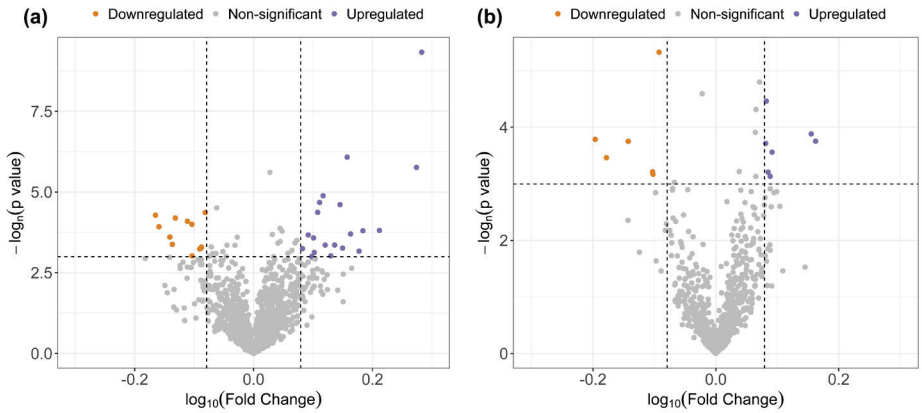

Figure 2. Untargeted plasma metabolomic (a) and lipidomic (b) profiling analysis. Volcano plots of the FC (x-axis) and p value (y-axis) for each detected metabolite and lipid in the comparison of non-dippers vs. dippers. Purple dots represent significantly downregulated (FC <0.83) molecules, and orange dots represent significantly upregulated (FC >1.25) molecules in non-dipper patients. The results are adjusted for confounding factors (age, sex, BMI, and antihypertensive medications). The threshold used to define statistical significance was p value <0.05. Abbreviations: BMI: body mass index; FC: fold change.

Table 2. Potential identities of the significantly differentially expressed metabolites and lipids between non-dipper and dipper OSA patients. The methodological approach used to detect the

molecules is represented as M for metabolomics and L for lipidomics. All compounds were putatively annotated based on physicochemical properties and/or spectral similarity with public/commercial spectral libraries: (a) ID based on exact mass, RT, and MS/MS spectrum; (b) ID based on exact mass and RT. Abbreviations: CL: cardiolipin; DG: diacylglycerol; PG: phosphatidylglycerol; PA: phosphatidic acid; PC: phosphatidylcholine; PS: phosphatidylserine; RT: retention time.

Mass	RT (min)	Method	Regulation Dipper vs. Non-dipper	Putative identification	Class	Reliability
791.6017	11.42984	M	Up	PC(38:4)	GP	b
598.2597	8.905328	M	Up	LysoPG(22:4)	GP	b
791.5651	10.62096	M	Up	PS(36:0)	GP	b
598.4338	11.2998	M	Up	PA(P-31:1)	GP	b
811.5371	14.02962	M	Up	PS(38:4)	GP	a
514.31245	10.82806	M	Up	LysoPA(22:1)	GP	a
760.4527	11.71748	M	Down	OxPA(40:8)	GP	b
658.4229	11.58019	M	Down	CL(62:4)	GP	a
515.2295	11.71635	M	Down	OxLysoPC(12:0)	GP	b
807.5784	7.231465	L	Down	PC(38:5)	GP	a
465.3101	8.988933	M	Up	Glycocholic acid	ST	a
568.3252	9.260691	M	Up	Deoxycholic acid 3-glucuronide	ST	b
449.3152	8.721428	M	Up	Glycohyodeoxycholic acid	ST	b
499.2975	10.32784	M	Up	Taurochenodesoxycholic acid	ST	a
318.26	11.57911	M	Up	Pregnanetriol	ST	b
370.1825	8.089659	M	Down	Androsterone sulfate	ST	a
602.4646	11.71269	M	Up	DG(32:1)	GL	b
616.5038	8.262866	L	Up	DG(36:4)	GL	b
635.5499	7.994549	L	Up	DG(36:3)	GL	a
240.1008	7.275254	M	Down	3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid	FA	b
1079.255	7.688919	L	Down	(3S)-3-Hydroxylinoleoyl-CoA	FA	b
160.1219	0.5020314	M	Down	N6-Methyl-L-Lysine	AA	a
368.1674	8.3971	M	Down	L-Tryptophyl-L-Lysine	AA	a
412.1364	11.15013	M	Up	Penicilloic acid	Drug	a
256.0955	4.755068	M	Down	m-chlorophenylpiperazine (m-CPP)	Drug	b
827.1027	0.5837527	M	Up	Unknown		
822.2788	11.38875	M	Up	Unknown		
816.2971	11.38298	M	Up	Unknown		
1108.853	11.57688	M	Up	Unknown		
764.4844	12.07795	M	Up	Unknown		
1212.914	11.70673	M	Up	Unknown		
1249.356	9.835346	L	Up	Unknown		

1338.199	10.34988	L	Up	Unknown
1171.264	7.82933	L	Up	Unknown
1266.198	10.65473	L	Up	Unknown
1384.454	9.951343	L	Up	Unknown
474.2918	12.98659	M	Down	Unknown
606.0906	12.08467	M	Down	Unknown
1025.551	13.29278	M	Down	Unknown
542.1265	8.394325	M	Down	Unknown
166.9866	0.9055392	L	Down	Unknown
665.0966	5.214503	L	Down	Unknown
1428.382	10.15159	L	Down	Unknown
305.3186	2.950193	L	Down	Unknown

3.3. Plasma metabotype associated with impaired BP dipping in OSA

Once we had established the differentially expressed metabolites and lipids in plasma based on dipping status, we sought to evaluate the linear association of this circulating profile with ambulatory measures of BP. As depicted in Figure 3, the identi-fied molecular profile was broadly associated with ABPM variables, not only with the DR of mean BP but also with the DRs of systolic and diastolic BP and especially with nighttime BP values.

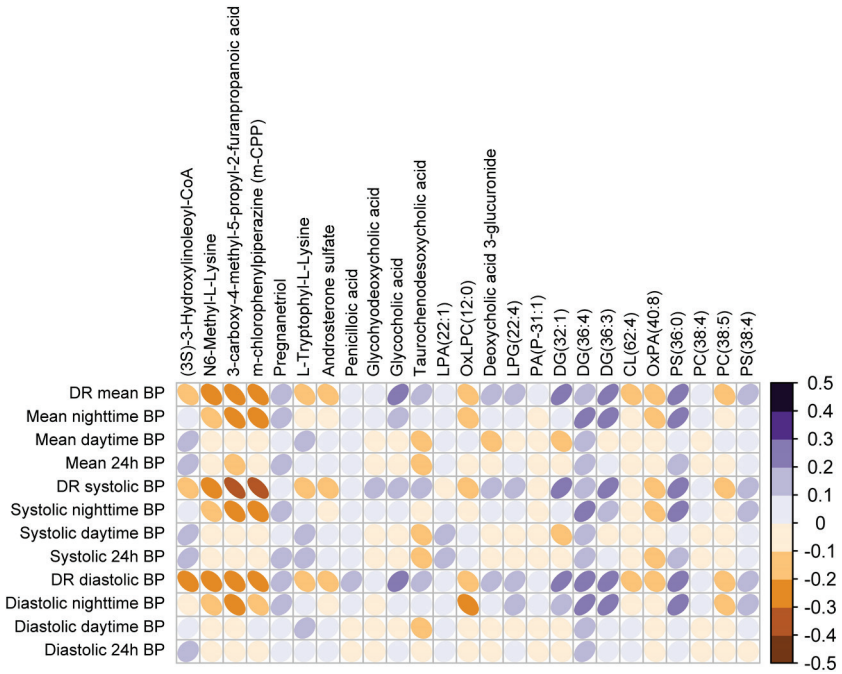

Figure 3. Adjusted linear associations between ABPM parameters and the identified metabolites and lipids. The color scale illustrates the degree of correlation and ranges from orange to purple, indicating negative to positive correlations, respectively. The results are adjusted for confounding factors (age, sex, BMI, and antihypertensive medications). Abbreviations: ABPM: ambulatory blood pressure monitoring; BP: blood pressure; CL: cardiolipin; DG: diacylglycerol; DR: dipping ratio; PG: phosphatidylglycerol; PA: phosphatidic acid; PC: phosphatidylcholine; PS: phospha-tidylserine.

A multivariate analysis based on PLS-DA was applied to the differentially detected metabolites and lipids. Figure 4a shows that the plasma metabolipidomic levels were able to separate patients with a dipping profile from those with a non-dipping profile, depicting a differential plasma metabotype associated with impaired BP dipping. Given that the first component of PLS-DA was the key contributor for separating the study groups (non-dippers vs. dippers), we performed an in-depth study on the loading of each feature to this component (Figure 4b). As illustrated in Figure 4c, the identified metabotype was significantly associated with the DR as a continuous variable.

We then explored the association of the metabolipidomic profile generated by PLS-DA with OSA severity markers, including the AHI as the primary disease-defining metric, the TSat90 as a hallmark of nocturnal hypoxemia, and the respiratory arousal index as a hallmark of sleep fragmentation. A positive dose–response relationship was found between the parameters of OSA severity and the plasma metabolipidomic sig-nature (Figure S1).

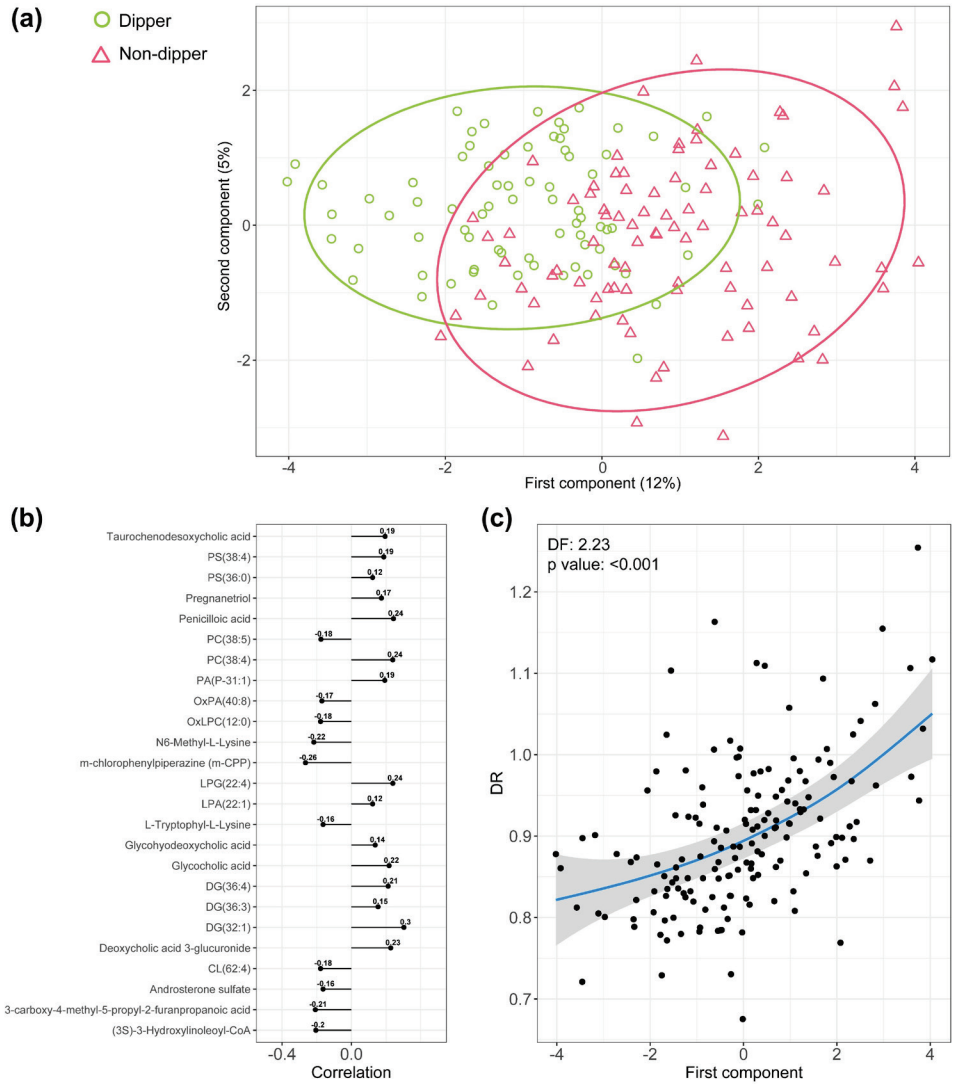

Figure 4. Plasma metabotype associated with impaired BP dipping in OSA. (a) PLS-DA discriminating between OSA patients with a dipping BP pattern and OSA patients with a non-dipping BP pattern. Each point represents a patient. Dipper patients appear in green, and non-dipper patients appear in pink. (b) Loading of each identified metabolite and lipid to the first component of PLS-DA. (c) GAMs with penalized thin plate regression splines representing the first component of PLS-DA (x-axis) and the DR as a continuous variable (y-axis). Each point represents a patient. The results are adjusted for con-founding factors (age, sex, BMI, and antihypertensive medications). Abbreviations: BMI: body mass index; BP: blood pres-sure; CL: cardiolipin; DF: estimated degrees of freedom; DG: diacylglycerol; GAM: generalized additive model; OSA: ob-structive sleep apnea; PA: phosphatidic acid; PC: phosphatidylcholine; PG: phosphatidylglycerol; PLS-DA: partial least-squares discriminant analysis; PS: phosphatidylserine.

3.4. Association of the metabolipidomic fingerprint with changes in the DR after OSA treatment with CPAP

Treatment recommendations for patients diagnosed with OSA were based on usual clinical practice. A total of 84 patients were treated with CPAP and evaluated after 6 months of therapy. The median [p25;p75] of the CPAP use value was 4.87 [3.67; 6.42], with good compiler patients (average use ≥4 h/day) representing 68.3% of all treated patients. The changes in the ABPM parameters between the beginning of the study and the posttreatment period are presented in Table S3. In brief, slight reductions in all ABPM variables (24 h BPs, daytime BPs, and nighttime BPs) were observed. The observed reduction in the mean nocturnal BP after CPAP treatment was -3.49 mmHg (95% CI: [-6.30;-0.68]). The variation in the DR was 0.04 (95% CI: [0.00;0.08]) for patients who presented a dipping pattern at baseline and -0.06 (95% CI: [-0.09;-0.03]) for those who presented a non-dipper profile at baseline.

