Universitat de Lleida

Síndrome de Apnea-Hipopnea del Sueño y Factores de Riesgo Cardiovascular

Cristina Esquinas López

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SÍNDROME DE APNEA-HIPOPNEA DEL SUEÑO Y FACTORES DE RIESGO CARDIOVASCULAR

TESIS DOCTORAL

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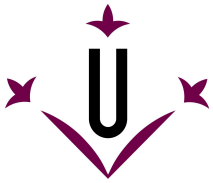

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Octubre de 2013

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Lleida, 15 de septiembre del 2013

Dr. Ferrán Barbé Illa, profesor titular de la Universitat de Lleida.
Dra. Antonia Barceló, doctor en Medicina por la Universidad de les Illes Balears, como directores del trabajo de Tesis Doctoral "Síndrome de Apnea-Hipopnea del Sueño y Factores de Riesgo Cardiovascular", realizado por la Sra. Cristina Esquinas López.

CERTIFICAMOS

Que en el trabajo presentado para optar al Grado de Doctor de la Universidad de Lleida, se han alcanzado los objetivos fijados al inicio de la Tesis. El trabajo de la presente tesis se ha realizado en el Departamento de Medicina de la Universitat de Lleida. La memoria que se presenta constituye un trabajo compacto que da respuesta a toda una serie de interrogantes planteados en torno a la importancia del síndrome de apnea-hipopnea del sueño y su relación con los factores de riesgo cardiovascular.

Por tanto, consideramos apto este trabajo para proceder a su lectura y defensa ante la comisión correspondiente.

Por que así conste firmamos la presente certificación en Lleida a 15 de septiembre de 2013.

Dr. Ferrán Barbé Illa

Dra. Antonia Barceló

A Luis,
A mis padres.

Lo poco que he aprendido carece de valor,
comparado con lo que ignoro y no desespero en aprender.

René Descartes (1596-1650) Filósofo y matemático francés.

El que da, no debe volver a acordarse; pero el que recibe nunca debe olvidar.

— Proverbio Hebreo

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Hace algo más de diez años decidí iniciarme en el mundo de la investigación durante mi paso por el Hospital Clínic. Un lugar muy especial para mí, pues allí se me abrieron las puertas a un camino que me ha dado muchas satisfacciones. Una de ellas, es la presentación de esta tesis doctoral. Me siento afortunada por trabajar en la disciplina para la que me he formado y sobretodo, con la que disfruto.

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Y sin duda hoy no sería lo que soy, ni estaría presentando este trabajo sin el esfuerzo y apoyo de las dos personas más importantes en mi vida. Gracias papa y mama. Os quiero. Para vosotros va esta tesis.

Cristina Esquinas

Octubre 2013

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ACRÓNIMOS

AASM	American Academy of Sleep Medicine
AG	ácidos grasos
AIT	análisis por intención de tratar
CPAP	presión positiva continua en la vía aérea *(siglas en inglés de Continuous positive airway pressure)*
DM 2	diabetes mellitus tipo 2
CEC	Comité de Eventos Clínicos
EDS	somnolencia diurna excesiva *(siglas en inglés de Excessive daytime sleepiness)*
HDL	lipoproteína de alta densidad *(siglas en inglés de High density lipoprotein)*
HOMA	modelo de evaluación homeostática *(siglas en inglés de Homestatic Model Assessment)*
HTA	hipertensión arterial
IAH	índice de apnea-hipopnea
IC	intervalo de confianza
IM	infarto de miocardio
IMC	índice de masa corporal
NCEP-ATP III	III programa nacional de educación sobre el colesterol del panel de expertos en la detección, evaluación y tratamiento de la hipercolesterolemia en adultos
NF-kb	factor nuclear kβ
NO	óxido nítrico *(siglas en inglés de nitric oxide)*
OR	Odds Ratio
PCR	poligrafía cardiorrespiratoria
PP	por protocolo
PR	poligrafía respiratoria
PSG	polisomnografía
PTH	hormona paratiroidea
RI	resistencia a la insulina
ROS	especies reactivas de oxígeno *(siglas en inglés de Reactive oxygen species)*
SAHS	síndrome apnea-hipopnea del sueño

SCA	síndrome coronario agudo
SEPAR	Sociedad Española de Neumología y Cirugía Torácica
SM	síndrome metabólico
VAS	vía aérea superior

Parte I

TESIS

1

PRESENTACIÓN

La presente tesis doctoral se estructura según las directrices de la normativa para la presentación de tesis doctorales en formato artículos, aprobada por el Acuerdo num. 19/2002 de la Junta de Gobierno del 26 de febrero del 2002 de la Universidad de Lleida.

Los estudios presentados en la presente tesis doctoral pertenecen a una misma línea de investigación iniciada en 2010 con el fin de profundizar en la relación entre el síndrome de apnea-hipopnea del sueño y los factores de riesgo cardiovascular. Los resultados obtenidos han aportado información relevante en este campo y han dado lugar a cuatro artículos publicados en revistas internacionales:

1. Bonsignore MR, Esquinas C, Barceló A, Sanchez-de-la-Torre M, Paternó A, Duran-Cantolla J, Marín JM, Barbé F. Metabolic syndrome, insulin resistance and sleepiness in real-life obstructive sleep apnoea. Eur Respir J. 2012 May;39:1136-43.

2. Barceló A, Piérola J, de la Peña M, Esquinas C, Fuster A, Sanchez M, Carrera M, Alonso-Fernandez A, Ladaria A, Bosch M, Barbé F. Free fatty acids and the metabolic syndrome in patients with obstructive sleep apnoea. Eur Respir J. 2011 Jun;37:1418-23.

3. Barceló A, Esquinas C, Piérola J, De la Peña M, Sánchez-de-la-Torre M, Montserrat JM, Marín JM, Duran J, Arqué M, Bauça JM, Barbé F. Vitamin D Status and Parathyroid Hormone Levels in Patients with Obstructive Sleep Apnea. Respiration. 2012 Nov 15. [Epub ahead of print]

4. Esquinas C, Sánchez-de-la Torre M, Aldomá A, Florés M, Martínez M, Barceló A, Barbé F. Rationale and methodology of the impact of continuous positive airway pressure CPAP on patients with ACS and non-sleepy OSA: The ISAACC trial. Clin Cardiol. 2013 Sep;36:495-501.

En los tres primeros estudios, la doctoranda ha sido la responsable de todos los aspectos referentes a la metodología de investigación y del análisis estadístico de los resultados, con un papel relevante en la interpretación de los resultados y extracción de conclusiones, y en la redacción del manuscrito.

En el cuarto artículo la doctoranda ha desarrollado todo el trabajo que se describe en la publicación. Este artículo ha permitido la realización de un estudio multicéntrico, prospectivo, aleatorizado, abierto y controlado, cuyo objetivo primario es evaluar el impacto del tratamiento con presión positiva continua en la vía aérea *(siglas en inglés de Continuous positive airway pressure)* (CPAP) sobre la aparición de nuevos eventos cardiovasculares en pacientes con síndrome apnea-hipopnea del sueño (SAHS) y síndrome coronario agudo (SCA) siendo la doctoranda la coordinadora nacional de este estudio.

2 RESUMEN

2.1 RESUMEN

El síndrome apnea-hipopnea del sueño (SAHS) es una enfermedad muy prevalente que afecta al 4-6 % de los varones y al 2-4 % de las mujeres en la población general adulta de edades medias. Los pacientes que padecen este síndrome tienen episodios repetidos de colapso de la vía aérea superior (VAS) durante el sueño que da lugar a episodios de hipoxia-reoxigenación, despertares transitorios *(arousals)*, incremento de la actividad neurovegetativa y cambios en la presión intrapleural. Todo este conjunto de fenómenos fisiopatológicos dan lugar a diversas comorbilidades cardiovasculares, neurológicas y metabólicas. La enfermedad cardiovascular es la mas notoria, y se ha relacionado con los episodios de hipoxia/normoxia o con la actividad del sistema neurovegetativo. Los aspectos metabólicos no son tan conocidos en la actualidad.

El objetivo principal de los trabajos presentados en esta tesis es doble: por una parte, analizar aspectos básicos de la relación existente entre el SAHS y la enfermedad cardiovascular y metabólica; por otra en pacientes con SAHS se desarrolla y diseña detalladamente un protocolo que tiene como objetivo estudiar el impacto del tratamiento con presión positiva continua en la vía aérea *(siglas en inglés de Continuous positive airway pressure)* (CPAP) sobre la aparición de nuevos eventos cardiovasculares en pacientes con SAHS y síndrome coronario agudo (SCA). Se han realizado cuatro estudios: tres de ellos de mecanismos e investigación básica, y por último, un cuarto de carácter clínico.

El primer estudio (Bonsignore MR, et al. Eur Respir J. 2012) incluyó un total de 529 pacientes con SAHS, y mostró que en los pacientes con SAHS la prevalencia de síndrome metabólico (SM) es muy elevada. Además, el 38,2 % de los pacientes restantes presentaba al menos uno o dos componentes del SM. El número de componentes del SM correlacionó positivamente con la presencia de resistencia a la insulina (RI). El segundo estudio (Barceló A, et al. Eur Respir J. 2011), incluyó un total de 119 pacientes con SAHS y de 119 controles con una distribución similar de sexo, edad e índice de masa corporal (IMC). Este estudio mostró una elevada concentración plasmática de ácidos grasos (AG) libres en los pacientes con SAHS, existiendo una correlación positiva entre diversos índices de gravedad del SAHS y la concentración plasmática de AG. El tercer estudio (Barceló A, et al. Respiration. 2012), incluyó 826 sujetos con nuevo diagnóstico de SAHS. El estudio reveló una alta prevalencia de déficit de vitamina D en los pacientes con SAHS. Se observó una relación inversa entre la concentración plasmática de vitamina D y la presencia de SM y diabetes. Una concentración plasmática elevada de hormona paratiroidea (PTH) se relacionaba con un aumento de la prevalencia de obesidad e hipertensión. En el cuarto estudio (Esquinas C, et al. Clin Cardiol. 2013), se detalla en profundidad el diseño y metodología de un estudio multicéntrico, prospectivo, aleatorizado, abierto y controlado, en el que se incluyen, de forma consecutiva, los pacientes con diagnóstico de SCA y sin somnolencia diurna (escala de Epworth$\leqslant$10) que son ingresados en las Unidades Coronarias de 15 hospitales universitarios en España. Los pacientes son sometidos a un estudio del sueño mediante poligrafía cardiorrespiratoria (PCR). Aquéllos con un índice de apnea-hipopnea (IAH)$\geqslant$15/h son aleatorizados a tratamiento con

CPAP (grupo 1: 632 pacientes) o a tratamiento conservador (grupo 2: 632 pacientes). Los pacientes con IAH<15/h (Grupo 3: 632 pacientes) son monitorizados como grupo de referencia. Se realizan visitas de seguimiento al mes y a los 3, 6 y 12 meses tras la aleatorización, y posteriormente cada 6 meses tras el primer año se seguimiento.

2.2 SUMMARY

Sleep Apnea-Hypopnea Syndrome (SAHS) is a common disorder that affects 4-6% of males and 2-4% of women in the general adult population in middle age. Patients with this diagnosis present repeated episodes of collapse of the upper airway (UA) during sleep. The repercussions of SAHS (episodes of hypoxia-reoxygenation, arousals, increased neurovegetative activity and changes in intrapleural pressure). This set of pathophysiological phenomena lead to various cardiovascular comorbidities, neurological and metabolic diseases. Cardiovascular disease is the most outstanding, being associated with episodes of hypoxia/normoxia of neurovegetative system activity. The metabolic aspects are not presently known.

The main objective of this thesis is twofold: firstly, to analyze the basic aspects of the relationship between SAHS and cardiovascular and metabolic disease; secondly, to develop and design a detailed protocol that aims to study the impact of treatment with continuous positive airway pressure airway (CPAP) on the rate of cardiovascular events in patients with SAHS and acute coronary syndrome (ACS). Four studies have been undertaken: three in basic research, the fourth in clinical research.

The first study (Bonsignore MR, et al. Eur Respir J. 2012) included a total of 529 patients with SAHS. The results show that the metabolic syndrome (MS) is very frequently found in patients with SAHS, as it was detected in about half the patients at the time of diagnosis, with 38.2% presenting one or two components of MS. The number of components of MS positively correlates with the presence of insulin resistance (IR) in SAHS patients. The second study (Barceló A, et al. Eur Respir J. 2011) included a total of 119 patients with SAHS and 119 controls paired according to gender, age and body mass index (BMI). It was found that the levels of fatty acids (FA) are high in patients with SAHS and there was a positive correlation between the indexes of SAHS severity and the levels of FA. The third study (Barceló A, et al. Respiration. 2012) was performed on 826 subjects with a new diagnosis of SAHS. The study revealed a high prevalence of vitamin D deficiency in patients with SAHS. There was an inverse relationship between vitamin D levels and the presence of MS and diabetes while the high levels of parathyroid hormone (PTH) are associated with an increased prevalence of obesity and hypertension. The final article (Esquinas C, et al. Clin Cardiol. 2013) presented the design and methodology for a multicenter, prospective, randomized, controlled study to evaluate the impact of sleep apnea and its treatment with CPAP on the clinical course of patients with ACS. This study has included consecutive patients admitted to the Coronary Unit of one of 15 university hospitals in Spain with a diagnosis of ACS but no daytime sleepiness (Epworth scale $\leqslant 10$). All the patients have been subjected to a sleep study using cardiorespiratory polygraphy (CRP). Patients with an apnea-hypopnea index AHI$\geqslant$15/h have been randomized to a treatment with CPAP (group 1: 632 patients) or conservative treatment (group 2: 632 patients). Patients with an AHI<15/h (group 3: 632 patients) have been monitored as a reference group. Follow-up visits will be made at 1 month, 3 months, 6 months and 12 months after the randomization, and every 6 months after the first year of follow-up.

2.3 RESUM

La síndrome de l'apnea - hipopnea del son (SAHS) és un trastorn comú que afecta entre el 4-6% dels homes i al 2-4% de les dones en la població general adulta. Els pacients amb aquesta síndrome tenen episodis repetits de col · lapse de la via aèria superior (VAS) durant el son donant lloc a episodis d'hipòxia - reoxigenació, despertars *(arousals)*, increment de l'activitat neurovegetativa i canvis en la pressió intrapleural). Tot aquest conjunt de fenòmens fisiopatològics donen lloc a diverses comorbiditats cardiovasculars, neurològiques i metabòliques. La malaltia cardiovascular és una de les més importants i s'ha relacionat amb episodis de hipòxia-normòxia o amb l'activitat del sistema neurovegetatiu. Els aspectes metabòlics no són tan coneguts en l'actualitat.

L'objectiu principal dels estudis presentats és doble: per una banda, analitzar aspectes bàsics de la relació existent entre la SAHS i la malaltia cardiovascular i metabòlica; per altra banda, en pacients amb SAHS es desenvolupa i dissenya detalladament un protocol que té com a objectiu estudiar l'impacte del tractament amb pressió positiva contínua en la via aèria superior (CPAP) sobre l'aparició de nous episodis cardiovasculars en pacients amb SAHS i síndrome coronària aguda (SCA). S'han realitzat quatre estudis: tres en investigació bàsica i mecanismes i l'altre clínic.

El primer estudi (Bonsignore MR, et al. Eur Respir J. 2012) va incloure un total de 529 pacients amb SAHS. Els resultats mostren que la presència de síndrome metabòlica (SM) és molt freqüent en pacients amb SAHS . A més, un 38,2% dels pacients presentava un o dos components de SM . El número de components de la SM correlaciona de manera positiva amb la presència de resistència a la insulina (RI). En el segon estudi (Barceló A, et al. Eur Respir J. 2011) va incloure un total de 119 pacients amb SAHS i 119 controls aparellats per sexe, edat i índex de massa corporal (IMC) . S'observa que la concentració plasmàtica d'àcids grassos (AG) lliures és elevada en els pacients amb SAHS i existeix una correlació positiva entre els índexs de gravetat de la SAHS i la concentració plasmàtica d'AG . El tercer estudi (Barceló A, et al. Respiration. 2012) mostra els resultats d'un estudi realitzat en 826 subjectes amb nou diagnòstic de SAHS . El dèficit de vitamina D s'associa de manera inversa amb la prevalença de SM , i diabetis . Una concentració plasmàtica elevada d'hormona paratiroïdal (PTH) es relaciona amb un augment de la prevalença d'obesitat i hipertensió . En l'últim article (Esquinas C, et al. Clin Cardiol. 2013), es presenta el disseny i la metodologia d'un estudi multicèntric, prospectiu, aleatoritzat i controlat. En aquest estudi s'estan incloent de manera consecutiva dels pacients que són ingressats a la unitat coronària amb diagnòstic de SCA i sense somnolència diürna (escala d'epworth $\leqslant$10) en 15 hospitals universitaris a Espanya . Tots els pacients es sotmeten a un estudi del son mitjançant poligrafia cardiorespiratòria (PCR) . Els pacients amb un índex d'apnea - hipopnea (IAH)$\geqslant$15/h son aleatoritzats al tractament amb CPAP (grup 1: 632 pacients) o tractament conservador (grup 2: 632 pacients) . Els pacients amb IAH<15/h (grup 3 : 600 pacients) són monitoritzats com grup de referència. Es realitzen les visites de seguiment a 1 , 3 , 6 i 12 mesos després de la aleatorització i cada 6 mesos després del primer any de seguiment.

3 INTRODUCCIÓN

3.1 SÍNDROME DE APNEA-HIPOPNEA DEL SUEÑO

El ser humano pasa aproximadamente un tercio de su vida durmiendo. Sin embargo, muchos aspectos relacionados con los eventos que ocurren durante el sueño son aún desconocidos. Sabemos que el sueño no es un proceso pasivo. Es un proceso activo que tiene su propio "programa" de secuencia de estadios, caracterizados por patrones neurofisiológicos definidos, durante los cuales se desarrollan diferentes funciones autonómicas. Nuestro conocimiento acerca de los mecanismos y las fases del sueño aumenta cada vez más. Muchas funciones, como la consolidación de la memoria, el humor y el carácter, así como otras endocrinas e inmunológicas están relacionadas con un buen descanso nocturno.

En la primera mitad del siglo XIX, Charles Dickens describió en su obra "Los papeles del club Pickwick" a un personaje obeso, roncador y con una clara hipersomnia diurna. Se trata de la primera referencia histórica de la que se tiene constancia de un sujeto con un trastorno respiratorio del sueño. Años después, la asociación entre obesidad hipercapnia e hipoxemia recibió la denominación de "Síndrome de Pickwick". A partir de 1960 empieza a desarrollarse ampliamente la polisomnografía (PSG), lo que permitió describir qué sujetos obesos afectos de hipersomnia diurna tenían un patrón respiratorio anormal durante el sueño, caracterizado por la presencia de frecuentes episodios de ausencia de respiración (apneas). En la década de los 70 Guilleminault acuñó el término "síndrome de apnea del sueño" [2] y, en 1981, Sullivan aportó un tratamiento eficaz para el mismo basado en la aplicación de presión positiva continua en la vía aérea *(siglas en inglés de Continuous positive airway pressure)* (CPAP) por vía nasal [3]. En la actualidad se conoce una gran variedad de trastornos del sueño. Aunque muchos de ellos apenas han sido reconocidos por el colectivo médico, ha sido en las últimas dos décadas cuando la comunidad científica ha empezado a mostrar interés por ellos, fomentando un incremento en la investigación y consecuentemente, en los conocimientos adquiridos acerca de estos trastornos.

La presente tesis se centra en uno de los trastornos del sueño más frecuentes: el síndrome apnea-hipopnea del sueño (SAHS). El SAHS se caracteriza por la presencia de episodios repetidos de obstrucción total (apneas) o parcial (hipopneas) al flujo aéreo oronasal durante el sueño. Los episodios se acompañan típicamente de desaturación de la oxihemoglobina, aumento del flujo simpático y pueden finalizar con un breve microdespertar *(arousal)*, lo que ocasiona la fragmentación del sueño y disrupción de su arquitectura [4, 5, 6]. Los síntomas principales son somnolencia diurna excesiva *(siglas en inglés de Excessive daytime sleepiness)* (EDS), ronquidos, apneas observadas así como desarrollo de trastornos neurocognitivos, cardiorrespiratorios, inflamatorios y metabólicos secundarios. El SAHS constituye un problema de salud pública en los países desarrollados. Diferentes estudios epidemiológicos llevados a cabo en los Estados Unidos y en Europa han evidenciado que el SAHS es una enfermedad muy prevalente que afecta al 4-6 % de los varones y al 2-4 % de las mujeres en la población general adulta de edades medias [7]. La prevalencia aumenta con la edad [8, 9, 10, 11], y ésta es 2 o 3 veces superior en varones que en mujeres [8], aunque en éste se incrementa significativamente en la menopausia [10]. El principal factor de riesgo para el desarrollo del SAHS en la edad adulta es la obesidad [8, 9, 10, 11, 12]. Otros factores de

riesgo son las anomalías de la vía aérea superior (VAS), las alteraciones craneofaciales, las endocrinopatías (hipotiroidismo, acromegalia), la menopausia y los factores genéticos [13].

La primera descripción del SAHS fue popularizada por Guilleminault en el año 1976. Se trata de un síndrome caracterizado por la presencia de apneas obstructivas (detectadas mediante PSG nocturna), acompañadas de hipersomnia diurna [2]. Esta descripción inicial del SAHS incluía una duración mínima de dichas apneas obstructivas de 10 segundos, considerándose como punto de corte un número de apneas por hora de sueño, es decir, un índice de apnea-hipopnea (IAH)>5/h. La American Academy of Sleep Medicine (AASM) estableció un documento de consenso en el año 1999 con el objetivo de estandarizar la evaluación de los eventos respiratorios, aunque más dirigido a la investigación clínica que a la práctica clínica habitual, considerándose anormal un IAH>5/h. Este consenso clasifica además el SAHS de la siguiente manera: leve (IAH=5-15/h), moderado (IAH=15-30/h) y grave (IAH>30/h con síntomas asociados) [14]. La presencia de un número anormal de apneas/hipopneas durante el sueño asociado con síntomas relacionados con la enfermedad establece el diagnóstico de SAHS y permite cuantificar su gravedad. Sin embargo, la heterogeneidad de dispositivos disponibles en el mercado y los diferentes sensores utilizados para el registro del flujo aéreo en boca y nariz (sonda nasal o termistor oronasal), con diferente sensibilidad según se utilicen para la detección de apneas o hipopneas, hace que diversos autores también limiten la patología en nuestra práctica clínica habitual en un IAH$\geqslant$10/h si se acompaña de sintomatología. Es importante tener en cuenta que además existe cierta controversia en la literatura a la hora de establecer definiciones estandarizadas de hipopneas [15].

El SAHS se diagnostica y se trata en las Unidades de Sueño, donde se realiza una historia clínica enfocada hacia los trastornos del sueño y una exploración de la VAS. Es necesario realizar un diagnóstico diferencial adecuado, ya que hay más de 80 trastornos del sueño, muchos de los cuales cursan con somnolencia diurna. Hay que tener en cuenta que también otros procesos, como los hábitos de mal dormir o la depresión, pueden producir somnolencia. La prueba de referencia para establecer el diagnóstico del SAHS es la PSG nocturna. Se trata del registro de una serie de variables neurofisiológicas, respiratorias y cardíacas que nos permiten conocer la cantidad y la calidad del sueño, así como la repercusión de las apneas e hipopneas en éste [16, 17, 18, 19, 20]. Los parámetros que se evalúan en la PSG nocturna son los siguientes:

- Flujo aéreo naso-bucal: su registro se realiza a través de unas resistencias térmicas o termistores que detectan las diferencias de temperatura entre el aire espirado e inspirado.
- Medición de las oscilaciones de la presión nasal a través de una cánula similar a las que se utilizan con el oxígeno. A partir de este parámetro se estima el flujo.
- Esfuerzo ventilatorio: se mide mediante la colocación de unas bandas torácicas y abdominales que son sensibles al estiramiento.
- Intercambio gaseoso: la saturación de oxígeno arterial se mide a través de la oximetría cutánea.
- Ritmo cardíaco: a través de un electrocardiograma se registran los cambios de ritmo cardíaco asociados a trastornos del sueño.
- Arquitectura del sueño: Con el fin de valorar la calidad y composición del sueño, se analizan los registros del electroencefalograma, del electrooculográma y del electro-

miograma submentoniano. Otros parámetros útiles para el diagnóstico del SAHS son la monitorización del movimiento de las extremidades (mediante electromiograma tibial) o los ruidos respiratorios (mediante micrófono).

La poligrafía respiratoria (PR), que evalúa las variables respiratorias y cardíacas mediante estudios más simples, es una alternativa a la PSG de utilidad en los casos en los que exista una alta sospecha de SAHS. Puede realizarse tanto en el hospital como en el domicilio de los pacientes [21, 22, 23].

El tratamiento en los pacientes con SAHS debe ir encaminado a resolver los síntomas asociados, a normalizar la estructura del sueño, el IAH y la oxigenación, y a reducir el riesgo de complicaciones sistémicas. Existen diversas alternativas terapéuticas, resumidas en la Figura 3.1. que abarcan desde medidas generales, hasta técnicas quirúrgicas. También se han sugerido algunos fármacos, aunque el pilar terapéutico del SAHS es el tratamiento con CPAP.

Tratamiento del SAHS	
Medidas generales	**Tratamiento farmacológico**
Higiene del sueño	Modafinil
Evitar tabaco y alcohol	Medroxiprogesterona
Evitar determinados fármacos	Tiroxina
Pérdida de peso	Acetazolamida
Tratamiento postural	Teofilina
Tratamiento de enfermedades asociadas	Antagonista de los opiáceos
Tratamiento de alteraciones nasales	Nicotina
	Protriptilina
Medidas quirúrgicas	IRS
Traqueotomía	AntiHTA
Cirugía nasal	
Adenoamigdalectomía	**CPAP o BIPAP**
Uvulopalatofaringoplastia	
	Otros
Dispositivos orales	Neuroestimuladores del nervio hipogloso
Retenedores de la lengua	Marcapasos auricular cardiaco
DAM	Oxigenoterapia
Tubo nasofaríngeo	

Figura 3.1: Alternativas en el tratamiento del SAHS

La CPAP se administra mediante un dispositivo y su fin es estabilizar la vía aérea y evitar su colapso, corrigiendo así el IAH de forma que se consiga normalizar la estructura del sueño. Los dispositivos de CPAP constan de una turbina que genera un flujo de aire a presión positiva, una mascarilla (nasal o nasobucal) que se fija a la cara con unos arneses y una fuga intencional para evitar la reinhalación. Este flujo de aire generado por la turbina, transmitido a la vía aérea mediante una tubuladura y la mascarilla, actúa a modo de cuña neumática impidiendo el colapso de la faringe durante el sueño. Cada paciente precisa una presión determinada, por lo que ésta debe de determinarse individualmente mediante un estudio nocturno [24]. Clásicamente, la elección de la presión se ha llevado a cabo con titulación manual mediante PSG, aunque también se han utilizado fórmulas de predicción con buenos resultados [25]. En los últimos años se ha comenzado a utilizar la titulación con autoCPAP no vigilada en ciertos tipos de pacientes (con SAHS moderado o grave, roncadores, y que no presenten comorbilidades importantes), aunque aún no está claro el número de noches que el paciente debe usar el dispositivo antes de elegir una presión óptima adecuada para cada caso [26, 27, 28, 29]. La CPAP es el tratamiento aceptado en pacientes con SAHS sintomáticos, o con factores de riesgo cardiovascular notorios. En pacientes asintomáticos o sin factores de riesgo, la evidencia es menos clara, y hay que individualizar cada caso. Varios trabajos han encontrado efectos positivos tras el uso de CPAP en casos leves o moderados

que presentan clínica de somnolencia o factores de riesgo cardiovascular [30, 31, 32].

En resumen, el SAHS es muy prevalente pero altamente infradiagnosticado, sólo un 10 % de los pacientes con SAHS son diagnosticados y tratados [7]. Actualmente se dispone de un diagnóstico diferencial y tratamiento eficaz. El SAHS se relaciona con un aumento significativo de la morbilidad cardiovascular, hipertensión arterial (HTA), aumento del riesgo de accidente de tráfico y fallos cognitivos [33, 34, 35, 36]. Esta estrecha relación del SAHS con la morbilidad cardiovascular hace que se haya convertido en un importante problema de salud pública [37, 38, 39]. Por este motivo, en los últimos años se ha experimentado un incremento en el conocimiento del SAHS, tanto por parte de la población como por parte de los profesionales sanitarios.

3.2 ENFERMEDAD CARDIOVASCULAR Y SAHS

3.2.1 *Implicaciones cardiovasculares del SAHS*

Existe evidencia científica que sugiere que los pacientes con SAHS tienen un mayor riesgo de enfermedades cardiovasculares, incluida la muerte prematura por eventos vasculares [40, 41, 42, 43, 44]. Los eventos asociados con el colapso de las VAS conducen a microdespertares transitorios *(arousals)*, presiones intratorácicas negativas, episodios intermitentes de hipoxia y reoxigenación, y a un incremento de la actividad neurovegetativa. Estos eventos tienen lugar en ciclos repetitivos durante el sueño e inducen la activación de diversas vías (mecanismos intermedios) que predisponen a la aterosclerosis. La investigación básica, epidemiológica y los datos clínicos respaldan la noción de que el SAHS podría influir en el inicio y progresión de varias enfermedades cardiovasculares.

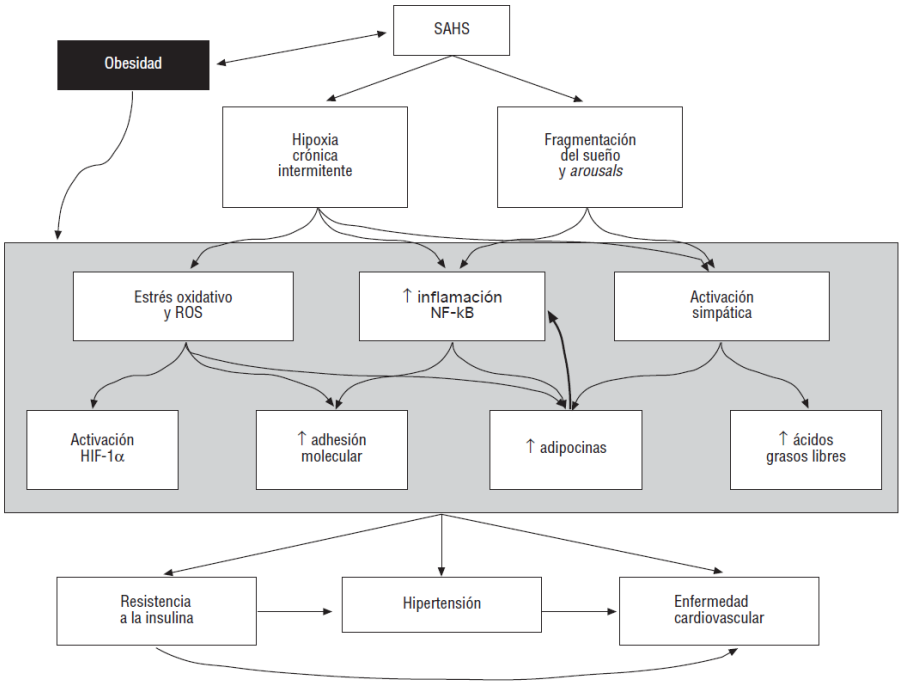

Figura 3.2: Representación esquemática de la patogenia del SAHS en la aparición de acontecimientos cardiovasculares. Modificado de Pack y Gislason [1].

Se han observado diferentes mecanismos intermedios que enlazan el SAHS con la enfermedad cardiovascular. Los mecanismos más importantes son: el estrés oxidativo, la activación simpática, la inflamación, la hipercoagulabilidad, la disfunción endotelial y las alteraciones metabólicas Figura 3.2. Aunque a continuación se describen de forma independiente, estos mecanismos intermedios, están estrechamente relacionados entre sí y se manifiestan simultáneamente en pacientes con SAHS. El estudio de mecanismos metabólicos es una de las partes importantes de esta tesis.

3.2.2 *Mecanismos intermedios*

3.2.2.1 *Estrés oxidativo*

El estrés oxidativo surge por un desequilibrio entre la producción de especies reactivas de oxígeno *(siglas en inglés de Reactive oxygen species)* (ROS) y el sistema biológico capaz de eliminarlos. Las ROS pueden reaccionar con lípidos, proteínas y ácidos nucléicos, causando daños en estas macromoléculas. Esta es la base patógena de enfermedades crónicas relacionadas con la edad como el cáncer, las enfermedades cardiovasculares, la diabetes, la inflamación crónica y los trastornos neurodegenerativos. En los pacientes con SAHS, diversos estudios, han mostrado un aumento en la producción de ROS en ciertas subpoblaciones de monocitos y granulocitos [45]. Un estudio clínico aleatorizado ha demostrado que en los pacientes con SAHS los niveles de 8-isoprostano son significativamente superiores, mientras que los niveles de óxido nítrico *(siglas en inglés de nitric oxide)* (NO) eran inferiores a los de sujetos sanos. Estos niveles de ambos biomarcadores se normalizaron con el tratamiento con CPAP [46]. Junto al aumento de la producción de ROS, algunos autores han sugerido que la apnea del sueño podría aumentar el estrés oxidativo mediante la reducción de la capacidad antioxidante [47].