We next tested whether the baseline metabolipidomic signature of impaired BP dipping was able to explain the modulation in the DR after 6 months of OSA treatment with CPAP. As displayed in Figure 5, the molecular profile identified at baseline was linearly associated with changes in the DR after CPAP use (Figure 5). Higher loadings of the first component were associated with a post-treatment decrease in the DR (mainly representing non-dipper patients), whereas lower loadings of the first component at baseline were related to an increase in the DR after CPAP treatment (primarily representing non-dipper patients).

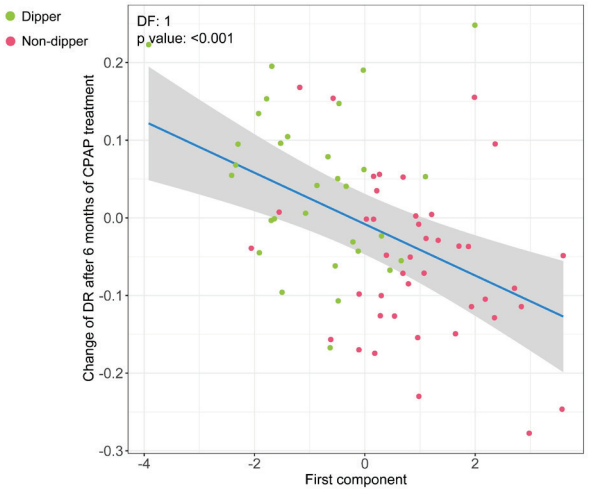

Figure 5. Association of the baseline metabolipidomic signature of impaired BP dipping with the variation in the DR after 6 months of OSA treatment with CPAP. GAMs with penalized thin plate regression splines illustrating the first component of PLS-DA (x-axis) and the change in the DR after CPAP treatment (y-axis). Each point represents a patient. Patients with a dipping pattern at baseline appear in green, and those with a baseline non-dipping pattern appear in pink. The results are adjusted for confounding factors (age, sex, BMI, and antihypertensive medication use). Abbreviations: BMI: body mass index; BP: blood pressure; CPAP: continuous positive airway pressure; DF: estimated degrees of freedom; GAM: generalized additive model; OSA: obstructive sleep apnea.

3.5. Pathway enrichment analysis

Finally, to gain an understanding of the molecular mechanisms in which the iden-tified metabolipidomic profile may be involved, we performed a bioinformatic in silico analysis using the online software MetaboAnalyst. Four enriched pathways, namely, glycerophospholipid metabolism, primary bile acid biosynthesis, linoleic acid metabo-lism, and alpha-linolenic acid metabolism, met the criteria at the defined significance level (Figure 6). Additionally, a number of metabolite sets involved in multiple systemic metabolic pathways were found to be significantly enriched in the bioinformatic anal-ysis (Figure S2).

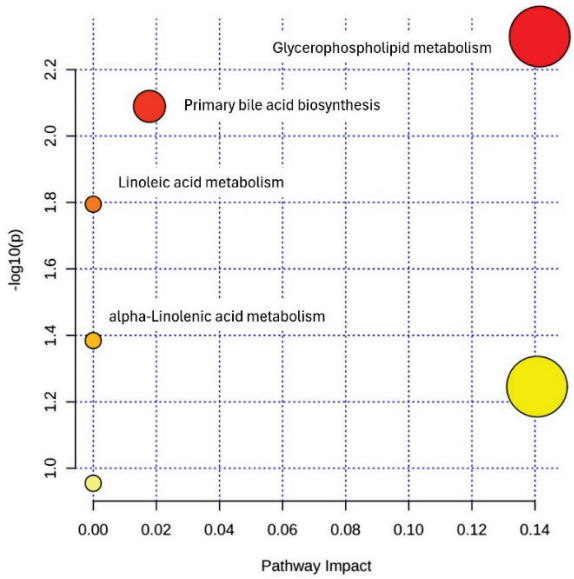

Figure 6. Pathway enrichment analysis including the metabolipidomic features identified as rel-evant to circadian BP control in OSA. Scatter plot presenting the enriched metabolic pathways in which the identified metabolites and lipids may be involved. Each circle represents a pathway. The color gradient indicates the significance of the pathway according to p value, with yellow indicating higher p values and red indicating lower p values (y-axis). The size of the circle rep-resents the

impact score of the pathway based on the number of molecules contained in the pathway (x-axis). Significantly affected pathways with a p value < 0.05 appear with their name.

4. Discussion

This study comprehensively explores the blood metabolomic and lipidomic land-scape underlying the circadian variation of BP in patients with OSA. We report the following findings: (i) a set of plasma metabolites and lipids were differentially detected in OSA patients with abnormal nocturnal BP dipping; (ii) the circulating levels of this profile were linearly associated with ambulatory measures of BP; (iii) multivariate analysis defined a circulating metabotype associated with impaired BP dipping, which was highly correlated with OSA severity measures; (iv) the identified baseline molec-ular signature showed a dose–response relation with the modulation of BP dipping after OSA treatment with CPAP; and (v) the metabolipidomic profile, which was mainly composed of lipidic species, was implicated in multiple systemic biological pathways, notably in the metabolism of glycerophospholipids and bile acids.

Apnea and hypopnea, along with the consequent compensatory hyperpnea, are associated with decreased parasympathetic and increased sympathetic activity [54]. Elevated sympathetic outflow, most notably occurring at the end of respiratory events, antagonizes the natural BP dipping phenomenon, causing surges in intravascular pressure. The loss or disruption of nocturnal hemodynamic dipping causes important adverse health consequences. Nevertheless, there is a limited understanding of the molecular components connecting OSA to the disruption of circadian BP rhythmicity.

Biofluids provide a close representation of the metabolic activity of the organs from which they are derived or the organs they bathe. Since blood irrigates all organs and tissues, blood-based profiling analyses can serve as a reliable metabolic proxy for the entire organism [55]. Untargeted metabolomics analysis has demonstrated wide-ranging applicability in revealing unanticipated metabolic changes across various biological conditions due to its sensitivity, high-throughput capabilities, and minimal sample needs. Although small in number, some studies using metabolomic and lip-idomic approaches have been conducted to evaluate biomarkers and explore the physiopathological mechanisms of BP and hypertension [56–59]. However, to date, no study has specifically addressed the impact of the 24-h circadian variation in BP on the circulating metabolipidome.

A metabotype, or metabolic phenotype, can be defined as a group of individuals characterized by similarities in metabolic profiles [26], which in turn result from interactions among lifestyle, the gut microbiome, genetics, and environmental factors. Here, we identified a circulating metabotype associated with impaired BP dipping in OSA, which was associated with OSA severity markers and BP dipping changes after OSA treatment. Among the two main hallmarks of OSA, namely, nocturnal hypoxemia and sleep fragmentation, we found that the latter showed the strongest correlation with the identified metabotype. This finding aligns with our previous research, where a comprehensive characterization of the polysomnographic determinants of non-dipping in OSA patients revealed that the respiratory arousal index was a key parameter related to the loss of nocturnal dipping [35]. Recurrent arousals from sleep could have a significant impact in the levels of the metabolites and lipids that are detectable in peripheral blood. Our results indicate that sleep fragmentation represents a relevant mechanistic path-way underpinning the link between OSA and the dysregulation of BP rhythmicity, which may be reflected in a specific plasma metabotype.

The specific blood-based metabotype identified here primarily involved glycerophospholipids (10 out of 25), sterols (6), glycerolipids (3), fatty acids (2), and amino acids/peptides (2). This suggests that the metabolic impact of a non-dipping BP profile in OSA would be mainly manifested as dysregulation of phospholipid, sterol, and fatty acid metabolism. Globally, three functional categories associated with the different lipid classes could be inferred: metabolic intermediates, bioenergetic compounds, and bioactive lipids. Importantly, most of these lipid species could be involved in the circadian regulation of BP rhythms.

Among glycerolipids, diacylglycerols represent the main subclass of lipids associ-ated with the non-dipper condition in OSA. Vasopressins are known to bind G-protein coupled receptors and activate intracellular phospholipase-C [60], generating diacyl-glycerol as an end-product of the reaction, which, along with the secondary messenger inositol-1,4,5-triphosphate, regulates the cytosolic concentration of calcium ions and protein kinase C (PKC) activity. Elevated PKC activity is known to promote oxidative stress through the stimulation of ROS-producing enzymes [61,62], and to increase ex-pression of inflammatory factors [63], which can exacerbate the cellular oxidative en-vironment and contribute to endothelial dysfunction in OSA. Indeed, vascular endo-thelial

inflammation and enhanced endothelial oxidative stress provide a starting point to elucidate the mechanisms mediating the association between OSA and BP impair-ments [64]. Increased expression of these lipid classes in non-dippers suggests an up-regulation of the diacylglycerol axis. Therefore, it is biologically plausible to postulate a pathophysiological role for diacylglycerols in the regulation of nocturnal BP [65], although evidence demonstrating a direct relation between the plasma content of diacylglycerols and the activation of intracellular signaling pathways involving diacylglycer-ols is currently lacking. Additionally, in line with our findings, DG(36:3) has been pre-viously described as a cross-species lipid marker of sleep restriction and sleep duration [66], reinforcing the role of OSA in the observed associations.

Glycerophospholipids are the class of lipids that have been shown to be the most affected by the non-dipping pattern in OSA, with 10 lipid species found to be differentially expressed compared to dipper patients: lysophospholipids (3), phosphatidic acid (2), phosphatidylcholine (2), phosphatidylserine (2), and cardiolipin (1). All these glycerophospholipid subclasses, along with diacylglycerols, exhibit a strongly interdependent relationship, either as precursors or products, in their biosynthesis pathways [67], suggesting a potential alteration in phospholipid biosynthesis in patients with absent nocturnal BP dipping. Interestingly, the phosphatidylcholine and serine forms, as well as cardiolipin, have been demonstrated to have biosynthetic, structural, and functional links with mitochondria [68]. Alterations in the expression of these lipids could be the result of an alteration in the bioenergetic capacity of the affected cell types, with subsequent effects on BP regulation. Reinforcing this idea, lower levels of an acyl-coenzyme A, which is the form in which fatty acids are used in mitochondrial be-ta-oxidation, were detected in non-dippers. Mitochondrial dysfunction is a major source of ROS production [69]. ROS can act as signaling molecules that play important regu-latory roles in a plethora of physiological processes [70], potentially including the cir-cadian regulation of BP. Two additional considerations should be noted. First, the af-fected phosphatidylcholine and serine species are characterized by 4 and 5 double bonds. This suggests the presence in their structure of 20:4 (arachidonic acid) and 20:5 (eicosapentaenoic acid) fatty acids, which are both precursors for the biosynthesis pathways of eicosanoids, which, among several functions, are known to play a role in vasodilatation and BP fluctuation [71,72]. The second is the involvement of various lysophospholipids and phosphatidic acids,

which, in addition to being intermediates in the biosynthesis of glycerophospholipid precursors, are also known to be bioactive li-pids that may also participate in the regulation of BP [73].

Sterols are another relevant class of lipids that were differentially detected in non-dipper patients. Six differentially abundant sterols were identified: 2 were deriva-tive metabolites of sex hormones, and the 4 remaining sterols were bile acids and con-jugates. Available evidence suggests that these molecules could affect BP control, acting as bioactive lipids. In this sense, bile acids are known to be able to reduce BP by atten-uating vascular reactivity [74], while conjugated bile acids are known to be inversely correlated with BP in humans and rats [75]. Furthermore, bile acids may function as bioactive lipids that activate different nuclear receptors [76] regulating hypoxia induci-ble factor-1-alpha (HIF-1-alpha) under hypoxic conditions [77], suggesting the activa-tion of adaptive mechanisms directed to counter OSA-derived injury. Additionally, some forms of bile acids have been associated with reduced oxidative stress and de-creased inflammation in many in-vitro and in-vivo models of various diseases, mostly due to a cytoprotective effect. The mechanisms underlying this cytoprotective activity have been mainly attributed to alleviation of endoplasmic reticulum stress and stabili-zation of the unfolded protein response [78]. Although the underlying molecular mechanisms remain unknown, our findings and observations from other authors sug-gest a potential adaptative protective role for bile acid metabolism in the alteration of BP regulation in OSA patients.

Overall, the metabolipidomic signature identified in this study could hold potential implications in the circadian control of BP, and may represent the first steps to establish the molecular bases underlying the dysregulation of nocturnal BP control, frequently observed in OSA patients. Additionally, the metabolipidomic signature identified in this study could potentially constitute a source of therapeutic targets.

Our study has certain limitations that should be noted. First, due to the observa-tional design of the study, cause–effect relationships remain unproven. Second, alt-hough we adjusted the analyses for various confounding factors, individual biologic conditions and/or lifestyle behaviors, such as shift work routines, diet, caffeine intake, psychological stress, or antihypertensive drug intake timing, may have impacted the observed associations. Third, due to the incipient state of this research field, an explor-atory and

unbiased methodological approach was applied, aiming to gain new insights from which new hypotheses might be developed. Therefore, further studies are needed to determine the validity of our findings in another independent group of individuals.

The strengths of this study include the evaluation of a relatively large cohort of consecutive participants who were referred to the sleep unit for suspected OSA. This resulted in a realistic degree of heterogeneity within the OSA study population and facilitated the evaluation of the entire spectrum of disease severity. Other strengths include the use of 24-h ABPM and PSG, which are gold-standard techniques for identi-fying the circadian patterns of BP and diagnosing OSA, respectively. In addition, the ABPM and PSG data enabled us to explore the relationships among BP variables, OSA variables, and circulating metabolomic and lipidomic levels. This approach mitigates the potential for identifying misleading or nonrepresentative associations based solely on dipping status and OSA status. Finally, the longitudinal design of the study and the consequent evaluation of the relation between the metabolipidomic signature and the effect of CPAP treatment on the evolution of the DR reinforce the etiological role of OSA in the observed associations.