3.2.2.2 *Activación simpática*

El aumento de la actividad del sistema nervioso simpático contrae los vasos sanguíneos y aumenta el gasto cardíaco. El grado de activación del sistema nervioso simpático se correlaciona con la gravedad del aumento de la presión arterial y es más pronunciado en el contexto de enfermedades metabólicas como la diabetes, la obesidad y el síndrome metabólico (SM) [48]. Hay pruebas robustas que asocian el SAHS con un aumento de la actividad simpática [49]. La hipoxia y la hipercapnia actúan sinérgicamente para aumentar la actividad simpática, y este efecto es especialmente notable durante la apnea [50]. Una de las características de los pacientes con SAHS es la alta frecuencia de despertares durante el sueño, lo que induce un marcado aumento de la actividad simpática durante cada episodio apneico.

3.2.2.3 *Inflamación*

El SAHS parece estar asociado a la inflamación local y sistémica. Los ronquidos pueden ocasionar, al menos de forma parcial, cambios inflamatorios a nivel local. El análisis histológico de los tejidos obtenidos a partir de pacientes con SAHS que han sido sometidos a una uvulopalatofaringoplastia, ha revelado la presencia de edema subepitelial, la infiltración excesiva de células plasmáticas y la reducción del área superficial de las papilas del tejido conectivo, que proporcionan anclaje para el epitelio. Los leucocitos polimorfonucleares también están aumentados en el líquido de lavado nasal de los pacientes con SAHS [51]. Además de inflamación local, existe evidencia de inflamación sistémica en pacientes con

SAHS. La inflamación crónica está estrechamente relacionada con la formación y la progresión de la aterosclerosis. En pacientes con SAHS se ha detectado la activación de factores de transcripción sensibles a oxígeno tales como factor inducible por hipoxia-1 y factor nuclear kβ (NF-kb). La transcripción del factor NF-κß es clave en la regulación de la inflamación, la respuesta inmune y la supervivencia celular. Además, varios estudios en pacientes con SAHS han descrito un incremento en los niveles plasmáticos de otros marcadores inflamatorios como las citocinas (incluyendo la interleucina-6 y el factor de necrosis tumoral alfa), las metaloproteinasas de la matriz y las proteínas de fase aguda, así como de moléculas de adhesión endoteliales como la molécula de adhesión intercelular 1 y las moléculas de adhesión celular vascular [52]. La obesidad es la comorbilidad más común en el SAHS, está presente en más de 50 % de los pacientes y se considera una situación inflamatoria crónica en sí misma. Por este motivo, la obesidad puede representar el factor de confusión más importante en la relación de la apnea del sueño con la inflamación. En este sentido, existen estudios contradictorios respecto a los marcadores inflamatorios y su relación con la obesidad y el SAHS [53, 54]

3.2.2.4 *Alteraciones de la coagulación*

Existe evidencia científica que apoya que los sujetos con SAHS muestran hipercoagulabilidad, lo que puede contribuir a un aumento del riesgo de eventos cardiovasculares. Los pacientes con SAHS muestran un aumento en la concentración plasmática de fibrinógeno, así como una baja actividad fibrinolítica. Además, algunos estudios han mostrado niveles elevados de los factores de coagulación XIIa, VIIa y de complejo trombina-antitrombina [55]. Los pacientes con SAHS presentan una mayor activación y agregación plaquetaria. Algunos estudios de intervención han mostrado que el tratamiento con CPAP reduce la coagulabilidad y el riesgo de trombosis en pacientes con SAHS [56, 57, 58, 59].

3.2.2.5 *Disfunción endotelial*

El endotelio vascular es una monocapa celular que recubre la zona interna de los vasos sanguíneos, está involucrado en el control del tono vasomotor y es el principal regulador de la hemostasia vascular. Se ha observado que la disfunción endotelial se produce en respuesta a los factores de riesgo cardiovasculares y parece acelerar el desarrollo de la aterosclerosis [60]. La valoración de la función endotelial en el SAHS incluye la evaluación funcional de las respuestas vasculares (mediante registro de los cambios en el flujo sanguíneo en respuesta a los vasodilatadores dependientes del endotelio o hipoxemia), la cuantificación de los niveles de células endoteliales apoptóticas circulantes y la valoración de los índices plasmáticos de varios biomarcadores endoteliales [61]. Por otra parte, el aumento de la activación simpática y el estrés oxidativo, dos condiciones que, como se ha citado previamente se encuentran comúnmente en el SAHS, pueden contribuir al desarrollo de la disfunción endotelial.

3.2.3 *Alteraciones metabólicas*

Factores relacionados con el SAHS como aumento de la actividad simpática, fragmentación del sueño y la hipoxia intermitente han demostrado contribuir al desarrollo de alteraciones metabólicas. El SM es un conjunto de factores de riesgo que se ha asociado con la aparición de resistencia a la insulina (RI), el aumento de riesgo de diabetes mellitus tipo 2 (DM 2) y de mortalidad cardiovascular o por cualquier causa. A pesar de estas evidencias, su valor predictivo de riesgo cardiovascular es todavía objeto de debate. En los últimos

años, el concepto de SM ha ganado popularidad y su conocimiento ha mejorado el manejo clínico de los pacientes con obesidad. Según la última definición del III programa nacional de educación sobre el colesterol del panel de expertos en la detección, evaluación y tratamiento de la hipercolesterolemia en adultos (NCEP-ATP III) [62], el SM se diagnostica cuando se observan alteraciones en al menos tres de los siguientes componentes individuales: perímetro de cintura elevado, como un indicador de obesidad central, aumento de la presión arterial, hiperglucemia en ayunas, aumento del nivel de triglicéridos y disminución de la lipoproteína de alta densidad *(siglas en inglés de High density lipoprotein)* (HDL). Los pacientes con SAHS presentan anomalías en cada uno de los componentes del SM, además de otras alteraciones anteriormente citadas como son activación simpática, disfunción endotelial, inflamación sistémica, hipercoagulabilidad y RI [63]. Sin embargo, no existe un patrón claro que establezca la relación entre el SAHS y el SM o sus componentes individuales.

Los pacientes con obesidad presentan concentraciones plasmáticas elevadas de ácidos grasos (AG) libres. Los AG se liberan principalmente de los depósitos de triglicéridos del tejido adiposo y tienen un papel fisiológico importante en la producción de energía [64]. Trabajos anteriores han puesto de manifiesto el papel de los AG en el desarrollo de RI y de alteraciones diversas relacionadas con el SM [65, 66]. Además, los AG también pueden contribuir al estrés oxidativo, a la inflamación y la disfunción endotelial [67, 68, 69]. A pesar del papel central de los AG en los procesos fisiopatológicos del SM, no existen estudios que avalen su relación con el SM en el SAHS.

Otra alteración metabólica que puede estar ligada a la enfermedad cardiovascular es la alteración en la concentración plasmática de vitamina D. La vitamina D es una proteína liposoluble que se ha relacionado clásicamente con el metabolismo fosfocálcico, pero se han encontrado receptores de vitamina D o de sus metabolitos en diferentes células del organismo, lo que indica que puede estar implicada en otros mecanismos fisiológicos. Diversos estudios han mostrado un incremento de déficit de vitamina D a nivel poblacional [70, 71, 72, 73], cuyas consecuencias se desconocen [73, 74, 75]. La vitamina D y la hormona paratiroidea (PTH) participan en el metabolismo óseo y en la homeostasis del calcio. Además, en los últimos años se ha evidenciado que la vitamina D tiene efectos pleiotrópicos y desempeña un papel importante en una amplia gama de funciones de órganos [70]. Diversos estudios sugieren que una concentración plasmática baja de vitamina D puede estar inversamente relacionada con la obesidad, la intolerancia a la glucosa, el SM y la presencia de enfermedades cardiovasculares [72, 76, 77, 78, 79]. Datos de estudios previos muestran que la concentración plasmática de vitamina D se asocia inversamente con la RI. Por otra parte, la PTH elevada secundaria a un nivel bajo de vitamina D también puede aumentar el riesgo de desarrollar componentes del SM, incluyendo la HTA, la DM 2 o la obesidad [77, 80].

Los pacientes con SAHS pueden presentar alteraciones a nivel metabólico que podrían contribuir a la aparición y/o peor pronóstico de la enfermedad cardiovascular. A pesar de ello, no existen estudios bien diseñados que analicen detalladamente la presencia de los factores de riesgo cardiovascular como SM, la alteración de la concentración plasmática de AG libres o la presencia de déficit de vitamina D en los pacientes con SAHS. Algunas investigaciones al respecto muestran datos poco concluyentes o contradictorios. La presente tesis doctoral se centrará en dar respuesta a estas hipótesis.

3.2.4 *Impacto del SAHS y de su tratamiento con CPAP en la evolución cardiovascular*

El tratamiento con CPAP revierte las apneas obstructivas, evita los episodios de hipoxia-reoxigenación, previene los episodios recurrentes de presión negativa intratorácica y reduce la actividad simpática y la presión arterial [81, 82]. Varios estudios han evaluado el efecto de la CPAP sobre la incidencia de HTA y los eventos cardiovasculares en pacientes con SAHS [83, 84]. Clínicamente, el tratamiento con CPAP revierte la EDS y mejora la calidad de vida de los pacientes [85], pero los datos sobre el impacto de la CPAP sobre la morbilidad y la mortalidad vascular se basan principalmente en estudios observacionales [86, 87]. En estos momentos no hay datos suficientes para apoyar el uso del tratamiento con CPAP en la prevención primaria o secundaria de la enfermedad cardiovascular. En este sentido se necesitan estudios de investigación aleatorizados, multicéntricos y con un tamaño de muestra adecuado que evidencien estas observaciones.

4 HIPÓTESIS Y OBJETIVOS

4.1 HIPÓTESIS GENERAL DE LA TESIS

Los fenómenos que se producen durante las apneas obstructivas (episodios de hipoxia-reoxigenación, despertares, cambios en la presión intrapleural y aumento de la actividad neurovegetativa) podrían favorecer el desarrollo de alteraciones metabólicas que se relacionarían con un aumento del riesgo cardiovascular, y como consecuencia ser al menos un factor que empeorara su pronóstico y evolución.

4.2 OBJETIVO GENERAL DE LA TESIS

Estudiar las bases fisiopatológicas y clínicas que relacionan el síndrome apnea-hipopnea del sueño (SAHS) y la enfermedad cardiovascular.

ESPECÍFICOS

1. Estudiar las alteraciones metabólicas que presentan los pacientes con SAHS y su posible relación con la comorbilidad cardiovascular.
   - Analizar la prevalencia de síndrome metabólico (SM) en una muestra de pacientes con SAHS y determinar las relaciones entre las variables de sueño, resistencia a la insulina (RI) y otras alteraciones metabólicas.
   - Determinar los niveles plasmáticos de ácidos grasos (AG) libres en los pacientes con SAHS y su relación con la presencia del SM.
   - Determinar la concentración plasmática de vitamina D y de hormona paratiroidea (PTH) en pacientes con SAHS y su relación con la presencia de diabetes mellitus tipo 2 (DM 2), hipertensión arterial (HTA) y SM.
2. Diseñar un estudio multicéntrico, aleatorizado, abierto y controlado en pacientes con SAHS que tiene como objetivo estudiar el impacto del tratamiento con presión positiva continua en la vía aérea *(siglas en inglés de Continuous positive airway pressure)* (CPAP) sobre la aparición de nuevos eventos cardiovasculares en pacientes con SAHS y síndrome coronario agudo (SCA).

5 ESTUDIO 1

Bonsignore MR, et al. Eur Respir J. 2012

5.1 HIPÓTESIS

Los pacientes con síndrome apnea-hipopnea del sueño (SAHS) presentan una elevada prevalencia de síndrome metabólico (SM). El SAHS y su gravedad se relacionan con la presencia de alteraciones metabólicas como el SM y la resistencia a la insulina (RI).

5.2 OBJETIVOS

- Determinar la prevalencia de SM en una cohorte de 535 pacientes con diagnóstico de SAHS y evaluar la relación existente entre las variables de sueño, la RI y otras alteraciones metabólicas.
- Determinar la relación entre la presencia de SM y sus componentes con la presencia de somnolencia diurna excesiva *(siglas en inglés de Excessive daytime sleepiness)* (EDS).

5.3 METODOLOGÍA

Este estudio de tipo transversal se realizó entre 2005-2007 en 535 pacientes con nuevo diagnóstico de SAHS del Hospital Son Dureta (Mallorca). Seis pacientes se excluyeron del análisis por no presentar datos que permitiesen valorar la presencia de SM.

El diagnóstico de SAHS se estableció a partir de un índice de apnea-hipopnea (IAH)$\geqslant$10/h. Los pacientes fueron clasificados en tres grupos en función de su IAH como leve (IAH<15/h), moderado (IAH=15-30/h) y grave (IAH>30/h). Se recogieron datos sociodemográficos, antropométricos (perímetro de cuello y de cintura, como marcador de obesidad central, e índice de masa corporal (IMC), variables de sueño (IAH, índice de *arousal*, saturación de oxígeno media y mínima) y marcadores bioquímicos. El diagnóstico de SM se realizó de acuerdo con el criterio III programa nacional de educación sobre el colesterol del panel de expertos en la detección, evaluación y tratamiento de la hipercolesterolemia en adultos (NCEP-ATP III) [62], que requiere la presencia de al menos tres de las siguientes condiciones: perímetro de cintura elevado ($\geqslant$94 cm en varones y $\geqslant$80 cm en mujeres), aumento de la presión arterial (>130/85 mmHg) o toma de fármacos para la hipertensión, hiperglucemia en ayunas (>100 mg/dl) o toma de hipoglucemiantes, triglicéridos aumentados ($\geqslant$150 mg/dl) o tratamiento para la dislipemia y disminución en la concentración de lipoproteína de alta densidad *(siglas en inglés de High density lipoprotein)* (HDL) (<50 mg/dl en varones y <40 mg/dl en mujeres) o tratamiento para la dislipemia. En 288 sujetos que carecían de diagnóstico previo de diabetes se determinó el nivel de insulina plasmática. La presencia de RI se calculó mediante el índice del modelo de evaluación homeostática *(siglas en inglés de Homestatic Model Assessment)* (HOMA) [88]. La EDS se definió a partir de la puntuación de la escala de Epworth validada al castellano (presencia de somnolencia $\geqslant$10 puntos) [89].

Tras realizar un riguroso control de los datos obtenidos, se procedió a realizar un estudio de normalidad. Se utilizaron las medias (desviación estándar) en el caso de las variables

cuantitativas o porcentajes válidos en el caso de las cualitativas para dar la información descriptiva univariada. En el análisis bivariado se utilizó el test T Student para datos no apareados o el test U Mann Whitney, en el caso de las variables que no presentaban una distribución normal, para comparar las características de : i) los pacientes en función del sexo; ii) los pacientes que no presentaban ningún componente de SM *vs.* el resto; iii) los pacientes con y sin presencia de SM y iv) los pacientes con y sin EDS. La comparación entre variables cualitativas se realizó mediante el test Chi cuadrado (o Test de Fisher en el caso de frecuencias <5). Algunas variables que no se distribuían normalmente, como es el caso del índice HOMA y los niveles de triglicéridos, se transformaron logarítmicamente para su análisis. Las relaciones lineales entre variables cuantitativas se analizaron mediante el test no paramétrico de Spearman, ya que algunas de las variables y biomarcadores presentaban cierta variabilidad. La tendencia lineal en el caso de las variables cualitativas se estudió mediante el test de Tau C de kendall.

Se realizó un estudio de las variables de confusión e interacción. Se incluyeron en los modelos multivariados aquellas variables clínicamente relevantes (cambio en la Odds Ratio (OR)>10 %). La interacción entre las variables se determinó a partir de las razones de verosimilitudes. Se construyeron modelos de regresión lineal crudos y ajustados por edad, consumo de tabaco activo, IMC y sexo para evaluar la relación entre el IAH y la saturación de oxígeno (variables independientes en los modelos) con cada uno de los componentes de SM (variables dependientes). Finalmente se construyó un modelo de regresión logística para identificar las variables relacionadas con la presencia de EDS a partir de un modelo por pasos. Se incluyeron en este modelo las variables que presentaban un valor p<0,20 en el análisis bivariado. Se asumió un valor p<0,05 como significativo en todos los análisis.

5.4 PRINCIPALES RESULTADOS

1. Se consideró un total de 529 pacientes con SAHS para el análisis (424 varones y 105 mujeres). Se observaron diferencias en función del sexo en el perímetro del cuello (42,8±3,5 cm *vs.* 37,3±3,8 cm, p<0,001) y en el IAH (44,8±26,7/h *vs.* 37,7±30,7/h, p=0,02), siendo superiores los valores en los varones.

2. La prevalencia de SM en la muestra de acuerdo con el criterio NCEP-ATP III fue del 52 %.

3. La gravedad del SAHS se relacionó significativamente con la presencia de SM Figura 5.1, siendo la prevalencia de SM superior en pacientes con SAHS grave. En la muestra, 55 sujetos no presentaban ningún componente del SM.