5. Conclusions

To our knowledge, this study is the first to apply a systemic metabolipidomic pro-filing strategy to enhance our understanding of the molecular mechanisms underlying the alteration of nocturnal hemodynamic dipping in OSA. We identified a specific plasma metabotype of impaired BP dipping, which was associated with poly-somnographic parameters of OSA severity and BP dipping variations after OSA treat-ment with CPAP. The identified metabotype was primarily composed of lipidic species, notably glycerophospholipids, sterols, and glycerolipids. The affected lipidic classes could have dual cytoprotective/physiopathologic implications for the circadian control of BP rhythmicity, through oxidative stress-related pathways. This exploratory study provides hypothesis-generating data and may form the basis for future investigations. However, further research involving additional cohorts and functional analyses are needed to corroborate these findings.

Author Contributions: MSdT is the guarantor of the study. LP, IDB, and MSdT contributed to the study design. LP, GT, OM, RV, and FB contributed to data acquisition. LP, IDB, EGL,

MJ, JS, RP, FB, and MSdT contributed to data analysis and interpretation. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of the University Hospital Arnau de Vilanova-Santa María of Lleida, Spain (approval number 1153/1411).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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SUPPLEMENTARY INFORMATION

Metabolipidomic Analysis in Patients with Obstructive Sleep Apnea Discloses a Circulating Metabotype of Non-Dipping Blood Pressure

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Figure S1. Association of the plasma metabolipidomic signature of impaired BP dipping with OSA severity parameters. GAM models with penalized thin plate regression splines illustrating the first component (x-axis) and the OSA severity parameters (y-axis): (a) AHI, (b) TSat90, and (c) respiratory arousal index. Each point represents a patient. Dipper patients appear in green, and non-dipper patients appear in pink. The results are adjusted for confounding factors (age, sex, BMI, and antihypertensive medications). Abbreviations: AHI: apnea-hypopnea index; BMI: body mass index; BP: blood pressure; DF: estimated degrees of freedom; GAM: generalized additive model; OSA: obstructive sleep apnea; TSat90: time with oxygen saturation <90%.

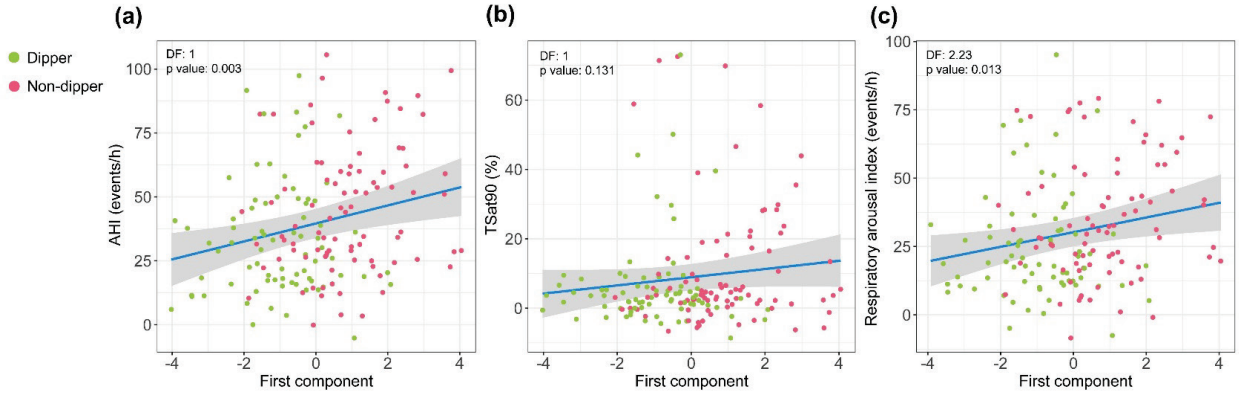

Figure S2. Metabolite set enrichment analysis including the metabolipidomic features identified as relevant to circadian BP control in OSA. Dot plot presenting the top 25 significantly enriched metabolite sets in which the identified metabolites and lipids may be involved. Each circle represents a metabolite set. The color gradient indicates the significance of the set according to p value, with yellow indicating higher p values and red indicating lower p values (y-axis). The size of the circle represents the enrichment ratio of the metabolite set, computed by observed hits / expected hits (x-axis).

Table S1. Differentially expressed features in the metabolomic analysis. Results are adjusted for confounders (age, sex, BMI, and antihypertensive medications). Metabolites with FC <0.8 (downregulated) or >1.25 (upregulated) and p value <0.05 were considered. Features are represented as exact mass and RT. Definition of abbreviations: BMI = body mass index; FC = fold change; OSA = obstructive sleep apnea; RT = retention time.

Metabolomics		Ionization	FC	p-value
Molecular mass	RT (min)			
602.4646	11.71269	-	1.918	<0.001
827.1027	0.5837527	-	1.437	0.002
465.3101	8.988933	-	1.879	0.003
791.6017	11.42984	-	1.309	0.008
598.2597	8.905328	+	1.291	0.009
568.3252	9.260691	-	1.398	0.010
822.2788	11.38875	-	1.282	0.013
474.2918	12.98659	-	0.829	0.013
256.0955	4.755068	-	0.684	0.014
160.1219	0.5020314	-	0.738	0.015
370.1825	8.089659	-	0.774	0.017
760.4527	11.71748	-	0.788	0.018
240.1008	7.275254	-	0.693	0.020
816.2971	11.38298	-	1.628	0.022
449.3152	8.721428	-	1.527	0.022
499.2975	10.32784	-	1.456	0.025
318.26	11.57911	-	1.236	0.026
606.0906	12.08467	-	0.723	0.027
791.5651	10.62096	-	1.261	0.028
658.4229	11.58019	-	0.730	0.034
598.4338	11.2998	+	1.369	0.035
1108.853	11.57688	-	1.320	0.035
1025.551	13.29278	-	0.817	0.037
515.2295	11.71635	-	0.816	0.038
811.5371	14.02962	-	1.412	0.038
764.4844	12.07795	-	1.209	0.039
368.1674	8.3971	-	0.812	0.039
412.1364	11.15013	-	1.504	0.042
514.31245	10.82806	+	1.265	0.044
1212.914	11.70673	-	1.348	0.049
542.1265	8.394325	-	0.788	0.049

Table S2. Differentially expressed features in the lipidomic analysis. Results are adjusted for confounders (age, sex, BMI, and antihypertensive medications). Lipids with FC <0.8 (downregulated) or >1.25 (upregulated) and p value <0.05 were considered. Features are represented as exact mass and RT. Definition of abbreviations: BMI = body mass index; FC = fold change; OSA = obstructive sleep apnea; RT = retention time.

Lipidomics		Ionization	FC	p-value
Molecular mass	RT (min)			
166.9866	0.9055392	+	0.808	0.005
1249.356	9.835346	+	1.208	0.012
1338.199	10.34988	+	1.430	0.021
807.5784	7.231465	+	0.636	0.023
1171.264	7.82933	-	1.453	0.023
1079.255	7.688919	+	0.720	0.024
1266.198	10.65473	+	1.205	0.024
616.5038	8.262866	+	1.235	0.028
665.0966	5.214503	+	0.664	0.031
1428.382	10.15159	+	0.789	0.040
635.5499	7.994549	+	1.216	0.041
305.3186	2.950193	+	0.790	0.042
1384.454	9.951343	+	1.226	0.044

Table S3. Changes in the ABPM parameters after 6 months of OSA treatment with CPAP. Data are presented as the mean [95% CI]. P values <0.05 are presented in bold. Abbreviations: ABPM: ambulatory blood pressure monitoring; BP: blood pressure; CI: confidence interval; CPAP: continuous positive airway pressure; DR: dipping ratio.

	OSA patients treated with CPAP N = 84
Dipping ratios	
24h DR	-0.02 [-0.04;0.01]
Systolic DR	-0.01 [-0.03;0.02]
Diastolic DR	0.02 [-0.01;0.04]
Nighttime BP	
Mean (mmHg)	**-3.49 [-6.30;-0.68]**
Systolic (mmHg)	-3.69 [-7.75;0.37]
Diastolic (mmHg)	-1.91 [-4.07;0.24]
Daytime BP	
Mean (mmHg)	-1.58 [-4.45;1.29]
Systolic (mmHg)	-2.99 [-6.45;0.46]
Diastolic (mmHg)	-1.15 [-3.35;1.04]
24h BP	
Mean (mmHg)	-2.12 [-4.49;0.25]
Systolic (mmHg)	**-4.00 [-7.33;-0.66]**
Diastolic (mmHg)	-1.44 [-3.19;0.31]

STUDY 5

Hypoxic Burden to Guide CPAP Treatment Allocation in Patients with Obstructive Sleep Apnoea: a *post-hoc* Study of the ISAACC Trial

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Hypoxic burden to guide CPAP treatment allocation in patients with obstructive sleep apnoea: a *post hoc* study of the ISAACC trial

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Hypoxic burden stands out as an obstructive sleep apnoea severity metric with potential clinical utility to identify patients most likely to benefit from continuous positive airway pressure treatment for secondary cardiovascular prevention https://bit.ly/3szv4hf

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Abstract

Background Hypoxic burden (HB) has emerged as a strong predictor of cardiovascular risk in obstructive sleep apnoea (OSA). We aimed to assess the potential of HB to predict the cardiovascular benefit of treating OSA with continuous positive airway pressure (CPAP).

Methods This was a *post hoc* analysis of the ISAACC trial (ClinicalTrials.gov: NCT01335087) including non-sleepy patients with acute coronary syndrome (ACS) diagnosed with OSA (apnoea–hypopnoea index $\geqslant$15 events·h^{-1}) by respiratory polygraphy. Patients were randomised to CPAP or usual care and followed for a minimum of 1 year. HB was calculated as the total area under all automatically identified desaturations divided by total sleep time. Patients were categorised as having high or low baseline HB according to the median value (73.1%min·h^{-1}). Multivariable Cox regression models were used to assess whether the effect of CPAP on the incidence of cardiovascular outcomes was dependent on the baseline HB level.

Results The population (362 patients assigned to CPAP and 365 patients assigned to usual care) was middle-aged (mean age 59.7 years), overweight/obese and mostly male (84.5%). A significant interaction was found between the treatment arm and the HB categories. In the high HB group, CPAP treatment was associated with a significant reduction in the incidence of cardiovascular events (HR 0.57 (95% CI 0.34–0.96)). In the low HB group, CPAP-treated patients exhibited a trend toward a higher risk of

cardiovascular outcomes than those receiving usual care (HR 1.33 (95% CI 0.79–2.25)). The differential effect of the treatment depending on the baseline HB level followed a dose–response relationship.
Conclusion In non-sleepy ACS patients with OSA, high HB levels were associated with a long-term protective effect of CPAP on cardiovascular prognosis.

Introduction

Obstructive sleep apnoea (OSA) is a chronic sleep disorder affecting approximately 1 billion people worldwide [1]. OSA is characterised by recurrent episodes of upper airway collapse leading to large intrathoracic pressure swings and repetitive cycles of oxygen desaturation and hypercapnia followed by re-oxygenation and/or arousal from sleep [2]. These perturbations initiate a cascade of downstream consequences, including sympathetic activation, inflammation, oxidative stress, metabolic dysregulation and endothelial dysfunction, which can ultimately trigger cardiovascular disease (CVD) [3].

Untreated OSA is associated with wide-ranging complications, especially in the cardiovascular sphere [4–10]. Together with lifestyle interventions, treatment with continuous positive airway pressure (CPAP) constitutes the first-line therapy for moderate-to-severe or symptomatic OSA patients. Previous evidence from randomised controlled trials (RCTs) has shown a favourable impact of CPAP treatment on a variety of CVD drivers, such as high blood pressure [11], inflammation [12], endothelial dysfunction [13] and atherosclerosis [14]. Nonetheless, despite the cumulative clinical and experimental data pointing to OSA as a modifiable risk factor for CVD [15], the effect of CPAP treatment on cardiovascular end-points remains controversial [16]. In the past decade, three large secondary prevention RCTs have explored the cardiovascular benefits of CPAP, failing to demonstrate a positive long-term effect of the treatment on the incidence of adverse cardiovascular outcomes [17–19]. The high variability of response among the available studies emphasises the existence of substantial heterogeneity in the impact of CPAP therapy on cardiovascular consequences [20]. This clinical scenario invites us to question whether specific subgroups of patients with OSA may benefit more from the therapy than others.

The current framework for the management of OSA reflects a “one size fits all” approach, where a single parameter, the apnoea–hypopnoea index (AHI), is used to define the presence, categorise the severity and guide the physician prescription for therapy. This generalist way of addressing a disease as complex as OSA can lead to important repercussions for patients and healthcare systems. Compelling data indicate that the AHI alone does not capture the heterogeneity of OSA and its underlying physiopathological consequences, particularly for the cardiovascular system [21]. Under this paradigm, substantial efforts are being made to develop alternative metrics that could effectively characterise OSA and predict adverse outcomes. A growing list of candidates have been proposed in recent years [22]. Promisingly, hypoxic burden (HB) has emerged as a strong predictor of CVD-related morbidity and mortality in several population-based and clinical cohorts [23–28]. However, the extent to which HB can be used to guide CPAP therapeutic decision making remains to be determined. In this study, we sought to assess the potential of HB to identify a specific subgroup of OSA patients who exhibit cardiovascular benefit from CPAP therapy.

Methods

Study population

This is a *post hoc* analysis of the ISAACC study (ClinicalTrials.gov: NCT01335087), a multicentre, open-label, parallel, prospective RCT [17]. A detailed description of the study aims and protocol, including inclusion and exclusion criteria, is outlined elsewhere [29]. Briefly, consecutive adult subjects admitted for acute coronary syndrome (ACS) to the coronary care unit or cardiology hospitalisation ward were eligible to participate in the study if they did not exhibit excessive daytime sleepiness, defined as Epworth Sleepiness Scale (ESS) $\leqslant$10. ACS was defined as the acute presentation of coronary disease with or without ST segment elevation infarction, unstable angina or type 1 myocardial infarction. Recruitment took place throughout 15 hospitals in Spain between 2011 and 2018, with the last end-of-study visit conducted in 2019. The clinical research ethics committee of each participating centre approved the trial protocol (approval number in the coordinating centre: 2010/852) and every enrolled patient provided informed written consent to participate in the study.

Procedures and outcomes of the ISAACC trial

Baseline clinical evaluation

In the initial visit, detailed information regarding sociodemographic characteristics, health behaviours and medical history, including comorbidities and prescribed medications, was collected from all patients by trained clinicians. General physical and anthropometric parameters were recorded, and the degree of self-reported daytime sleepiness was assessed with the validated Spanish version of the ESS.

Sleep study

All patients who satisfied the selection criteria underwent respiratory polygraphy (Embletta; ResMed, Bella Vista, Australia) during the first 24–72 h after the time of hospitalisation for ACS. Sleep recordings included at least oronasal flow measurement (nasal pressure and thermistor), thoracoabdominal respiratory inductance plethysmography, heart rate measurement and fingertip pulse oximetry. All methods were performed according to the national clinical practice guidelines and regulations [30]. Apnoeas were defined as an interruption in oronasal airflow for ⩾10 s. Hypopnoeas were defined as a reduction in oronasal airflow for ⩾10 s associated with a decrease in arterial oxygen saturation (S_{aO_2}) ⩾4%. Hypoxaemia parameters included the oxygen desaturation index (ODI), defined as the number of episodes of S_{aO_2} decrease ⩾4%, and the percentage of total sleep time spent with S_{aO_2} <90% (T_{Sat90}). The definition of the study groups in the ISAACC trial was based on the AHI, which represents the average number of apnoea plus hypopnoea events per hour of sleep. Patients diagnosed with OSA (AHI ⩾15 events·h^{-1}) were randomly assigned (1:1) to the CPAP group or the usual care group. Usual care instructions comprised education on lifestyle and behavioural modifications.

Follow-up evaluations

The primary end-point of the ISAACC trial was a composite of death from any cardiovascular cause or non-fatal events, including acute myocardial infarction, non-fatal stroke, hospital admission for heart failure and new hospitalisations for unstable angina or transient ischaemic attack. Follow-up visits were scheduled for all patients at 1, 3, 6, 12, 18, 24, 30 and 36 months and annually thereafter. All patients were monitored and followed up for a minimum of 1 year. Each clinic visit included a physical examination, detailed record of current medication use, unhealthy life habits and assessment of the rate of cardiovascular events. A blinded committee adjudicated both fatal and non-fatal cardiovascular outcomes specified in the protocol. The follow-up time was defined as the time between the baseline visit and the end of the study or the occurrence of a cardiovascular event, whichever occurred first. Each patient's level of adherence to the treatment was measured by means of the internal clock of the CPAP device, dividing the hours of CPAP use by the days of treatment. Good compliance was defined as the use of the CPAP device for an average of ⩾4 h·day^{-1}.

Calculation of the oximetry-based HB

The original HB calculation method has been modified to calculate the HB using automatically identified desaturations from pulse oximetry, regardless of scored events. In the Sleep Heart Health Study (SHHS), this method has been proven to have a strong correlation with the HB obtained through manually scored apnoeas and hypopnoeas, and was significantly associated with CVD mortality, hypertension and excessive daytime sleepiness with similar effect sizes [31]. Similar to our published algorithm, all oxygen desaturation levels exceeding a 2% drop were automatically identified from the S_{aO_2} signal. The identified desaturations were then synchronised with respect to the minimum oxygen saturation and were ensemble averaged to obtain the individual-specific search window, defined as the time interval between the two peaks around the minimum value of the ensemble average signal (figure 1). HB was then determined by summing all the individual areas under the desaturation curve restricted by the search window and dividing by the total sleep time [31].

Statistical analysis

Patients were categorised according to the baseline HB level as the "low HB group" or "high HB group" when the HB was below or above the median value of the population (⩽73.1%min·h^{-1} or >73.1%min·h^{-1}, respectively). To test the primary hypothesis that the effect of CPAP treatment on the primary outcome is moderated by the baseline HB level (*i.e.* that the effect of CPAP differs between those patients with high HB and those with low HB at baseline), a Cox regression model was used to assess the interaction between treatment arm and HB category (high HB×CPAP, with high HB being HB above the median HB). Kaplan–Meier survival curves were plotted to illustrate incident CVD per treatment arm in the overall sample and in the subgroups with low or high HB. Log-rank tests were used to assess differences between survival curves. Additional multivariable Cox regression analyses were performed after adjusting for confounders (age, sex, body mass index and presence of CVD prior to admission to the study). For comparison, these analyses were repeated for the AHI, ODI and T_{Sat90}. Additionally, in an exploratory analysis, the CPAP group was divided into two subgroups based on median adherence to CPAP ("lower" or "higher" adherence subgroups). In the overall sample as well as in the low and high HB groups, the Kaplan–Meier survival curves compared the incidence of CVD of lower and higher adherence subgroups with that of the usual care group. Finally, in another exploratory analysis, to assess the dose–response relationship between HB and incident CVD in each treatment arm, HB was modelled continuously and the risk of incident CVD was predicted (relative to the average baseline characteristics of the OSA patients in

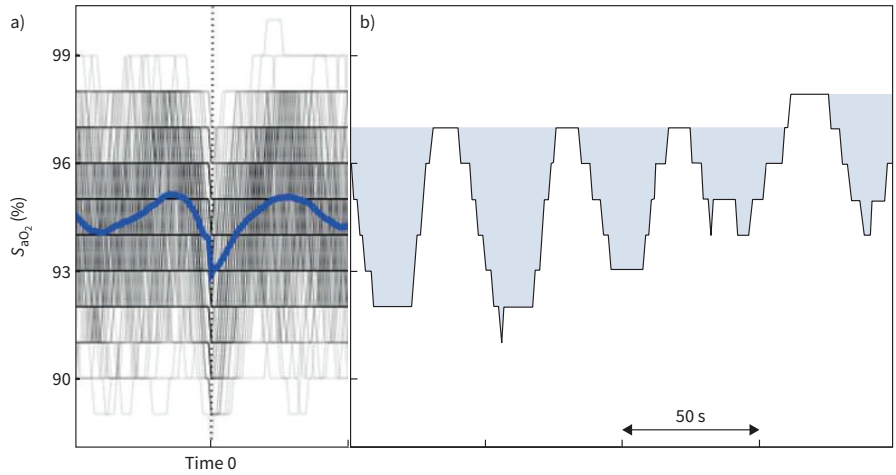

FIGURE 1 Calculation of the oximetry-based hypoxic burden (HB). a) All automatically detected desaturations are synchronised at their minimum saturation levels (time 0) and ensemble averaged to obtain the search window (duration between two maximum peaks around time 0). b) HB is calculated as the normalised summation of the coloured arterial oxygen saturation (S_{aO_2}) areas within the search window for all desaturation episodes divided by total sleep time.

the ISAACC trial). All statistical analyses were conducted using the R statistical package (www.r-project.org) and statistical significance was accepted at $p<0.05$ for the primary analysis.

Results

Description of the study cohort

Of the 1255 patients with OSA enrolled in the ISAACC trial, all patients with available respiratory polygraphy data were included in this *post hoc* analysis. After excluding 33 patients due to irregularities in the sleep recording and 33 due to inadequate S_{aO_2} signal quality, the final study cohort comprised 727 OSA patients (figure 2). No clinically relevant differences were observed between the included and excluded patients regarding baseline clinical and sleep characteristics, percentage of individuals allocated to each treatment arm or incidence of outcomes during the follow-up (supplementary table S1).

The analytic sample comprised individuals who were mainly middle-aged (mean age 59.7 years) and overweight/obese, and most participants were male (84.5%). Baseline characteristics of the study cohort by categories of HB and treatment allocation are summarised in table 1. A total of 365 individuals were assigned to the usual care arm and 362 individuals were assigned to the CPAP treatment arm. The treatment groups were similar in terms of sociodemographic/anthropometric characteristics, unhealthy life habits, comorbidities, prescribed medications and sleep parameters, including HB (supplementary table S2).

As expected, patients in the high HB groups presented with more severe OSA than those in the low HB groups, reflected in greater values of AHI and the other respiratory polygraphy parameters. Likewise, patients with high HB values were more obese than those with low HB values (supplementary table S3). Among CPAP-treated individuals, 32.0% were good compliers in the low HB group, while 44.1% were good compliers in the high HB group ($p=0.025$).

Differential effect of CPAP treatment on cardiovascular prevention according to the baseline HB level

Over a mean±SD follow-up of 32.31±22.46 months, a total of 117 OSA subjects experienced a major adverse cardiovascular event. A significant interaction was found between CPAP treatment and the HB categories ($p=0.023$), indicating a differential effect of the treatment depending on the baseline HB level. The results were maintained after adjustment for confounding factors ($p=0.035$). Figure 3 and table 2 compare the impact of CPAP treatment *versus* usual care on the incidence of the primary end-point by categories of pre-intervention HB. While there was no association of CPAP therapy with cardiovascular prevention in the entire cohort, substantial heterogeneity in the effect of the treatment was observed when the population was stratified based on the HB. In the high HB group, there was a significant CPAP-related

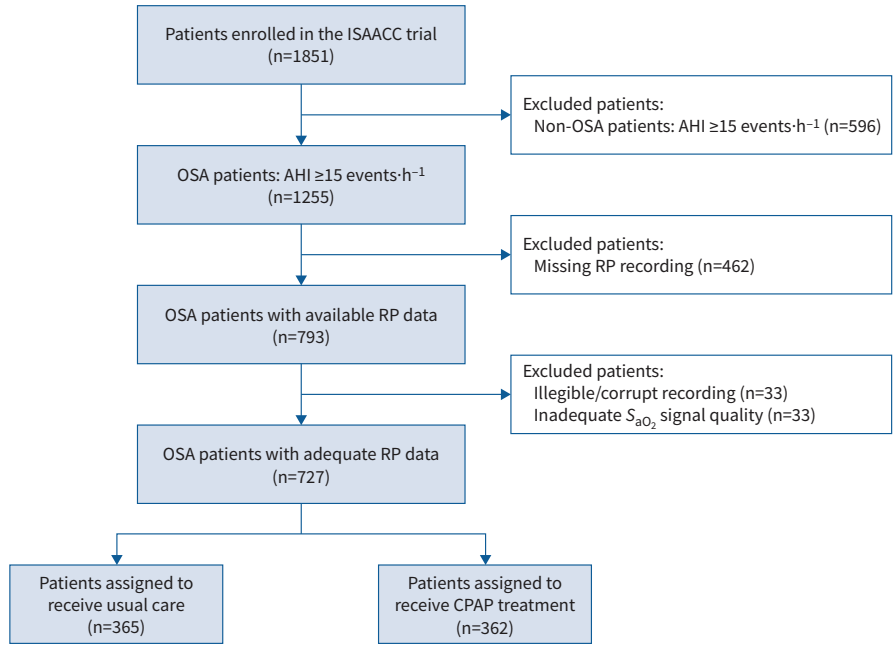

FIGURE 2 Study flowchart. Of the 1851 participants enrolled in the ISAACC trial, 1255 patients with obstructive sleep apnoea (OSA) were considered for the present *post hoc* analysis. Respiratory polygraphy (RP) data were available for a total of 793 patients, of whom 33 had irregularities in the sleep recording and 33 involved inadequate arterial oxygen saturation (S_{aO_2}) signal quality. The final study cohort comprised 727 OSA patients. AHI: apnoea–hypopnoea index; CPAP: continuous positive airway pressure.

reduction in the incidence of adverse cardiovascular events during the follow-up (HR 0.57 (95% CI 0.34–0.96); p=0.035). Conversely, in the low HB category, CPAP-treated patients exhibited a trend toward a higher risk of cardiovascular outcomes during the follow-up than those receiving usual care (HR 1.33 (95% CI 0.79–2.25); p=0.281). The magnitude of the effect size was maintained after adjustment for confounders.

Furthermore, we evaluated the dose–response relationship between the baseline HB as a continuous variable and the incidence of cardiovascular events during the follow-up, according to the treatment arm (figure 4). Participants who received CPAP treatment had a reduced cardiovascular risk as the HB level increased. The opposite trend was observed in the usual care group, where the risk for cardiovascular events increased as the HB level increased.

Considering the higher values of CPAP compliance in the high HB group, we additionally explored the potential association between adherence to CPAP treatment and cardiovascular prognosis. In the low HB group, participants with higher adherence to CPAP treatment exhibited a non-significant higher cardiovascular risk, whereas in the high HB group, both adherence groups showed the same trend of protection against cardiovascular events compared with the usual care group (supplementary figure S1).

Notably, we did not find an association between CPAP treatment and cardiovascular prevention according to the baseline levels of AHI or other conventional OSA severity metrics, such as the ODI or T_{Sat90} (supplementary figure S2).

Secondary analyses were used to assess the effect of CPAP on the cardiac components of the primary end-point (*i.e.* cardiovascular death, non-fatal acute myocardial infarction and new hospitalisation for unstable angina) in the low and high HB groups (table 3). As with the primary outcome, there was a significant interaction between the treatment and HB categories (p=0.025). CPAP therapy showed a stronger protective effect against cardiac events only in participants with high HB (HR 0.52 (95% CI 0.29–0.92); p=0.025). This result did not change after adjustment for covariates.