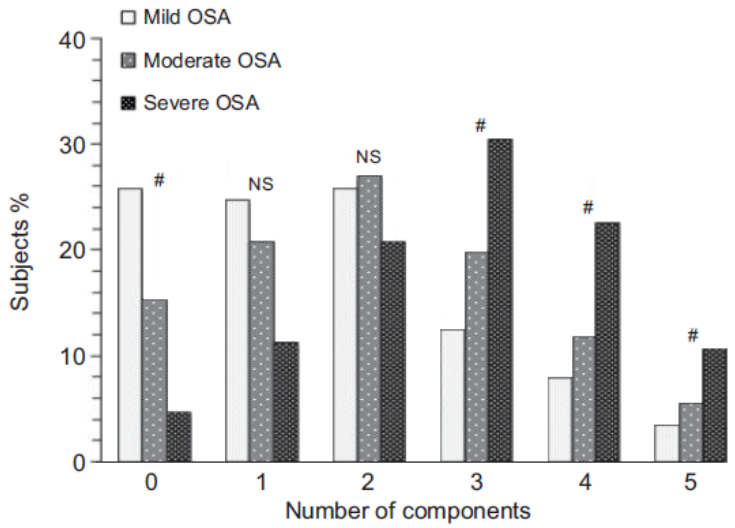

Figura 5.1: Gravedad del SAHS y número de componentes de SM.
SAHS leve (IAH<15/h), moderado (IAH 15-30/h) y grave (IAH>30/h).

4. Los marcadores de gravedad del SAHS (IAH y saturación de oxígeno media) mostraron relación con cada uno de los componentes de SM en los modelos crudos. Tras ajustar por las variables de confusión (edad, consumo de tabaco, IMC y sexo) sólo la presión sistólica (coeficiente beta=0,395, p=0,001) y diastólica (beta=0,495, p=0,001) se relacionaba con el IAH. En el caso de la saturación de oxígeno media, sólo el perímetro de cintura (beta=-0,076, p=0,03) y la presión sistólica (beta=-0,033, p=0,006) mantenían la relación tras ajustar.

5. Se observó tendencia lineal positiva entre el índice HOMA y el número de componentes de SM (r=0,455, p<0,001) Figura 5.2. Las correlaciones entre marcadores de gravedad del SAHS y el índice HOMA perdieron la significación tras ajustar por el IMC.

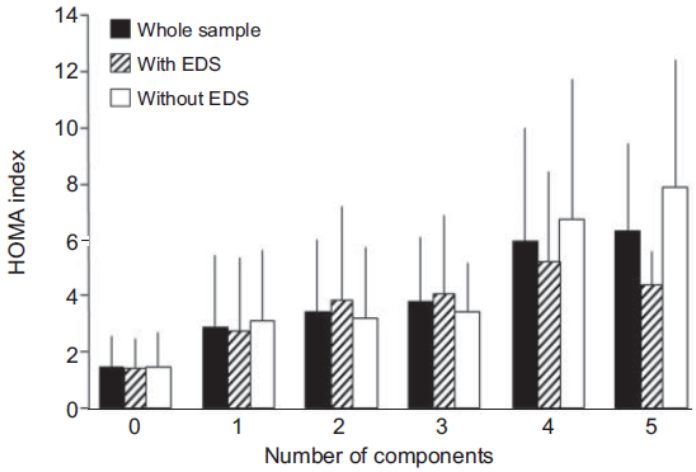

Figura 5.2: RI, medida por el índice HOMA.
Medida en 288 pacientes. (r=0,455, p<0,001).

6. La prevalencia de SM fue similar en el grupo de pacientes con y sin EDS (p=0,235). De la misma forma, tampoco se observaron diferencias significativas en la presencia de

EDS entre los pacientes que no presentaban ningún componente de SM y los que sí presentaban alguno de estos (9,9±5,1 *vs.* 9,7±5,1; p>0,05). Las relaciones entre el índice HOMA y el número de componentes de SM fueron comparables entre los pacientes con y sin EDS (r=0,466, p<0,001 y r=0,426, p<0,001, respectivamente) Figura 5.2.

7. El sexo (OR=1,033, IC95 %=1,013-1,054, p=0,001), el perímetro de cuello (OR=1,174, IC95 %=1,057-1,304, p=0,003), el índice de *arousal* (OR=1,027, IC95 %=1,005-1,048, p=0,014), el IMC (OR 1,083, IC95 %=1,010-1,162, p=0,03) y la saturación mínima de oxígeno (OR=0,953, IC95 %=0,910-0,997, p=0,036) se asociaron significativamente con el SM en un modelo de regresión múltiple multivariado. Al realizar el análisis estratificado en función del sexo, en los hombres, los marcadores de obesidad central y la hipoxia intermitente se asociaron significativamente con el SM, mientras que en las mujeres sólo se relacionaron la edad y el índice de *arousal*. En este modelo la EDS no se relacionó con el SM.

8. Al analizar las variables relacionadas con la EDS, la edad (OR=0,979, IC95 %=0,963-0,995, p=0,01) y la saturación de oxígeno media (OR=0,917, IC95 %=0,869-0,968, p=0,002) se relacionaron negativamente con la somnolencia, y explicaron el 20 % de su variabilidad.

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Metabolic syndrome, insulin resistance and sleepiness in real-life obstructive sleep apnoea

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ABSTRACT: The metabolic syndrome shows a variable prevalence in obstructive sleep apnoea (OSA), and its association with insulin resistance or excessive daytime sleepiness in OSA is unclear. This study assessed the following in consecutive patients with newly diagnosed OSA: 1) the prevalence of metabolic syndrome; and 2) its association with insulin resistance and daytime sleepiness.

Metabolic syndrome (National Cholesterol Education Program Adult Treatment Panel (NCEP-ATP) III criteria), insulin resistance (Homeostatic Model Assessment (HOMA) index, n=288) and daytime sleepiness (Epworth Sleepiness Scale) were assessed in 529 OSA patients.

The prevalence of metabolic syndrome was 51.2%, which increased with OSA severity. Each metabolic syndrome component correlated with apnoea/hypopnoea index, but only blood pressure retained significance after correction for confounders. Both obesity and OSA contributed to metabolic abnormalities, with different sex-related patterns, since diagnosis of metabolic syndrome was significantly associated with neck circumference, age, body mass index and lowest arterial oxygen saturation in males, and with age and arousal index in females. The number of metabolic syndrome components increased with HOMA index ($p<0.001$). Prevalence of sleepiness was the same in patients with and without metabolic syndrome.

The metabolic syndrome occurs in about half of ''real-life'' OSA patients, irrespective of daytime sleepiness, and is a reliable marker of insulin resistance.

KEYWORDS: Epidemiology, intermittent hypoxia, metabolism, sex

Obstructive sleep apnoea (OSA) is often associated with obesity, hypertension and other cardiovascular risk factors [1], and untreated patients with severe OSA show an increased risk for cardiovascular morbidity and mortality [2, 3]. However, since OSA and obesity frequently coexist, their respective role in increased cardiovascular risk is still debated.

Several studies have shown that insulin resistance occurs in OSA patients and directly correlates with OSA severity (for review see [4]). Besides obesity, OSA may play an independent role in the pathogenesis of insulin resistance, since intermittent hypoxia was shown to cause insulin resistance in healthy humans [5]. However, the available data are somewhat controversial, since the association of OSA and insulin resistance was mostly accounted for by obesity in at least four studies [6–9], and short-term treatment of OSA with continuous positive airway pressure (CPAP) failed to improve metabolic abnormalities [4].

The metabolic syndrome is a cluster of risk factors associated with insulin resistance, increased risk for type 2 diabetes [10] and increased overall and cardiovascular mortality [11]. Although its value in cardiovascular risk prediction is debated, the concept of metabolic syndrome has gained popularity and improved clinicians' awareness of metabolic problems in obese subjects [12]. According to the latest National Cholesterol Education Program Adult Treatment Panel (NCEP-ATP) III definition [13], the metabolic syndrome is diagnosed when at least three of the following conditions occur: increased waist circumference, as a marker of central obesity; increased blood pressure; fasting hyperglycaemia; increased serum triglyceride and decreased high-density lipoprotein (HDL) cholesterol concentrations.

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Prevalence of the metabolic syndrome in OSA patients according to the NCEP-ATP III definition was found to range between 23% and 87% [14–19]. Most studies included small numbers of patients and did not assess insulin resistance in conjunction with metabolic syndrome. One case–control study [7] and a cross-sectional population study [20] suggested that metabolic syndrome, but not insulin resistance, was associated with OSA. Surprisingly, the value of metabolic syndrome in predicting insulin resistance has not been specifically tested in OSA patients. Therefore, our first aim was to assess the prevalence of the metabolic syndrome in a large sample of consecutive OSA patients at diagnosis and to compute relationships between sleep characteristics, insulin resistance and metabolic abnormalities.

Excessive daytime sleepiness (EDS) is a major symptom of OSA. EDS in OSA patients was reported to be associated with hypertension [21], altered autonomic modulation [22] and type 2 diabetes [23]. EDS was among the factors significantly associated with OSA and metabolic syndrome in the recent study by AGRAWAL *et al.* [18]. Two case–control studies have found that EDS predicted insulin resistance in OSA patients independently of obesity [24, 25]; only sleepy patients showed improved insulin sensitivity after CPAP treatment for 3 months [24]. Conversely, other studies have found a similar degree of subjective sleepiness in metabolic syndrome patients with or without OSA [26]. Therefore, the second aim of the current investigation was to assess the characteristics of OSA patients reporting daytime sleepiness and whether EDS is associated with metabolic syndrome in a large series of OSA patients.

METHODS

Patients

Consecutive patients referred to the Sleep Laboratory, Hospital Son Dureta, Palma de Mallorca, Spain, in the years 2005–2007 were studied (n=535). The inclusion criteria were age >18 yrs, diagnosis of OSA and wish to participate to the study. No eligible patient refused to participate. Six patients were excluded due to missing data, reducing the sample to 529 patients. The study protocol was approved by the local Institutional Review Board (approval number IB741/09PI), and all participants gave their informed written consent.

Sleep study

OSA was diagnosed by full polysomnography (E-Series Compumedics, Abbotsford, Australia) that included recording of oronasal flow, thoraco-abdominal movements, ECG, submental and pretibial electromyography, electrooculogram, electroencephalogram and pulse oximetry, as previously described [22]. Apnoea was defined by absence of airflow lasting ⩾10 s. Hypopnoea was defined as any airflow reduction lasting ⩾10 s associated with either oxygen desaturation ⩾4% or arousal and oxygen desaturation ⩾3%. The apnoea/hypopnoea index (AHI) and the arousal index (ArI) were defined as the number of apnoeas and hypopnoeas, and of arousals, respectively, per hour of sleep. OSA was diagnosed if AHI was ⩾10 events·h^{-1}. EDS, quantified by the Epworth Sleepiness Scale (ESS), was defined as an ESS score ⩾10.

Metabolic syndrome

As a general measure of obesity, body mass index (BMI) was defined as weight divided by $height^2$ (in kg·m^{-2}). Neck and waist circumferences (in cm) were also measured. The metabolic syndrome was diagnosed based on the presence of three or more of the following factors: waist circumference ⩾80 cm in females and ⩾94 cm in males (all patients were Caucasian); serum triglycerides ⩾150 mg·dL^{-1} or lipid-lowering treatment; HDL cholesterol <40 mg·dL^{-1} in males, <50 mg·dL^{-1} in females, or lipid-lowering treatment; increased blood pressure or anti-hypertensive treatment; and fasting blood glucose >100 mg·dL^{-1} or anti-diabetic treatment [13].

Office blood pressure was measured by a standard mercury sphygmomanometer while the subject was quietly seated after ⩾5 min of rest. Increased blood pressure was recorded if systolic blood pressure was >130 mmHg or diastolic pressure was >85 mmHg, or the patient was on anti-hypertensive treatment.

Fasting venous blood samples were obtained in the morning after polysomnography. Glucose, triglycerides, total cholesterol and HDL cholesterol were determined by standard enzymatic methods on a Hitachi Modular analyser (Roche Diagnostics, Indianapolis, IN, USA). In 288 patients without previously known diabetes, plasma insulin concentration was measured by chemiluminescent assays on an Immulite 2000 analyser (Siemens Medical Solutions Diagnostics, New York, NY, USA). Insulin resistance was calculated using the Homeostatic Model Assessment (HOMA) index [27].

Statistical analysis

Data are presented as mean±SD; categorical data are shown as percentage of positive patients.

Unpaired t-tests (for numerical variables) or nonparametric Mann–-Whitney U-tests (for variables that were not normally distributed) were used to compare: 1) patient characteristics according to sex; 2) patients without any metabolic syndrome components *versus* all other patients; 3) patients with and without a diagnosis of metabolic syndrome; and 4) patients with and without EDS. Frequencies were compared by the Chi-squared test for categorical variables (Fisher's exact test with observed frequencies <5). Due to non-normal distribution, HOMA index and serum triglyceride values were analysed after logarithmic transformation.

Trends were analysed by the Spearman rank test, or the Kendall Tau-c test for categorical variables. Multiple linear regression was used to assess the relationship between AHI, ArI and arterial oxygen saturation (S_{a,O_2}) as independent variables and each metabolic syndrome component as dependent variable.

The multivariate logistic regression model was used to assess determinants of metabolic syndrome and EDS. To this aim, we used anthropometric and sleep variables together with the variables showing $p<0.20$ in bivariate analysis. Variables were selected using a stepwise approach. A p-value <0.05 was considered significant. The SPSS version 17 software (SPSS Inc., Chicago, IL, USA) was used for all analyses.

RESULTS

Metabolic syndrome

Total sample and sex-specific characteristics are reported in table 1. OSA patients were mostly male and obese. Females accounted for about one-fifth of the total sample (n=105). Neck

TABLE 1 Anthropometric and sleep characteristics of the sample

	All patients	Males	Females
Subjects n	529	424	105
Age yrs	51.3±12.8	50.9±13.0	52.9±12.0
Hypertension	35.2	36.6	29.5
Dyslipidaemia	32.5	35	30.0
Diabetes mellitus	17.0	16.5	19
BMI kg·m^{-2}	30.8±6.0	30.6±5.4	31.6±7.9
Obesity#	49	48.3	51.4
Neck circumference cm	41.6±4.1	42.7±3.5	37.3±3.8***
AHI events·h^{-1}	43.4±27.6	44.8±26.7	37.7±30.7+
ArI events·h^{-1}	51.5±23.2	52.4±22.6	47.8±25.3
Mean nocturnal S_{a,O_2} %	92.3±4.0	92.1±4.0	93.0±4.0
Lowest S_{a,O_2} %	80.8±9.3	80.5±9.3	81.6±9.4
ESS score	9.7±5.1	9.8±5.0	9.5±5.5
Daytime sleepiness¶	51.8	51.4	53.3
Diagnosis of metabolic syndrome	51.2	51.4	50.5
High blood pressure	54.6	55.4	51.4
High waist circumference	72	70.8	77.1
Low HDL cholesterol	26.8	26.7	27.6
High triglyceride level	45.9	47.6	39
High fasting blood glucose	49.9	50.7	46.7

Data are presented as mean±SD or %, unless otherwise stated. BMI: body mass index; AHI: apnoea/hypopnoea index; ArI: arousal index; S_{a,O_2}: arterial oxygen saturation; ESS: Epworth Sleepiness Scale; HDL: high-density lipoprotein. #: BMI ⩾30 kg·m^{-2}; ¶: ESS ⩾10. ***: p<0.001; +: p=0.02 for difference between sexes by unpaired t-test or Chi-squared analysis.

circumference and AHI were significantly higher in males than in females. Current smokers accounted for 29% of the subjects, while chronic obstructive pulmonary disease or cardiovascular disease (coronary artery disease and heart failure) occurred in 6.6% and 5.9% of the subjects, respectively, without differences between sexes (data not shown).

The overall prevalence of the metabolic syndrome was 51.2%. The number of metabolic syndrome components increased with OSA severity (table 2 and fig. 1). Mild, moderate and severe OSA patients showed significantly different distributions of metabolic syndrome components, with mild patients often being free of any metabolic syndrome component, and severe patients frequently showing three to five metabolic syndrome components. Patients with one or two metabolic syndrome components were classified with all degrees of OSA severity without any specific distribution pattern (fig. 1).

The distribution and prevalence of metabolic syndrome components in the sample are shown in figure 2. The most common combinations of metabolic syndrome components included increased waist circumference, hypertension and abnormal fasting blood glucose (table S1).

Some patients (n=55) showed no metabolic syndrome components. Compared with patients with one or more metabolic syndrome components, they were non-obese, had mild to moderate OSA, were significantly younger (table S2), and included more active smokers (data not shown).

Markers of OSA severity (AHI and mean S_{a,O_2}) (table 3) showed significant unadjusted linear relationships with each component of the metabolic syndrome. Most of the significance was lost for AHI after adjustments for age, BMI, smoking and

TABLE 2 Progressive metabolic impairment is associated with increasing obstructive sleep apnoea severity but not with increasing excessive daytime sleepiness

	Number of metabolic syndrome components						Rho/tau
	0	1	2	3	4	5	
Subjects n	55	81	121	134	94	44	
Age yrs	40.9±13.1	46.6±12.0	52.5±12.5	52.9±11.8	54.3±11.5	55.9±13.4	0.305***
Diabetes mellitus	0	0	10	20.5	36.4	47.7	0.338***
BMI kg·m^{-2}	24.5±2.7	28.2±4.6	29.7±5.2	32.5±5.5	34.2±6.1	34.4±5.6	0.548***
Obesity#	3.7	28.0	48.6	68.6	81	84.6	0.517***
Neck circumference cm	37.5±3.2	39.6±3.7	41.1±3.3	42.3±3.7	44.2±4.1	43.8±4.0	0.462***
AHI events·h^{-1}	22.9±17.6	31.5±20.8	36.8±23.5	49.9±26.8	56.2±28.1	61.9±31.2	0.428***
ArI events·h^{-1}	34.3±16.9	41.6±17.0	46.1±20.7	56.2±22.6	64.2±23.5	65.0±22.4	0.428***
Mean nocturnal S_{a,O_2} %	95.3±2.0	94.1±2.3	93.2±2.8	91.5±4.1	90.2±4.6	90.2±4.9	-0.466***
Lowest S_{a,O_2} %	87.1±5.7	84.6±6.7	83.1±7.2	78.2±9.4	77.6±9.8	74.3±11.5	-0.419***
ESS score	9.9±5.2	9.7±5.3	9.0±4.9	9.7±5.1	10.5±4.9	9.7±5.6	0.036
Systolic blood pressure mmHg	113.3±8.5	121.7±13.7	130.3±16.9	135.0±15.2	139.3±17.7	144.1±10.5	0.522***
Diastolic blood pressure mmHg	68.0±9.0	74.6±9.4	79.6±11.5	82.6±12.3	85.2±11.2	86.8±10.7	0.429***
Waist circumference cm	88.9±9.3	99.8±11.5	105.3±11.1	112.4±12.0	115.4±13.1	114.8±11.5	0.575***
HDL cholesterol mg·dL^{-1}	60.1±13.6	57.1±14.9	57.4±22.7	51.3±10.6	49.8±22.3	41.6±11.7	-0.363***
Triglycerides mg·dL^{-1}	85.6±31.4	113.3±58.0	129.8±54.2	160.0±74.3	205.5±94.8	245.8±132.1	0.598***
Fasting blood glucose mg·dL^{-1}	88.5±6.6	91.8±6.5	101.8±26.1	110.1±24.7	117.7±27.5	128.7±30.0	0.614***

Data are presented as mean±SD or %, unless otherwise stated. BMI: body mass index; AHI: apnoea/hypopnoea index; ArI: arousal index; S_{a,O_2}: arterial oxygen saturation; ESS: Epworth Sleepiness Scale; HDL: high-density lipoprotein. #: BMI ⩾30 kg·m^{-2}. ***: p<0.001 for trend.

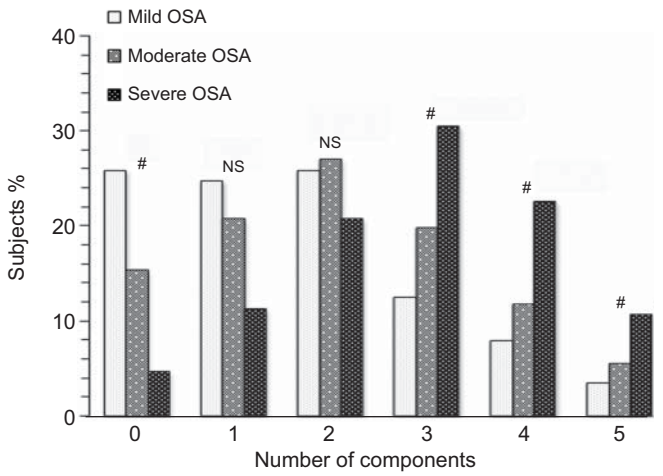

FIGURE 1. Obstructive sleep apnoea (OSA) severity and number of metabolic syndrome components. Patients with mild OSA (apnoea/hypopnoea index (AHI) <15 events·h^{-1}) were often free of any metabolic syndrome component. Conversely, patients with severe OSA (AHI >30 events·h^{-1}) had a metabolic syndrome diagnosis (*i.e.* three or more components) more frequently than the other two groups. Distribution of mild, moderate and severe OSA did not differ in patients with one or two metabolic syndrome components. #: p<0.0001 by Chi-squared test; NS: nonsignificant.

sex, except for systolic and diastolic blood pressure. Mean S_{a,O_2} remained significantly associated with waist circumference and diastolic blood pressure after adjustments. ArI and lowest S_{a,O_2} were also analysed, with results similar to those obtained for AHI and mean S_{a,O_2}, respectively (table S3).

Insulin resistance

Insulin resistance, estimated by the HOMA index in 288 patients, increased with increasing number of metabolic syndrome components (Spearman's rho 0.455; p<0.001) (fig. 3 and table 4). The HOMA index correlated positively with AHI and ArI, and negatively with lowest or mean S_{a,O_2} in unadjusted bivariate analysis (p<0.0001) (data not shown). All such relationships became nonsignificant after adjustment for BMI and were unaffected by sex (data not shown).

Sleepiness

The prevalence of the metabolic syndrome, or of each of its components, was similar in patients with and without EDS (fig. 2). The relationship between HOMA index and number of metabolic syndrome components was comparable in OSA patients with (0.466; p<0.001) and without EDS (0.426; p<0.001) (fig. 3). ESS and HOMA index did not show any significant relationship. Patients free from any metabolic syndrome component showed a similar EDS to the rest of the sample (table S2). No difference was found in EDS between patients with and without a metabolic syndrome diagnosis (table 5). Patients with EDS were younger, showed a slightly higher waist circumference and worse polysomnographic variables, but similar blood pressure and other metabolic variables compared with nonsleepy patients (table 5).

Logistic regression analysis

To assess the factors associated with the metabolic syndrome in the whole sample, the following variables were entered into the model: EDS, BMI, neck circumference, sex, age, AHI, ArI, apnoea index, lowest and mean nocturnal S_{a,O_2}. The metabolic syndrome in OSA was significantly associated with the following: sex (OR 1.033, 95% CI 1.013–1.054; p=0.001); neck circumference (OR 1.174, 95% CI 1.057–1.304; p=0.003); ArI (OR 1.027, 95% CI 1.005–1.048; p=0.014); BMI (OR 1.083, 95% CI 1.010–1.162; p=0.03); lowest nocturnal S_{a,O_2} (OR 0.953, 95% CI 0.910–0.997; p=0.036). Age showed a strong trend (OR 1.033, 95% CI 0.998–5.3; p=0.05), while EDS did not contribute significantly (OR 1.220, 95% CI 0.780–1.907; p=nonsignificant).

To better explore sex-related differences, the analysis was repeated separately in males and females. In males, the metabolic syndrome was significantly associated with neck circumference, age, BMI and lowest nocturnal S_{a,O_2}, with the regression accounting for 42.3% of the variability. In females, ArI and age explained 52.4% of metabolic syndrome variability, and lowest nocturnal S_{a,O_2} showed a strong trend for association with metabolic syndrome (p=0.053).

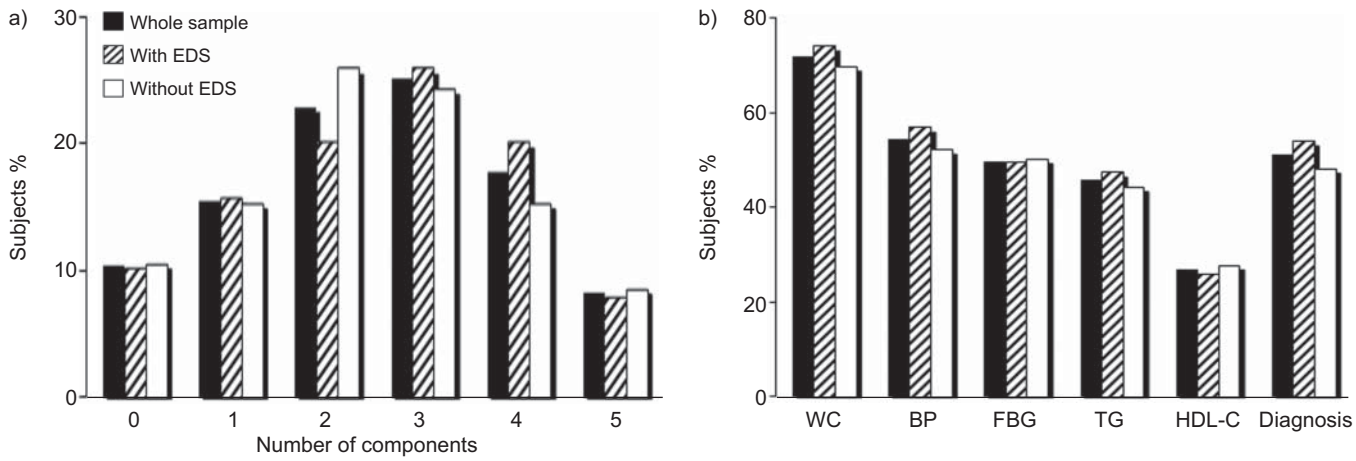

FIGURE 2. Distribution of a) the number of metabolic syndrome components and b) the prevalence of each metabolic syndrome component and of metabolic syndrome diagnosis. No significant difference was found between patients with and without excessive daytime sleepiness (EDS) for any variable. WC: increased waist circumference; BP: elevated blood pressure; FBG: elevated fasting blood glucose; TG: elevated triglycerides; HDL-C: decreased high-density lipoprotein cholesterol.

TABLE 3 Relationships between each metabolic syndrome component and obstructive sleep apnoea severity assessed by apnoea/hypopnoea index (AHI) or mean nocturnal arterial oxygen saturation (S_{a,O_2})

	AHI						Mean S_{a,O_2}					
	Unadjusted		Model 1#		Model 2¶		Unadjusted		Model 1#		Model 2¶	
	β	p-value	β	p-value	β	p-value	β	p-value	β	p-value	β	p-value
Waist circumference	1.067	<0.001	0.409	<0.001	0.258	NS	-0.155	<0.001	-0.076	<0.001	-0.050	0.03
Systolic blood pressure	0.627	<0.001	0.414	<0.001	0.395	0.001	-0.056	<0.001	-0.020	0.03	-0.016	NS
Diastolic blood pressure	0.752	<0.001	0.524	<0.001	0.495	0.001	-0.069	<0.001	-0.039	<0.001	-0.033	0.006
Fasting blood glucose	0.214	<0.001	0.013	NS	0.012	NS	-0.036	<0.001	-0.009	NS	-0.009	NS
Serum triglycerides+	10.401	<0.001	1.517	NS	0.575	NS	-1.952	<0.001	-0.599	0.04	-0.442	NS
HDL cholesterol	-0.161	0.02	-0.010	NS	0.037	NS	0.037	<0.001	0.018	0.03	0.011	NS

HDL: high-density lipoprotein. #: adjusted for age, smoking and body mass index (BMI); ¶: adjusted for age, smoking, BMI and sex; +: log-transformed. NS: nonsignificant.

Factors associated with EDS in the entire sample were also analysed. Age (OR 0.979, 95% CI 0.963–0.995; p=0.01) and mean nocturnal S_{a,O_2} (OR 0.917, 95% CI 0.869–0.968; p=0.002) were negatively associated with EDS, and explained 20% of EDS variability.

DISCUSSION

In a large ''real-life'' sample of OSA patients at diagnosis, the metabolic syndrome according to NCEP-ATP III criteria occurred in about half of the subjects, and severe OSA was significantly associated with a diagnosis of metabolic syndrome, *i.e.* occurrence of three or more components of the syndrome. An increase in the number of metabolic syndrome components was associated with worse insulin resistance; correlations between markers of OSA severity and the HOMA index became nonsignificant after adjusting for BMI, indicating a major role of obesity in the relationship between OSA and insulin resistance. Each of the metabolic syndrome components showed crude linear relationships with markers of OSA severity but, after adjustment for confounders, AHI remained significantly correlated only to blood pressure, and mean S_{a,O_2} remained correlated only to waist circumference and diastolic blood pressure. Therefore, the metabolic syndrome was associated with both obesity- and OSA-related variables. EDS was shown to be a poor clinical marker of metabolic abnormalities, and nocturnal intermittent hypoxia and age explained a small fraction of EDS variability.

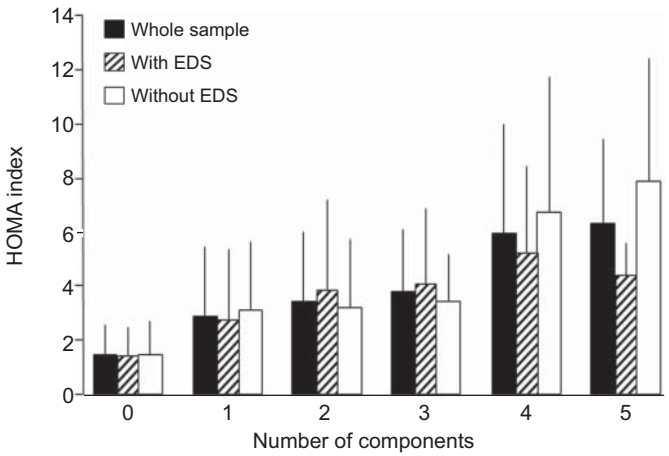

FIGURE 3. Insulin resistance, assessed as Homeostatic Model Assessment (HOMA) index in 288 patients, linearly increased with the number of metabolic syndrome components (Spearman's rho 0.455; p<0.001). No significant differences were found between patients with and without excessive daytime sleepiness (EDS). The number of subjects in each group can be found in table 4.

Prevalence of the metabolic syndrome

Clinical studies in OSA patients have reported variable prevalence of the metabolic syndrome according to NCEP-ATP III criteria, with values ranging between 23% and 87% [14–19]. The largest published series (819 Japanese patients) found prevalence of the metabolic syndrome to be 49.5% in males and 32% in females [15]. Our results are similar for male patients, but the prevalence rate of metabolic syndrome in our female patients was higher, possibly due to more severe obesity in our sample.

Case–control studies have reported variable prevalence rates of the metabolic syndrome, or of insulin resistance and metabolic syndrome components [7, 28]. Increased waist circumference, hypertension and increased fasting blood glucose were the

TABLE 4 Subjects assessed by the Homeostatic Model Assessment (HOMA) index (see fig. 3)

Number of components	Whole sample	With EDS	Without EDS
0	43	24	19
1	51	24	27
2	80	40	40
3	72	43	29
4	34	20	14
5	8	4	4

Data are presented as n. Subjects were grouped according to the number of metabolic syndrome components and assessment of excessive daytime sleepiness (EDS).

TABLE 5 Comparisons in the entire sample# between patients with and without a diagnosis of metabolic syndrome¶ and with and without excessive daytime sleepiness (EDS)+

	Metabolic syndrome		EDS	
	Without	With	Without	With
Subjects n	258	271	255	274
Age yrs	48.1±13.3	54.4±11.0*	52.6±13.1	50.1±13.2*
BMI kg·m^{-2}	28.1±4.9	33.7±3.9*	30.3±5.2	31.2±6.6
Neck circumference cm	39.9±3.6	43.1±3.9*	41.3±4.0	41.8±4.2
AHI events·h^{-1}	32.2±22.1	54.0±28*	40.3±25.9	46.2±28.9*
ArI events·h^{-1}	42.1±19.3	60.4±23.2*	49.5±21.8	53.3±24.3
Lowest S_{a,O_2} %	84.5±7.7	77.1±10.1*	82.0±8.9	79.6±9.7*
ESS score	9.4±5.0	10.0±5.1	5.3±2.4	13.9±3.0
Systolic blood pressure mmHg	124.0±15.3	138.2±16.6*	130.7±18.0	131.5±16.4
Diastolic blood pressure mmHg	75.8±10.8	85.6±12.2*	79.1±11.7	80.8±12.8
Waist circumference cm	100.1±12.5	113.8±12.3*	105.8±13.0	108.4±15.1*
HDL cholesterol mg·dL^{-1}	58±19	49±16*	54±18	53±18
Triglycerides mg·dL^{-1}	115±54	189±98*	151±89	155±87
Fasting blood glucose mg·dL^{-1}	95.0±19.3	115.8±27.3	105.8±13.0	108.4±15.2

Data are presented as mean±SD, unless otherwise stated. BMI: body mass index; AHI: apnoea/hypopnoea index; ArI: arousal index; S_{a,O_2}: arterial oxygen saturation; ESS: Epworth Sleepiness Scale; HDL: high-density lipoprotein. #: n=529; ¶: three or more components; +: ESS ⩾10. *: significant difference for with *versus* without ($p<0.05$).

commonest metabolic syndrome components in the present study; these findings agree with those reported by KONO *et al.* [28]. Conversely, the study by SASANABE *et al*. [15] reported dyslipidaemia as the third most frequent finding. Finally, population-based studies yielded a prevalence of metabolic syndrome in OSA of between 26% and 35% [29–31]. A recent population study reported a 44% prevalence of metabolic syndrome in females with AHI >15 events·h^{-1} [32].