TABLE 1 Baseline characteristics of the study cohort by categories of hypoxic burden (HB) and treatment allocation

	Usual care		CPAP treatment	
	Low HB (n=187)	High HB (n=178)	Low HB (n=177)	High HB (n=185)
Clinical data				
Demographic/anthropometric				
Age (years)	60.0 (53.0–66.0)	59.0 (53.0–68.0)	60.0 (53.0–67.0)	58.0 (52.0–65.0)
Sex				
Male	161 (86.1)	151 (84.8)	143 (80.8)	159 (85.9)
Female	26 (13.9)	27 (15.2)	34 (19.2)	26 (14.1)
BMI (kg·m^{-2})	28.3 (25.7–30.8)	30.1 (27.9–32.8)	27.8 (25.5–30.8)	29.4 (26.8–33.1)
Smoking status				
Never	46 (24.6)	40 (22.5)	41 (23.2)	46 (24.9)
Former	50 (26.7)	53 (29.8)	45 (25.4)	47 (25.4)
Current	91 (48.7)	85 (47.8)	91 (51.4)	92 (49.7)
Drinking status				
Never	142 (78.9)	132 (75.9)	124 (71.3)	138 (76.2)
Former	2 (1.11)	2 (1.15)	1 (0.57)	1 (0.55)
Current	36 (20.0)	40 (23.0)	49 (28.2)	42 (23.2)
Comorbidities				
Diabetes	38 (20.3)	50 (28.1)	52 (29.4)	54 (29.2)
Hypertension	95 (50.8)	111 (62.4)	97 (54.8)	105 (56.8)
Dyslipidaemia	104 (55.6)	101 (56.7)	110 (62.1)	109 (58.9)
Chronic pneumopathy	13 (6.95)	14 (7.87)	11 (6.21)	10 (5.41)
Previous CVD	29 (15.5)	42 (23.6)	33 (18.6)	37 (20.0)
Medication use				
Insulin	7 (3.74)	11 (6.18)	6 (3.39)	13 (7.03)
Oral antidiabetic drugs	30 (16.0)	42 (23.6)	37 (20.9)	48 (25.9)
ACE inhibitors	41 (21.9)	47 (26.4)	40 (22.6)	38 (20.5)
β-blockers	37 (19.8)	37 (20.8)	28 (15.8)	40 (21.6)
Diuretic agents	30 (16.0)	38 (21.3)	35 (19.8)	34 (18.4)
Calcium-channel blockers	20 (10.7)	36 (20.2)	23 (13.0)	22 (11.9)
Angiotensin II receptor blockers	24 (12.8)	30 (16.9)	28 (15.8)	35 (18.9)
Lipid-lowering drugs	60 (32.1)	69 (38.8)	67 (37.9)	78 (42.2)
Antiplatelet drugs	39 (20.9)	43 (24.2)	40 (22.6)	42 (22.7)
Anticoagulants	9 (4.81)	8 (4.49)	7 (3.95)	8 (4.32)
Sleep data				
AHI (events·h^{-1})	22.0 (17.2–33.0)	42.0 (32.0–55.5)	23.0 (18.2–29.4)	44.0 (30.0–59.0)
ODI (events·h^{-1})	19.1 (14.4–26.7)	41.2 (32.6–54.3)	18.0 (12.8–24.6)	37.7 (25.6–52.6)
Mean S_{aO_2} (%)	93.7 (92.3–94.9)	92.4 (90.8–93.6)	93.3 (92.0–94.4)	92.7 (91.3–93.6)
Minimum S_{aO_2} (%)	87.0 (82.0–88.0)	82.0 (76.0–85.0)	85.0 (81.0–88.0)	82.0 (78.0–85.0)
T_{Sat90} (%)	0.75 (0.10–3.65)	8.95 (2.68–27.3)	1.30 (0.20–6.40)	6.60 (1.60–22.0)
HB (%min·h^{-1})	50.6 (40.6–61.2)	106 (85.0–142)	53.3 (43.3–63.1)	102 (84.4–139)
Somnolence (ESS)	4.00 (3.00–6.00)	5.00 (4.00–8.00)	5.00 (3.00–6.00)	5.00 (3.00–8.00)

Data are presented as median (interquartile range) or n (%). CPAP: continuous positive airway pressure; BMI: body mass index; CVD: cardiovascular disease; ACE: angiotensin-converting enzyme; AHI: apnoea–hypopnoea index; ODI: oxygen desaturation index; S_{aO_2}: arterial oxygen saturation; T_{Sat90}: percentage of total sleep time with S_{aO_2} <90%; ESS: Epworth Sleepiness Scale. The pre-intervention HB was categorised as "low" or "high" based on the median value (⩽73.1%min·h^{-1} or >73.1%min·h^{-1}, respectively).

Discussion

In the present *post hoc* analysis of the ISAACC trial, we report that in non-sleepy patients with ACS, there is a differential pattern of response to OSA treatment with CPAP depending on the baseline HB level. We observed that OSA patients with a high baseline HB not only had an increased risk for developing future adverse cardiovascular outcomes but also exhibited a significant long-term protective effect of CPAP on cardiovascular prognosis compared with those with a low baseline HB. These results suggest that this novel OSA severity metric could be useful to identify those patients who are most likely to experience a reduction in cardiovascular risk following treatment with CPAP.

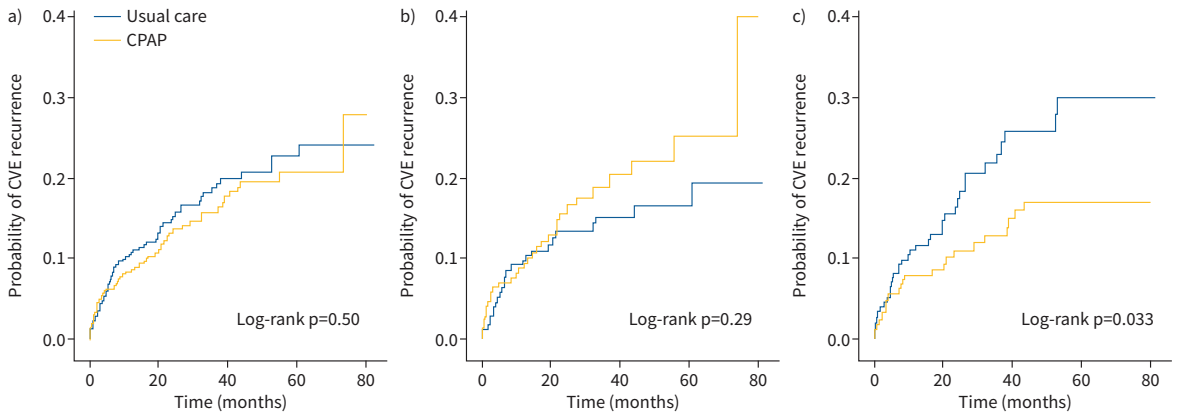

FIGURE 3 Differential patterns of cardiovascular response to continuous positive airway pressure (CPAP) treatment according to the baseline hypoxic burden (HB) level. Unadjusted Kaplan–Meier curves showing the cumulative incidence of cardiovascular events (CVEs) in the CPAP *versus* usual care groups for a) all participants (n=727), b) participants with low HB (n=364) and c) participants with high HB (n=363). The pre-intervention HB was categorised as "low" or "high" based on the median value (⩽73.1%min·h^{-1} or >73.1%min·h^{-1}, respectively).

The potential benefits of successfully treating OSA range from improving clinical symptomatology and reducing its associated morbidity and mortality to decreasing healthcare resource utilisation and consequent costs. CPAP is currently the mainstay of therapy for adults with OSA. There is high-quality evidence indicating that CPAP therapy reduces the frequency of respiratory events during sleep, decreases daytime sleepiness [32], improves systemic blood pressure [33], lowers the risk of traffic accidents [34] and improves quality of life [35, 36] across a range of disease severities. In contrast, RCTs have been unsuccessful in demonstrating a positive effect of treating OSA with CPAP on primary and secondary cardiovascular outcomes [37]. Several authors have discussed potential explanations that may shed light on these conflicting results [38, 39]. A substantial line of reasoning is based on questioning the use of the AHI as the "holy grail" to assess OSA [40]. Despite the standard use of this metric as the primary diagnostic, prognostic and treatment-deciding criterion for OSA [41, 42], available literature indicates that the AHI is an oversimplification of a complex and diverse disease phenomenon [40]. In recent years, the sleep medicine community has pointed to the need for a more tailored approach to optimise the management of this heterogeneous disease.

In the era of precision medicine, it has been a research priority to develop additional markers that fully capture the impact of OSA on different health outcomes, especially those that accurately foresee cardiovascular risk. A considerable number of alternatives have been proposed, where HB emerges as a

TABLE 2 Cox regression models evaluating the effect of continuous positive airway pressure (CPAP) treatment on the primary end-point according to the baseline hypoxic burden (HB) level

	Events	Follow-up (months)	Unadjusted HR (95% CI)	p-value	Adjusted[#] HR (95% CI)	p-value
Low HB						
Usual care	26 (13.9)	33.34 (15.03–54.04)	Reference		Reference	
CPAP	31 (17.4)	24.34 (13.80–43.37)	1.33 (0.79–2.25)	0.281	1.31 (0.78–2.22)	0.308
High HB						
Usual care	36 (20.3)	24.26 (12.27–41.86)	Reference		Reference	
CPAP	24 (13.0)	32.00 (13.27–58.22)	0.57 (0.34–0.96)	**0.035**	0.61 (0.36–1.03)	0.063

Data are presented as n (%) or median (interquartile range), unless otherwise stated. The pre-intervention HB was categorised as "low" or "high" based on the median value (⩽73.1%min·h^{-1} or >73.1%min·h^{-1}, respectively). Usual care and low baseline HB are the reference categories for the CPAP and high HB groups, respectively. [#]: adjustment for age, sex, body mass index and previous cardiovascular disease. Significant p-values (p<0.05) are presented in bold.

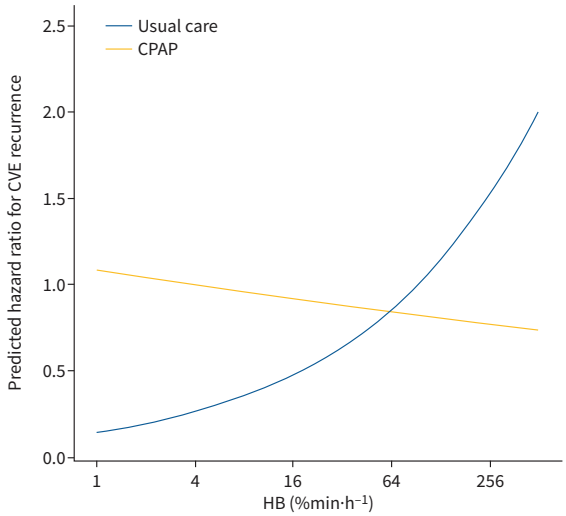

FIGURE 4 Dose–response relationship between baseline hypoxic burden (HB) and predicted risk for cardiovascular events (CVEs) according to treatment arm. Unadjusted curves showing the predicted hazard ratio for the primary end-point in the continuous positive airway pressure (CPAP) *versus* usual care groups, as a function of the continuous HB level. The predicted hazard ratios were estimated using the following criteria: average age (59.7 years), male sex (majority), average body mass index (29.3 kg·m^{-3}) and absence of previous cardiovascular disease (majority). The *x*-axis is log-scaled.

physiologically informed metric that captures the depth, duration and frequency of night-time respiratory event-related hypoxaemia. Since its discovery [23], an increasing number of studies have demonstrated the capacity of HB for predicting CVD-related outcomes, including mortality [28], major cardiovascular events [23], stroke [25], heart failure [24], hypertension [26] and chronic kidney disease [27]. Nevertheless, to the best of our knowledge, there has been no study to date evaluating the potential of this OSA severity metric to predict the therapeutic benefit of CPAP [43]. In this secondary analysis of the ISAACC trial, we observed that the effect of OSA intervention with CPAP on the incidence of adverse cardiovascular outcomes was dependent on the baseline HB level. The cardiovascular response to CPAP treatment followed a dose–response relationship with the baseline HB levels and only those patients with a high HB exhibited a protective effect of the treatment, regardless of the level of adherence. In contrast, this protection conferred by CPAP against the development of CVD was not evidenced by stratifying the population by the AHI or other metrics of nocturnal hypoxaemia, including the ODI and T_{Sat90}. Our

TABLE 3 Cox regression models evaluating the effect of continuous positive airway pressure (CPAP) treatment on the cardiac end-point according to the baseline hypoxic burden (HB) level

	Events	Follow-up (months)	Unadjusted HR (95% CI)	p-value	Adjusted[#] HR 31(95% CI)	p-value
Low HB						
Usual care	18 (9.6)	34.91 (16.78–55.95)	Reference		Reference	
CPAP	22 (12.4)	25.96 (14.32–47.57)	1.36 (0.73–2.54)	0.331	1.36 (0.72–2.55)	0.339
High HB						
Usual care	31 (17.4)	24.6 (12.85–47.04)	Reference		Reference	
CPAP	19 (10.3)	34.29 (17.50–58.45)	0.52 (0.29–0.92)	0.025	0.53 (0.30–0.95)	0.034

Data are presented as n (%) or median (interquartile range), unless otherwise stated. The pre-intervention HB was categorised as "low" or "high" based on the median value (≤73.1%min·h^{-1} or >73.1%min·h^{-1}, respectively). Usual care and low baseline HB are the reference categories for the CPAP and high HB groups, respectively. [#]: adjustment for age, sex, body mass index and previous cardiovascular disease. Significant p-values (p<0.05) are presented in bold.

findings reinforce the need to move beyond the AHI and conventional OSA severity metrics to fully characterise the disease.

Due to the large heterogeneity of this disease, it is increasingly recognised that the effect of CPAP on different health outcomes may not be uniform across the entire spectrum of the OSA population. The reliance on a single metric that does not capture this heterogeneity may prove detrimental to our therapeutic efforts as inadequate treatment allocation may result in inefficient utilisation of healthcare resources. Therefore, the accurate identification of individuals who are most likely to respond to CPAP treatment represents a hot topic in the field. This would be especially interesting for the large proportion of patients with sleep disordered breathing not reporting daytime sleepiness [44], for whom the prescription of CPAP as the treatment of choice remains a subject of great debate [45, 46]. This study provides further evidence of the existence of a specific subpopulation of patients who may be impacted differently by OSA and consequently have a different response to treatment. Our results facilitate a step toward personalised, guided therapeutic decision making in OSA. This could reduce healthcare costs by avoiding the administration of treatment that is unnecessary or ineffective for individuals with little probability of response, especially considering the tendency toward a harmful effect of CPAP observed in patients with low HB. Furthermore, the HB could not only help to capture the variability in OSA and guide CPAP treatment allocation but also inform enrolment criteria for the design of future RCTs.

For a novel disease metric to be useful, it should be easily incorporated into routine clinical practice. In this study, we used a validated new approach to calculate the HB based on automatically identified oxygen desaturations [31]. This approach eliminates the need for manual scoring of respiratory events, which is time consuming and prone to inter-scorer technical variability, which is a reported cause of inconsistencies in the calculation of conventional OSA indices among different sleep laboratories [47]. Other metrics have arisen in recent years. In particular, it has been shown that individuals with greater respiratory event-related pulse rate response (ΔHR) were at increased risk of cardiovascular morbidity and mortality [47]. Additionally, this metric was demonstrated to predict CPAP benefit in the RICCADSA trial [48]. Here, the original manual scoring of respiratory events was unavailable for some subjects and therefore we were unable to compute the ΔHR. Nevertheless, the simplicity of our novel HB measurement enables its calculation using wearable technology that only records the S_{aO_2} signal, facilitating its inclusion as part of a clinical support system for OSA, not only to identify at-risk patients but also for targeted therapy.