Components of the metabolic syndrome and insulin resistance

The metabolic syndrome is considered to be the clinical manifestation of insulin resistance [10, 13]. Two studies, however, reported that insulin resistance may not be associated with metabolic syndrome in OSA patients. In the Turkish population, OSA in males was associated with metabolic syndrome but not with insulin resistance [20]. A case–control study reported similar results [7]. The linear increase of the HOMA index with an increasing number of metabolic syndrome components, as found in our study, suggests that this number, known as the metabolic index, may be a clinically useful indicator of the metabolic load in OSA patients. When the data were stratified for OSA severity, occurrence of one or two components of the metabolic syndrome showed similar frequencies in patients with mild, moderate or severe OSA, whereas a diagnosis of metabolic syndrome (*i.e.* three or more components) was more frequent in severe OSA, and absence of any metabolic syndrome component prevailed in mild OSA. These findings agree with those reported by THEORELL-HAGLÖW *et al*. [32] in a population-based study on females, indicating that the association between OSA and metabolic syndrome is especially strong in patients with severe OSA.

The number of metabolic syndrome components carries prognostic implications. Both all-cause and cardiovascular mortality increased with the number of metabolic syndrome components [11], hypertension being the most potent factor, followed by central obesity and hypertriglyceridaemia. Other studies found increased risk only in patients with three or more metabolic syndrome components [33], or reported that a diagnosis of metabolic syndrome was not superior to the sum of individual risk factors in predicting cardiovascular mortality [34], severity of vascular lesions or progression of atherosclerosis [35]. No longitudinal data are yet available on the prognostic significance of metabolic syndrome components in OSA patients.

Approximately 10% of our patients did not show any metabolic syndrome component. This subset differed from the rest of the sample, as the subjects were younger, non-obese and had mild OSA. It is possible that absence of metabolic defects represents an early stage in the natural history of OSA, but longitudinal studies are necessary to test this hypothesis. Alternatively, these patients may represent a distinct, still incompletely characterised, clinical OSA phenotype. Interestingly, the metabolic effects of intermittent hypoxia were recently shown to be quite small in lean mice [36]. If the same results were to be shown in humans with OSA, the patients without any metabolic syndrome component may represent a low-risk subgroup, with obvious consequences regarding treatment. Some studies, however, found metabolic abnormalities in non-obese OSA patients, although a diagnosis of metabolic syndrome was not fulfilled [19, 28, 37]. Conversely, both morbidly obese patients [38] and patients with the metabolic syndrome [26] showed worse metabolic variables associated with severe OSA compared with subjects without OSA.

Relationships between metabolic syndrome, OSA and obesity

Our study found that age, lowest S_{a,O_2}, BMI, neck circumference and ArI were significantly associated with metabolic

syndrome by multiple regression analysis, suggesting an independent role of OSA in addition to obesity. Neck circumference was found to independently predict cardiovascular risk in a large population-based study [39], but prevalence of OSA was not assessed, indicating the need to further study the impact of fat distribution on the complex relationship between OSA, obesity and metabolism. Sex may also play a role, as suggested by the results obtained by separate analysis of males and females. In males, markers of central obesity and intermittent hypoxia were significantly associated with metabolic syndrome, whereas in females only age and ArI were significant factors, possibly suggesting a major role of sleep fragmentation in females.

Sleepiness and metabolic syndrome

EDS has been proposed as a marker of OSA severity, especially for cardiovascular and metabolic outcomes [21–25]. Two case–control studies reported that EDS in OSA was associated with insulin resistance, suggesting that it could be used clinically as a marker of cardiometabolic abnormalities. Other studies, however, did not confirm such findings [26, 40]. In our study, EDS correlated negatively with age and mean nocturnal S_{a,O_2}, and did not affect the relationship between OSA and metabolic variables. The discrepancy between the results of case–control studies and our ''real-life'' study probably stems from the characteristics of the samples, since highly selected patients without comorbidities were examined in the studies by Barceló *et al*. [24] and Nena *et al*. [25]. We acknowledge that the ESS does not assess sleepiness objectively, and lack of multiple sleep latency test data is a major limitation of our study.

Conclusions

The metabolic syndrome according to NCEP-ATP III criteria occurs in about half of OSA patients at diagnosis, an additional 38.2% of patients showing one or two metabolic syndrome components. The number of metabolic syndrome components correlated with the HOMA index in OSA patients, and can be used as a clinical marker of insulin resistance. EDS, however, did not turn out to be a sensitive clinical marker of a detrimental metabolic profile in real-life OSA patients.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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Supplementary tables, ERJ 1511-2010.R2

Bonsignore et al. Metabolic Syndrome, Insulin Resistance and Sleepiness in Real-Life Obstructive Sleep Apnea

Table A. Frequency of different combinations of MetS components in the entire sample

MetS components n=1 (81 patients)

Waist circumference	48.8%
Elevated BP	26.2%
Triglycerides	13.4%
Glucose	7.3%
HDL-Cholesterol	3.7%

MetS components n= 2 (121 patients)

Waist + Elevated BP	33.9%
Waist + Glucose	19.8%
Waist + Tryglycerides	14.0%
Glucose + Elevated BP	11.6%
Waist + HDL-Cholesterol	5.8%
Glucose + Tryglicerides	5.8%
HDL-Cholesterol+Tryglicerides	5.0%
Other combinations	4.1%

MetS components n=3 (134 patients)

Waist + Elevated BP + Glucose	41.4%
Waist + Elevated BP + Tryglycerides	19.0%
Waist + Tryglycerides + Glucose	18.8%
Waist + Tryglycerides + HDL-Cholesterol	6.0%
Waist + Elevated BP + HDL-Cholesterol	4.5%
Glucose + Tryglycerides + HDL-Cholesterol	4.5%
Glucose + Elevated BP + Tryglycerides	3.0 %
Other combinations	2.8%

MetS components n=4 (94 patients)

Waist + Elevated BP + Glucose + Tryglycerides	43.0%
Waist + Glucose + Tryglycerides +HDL-Cholesterol	24.5%
Waist + Elevated BP + Tryglycerides + HDL-Cholesterol	19.1%
Waist + Elevated BP + Glucose + HDL-Cholesterol	7.4%
Other combinations	6.0%

Table B. Comparisons in the entire sample between patients without any MetS components and patients with one or more MetS components

All patients (n=529)	No MetS component (n=55, 79 % M)	1+ MetS components (n=474, % M)
Age (yr)	40.7 ± 13.0	52.6 ± 12.3*
Boby Mass Index (kg/m^2)	24.8 ± 2.6	31.6 ± 5.8*
Neck circumference (cm)	37.8 ± 3.2	42.0 ± 3.9*
AHI (events/h)	22.9 ±19.2	46.2 ± 27.6*
Arousal Index (events/h)	34.3 ± 16.8	53.5 ± 23.0*
Lowest SaO2 (%)	87.1 ± 5.4	80.1 ± 9.4*
Epworth Sleepiness Score	9.9 ± 5.1	9.7 ± 5.1
Systolic blood pressure (mmHg)	113.2 ± 12.0	133.7 ± 16.9*
Diastolic Blood Pressure (mmHg)	67.9 ± 9.7	82.4 ± 12.1*
Waist circumference (cm)	88.9 ± 9.0	108.0 ± 13.1*
HDL-Cholesterol (mg/dl)	60 ± 15	53 ± 18*
Triglycerides (mg/dl)	85 ±3 2	162 ± 89*
Fasting Blood Glucose (mg/dl)	90.2 ± 7.9	108.6 ± 26.7*

Table C. Summary of unadjusted and adjusted beta coefficients between each MetS component and arousal index or lowest nocturnal SaO2

	Arousal Index unadjusted	p	**Age, smoking and BMI**	p	**Age, smoking, BMI and gender**	p
Waist circumference	1.067	<0.001	0.409	<0.001	0.258	NS
Systolic Blood Pressure	0.627	<0.001	0.414	<0.001	0.395	0.001
Diastolic Blood Pressure	0.752	<0.001	0.524	<0.001	0.495	0.001
Fasting Blood Glucose	0.214	<0.001	0.013	NS	0.012	NS
Serum Tryglicerides (log-transf)	10.401	<0.001	1.517	NS	0.575	NS
HDL-cholesterol	-0.161	0.02	-0.010	NS	0.037	NS

	Lowest SaO2 unadjusted	p	**Age, smoking and BMI**	p	**Age, smoking, BMI and gender**	p
Waist circumference	-0.155	<0.001	-0.076	<0.001	-0.050	0.03
Systolic Blood Pressure	-0.056	<0.001	-0.020	0.03	-0.016	NS
Diastolic Blood Pressure	-0.069	<0.001	-0.039	<0.001	-0.033	0.006
Fasting Blood Glucose	-0.036	<0.001	-0.009	NS	-0.009	NS
Serum Tryglicerides (log-transf)	-1.952	<0.001	-0.599	0.04	-0.442	NS
HDL-cholesterol	0.037	<0.001	0.018	0.03	0.011	NS

6 ESTUDIO 2

Barceló A, et al. Eur Respir J. 2011

6.1 HIPÓTESIS

Los pacientes con síndrome apnea-hipopnea del sueño (SAHS) presentan niveles plasmáticos elevados de ácidos grasos (AG) libres; estos podrían contribuir a la presencia de síndrome metabólico (SM).

6.2 OBJETIVOS

- Evaluar los niveles plasmáticos de AG libres en pacientes con SAHS y determinar su posible relación en la presencia del SM.

6.3 METODOLOGÍA

En el segundo estudio de tipo trasversal, se incluyó de manera consecutiva un total de 119 pacientes con nuevo diagnóstico de SAHS y 119 sujetos como grupo control similar en cuanto a edad (±5 años), sexo e índice de masa corporal (IMC) (±3 $^{kg}/_{m^2}$) con el objetivo de evaluar si los niveles plasmáticos de AG libres eran superiores en el grupo SAHS y si estos niveles podían contribuir a la presencia de SM. El reclutamiento se realizó en la Unidad de Sueño del Hospital Son Dureta (Mallorca) durante los años 2008 y 2009.

El diagnóstico de SAHS se determinó a partir de un índice de apnea-hipopnea (IAH)$\geqslant$10/h. Los pacientes fueron clasificados en tres grupos en función de su IAH: pacientes con SAHS leve (IAH=10-20/h), moderado (IAH=21-40/h) y grave (IAH>40/h). El diagnóstico de SM se realizó de acuerdo con el criterio III programa nacional de educación sobre el colesterol del panel de expertos en la detección, evaluación y tratamiento de la hipercolesterolemia en adultos (NCEP-ATP III) [62], que requiere la presencia de al menos tres de las siguientes condiciones: perímetro de cintura elevado ($\geqslant$94 cm en varones y $\geqslant$80 cm en mujeres), aumento de la presión arterial (>130/85 mmHg) o toma de fármacos para la hipertensión, hiperglucemia en ayunas (>100 mg/dl) o toma de hipoglucemiantes, triglicéridos aumentados ($\geqslant$150 mg/dl) o tratamiento para la dislipemia y disminución en la concentración de lipoproteína de alta densidad *(siglas en inglés de High density lipoprotein)* (HDL) (<50 mg/dl en varones y <40 mg/dl en mujeres) o tratamiento para la dislipemia.

Se utilizó el test T Student para datos no apareados o el test de ANOVA (en el caso de las variables cuantitativas con más de 2 categorías). En algún caso se realizó un análisis *post-hoc* con múltiples comparaciones mediante el test de Scheffe. La comparación entre variables cualitativas se realizó mediante el test Chi cuadrado. Se construyó un modelo multivariado de regresión lineal para detectar las variables relacionadas con la concentración plasmática de AG (variable dependiente). En el modelo se incluyeron como variables independientes el grupo de estudio (SAHS, controles), la edad, el sexo, el IMC y el IAH. En todos los tests se consideró como significativo un valor de $p<0{,}05$.

6.4 PRINCIPALES RESULTADOS

1. La prevalencia de SM fue superior en los pacientes con SAHS que en los controles de la misma edad, sexo e IMC (38 % *vs.* 21 %, p=0,006).

2. La concentración plasmática de AG fue significativamente superior en el grupo SAHS (12,2±5 mg/dl *vs.* 10,5±5 mg/dl en el grupo control; p=0,015) Figura 6.1. Esta relación se mantuvo en los pacientes sin SM (p=0,04), mientras que en aquellos con SM no se detectaron diferencias en la concentración plasmática de AG entre los pacientes con SAHS y los controles (p=0,271). El grupo SAHS presentaba también concentraciones plasmáticas alteradas de glucosa, gamma glutamil transpeptidasa, proteína C reactiva y 8-isoprostanos Figura 6.1.

	Controls	OSAS	p-value
Subjects n	119	119	
Glucose mg·dL^{-1}	94±4	103±22	0.001
Triglycerides mg·dL^{-1}	124±51	147±94	0.079
Cholesterol mg·dL^{-1}	207±41	212±39	0.398
HDLc mg·dL^{-1}	56±15	55±16	0.505
Creatinine mg·dL^{-1}	0.88±0.2	0.96±0.3	0.692
Uric acid mg·dL^{-1}	6.2±4.7	6.1±3.2	0.336
AST U·L^{-1}	22±7	21±7	0.387
ALT U·L^{-1}	27±15	27±13	0.809
GGT U·L^{-1}	32±27	37±29	0.048
CRP U·L^{-1}	1.4 (0.5–3.2)	2.0 (0.9–3.6)	0.01
8-isoprostanes U·L^{-1}	4.3 (1.2–9.1)	11.4 (6.1–22.5)	0.001
FFAs mg·dL^{-1}	10.5±5	12.2±5	0.015

Data are presented as mean±SD or median (interquartile range), unless otherwise stated. OSAS: obstructive sleep apnoea syndrome; HDLc: high-density lipoprotein-cholesterol; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: γ-glutamyltransferase; CRP: C-reactive protein; FFA: free fatty acid.

Figura 6.1: Marcadores metabólicos y bioquímicos

3. En el grupo con SAHS, la concentración plasmática de AG se relacionaba linealmente de manera positiva con el IAH (r=0,210, p=0,026) y el índice de *arousal* (r=0,236, p=0,010). La concentración plasmática de AG también se relacionó con los niveles de gamma glutamil transpeptidasa (r=0,274, p=0,003) y los niveles de colesterol HDL (r=0,305, p=0,001).

4. El IAH se relacionaba con la concentración plasmática de AG de manera independientemente de la edad, el sexo, el IMC o la presencia de SM, (p=0,028) Figura 6.2. Los pacientes con SAHS grave presentaron una concentración plasmática de AG superior (13,3±5,2 mg/dl) en comparación con el grupo de SAHS moderado (11,4±4,2, p=0,004) o leve (10,5±4,0 mg/dl; p=0,004).

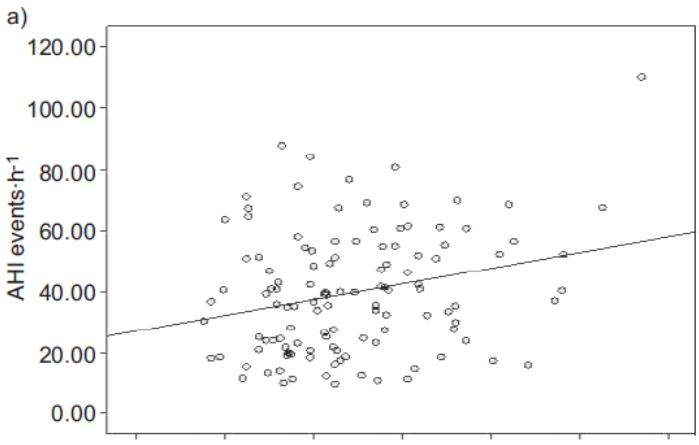

Figura 6.2: Relación entre los niveles de AG y el IAH.

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Free fatty acids and the metabolic syndrome in patients with obstructive sleep apnoea

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ABSTRACT: Obesity and metabolic syndrome (MS) occur frequently in patients with obstructive sleep apnoea syndrome (OSAS). We hypothesised that circulating free fatty acids (FFAs) are elevated in OSAS patients independently of obesity. This elevation may contribute to the development of MS in these patients.

We studied 119 OSAS patients and 119 controls. Participants were recruited and studied at sleep unit of our institution (Hospital Universitari Son Dureta, Palma de Mallorca, Spain) and were matched for sex, age and body mass index (BMI). The occurrence of MS was analysed by clinical criteria. Serum levels of FFAs, glucose, triglycerides, cholesterol, high-density lipoprotein–cholesterol, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, C-reactive protein and 8-isoprostanes were determined.

Prevalence of MS was higher in OSAS than in the control group (38 *versus* 21%; p=0.006). OSAS patients had higher FFAs levels than controls (mean±SD 12.2±4.9 *versus* 10.5±5.0 mg·dL^{-1}; p=0.015). Among subjects without MS, OSAS patients (OSAS+ MS-) showed higher levels of FFAs than controls (OSAS- MS-) (11.6±4.7 *versus* 10.0±4.4 mg·dL^{-1}; p=0.04). In a multiple regression model, after adjustment for age, sex, BMI and the presence of MS, FFAs were significantly associated with apnoea/hypopnoea index (p=0.04).