It has been proposed that OSA may have a different impact on the cerebrovascular system than on the coronary system [48], and therefore the weights of the cardiac and cerebrovascular components of the primary composite end-point could not be equally balanced. Based on this assumption, we additionally tested the prognostic value of HB focusing exclusively on the cardiac component of the primary end-point. This analysis yielded stronger associations between HB and the effect of CPAP treatment, with a calculated risk reduction of 47% in patients with high HB.

Strengths and limitations

The strengths of this study include its multicentre design, which enabled us to appropriately address the association of HB with the impact of CPAP treatment on long-term cardiovascular outcomes by evaluating a large number of patients with confirmed diagnoses of ACS and OSA, to appropriately address the association of HB with the impact of CPAP treatment on long-term cardiovascular outcomes. Standardised methods were applied for data collection across the participating centres. All sleep studies were performed with the same respiratory polygraphy model and were scored by certified technicians following international guidelines. Moreover, major adverse cardiovascular outcomes specified in the study protocol were documented and adjudicated by a blinded external committee.

Our study has several limitations that should be noted. First, although a causal relation of OSA to vascular disease has strong biological plausibility, due to the exploratory nature of this secondary analysis of an RCT, cause–effect relationships remain unproven. The results of this study indicate the possibility of identifying specific subgroups of OSA patients who could respond to CPAP treatment based on an oximetry-based metric. However, the validity of our findings should be further confirmed in independent cohorts. Second, the unavailability of raw respiratory polygraphy data for all OSA patients in the ISAACC cohort may pose a potential limitation. Although no clinically relevant differences were observed between the included and excluded patients, this aspect could have introduced bias into the results. Third, although the method for HB calculation applied here has been validated in the SHHS, the original approach for calculating HB could not be applied in this cohort due to the unavailability of all the original manually scored event files. Fourth, for ethical reasons, patients with excessive daytime sleepiness were excluded from the ISAACC trial. Therefore, the results should not be extrapolated to OSA patients with somnolence.

Fifth, the study design is limited to patients admitted to the hospital for ACS, making it necessary to explore the clinical value of HB in different clinical settings. Sixth, due to insufficient statistical power to individually evaluate the cerebrovascular composite end-point, further research is needed to determine the potential of HB to predict the effect of CPAP on cerebrovascular consequences. Finally, the under-representation of women and subjects with other ethnicities may limit the generalisability of the results to the global population. Future studies should confirm whether the results are valid for women and individuals with other racial backgrounds.

Conclusions

In patients with ACS, OSA treatment with CPAP prevented CVD-related events in patients with a high baseline HB. Although further validation work is needed, our exploratory findings highlight the potential for this oximetry-based metric to identify OSA patients without excessive daytime sleepiness who are more likely to respond to CPAP therapy in the long term. The HB represents a simple, inexpensive and easily accessible metric that could feasibly be translated into routine clinical practice to guide CPAP treatment allocation for cardiovascular prevention in asymptomatic OSA.

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Conflict of interest: M.Á. Martinez-Garcia received grants from VitalAire and Philips, Spain, and serves as a consultant for ResMed Inc., Australia, companies that develop products related to sleep apnoea. L. Messineo received a consultancy fee from Apnimed. A. Wellman works as a consultant for Apnimed, SomniFix and Nox, and has received grants from SomniFix and Sanofi; A. Wellman has a financial interest in Apnimed, a company developing pharmacological therapies for sleep apnoea. His interests were reviewed and are managed by Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies. S. Redline received grant support and consulting fees from Jazz Pharmaceuticals, and consulting fees from Apnimed and Lilly Pharma. S. Sands receives personal fees as a consultant for Nox Medical and Merck, outside the submitted work, and receives grant support from Apnimed and ProSomnus. F. Barbé received a research grant from ResMed Inc., Australia, a company that develops products related to sleep apnoea. A. Azarbarzin reports grant support from SomniFix and serves as a consultant for SomniFix, Respicardia, Eli Lilly and Apnimed. Apnimed is developing pharmacological treatments for obstructive sleep apnoea. A. Azarbarzin's interests were reviewed by Brigham and Women's Hospital and Mass General Brigham in accordance with their institutional policies. All other authors declare no competing interests.

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SUPPLEMENTARY INFORMATION

Hypoxic Burden to Guide CPAP Treatment Allocation in Patients with Obstructive Sleep Apnoea: A Post-hoc Study of the ISAACC Trial

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Figure S1. Cardiovascular response to CPAP treatment according to the baseline HB level and the mean adherence to the treatment. Unadjusted Kaplan–Meier curves showing the cumulative incidence of cardiovascular events in the Usual Care group, the lower CPAP adherence group, and the higher CPAP adherence group, for **(A)** all participants, **(B)** participants with low HB, and **(C)** participants with high HB. The pre-intervention HB was categorized as "low" and "high" when ≤ or > the median value (73.1 %min/h), respectively. The adherence to CPAP treatment was categorized as "lower" and "higher" when < or ≥ the median value of daily use (2.3 h/day), respectively. Definitions of abbreviations: CPAP = continuous positive airway pressure; HB = hypoxic burden.

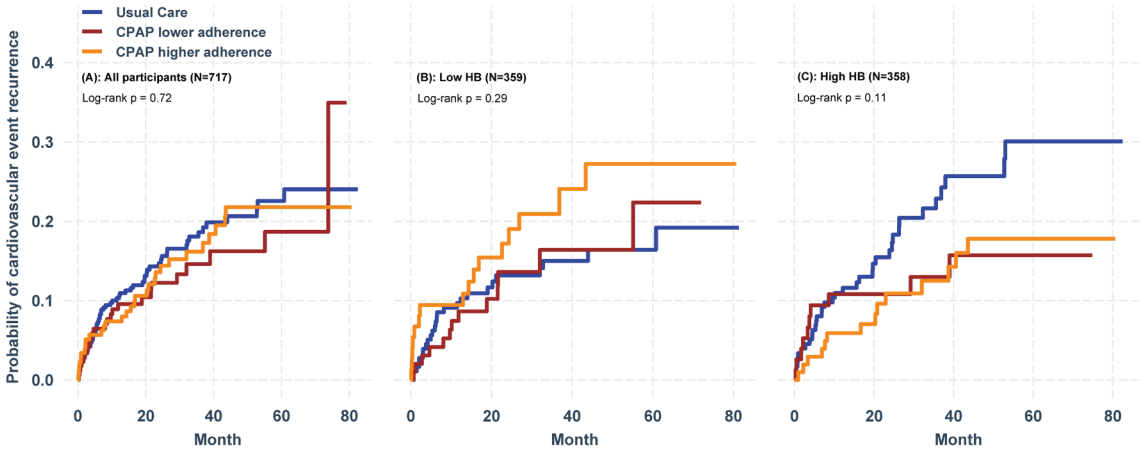


Figure S2. Cardiovascular response to CPAP treatment according to the conventional OSA severity metrics level. Unadjusted Kaplan–Meier curves showing the cumulative incidence of cardiovascular events in the CPAP *vs.* the Usual Care groups for **(A)** all participants, **(B)** participants with low OSA severity metric, and **(C)** participants with high OSA severity metric. The pre-intervention OSA severity measures were categorized as "low" and "high" when ≤ or > the median values, respectively. The median values were 30.8 events/h for AHI, 27.2 events/h for ODI and 3.3% for TSat90. Definitions of abbreviations: AHI = apnoea-hypopnoea index; CPAP = continuous positive airway pressure; ODI = oxygen desaturation index; TSat90 = percent of total sleep time spent with oxygen saturation <90%.

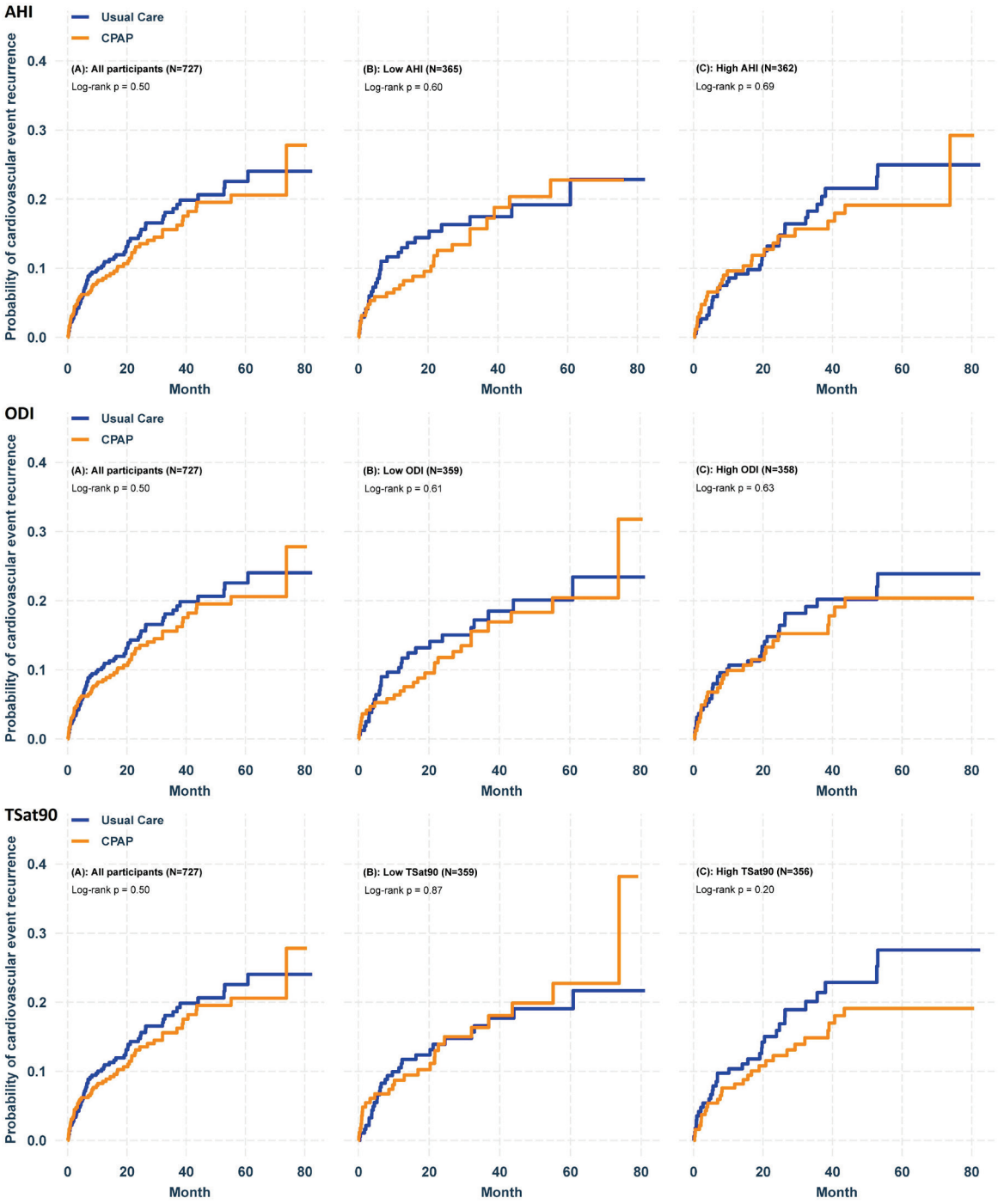

AHI
Usual Care
CPAP
(A): All participants (N=727)
Log-rank p = 0.50
(B): Low AHI (N=365)
Log-rank p = 0.60
(C): High AHI (N=362)
Log-rank p = 0.69
ODI
Usual Care
CPAP
(A): All participants (N=727)
Log-rank p = 0.50
(B): Low ODI (N=359)
Log-rank p = 0.61
(C): High ODI (N=358)
Log-rank p = 0.63
TSat90
Usual Care
CPAP
(A): All participants (N=727)
Log-rank p = 0.50
(B): Low TSat90 (N=359)
Log-rank p = 0.87
(C): High TSat90 (N=356)
Log-rank p = 0.20
Probability of cardiovascular event recurrence
0.0
0.1
0.2
0.3
0.4
0
20
40
60
80
Month

Table S1. Characteristics of the included *vs.* excluded participants from the ISAACC cohort. Data are presented as the median [p25;p75] for quantitative variables and n (%) for qualitative variables. Definitions of abbreviations: ACE = angiotensin-converting enzyme; AHI = apnoea-hypopnoea index; BMI = body mass index; CPAP = continuous positive airway pressure; CVD = cardiovascular disease; ESS = Epworth sleepiness scale; ODI = oxygen desaturation index; OSA = obstructive sleep apnoea; SaO_2 = oxygen saturation; TSat90 = time with SaO_2 <90%.