This study shows that FFAs are elevated in OSAS and could be one of the mechanisms involved in the metabolic complications of OSAS.

KEYWORDS: Free fatty acids, metabolic syndrome, sleep apnoea

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Obstructive sleep apnoea syndrome (OSAS) is a common disorder defined by the occurrence of repeated episodes of upper airway obstruction and airflow cessation (apnoeas) that normally lead to arterial hypoxaemia and sleep disruption [1, 2]. A number of clinical features, such as obesity, insulin resistance and the metabolic syndrome (MS) are often, but not invariably, present in these patients [3].

The relationship between obesity and the development of the MS in patients with OSAS is complex and poorly understood [3–5]. Obesity is generally regarded as a risk factor for both OSAS and MS [4]. However, factors other than obesity appear to play a significant role in the development of metabolic disturbances in patients with OSAS [6, 7], including sleep fragmentation and intermittent hypoxia.

Circulating free fatty acids (FFAs) are mainly released from triglyceride stores of the adipose tissue and serve as physiologically important energy substrates [8]. Previous work suggests an important role of FFAs in the development of insulin resistance and various disturbances related to MS [9, 10]. Additionally, FFAs could also contribute to oxidative stress, inflammation and endothelial dysfunction [11–13]. Despite the evidently central role of FFAs in pathophysiological processes leading to MS, there exist no studies investigating the relationship between FFAs and MS in OSAS

In this study, we hypothesised that FFAs are elevated in OSAS patients independently of obesity, and that this elevation may contribute to the development of MS in these patients. To test this hypothesis, we compared their concentration in patients with OSAS (with and without MS) and matched controls (with and without MS).

METHODS

Subjects and ethics

In this case–control study, we included 119 patients with OSAS and 119 controls.

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Participants were recruited from subjects who attended the sleep unit of our institution between January 2008 and December 2009. Patients and controls were selected based on the diagnosis of OSAS and were matched for sex, age (±5 yrs) and body mass index (BMI) (±3 kg·m^{-2}). No participant suffered from any other chronic disease (chronic obstructive pulmonary disease, liver cirrhosis, thyroid dysfunction, rheumatoid arthritis, chronic renal failure and/or psychiatric disorders). There were no differences between the number of patients and controls taking hypoglycaemic, hypolipaemiant and/or antihypertensive agents. No participant was regularly taking anti-inflammatory medication. The study was approved by the Ethics Committee of our institution and all participants gave signed consent after being fully informed of its goal and characteristics.

Measurements and definitions

The diagnosis of OSAS was established by full polysomnography (E-Series; Compumedics, Abbotsford, Australia) that included recording of oronasal flow, thoracoabdominal movements, ECG, submental and pretibial electromyography, electro-oculography, electroencephalography, and transcutaneous measurement of arterial oxygen saturation (S_{a,O_2}). Apnoea was defined by the absence of airflow for >10 s. Hypopnoea was defined as any airflow reduction that lasted >10 s and resulted in arousal or oxygen desaturation. We considered desaturation a decrease in S_{a,O_2} >4%. The apnoea/hypopnoea index (AHI) was defined as the sum of the number of apnoeas plus hypopnoeas per hour of sleep. The case or control status was defined by the AHI threshold of ⩾10 events·h^{-1}. Patients were classified into three groups according to their AHI as mild (AHI 10–20 events·h^{-1}), moderate (AHI 21–40 events·h^{-1}) and severe OSAS (AHI >40 events·h^{-1}).

Excessive daytime sleepiness was quantified subjectively by the Epworth Sleepiness Scale.

The occurrence of the MS was analysed according to the National Cholesterol Education Program Adult Treatment Panel III clinical criteria: 1) waist circumference ⩾102 cm in males and ⩾88 cm in females; 2) fasting glucose ⩾100 mg·dL^{-1} or patient on specific drug treatment; 3) triglycerides ⩾150 mg·dL^{-1} or patient on specific treatment; 4) high-density lipoprotein-cholesterol (HDLc) <40 mg·dL^{-1} in males and < 50 mg·dL^{-1} in females or patient on specific drug treatment; and 5) systolic blood pressure ⩾130 mmHg or diastolic blood pressure ⩾85 mmHg, or patient on specific drug treatment. MS was diagnosed if three out of these five factors were present.

After fasting overnight, venous blood samples were obtained between 08:00 and 10:00 h. Blood was centrifuged, and serum was immediately separated into aliquots and stored at -80°C until analysis.

Glucose, triglycerides, total cholesterol, HDLc, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase (GGT), uric acid and FFAs were determined by standard enzymatic methods on a Hitachi Modular analyser (Roche Diagnostics, Indianapolis, IN, USA). The plasma concentration of C-reactive protein (CRP) was measured by a commercial chemiluminiscent assay on a Immulite 2000 analyser (Siemens Medical Solutions Diagnostics, NY, USA). 8-isoprostanes were measured with an 8-isoprostane EIA kit (Cayman Chemicals Company, Ann Arbor, MI, USA).

Statistical analysis

Results are presented as %, median or mean±SD. Comparisons of group means were performed using unpaired t-tests (for comparison between any two groups) and using one-way ANOVA (for multiple-group comparison), followed by *post hoc* contrast when appropriate.

To determine the effect of sleep apnoea on FFAs, we used a multiple regression analysis, including all subjects, with study group, age, sex, BMI and AHI as the independent variables, and FFAs as the dependent variable.

The study was powered to detect a difference of 2.0 mg·dL^{-1} in FFAs, assuming a within-subject standard deviation of 2.0 in healthy subjects [13], at a significance level of 5% and with a power of 90%, which required 20 subjects in each group.

Correlations between variables were explored using the Spearman rank test. A p-value <0.05 was considered significant.

RESULTS

Characteristics of the study population are summarised in table 1. By design, sex, age and BMI were similar in patients and controls.

The prevalence of the MS was higher in the OSAS group than in the control group (p=0.006).

Metabolic and biochemical parameters are presented in table 2. Tables 3 and 4 show these parameters according to the presence or absence of the MS.

TABLE 1 Subject characteristics

	Controls	OSAS	p-value
Subjects n	119	119	
Age yrs	45±11	46±12	0.635
Males	87 (73)	88 (74)	0.883
BMI kg·m^{-2}	28±4	28±4	0.727
Waist circumference cm	101±11	101±11	0.889
Hypertension	22 (21)	26 (22)	0.470
Diabetes	4 (4)	9 (8)	0.169
Current smoker	36	36	0.951
MS	21%	38%	0.006
AHI events·h^{-1}	3.2 (1.8–4.5)	39 (23.2–53.5)	<0.001
Arousal index events·h^{-1}	22±13	47±18	<0.001
S_{a,O_2} %			
Mean	94±3	93±2	<0.001
Minimum	86±9	83±8	0.061
ESS score	7 (5–10)	11 (6–14)	<0.001

Data are presented as mean±SD, n (%) or median (interquartile range), unless otherwise stated. OSAS: obstructive sleep apnoea syndrome; BMI: body mass index; MS: metabolic syndrome; AHI: apnoea/hypopnoea index; S_{a,O_2}: arterial oxygen saturation; ESS: Epworth Sleepiness Scale.

TABLE 2 Metabolic and biochemical markers

	Controls	OSAS	p-value
Subjects n	119	119	
Glucose mg·dL^{-1}	94±4	103±22	0.001
Triglycerides mg·dL^{-1}	124±51	147±94	0.079
Cholesterol mg·dL^{-1}	207±41	212±39	0.398
HDLc mg·dL^{-1}	56±15	55±16	0.505
Creatinine mg·dL^{-1}	0.88±0.2	0.96±0.3	0.692
Uric acid mg·dL^{-1}	6.2±4.7	6.1±3.2	0.336
AST U·L^{-1}	22±7	21±7	0.387
ALT U·L^{-1}	27±15	27±13	0.809
GGT U·L^{-1}	32±27	37±29	0.048
CRP U·L^{-1}	1.4 (0.5–3.2)	2.0 (0.9–3.6)	0.01
8-isoprostanes U·L^{-1}	4.3 (1.2–9.1)	11.4 (6.1–22.5)	0.001
FFAs mg·dL^{-1}	10.5±5	12.2±5	0.015

Data are presented as mean±SD or median (interquartile range), unless otherwise stated. OSAS: obstructive sleep apnoea syndrome; HDLc: high-density lipoprotein-cholesterol; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: γ-glutamyltransferase; CRP: C-reactive protein; FFA: free fatty acid.

Compared with controls subjects, OSAS patients showed abnormal plasma levels of glucose, GGT, CRP and 8-isoprostanes (table 2).

Plasma levels of FFAs were significantly higher in OSAS patients than in subjects without OSAS (p=0.015). No significant differences in FFAs were detected between OSAS patients with MS (OSAS+ MS+) and controls with MS (OSAS- MS+) (p=0.271) (table 3). Nevertheless, among subjects without MS, OSAS patients (OSAS+ MS-) show higher levels of FFAs than controls (OSAS- MS-) (p=0.04) (table 4).

TABLE 3 Metabolic and biochemical markers in patients and controls with metabolic syndrome

	Controls	OSAS	p-value
Subjects n	23	45	
Glucose mg·dL^{-1}	101±15	116±27	0.013
Triglycerides mg·dL^{-1}	147±51	195±128	0.123
Cholesterol mg·dL^{-1}	218±45	217±32	0.981
HDLc mg·dL^{-1}	57±25	51±10	0.380
Creatinine mg·dL^{-1}	0.85±0.07	0.89±0.14	0.607
Uric acid mg·dL^{-1}	5.8±1.8	6.3±1.4	0.523
AST U·L^{-1}	23±8	21±8	0.349
ALT U·L^{-1}	30±15	30±15	0.789
GGT U·L^{-1}	43±35	44±35	0.957
CRP U·L^{-1}	1.3 (0.5–3.8)	2.0 (0.9–3.5)	0.894
8-isoprostanes U·L^{-1}	4.2 (2.3–7.6)	10.6 (4.7–24.0)	0.228
FFAs mg·dL^{-1}	11.5±5	13.1±5	0.271

Data are presented as mean±SD or median (interquartile range), unless otherwise stated. OSAS: obstructive sleep apnoea syndrome; HDLc: high-density lipoprotein-cholesterol; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: γ-glutamyltransferase; CRP: C-reactive protein; FFA: free fatty acid.

TABLE 4 Metabolic and biochemical markers in patients and controls without metabolic syndrome

	Controls	OSAS	p-value
Subjects n	86	74	
Glucose mg·dL^{-1}	92±15	95±12	0.204
Triglycerides mg·dL^{-1}	114±42	118±47	0.599
Cholesterol mg·dL^{-1}	201±37	207±43	0.313
HDLc mg·dL^{-1}	56±16	55±15	0.767
Creatinine mg·dL^{-1}	0.88±0.15	0.89±0.15	0.811
Uric acid mg·dL^{-1}	4.8±1.5	5.5±1.3	0.023
AST U·L^{-1}	21±6	21±7	0.723
ALT U·L^{-1}	26±15	25±11	0.676
GGT U·L^{-1}	29±25	33±25	0.358
CRP U·L^{-1}	1.4 (0.5–3.2)	2.0 (0.8–3.8)	0.01
8-isoprostanes U·L^{-1}	4.8 (1.4–9.4)	12.0 (6.7–21.2)	0.001
FFAs mg·dL^{-1}	10.0±4.4	11.6±4.7	0.04

Data are presented as mean±SD or median (interquartile range), unless otherwise stated. OSAS: obstructive sleep apnoea syndrome; HDLc: high-density lipoprotein-cholesterol; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: γ-glutamyltransferase; CRP: C-reactive protein; FFA: free fatty acid.

In the OSAS group, FFA levels were significantly related to AHI (r=0.210, p=0.026; fig. 1a) and the arousal index (r=0.236, p=0.010; fig. 1b). FFAs were also significantly related to GGT (r=0.274, p=0.003; fig. 2a) and HDLc levels (r=0.305, p=0.001; fig. 2b).

Associations between FFA levels and nocturnal oxygenation indices did not reach the level of statistical significance (mean S_{a,O_2} r=0.189 (p=0.083) and minimum S_{a,O_2} r=0.139 (p=0.141)).

In a multiple regression model, after adjustment for age, sex, BMI and the presence of MS, FFAs were significantly associated with AHI (p=0.028).

In addition, FFAs were significantly different between the three groups with mild, moderate and severe OSAS (ANOVA p=0.004) and higher in the severe OSAS group (13.3±5.2 mg·dL^{-1}) than in the moderate (11.4±4.2 mg·dL^{-1}; p<0.004) and the mild OSAS groups (10.5±4.0 mg·dL^{-1}; p<0.004).

In OSAS without MS, FFA levels were higher in the severe group (12.4±5.0 mg·dL^{-1}) than in the mild-to-moderate group (11.0±4.1 mg·dL^{-1}; p<0.01), but the correlation analysis between FFAs and AHI did not reach statistical significance (r=0.154; p=0.191). In this group, FFAs were also related to GGT (r=0.274; p=0.01) and HDLc (r=0.305; p=0.037).

DISCUSSION

The strengths of this study include assessment of associations between FFAs and OSAS, and the presence of the MS without the potential influence of confounding factors.

This study shows that: 1) the prevalence of the MS is higher in OSAS patients than subjects without OSAS of similar age, sex and BMI, suggesting that OSAS itself is a risk factor for the MS; 2) FFAs are elevated in patients with OSAS; and 3) AHI is independently associated with FFAs levels. These observations

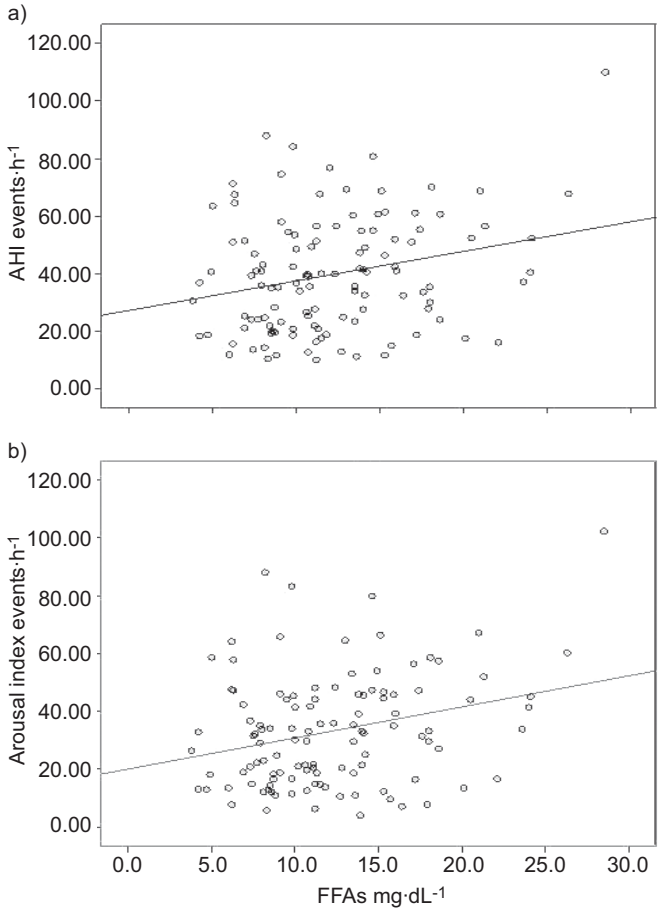

FIGURE 1. Relationship between free fatty acid (FFA) levels, and a) apnoea/hypopnoea index (AHI) (r=0.204, p=0.026) and b) arousal index (r=0.210, p=0.026) in the obstructive sleep apnoea syndrome population studied.

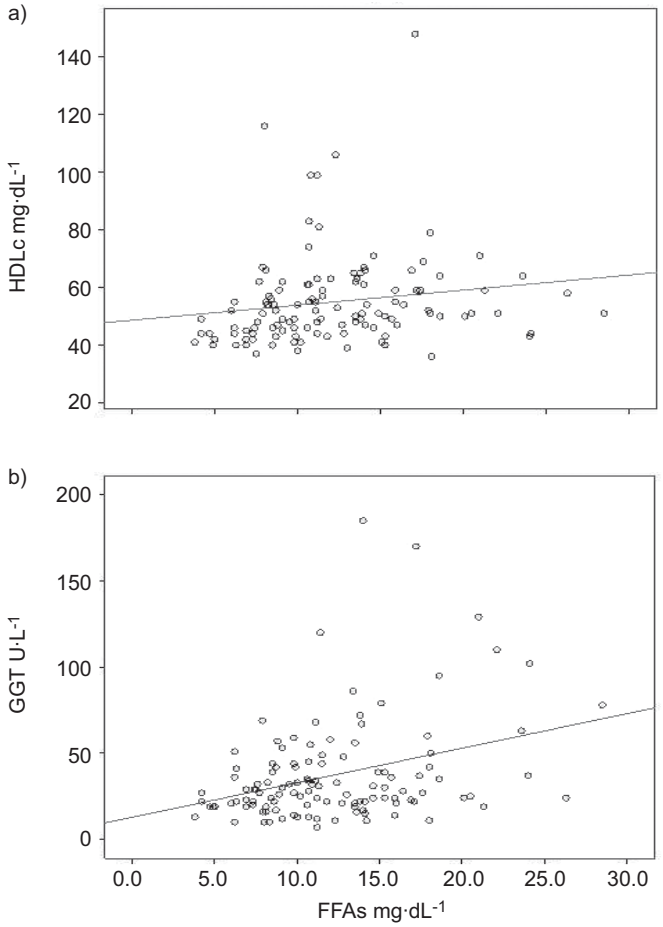

FIGURE 2. Relationship between free fatty acid (FFA) levels and a) high-density lipoprotein/cholesterol (HDLc) (r=0.305, p=0.001) and b) γ-glutamyltransferase (GGT) (r=0.274, p=0.003) in the obstructive sleep apnoea syndrome population studied.

suggest that FFA elevation could be one of the mechanisms involved in the metabolic complications of OSAS patients.