	INCLUDED n = 727	**EXCLUDED** n = 528
Clinical data		
Demographic/anthropometric		
Age (years)	59.0 [52.5;67.0]	60.0 [54.0;68.0]
Sex		
Male	614 (84.5%)	444 (84.1%)
Female	113 (15.5%)	84 (15.9%)
BMI (kg/m^2)	29.0 [26.4;31.9]	29.0 [26.4;31.8]
Smoking status		
Never	173 (23.8%)	134 (25.4%)
Former	195 (26.8%)	158 (29.9%)
Current	359 (49.4%)	236 (44.7%)
Drinking status		
Never	536 (75.6%)	347 (67.5%)
Former	6 (0.85%)	7 (1.36%)
Current	167 (23.6%)	160 (31.1%)
Comorbidities		
Diabetes	194 (26.7%)	135 (25.6%)
Hypertension	408 (56.1%)	299 (56.6%)
Dyslipidaemia	424 (58.3%)	294 (55.7%)
Chronic pneumopathy	48 (6.60%)	26 (4.92%)
Previous CVD	141 (19.4%)	117 (22.2%)
Medication use		
Insulin	37 (5.09%)	43 (8.14%)
Oral antidiabetic drug	157 (21.6%)	104 (19.7%)
ACE inhibitors	166 (22.8%)	134 (25.4%)
Beta-blockers	142 (19.5%)	115 (21.8%)
Diuretic agents	137 (18.8%)	93 (17.6%)
Calcium-channel blockers	101 (13.9%)	78 (14.8%)
Angiotensin II receptor blockers	117 (16.1%)	90 (17.0%)

Lipid-lowering drugs	274 (37.7%)	198 (37.5%)
Antiplatelet drugs	164 (22.6%)	129 (24.4%)
Anticoagulants	32 (4.40%)	33 (6.25%)
Sleep data		
AHI (events/h)	30.8 [21.0;45.8]	32.0 [21.4;46.9]
ODI (events/h)	27.2 [17.0;43.1]	25.7 [14.4;40.8]
Mean SaO_2 (%)	93.0 [91.9;94.0]	92.8 [91.4;94.0]
Minimum SaO_2 (%)	84.0 [79.0;87.0]	83.0 [79.0;86.0]
TSat90 (%)	3.30 [0.50;12.0]	4.10 [0.95;16.0]
Somnolence (ESS)	5.00 [3.00;7.00]	6.00 [4.00;8.00]
Treatment allocation		
CPAP	362 (49.8%)	267 (50.6%)
Usual Care	365 (50.2%)	261 (49.4%)
Incidence of primary endpoint		
No	610 (83.9%)	439 (83.1%)
Yes	117 (16.1%)	89 (16.9%)

Table S2. Baseline characteristics of the study cohort by treatment allocation. Data are presented as the median [p25;p75] for quantitative variables and n (%) for qualitative variables. Definitions of abbreviations: ACE = angiotensin-converting enzyme; AHI = apnoea-hypopnoea index; BMI = body mass index; CPAP = continuous positive airway pressure; CVD = cardiovascular disease; ESS = Epworth sleepiness scale; HB = hypoxic burden; ODI = oxygen desaturation index; OSA = obstructive sleep apnoea; SaO_2 = oxygen saturation; TSat90 = time with SaO_2 <90%.

	USUAL CARE n = 365	**CPAP TREATMENT n = 362**	**p value**
Clinical data			
Demographic/anthropometric			
Age (years)	59.0 [53.0;67.0]	59.0 [52.0;66.8]	0.314
Sex			0.508
Male	312 (85.5%)	302 (83.4%)	
Female	53 (14.5%)	60 (16.6%)	
BMI (kg/m^2)	29.1 [26.5;31.8]	28.7 [26.2;32.0]	0.429
Smoking status			0.687
Never	86 (23.6%)	87 (24.0%)	
Former	103 (28.2%)	92 (25.4%)	
Current	176 (48.2%)	183 (50.6%)	
Drinking status			0.367
Never	274 (77.4%)	262 (73.8%)	
Former	4 (1.13%)	2 (0.56%)	
Current	76 (21.5%)	91 (25.6%)	
Comorbidities			
Diabetes	88 (24.1%)	106 (29.3%)	0.136
Hypertension	206 (56.4%)	202 (55.8%)	0.922
Dyslipidaemia	205 (56.2%)	219 (60.5%)	0.267
Chronic pneumopathy	27 (7.40%)	21 (5.80%)	0.473
Previous CVD	71 (19.5%)	70 (19.3%)	1.000
Medication use			
Insulin	18 (4.93%)	19 (5.25%)	0.979
Oral antidiabetic drug	72 (19.7%)	85 (23.5%)	0.254
ACE inhibitors	88 (24.1%)	78 (21.5%)	0.463
Beta-blockers	74 (20.3%)	68 (18.8%)	0.680
Diuretic agents	68 (18.6%)	69 (19.1%)	0.957
Calcium-channel blockers	56 (15.3%)	45 (12.4%)	0.304
Angiotensin II receptor blockers	54 (14.8%)	63 (17.4%)	0.392

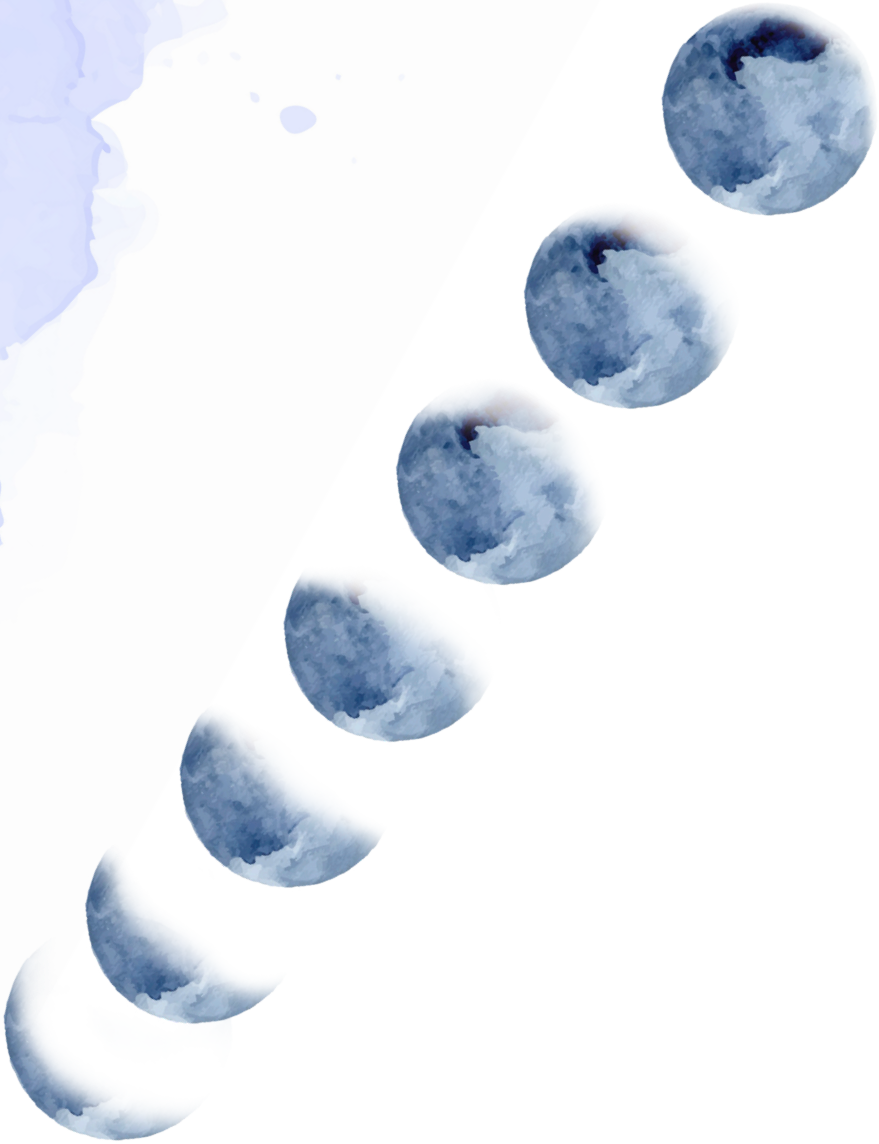
Lipid-lowering drugs	129 (35.3%)	145 (40.1%)	0.217
Antiplatelet drugs	82 (22.5%)	82 (22.7%)	1.000
Anticoagulants	17 (4.66%)	15 (4.14%)	0.875
Sleep data			
AHI (events/h)	32.7 [20.8;45.4]	29.8 [21.2;46.2]	0.835
ODI (events/h)	29.8 [17.5;44.5]	25.6 [16.8;42.2]	0.088
Mean SaO_2 (%)	93.0 [91.9;94.1]	93.0 [91.9;94.0]	0.591
Minimum SaO_2 (%)	84.0 [79.0;87.0]	83.0 [79.0;87.0]	0.316
TSat90 (%)	2.90 [0.40;12.0]	3.70 [0.70;11.9]	0.293
HB (%min/h)	70.9 [49.9;104]	74.5 [53.9;103]	0.270
Somnolence (ESS)	5.00 [3.00;7.00]	5.00 [3.00;7.00]	0.670

Table S3. Baseline characteristics of the study cohort by categories of baseline HB. Data are presented as the median [p25;p75] for quantitative variables and n (%) for qualitative variables. Significant p values (p <0.05) are presented in bold. Definitions of abbreviations: ACE = angiotensin-converting enzyme; AHI = apnoea-hypopnoea index; BMI = body mass index; CPAP = continuous positive airway pressure; CVD = cardiovascular disease; ESS = Epworth sleepiness scale; HB = hypoxic burden; ODI = oxygen desaturation index; OSA = obstructive sleep apnoea; SaO_2 = oxygen saturation; TSat90 = time with SaO_2 <90%.

	Low HB (HB ≤73.1 %min/h) n = 364	**High HB (HB >73.1 %min/h) n = 363**	**p value**
Clinical data			
Demographic/anthropometric			
Age (years)	60.0 [53.0;67.0]	59.0 [52.0;67.0]	0.849
Sex			0.55
Male	60 (16.5%)	53 (14.6%)	
Female	304 (83.5%)	310 (85.4%)	
BMI (kg/m^2)	28.1 [25.5;30.8]	29.8 [27.3;32.9]	**<0.001**
Smoking status			0.904
Never	87 (23.9%)	86 (23.7%)	
Former	95 (26.1%)	100 (27.5%)	
Current	182 (50.0%)	177 (48.8%)	
Drinking status			0.934
Never	266 (75.1%)	270 (76.1%)	
Former	3 (0.85%)	3 (0.85%)	
Current	85 (24.0%)	82 (23.1%)	
Comorbidities			
Diabetes	90 (24.7%)	104 (28.7%)	0.266
Hypertension	192 (52.7%)	216 (59.5%)	0.078
Dyslipidemia	214 (58.8%)	210 (57.9%)	0.856
Chronic pneumopathy	24 (6.59%)	24 (6.61%)	1.000
Previous CVD	62 (17.0%)	79 (21.8%)	0.129
Medication use			
Insulin	13 (3.57%)	24 (6.61%)	0.090
Oral antidiabetic drug	67 (18.4%)	90 (24.8%)	**0.045**
ACE inhibitors	81 (22.3%)	85 (23.4%)	0.775
Beta-blockers	65 (17.9%)	77 (21.2%)	0.295

Diuretic agents	65 (17.9%)	72 (19.8%)	0.557
Calcium-channel blockers	43 (11.8%)	58 (16.0%)	0.129
Angiotensin II receptor blockers	52 (14.3%)	65 (17.9%)	0.220
Lipid-lowering drugs	127 (34.9%)	147 (40.5%)	0.138
Antiplatelet drugs	79 (21.7%)	85 (23.4%)	0.643
Anticoagulants	16 (4.40%)	16 (4.41%)	1
Sleep data			
AHI (events/h)	22.8 [17.7;31.2]	42.7 [30.6;57.5]	**<0.001**
ODI (events/h)	18.4 [13.6;25.6]	39.6 [29.3;52.8]	**<0.001**
Mean SaO_2 (%)	93.6 [92.2;94.6]	92.6 [91.1;93.6]	**<0.001**
Minimum SaO_2 (%)	86.0 [81.5;88.0]	82.0 [77.0;85.0]	**<0.001**
TSat90 (%)	0.90 [0.10;4.77]	7.20 [2.10;24.6]	**<0.001**
HB (%min/h)	51.8 [41.1;62.2]	104 [84.8;140]	**<0.001**
Somnolence (ESS)	4.00 [3.00;6.00]	5.00 [3.00;8.00]	**<0.001**

DISCUSSION



DISCUSSION

This Doctoral Thesis addresses a chronic and heterogeneous sleep disorder with potentially severe health consequences. OSA affects nearly one billion individuals worldwide and its prevalence is anticipated to escalate in the near future, due to the aging of societies and the global obesity epidemic [320]. The socioeconomic impact of OSA together with its implication for the quality of life and health status of the patients, positions this disease as a major public health concern [321]. However, despite growing awareness in the last decades, it remains a significantly underdiagnosed and untreated condition.

Precision medicine fosters the development of more effective healthcare strategies, facilitating informed and evidence-based decisions that align with each patient's specific needs [322]. Implementing precision medicine approaches holds potential for better disease management, improved health outcomes, and minimized adverse effects for patients [323]. Within this Doctoral Thesis, we leverage advanced technologies and comprehensive analyses from different sources of data, including molecular data derived from omics analysis and physiological data derived from sleep tests, to improve the biological characterization and clinical management of OSA.

Adopting a precision medicine perspective, the research conducted in this Doctoral Thesis is directed towards: (i) identifying biomarker candidates to facilitate a more accessible and streamlined OSA diagnosis, (ii) enhancing prognostic insights into the aging-related, hemodynamic, and cardiovascular implications of OSA, and (iii) providing a more tailored approach to identify the patients who are more likely to benefit from CPAP treatment. The findings of this Doctoral Thesis in these three major areas of investigation are discussed individually in the following subsections.

Diagnosis

The high and concerning prevalence of OSA poses a challenge for diagnosis, as current diagnostic criteria require overnight monitoring of breathing during sleep, a procedure often hindered by financial constraints [271]. The limitations of the current OSA diagnosis justify efforts to explore new biomarkers for its early detection. In this Doctoral Thesis, we identify a signature comprising four plasma metabolites, which provided a powerful diagnostic performance, surpassing that of the STOP-Bang questionnaire, a commonly used tool for OSA screening. We provide a realistic setting of subjects with clinical suspicion of OSA, representing the most comprehensive concurrent metabolomic and lipidomic study to date, aiming to identify circulating molecular profiles associated with OSA, its severity, and its treatment. Our omics approach revealed that despite the corrections introduced to eliminate the effect of confounding variables, we were still able to identify a blood-based fingerprint of OSA, which strongly correlated with different polysomnographic measurements of disease severity. Moreover, CPAP treatment was associated with a partial modulation of this plasma profile, which would help to reinforce the etiological role of OSA in the observed associations.

The molecular profile that we identified here was mainly composed of lipidic species. Four functional categories associated with the different lipid classes could be inferred: membrane structural components, cellular protective antioxidants, lipids involved in mitochondrial bioenergetics, and bioactive lipids. Together, the results would suggest the activation of adaptive mechanisms to counter OSA-derived injury. Additionally, our results propose potential biomarker candidates for the accurate detection of OSA among individuals with suspicion of the disease, which could be incorporated into the usual clinical practice to help clinicians streamline disease detection using a routine blood test. It is essential to note that, due to the dynamic nature of metabolites, and the methodological challenges linked to their targeted quantification, the translation of metabolite-

based signatures to clinical practice requires additional steps in terms of simplification, standardization, and automatization.

Risk stratification

Aging-related consequences of OSA

With the current increase in life expectancy, delaying age-related diseases has become a crucial aspect of interest to society. Patients with OSA demonstrate susceptibility to aging-related impairments at an earlier age than anticipated [166]. Results from murine models support that OSA may lead to age-dependent premature aging, with a more marked affectation of young mice [180]. In this Doctoral Thesis, we observe that, in a large and multicenter cohort, OSA severity was independently associated with an increase in specific hallmarks of aging, following a dose–response relationship. Moreover, this association was more pronounced in younger patients (under 50 years of age), than in older ones.

Our data support the emerging concept that OSA may be a different phenotype in young *versus* old individuals [324], and raise awareness about the detrimental effects of OSA specifically in the young population. In this context, stratifying patients with OSA by age may lead to considerably better diagnoses and treatments in terms of personalized medicine, as young patients could represent a phenotype exhibiting higher susceptibility to OSA-related harm. Our findings led us to agree with the preceding proposal that OSA accelerates or potentiates aging mechanisms. Relatedly, a recent study showed that OSA patients displayed an acceleration of the systemic epigenetic age, which was reversible with the adherent use of CPAP treatment [325]. Together, the results would suggest the need for specific efforts directed towards an early diagnosis and early treatment of OSA, which could potentially slow the progression of cellular and molecular aging mechanisms, and maybe even delay the appearance of age-related disorders. However, studies elucidating the causative and mechanistic roles of OSA in the aging process are lacking.

Hemodynamic consequences of OSA

Apneas and hypopneas, along with the consequent compensatory hyperpneas, are associated with decreased parasympathetic and increased sympathetic activity. Elevated sympathetic outflow antagonizes the natural BP dipping phenomenon causing surges in intravascular pressure. The loss or disruption of nocturnal hemodynamic dipping causes important adverse health consequences. Nevertheless, there is a limited understanding of the physiopathological mechanisms connecting OSA to the disruption of circadian BP rhythmicity. Given the high prevalence of abnormal BP dipping in patients with OSA, and the prognostic implications of this pathological circadian pattern, there is an increasing interest in defining and understanding the factors that affect nocturnal BP dipping in patients with OSA. Applying appropriate therapeutic interventions in OSA patients with an increased risk for non-dipping BP could prevent subsequent cardiac and vascular damage.

In this Doctoral Thesis, a comprehensive characterization of the polysomnographic determinants of non-dipping BP in OSA patients revealed that the respiratory arousal index was the most relevant predictor of impaired BP dipping, beyond classical clinical risk factors and usual OSA metrics. Consistent with previous reports [326], we found that the risk of presenting a non-dipping circadian BP pattern increased with OSA severity. However, although repeated oxygen desaturations had traditionally been considered one of the main contributors to the loss of nocturnal BP dipping among patients with OSA [327–329], our results suggest that measures of sleep fragmentation could better predict OSA-related BP abnormalities. In the same line, it has been reported that hypoxemia alone does not explain BP elevations after obstructive events [330]. Indeed, other sleep disorders without effects on arterial SaO_2, such as chronic insomnia and restless legs syndrome, also increase BP values during sleep, diminishing the natural BP dipping phenomenon [331–335]. Additionally, other forms of arousal from sleep without airway obstruction or airflow limitation, such as

auditory arousals, have also been shown to induce transient BP fluctuations [336,337].

Furthermore, we provide the first study to explore the blood metabolipidomic landscape underlying the circadian variation of BP in patients with OSA. Biofluids provide a close representation of the metabolic activity of the organs from which they are derived or the organs they bathe. Since blood irrigates all organs and tissues, blood-based profiling analyses can serve as a reliable metabolic proxy for the entire organism [338]. In this Doctoral Thesis, we identified a specific plasma metabotype of non-dipping, which was remarkably associated with polysomnographic parameters of OSA severity and with BP dipping variations after OSA treatment with CPAP. The identified metabotype, primarily composed of lipidic species, was implicated in multiple systemic biological pathways which could have implications for the dysregulation of circadian BP rhythmicity among individuals with OSA. Interestingly, among the two main hallmarks of OSA, namely, nocturnal hypoxemia and sleep fragmentation, we found that the latter showed the strongest correlation with the identified metabotype.

These two studies provide insights into the potential physiopathological link between OSA and BP hemodynamics. Recurrent arousals from sleep, and the consequent overactivation of sympathetic activity, could have a significant impact in the levels of the metabolites and lipids that are detectable in peripheral blood. Our results indicate that sleep fragmentation may represent a relevant mechanistic pathway underpinning the link between OSA and the dysregulation of BP rhythmicity, which may be reflected in a specific plasma metabotype. These exploratory studies provide hypothesis-generating data and may form the basis for future investigations.

Response to treatment

Although OSA is widely recognized as an independent risk factor for cardiac and cerebrovascular diseases, the beneficial effects of CPAP therapy on cardiovascular outcomes in patients with established CVD have recently been challenged. Several authors have discussed potential explanations that may enlighten these conflicting results [339,340]. A substantial line of reasoning is that the existing RCTs have not enriched the enrolment to target subgroups of patients who are most likely to respond to the intervention. Moreover, treatment responses are difficult to predict from existing diagnostic variables and current diagnostic algorithms mainly based on the AHI, which is one-dimensional and not adapted to the heterogeneous OSA population. In recent years, the sleep medicine community has pointed to the need for a more tailored approach to optimize the therapeutic management of this heterogeneous disease.

Here, in a secondary analysis of the ISAACC RCT, we observed that, among non-sleepy patients with ACS, the effect of OSA treatment with CPAP on the incidence of adverse cardiovascular outcomes was dependent on the baseline level of HB. While there was no association of CPAP therapy with cardiovascular prevention in the entire cohort, as reported in the primary ISAACC study [243], substantial heterogeneity in the effect of the treatment was observed when the population was stratified based on the HB. The cardiovascular response to CPAP treatment followed a dose-response relationship with the baseline HB levels, and only those patients with high HB exhibited a protective effect of the treatment, regardless of the level of adherence. In contrast, this protection conferred by CPAP against the development of CVD was not evidenced by stratifying the population by AHI or other metrics of nocturnal hypoxemia, including ODI and TSat90. Our findings reinforce the need to move beyond the AHI and conventional OSA severity metrics to fully characterize the disease.

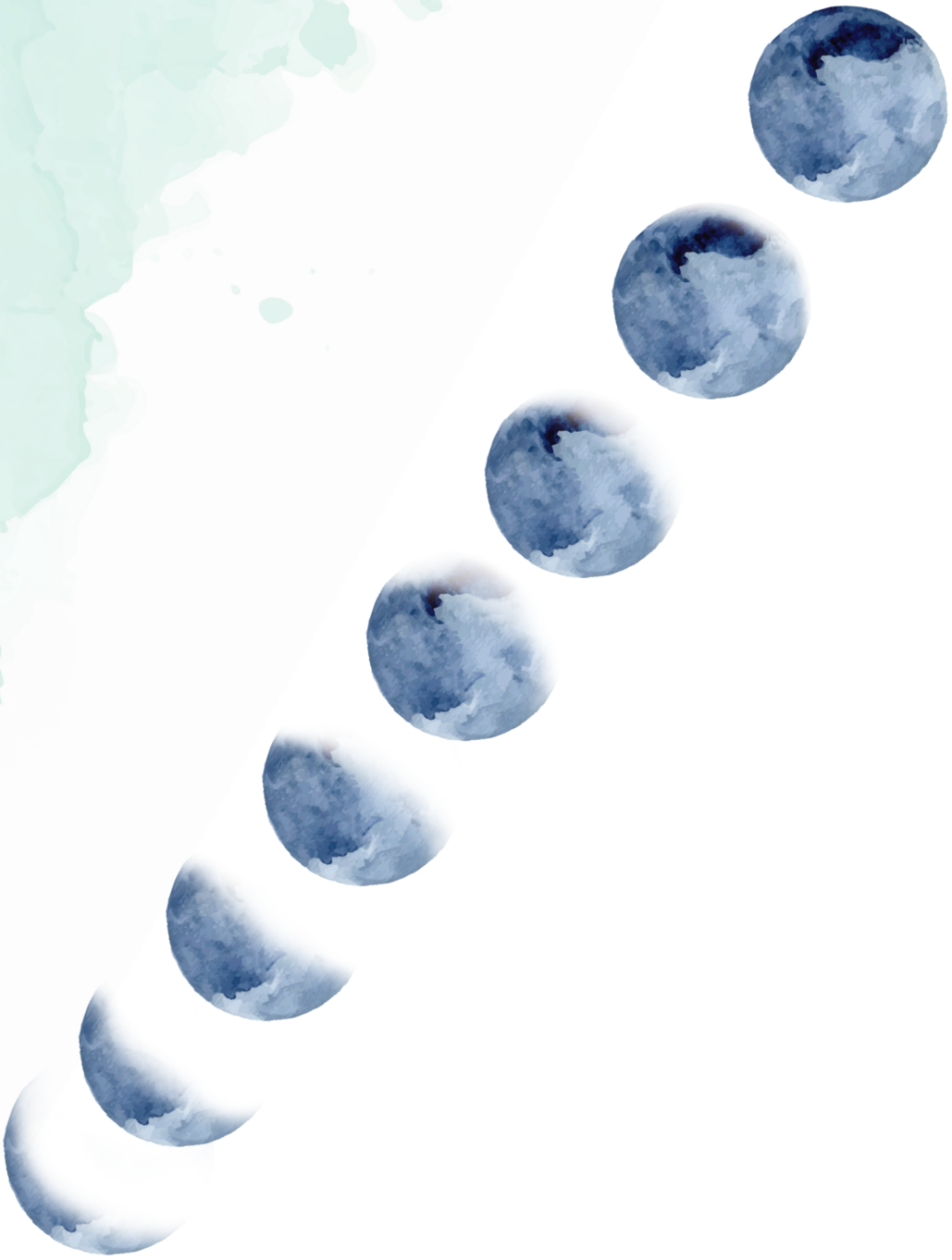
Due to the large heterogeneity of this disease, it is increasingly recognized that the effect of CPAP on different health outcomes may not be uniform across the

entire spectrum of the OSA population. The reliance on a single metric that does not capture this heterogeneity may prove detrimental to our therapeutic efforts as inadequate treatment allocation may result in inefficient utilization of healthcare resources. Our findings highlight the potential for this new biometric to identify a subgroup of OSA patients who are more likely to exhibit a long-term cardiovascular benefit from CPAP therapy. These results facilitate a step toward personalized therapeutic decision making in OSA. The simplicity of our novel HB measurement enables its calculation using wearable technology that only records SaO_2 signal, facilitating its inclusion as a part of a clinical support system for OSA, not only to identify at-risk patients but also for targeted therapy. Furthermore, the HB could not only help to capture the variability in OSA and guide CPAP treatment allocation, but also inform enrolment criteria for the design of future RCTs.

Concluding remarks

Based on the above findings, the combination of molecular information provided by -omics and physiological information derived from sleep data constitutes a promising approach to assist in medical decision-making in OSA. Nevertheless, it is crucial to acknowledge that additional work is required in this field. Further research is needed to clearly distinguish between correlative and causal observations, and current limitations are fundamental to elucidate the clinical applicability of the proposed biomarkers and biosignals. The use of real clinical settings, the validation of the findings in independent cohorts, the detailed evaluation of biomarker/biosignal performance, and the evaluation of cost-effectiveness, are essential steps to effectively translate these findings into a new paradigm for the management of patients with OSA.

CONCLUSIONS



CONCLUSIONS

Embracing a precision medicine perspective, this Doctoral Thesis aimed at deciphering novel biomarkers and biometrics for the characterization and clinical management of OSA, addresses three major areas of investigation: diagnosis, risk stratification, and response to treatment. This work, with a robust translational component, provides a valuable contribution to the understanding of the biological bases of the disease, while emphasizing the clinical utility.

The conclusions of this Doctoral Thesis can be outlined as follows:

1. A blood-based metabolipidomic fingerprint of OSA provides diagnosis biomarker candidates and suggests the activation of adaptive mechanisms in response to OSA-derived injury.

2. OSA severity is associated with a dose-response increase in specific hallmarks of aging, specifically in younger patients.

3. The respiratory arousal index is a key OSA-related parameter associated with non-dipping BP, highlighting the role of sleep fragmentation on the loss of circadian BP rhythmicity.

4. A specific plasma metabotype of non-dipping BP in individuals with OSA suggests systemic metabolic implications for the circadian control of BP in OSA.

5. Among non-sleepy patients with ACS, high baseline HB levels are associated with a long-term protective effect of CPAP treatment on cardiovascular prognosis.

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APPENDIX

Relation of scientific publications and book chapters resulting from the additional work performed during the completion of this Doctoral Thesis.

- Pinilla L; Barbé F; de Gonzalo-Calvo D. **MicroRNAs to Guide Medical Decision-Making in Obstructive Sleep Apnea: a Review**. *Sleep Med Rev*. 2021. Vol. 59, No. 101458. DOI: 10.1016/j.smrv.2021.101458.
- Pinilla L; Barbé F; Guerra J M; Llorente-Cortés V; de Gonzalo-Calvo D. **Epigenetics in Precision Medicine of Cardiovascular Disease**. *Epigenetics in Precision Medicine, Elsevier*. 2021. Vol. 30, Chapter 12, pp. 347 - 368. DOI: 10.1016/B978-0-12-823008-4.00010-X.
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- Polanco D; González J; Gracia-Lavedan E; Pinilla L; Plana R; Molina M; Pardina M; Barbé F. **Multidisciplinary Virtual Management of Pulmonary Nodules**. *Pulmonology*. 2022. DOI: 10.1016/j.pulmoe.2021.12.003.
- Zapater A; Benítez I D; Santamaria-Martos F; Pinilla L; Targa A; de Gonzalo-Calvo D; Torres G; Mínguez O; Cortijo A; Dalmases M; Barbé F; Sánchez-de-la-Torre M. **Endogenous Controls and microRNA Profile in Female Patients with Obstructive Sleep Apnea**. *Sci Rep-UK*. 2022. Vol. 12, No. 1, pp. 1916. DOI: 10.1038/s41598-022-05782-y.

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