The relationship between OSAS, obesity and the MS is complex and unclear [3].

Prevalence of the MS is higher in OSAS patients than in the general population [14, 15]. However, both OSAS and MS are associated with obesity, which is an important confounder for the independent effects of OSAS on metabolic variables [5, 16]. In our study, the prevalence of the MS was higher in OSAS patients than subjects without OSAS of similar age, sex and BMI, suggesting that OSAS itself is a risk factor for the MS. The MS is associated with increased risk for cardiovascular events, diabetes and non-alcoholic fatty liver disease [17–19]. The high prevalence of the MS in OSAS patients raises the possibility that some of the complications associated with the MS may be attributable to OSAS. In this sense, recent observations have shown that obstructive sleep apnoea has an incremental effect on markers of atherosclerosis in patients with metabolic syndrome [20].

The search for additional factors that may contribute to better a understanding of the links between OSAS and the MS is highly desirable. In this study, we evaluated whether FFAs may play a role in the development of the MS in OSAS.

Experimental studies in healthy subjects have demonstrated that an elevation in FFAs induces insulin resistance [21] . In addition, increased levels of FFAs in obese subjects were reported to contribute in the development of various disturbances related to the MS, such as insulin resistance, hypertension, dyslipidaemia and others [10, 11, 22].

Increased FFA supply in the liver is an initial step for the development of the characteristic disorders of the MS [12]. FFAs are released principally from adipose tissue through lipolysis of triglycerides [23]. Release of FFAs is regulated by the action of insulin and modulated by adrenergic activity [23, 24]. FFA concentrations are higher in obese individuals [8]. Nevertheless, there appears to be limited interindividual variability in plasma FFAs levels between people with similar BMI [10]. In our study, despite the similar anthropometric characteristics between OSAS and controls, FFAs were higher in the OSAS group, suggesting a high FFA flux originating from lipolysis in adipose tissue in these patients.

There are several mechanisms that support a relationship between OSAS and a dysfunctional adipose tissue, such as increased sympathetic activity, oxidative stress or adipose tissue inflammation [25–28] . Compared with controls subjects,

OSAS patients showed abnormal plasma levels of markers of inflammation and oxidative stress, such as CRP and 8-isoprostanes levels. However, these differences were independent of the presence of the MS. In our study, FFA levels were higher in OSAS patients without MS than in controls without MS. By contrast, despite the fact that FFA levels were higher in OSAS patients with MS than in controls with MS, the difference did not reach statistical significance. It is possible that the high variability in FFAs levels detected both in controls and patients with MS may explain the lack of significance.

We found a significant correlation between FFAs levels and AHI and arousal index, suggesting that sleep fragmentation and repetitive arousals may be involved in the FFA release into the circulation. However, the relationships between these mechanisms in OSAS and the regulation of FFAs, and their mediating role between OSAS and the MS needs further investigation. FFAs levels were different between the three groups with severe, moderate and mild OSAS. In OSAS patients without MS, FFA levels were also higher in the severe group than in mild-to-moderate group. Our results are concordant with a study by LAM *et al.* [29]. Those authors found that adipocyte fatty acid-binding protein levels correlated with obstructive sleep apnoea independently of obesity [29]. Multiple linear regression controlling for BMI, sex, age and the presence of MS confirmed an independent association between AHI and FFA levels.

Several lines of evidence support an independent association between OSAS and dysregulation of lipid metabolism [30, 31]. In our study, we observed a relationship between FFAs and HDLc, suggesting that the increased flux of FFAs to the liver may represent an important factor for the presence of dyslipidaemia in patients with OSAS. We speculate that elevated plasma FFAs could be one of the mechanisms involved in the metabolic and cardiovascular complications of OSAS patients.

Limitations

Some potential confounding factors, such as nutritional status, physical activity or the interaction between genetic variants, were not taken into account in our analysis.

Analyses were not adjusted for albumin. Since FFAs travel in serum bound to albumin, this may affect the results [32].

Increases of plasma FFAs cause endothelial dysfunction in healthy subjects [33]. Future studies should examine the role of FFAs using techniques to assess endothelial function and evaluate their long-term effect on the vascular bed in patients with OSAS.

However, we did not measure the levels of FFAs after continuous positive airway pressure treatment. Normalising plasma levels of FFAs levels can be expected to improve insulin resistance and other features of the MS. We think that future studies including these measurements are needed to determine the impact of all these observations on metabolic dysfunction of OSAS patients.

Conclusions

This study shows that FFAs are elevated in OSAS and may play a role in the pathogenesis of the MS in patients with OSAS.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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7 ESTUDIO 3

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7.1 HIPÓTESIS

Los pacientes con síndrome apnea-hipopnea del sueño (SAHS) pueden presentar niveles plasmáticos alterados de vitamina D y estos se relacionan con la presencia de factores de riesgo cardiovascular como la diabetes mellitus tipo 2 (DM 2), la hipertensión arterial (HTA) y el síndrome metabólico (SM).

7.2 OBJETIVOS

- Determinar la concentración plasmática de vitamina D y de hormona paratiroidea (PTH) en pacientes con SAHS y su relación con la presencia de DM 2, HTA, y SM.

7.3 METODOLOGÍA

Este estudio de tipo trasversal, incluyó un total de 826 pacientes con nuevo diagnóstico de SAHS de la Unidad de Trastornos del Sueño del Hospital de Son Espases (Mallorca) durante 2008-2010.

El diagnóstico de SAHS se estableció a partir de un índice de apnea-hipopnea (IAH)$\geqslant$10/h. Los pacientes fueron clasificados en dos grupos de gravedad en función de su IAH: SAHS leve-moderado (IAH<30, n=280) y grave (IAH$\geqslant$30, n=546). El diagnóstico de SM se realizó de acuerdo con el criterio III programa nacional de educación sobre el colesterol del panel de expertos en la detección, evaluación y tratamiento de la hipercolesterolemia en adultos (NCEP-ATP III) [62], que requiere la presencia de al menos tres de las siguientes condiciones: perímetro de cintura elevado ($\geqslant$94 cm en varones y $\geqslant$80 cm en mujeres), aumento de la presión arterial (>130/85 mmHg) o toma de fármacos para la hipertensión, hiperglucemia en ayunas (>100 mg/dl) o toma de hipoglucemiantes, triglicéridos aumentados ($\geqslant$150 mg/dl) o tratamiento para la dislipemia y disminución en la concentración de lipoproteína de alta densidad *(siglas en inglés de High density lipoprotein)* (HDL) (<50 mg/dl en varones y <40 mg/dl en mujeres) o tratamiento para la dislipemia.

Tras el análisis univariado realizado con el fin de determinar el comportamiento de las variables (normalidad), en algún caso se procedió a la transformación logarítmica antes de los análisis estadísticos con el objetivo de minimizar la variabilidad de los datos. Se utilizó el test T Student o ANOVA para comparar variables cuantitativas, y Chi cuadrado para las cualitativas (Test de Fisher si las frecuencias fueron <5). La relación lineal entre variables cuantitativas se realizó mediante el test no paramétrico de Spearman al existir cierta variabilidad en los datos. Finalmente se construyeron diferentes modelos de regresión logística para determinar la relación entre la concentración plasmática de vitamina D y de PTH (variables independientes) con la obesidad, DM 2, HTA, y SM (variables dependientes); la concentración plasmática de vitamina D se incluyó recodificada por niveles de gravedad (déficit grave $\leqslant$15 ng/ml; déficit moderado =16-30 ng/ml; niveles normales >30 ng/ml) y la de PTH se introdujo recodificada en terciles. Se obtuvieron las Odds Ratio (OR) e intervalo de

confianza (IC) al 95 % (IC95 %) ajustados por edad, sexo, y estación en el momento de la recolección de las muestras (primavera, verano, otoño, invierno). La principal fuente de vitamina D proviene de la radiación solar y por este motivo dependerá en función de la estación del año siendo el aporte superior en primavera y verano. Este dato debe tenerse en cuenta a la hora de analizar los resultados.

7.4 PRINCIPALES RESULTADOS

1. Se observó una alta prevalencia de déficit de vitamina D en los pacientes con SAHS, siendo de 55,3 % en varones un 63,2 % en mujeres.

2. La concentración plasmática de vitamina D en el grupo SAHS fue significativamente inferior en el subgrupo con SAHS grave (27,2±15,6 ng/ml) en comparación con el subgrupo con SAHS leve-moderado (30,7±14,2 ng/ml) sin mostrar una diferencia significativa (p=0,06). La concentración plasmática de PTH fue significativamente superior en el subgrupo con SAHS grave en comparación con el subgrupo con SAHS leve-moderado (58,5±28,1 pg/ml *vs.* 53,1±23,6 pg/ml, respectivamente; p=0,004).

3. Los pacientes con déficit de vitamina D eran mayores, presentaban un mayor perímetro de cuello y concentraciones plasmáticas más elevadas de glucosa, triglicéridos, colesterol y PTH con respecto a los pacientes con valores normales de vitamina D.

4. La concentración plasmática de PTH se relacionó positivamente de manera cruda con el IAH (r=0,210; p=0,002). Tras ajustar por las variables de interés, la concentración plasmática de PTH se relacionó negativamente con la saturación media (p=0,035) y mínima (p=0,043) de oxígeno.

5. El déficit de vitamina D se asoció con una prevalencia superior de SM y de DM 2 independientemente a la edad, el sexo o la estación del año Figura 7.1. En ambos casos se observa una tendencia lineal positiva, de forma que una concentración plasmática de vitamina D elevada disminuye la probabilidad de presentar DM 2 (tendencia lineal=0,038) o SM (p tendencia lineal<0,001).

	25(OH)D, ng/ml			
	≤15 (n = 105)	16–30 (n = 377)	>30 (n = 344)	p_{trend}
Diabetes[a]	1.00 (referent)	0.64 (0.37–1.01)	0.55 (0.33–0.94)	0.038
Obesity[a]	1.00 (referent)	1.24 (0.75–2.03)	0.92 (0.55–1.51)	0.314
Hypertension[a]	1.00 (referent)	1.03 (0.53–1.98)	0.87 (0.44–1.71)	0.630
MS[a]	1.00 (referent)	0.44 (0.27–0.67)	0.34 (0.21–0.56)	<0.001
High waist circumference[b]	1.00 (referent)	0.57 (0.31–1.06)	0.48 (0.25–0.89)	0.02
Hyperglycemia[b]	1.00 (referent)	0.74 (0.42–1.30)	0.75 (0.42–1.34)	0.324
Hypertriglyceridemia[b]	1.00 (referent)	0.38 (0.21–0.69)	0.37 (0.20–0.67)	<0.001
Low HDLc[b]	1.00 (referent)	0.62 (0.34–1.14)	0.47 (0.25–0.87)	0.018
High blood pressure[b]	1.00 (referent)	0.97 (0.51–1.85)	0.76 (0.39–1.49)	0.901

[a] Adjusted for age, season and sex; [b] adjusted for all variables in model 1 plus other components of MS.

Figura 7.1: OR ajustadas e IC95 % para DM 2, obesidad, HTA, SM y componentes de éste por categorías de vitamina D en pacientes con SAHS.

6. Una elevada concentración plasmática de PTH se asoció a una mayor prevalencia de obesidad independientemente de la edad, el sexo o la estación del año, (OR=2,05,

IC95 % 1,06-3,09; p_{trend}<0,001) y de HTA (OR=1,91, IC95 %=1,26-2,80; p_{trend}=0,002) Figura 7.2.

	PTH, pg/ml			
	≤42.7 (n = 272)	42.8–62.5 (n = 275)	≥62.6 (n = 274)	p_{trend}
Diabetes[a]	1.00 (referent)	1.28 (0.87–1.86)	1.23 (0.83–1.81)	0.592
Obesity[a]	1.00 (referent)	1.62 (1.12–2.32)	2.05 (1.06–3.09)	<0.001
Hypertension[a]	1.00 (referent)	1.68 (1.02–2.78)	1.83 (1.01–3.05)	0.049
MS[a]	1.00 (referent)	0.94 (0.66–1.36)	1.30 (0.92–1.98)	0.136
High waist circumference[b]	1.00 (referent)	1.84 (1.21–2.80)	1.30 (0.84–1.99)	0.238
Hyperglycemia[b]	1.00 (referent)	1.26 (0.83–1.89)	1.27 (0.83–1.95)	0.218
Hypertriglyceridemia[b]	1.00 (referent)	0.85 (0.56–1.28)	0.88 (0.57–1.37)	0.599
Low HDLc[b]	1.00 (referent)	1.25 (0.80–1.81)	0.99 (0.97–1.01)	0.556
High blood pressure[b]	1.00 (referent)	1.26 (0.83–1.91)	1.91 (1.26–2.80)	0.002

[a] Adjusted for age, season and sex; [b] adjusted for all variables in model 1 plus other components of MS.

Figura 7.2: OR ajustadas e IC95 % para DM 2, obesidad, HTA, SM y componentes de SM por categorías de PTH en pacientes con SAHS.

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Vitamin D Status and Parathyroid Hormone Levels in Patients with Obstructive Sleep Apnea

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Key Words
Vitamin D · Parathyroid hormone · Metabolic syndrome

Abstract
Background: Vitamin D insufficiency and high levels of parathyroid hormone (PTH) appear to be emerging risk factors for metabolic syndrome (MS), diabetes and cardiovascular disease, conditions that occur frequently in patients with obstructive sleep apnea syndrome (OSAS). ***Objectives:*** This study examined whether serum concentrations of 25-hydroxyvitamin D [25(OH)D] and PTH were associated with the presence of MS, diabetes and hypertension among an OSAS population. ***Methods:*** A total of 826 patients (635 men and 191 women) with newly diagnosed OSAS were studied. The occurrence of the MS was analyzed according to the National Cholesterol Education Program Adult Treatment Panel III clinical criteria. Serum levels of 25(OH)D, PTH, glucose, triglycerides, cholesterol, HDL cholesterol, creatinine and uric acid were determined. ***Results:*** In 55.3% of the men and in 63.2% of the women, the serum 25(OH)D level was less than 30 ng/ml (insufficient status). After adjusting for age, sex and seasonality, there was a significant trend of decreasing odds for diabetes [odds ratio (OR) 0.55, 95% confidence interval (CI) 0.33–0.94, $p_{trend} = 0.038$] and MS (OR 0.34, 95% CI 0.21–0.56, $p_{trend} < 0.001$) with increasing vitamin D levels. Higher PTH levels were associated with a higher prevalence of obesity (OR 2.05, 95% CI 1.06–3.09, $p_{trend} < 0.001$) and hypertension (OR 1.83, 95% CI 1.01–3.05, $p_{trend} = 0.049$). ***Conclusions:*** These data suggest an inverse association of 25(OH)D with diabetes and MS and a positive association of PTH with obesity and hypertension among patients with OSAS. Based on our observational study, the causative nature of the associations cannot be established. These findings require further examination in prospective studies including clinical trials.

Introduction

Obstructive sleep apnea syndrome (OSAS) is a common disorder defined by the occurrence of repeated episodes of upper airway obstruction and airflow cessation

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(apneas) that normally lead to arterial hypoxemia and sleep disruption [1, 2]. A number of clinical features, such as obesity, metabolic syndrome (MS) and cardiovascular disease are often present in these patients [3–5].

The recognition of the presence of widespread low vitamin D status is increasing [6–9]. The consequences of this growing epidemic of vitamin D insufficiency are still not well understood [9–11]. Vitamin D and parathyroid hormone (PTH) participate in bone metabolism and calcium homeostasis. In addition, in recent years it has become clear that vitamin D has pleiotropic effects and plays an important role in a broad range of organ functions [6]. A growing body of evidence suggests that low levels of 25-hydroxyvitamin D [25(OH)D] may be inversely associated with obesity, glucose intolerance, MS and cardiovascular disease [8, 12–15]. Vitamin D concentrations are inversely associated with surrogate measures of insulin resistance and it has been reported that administration of vitamin D improves insulin secretion. Furthermore, based on blood pressure reduction with vitamin D supplementation in patients with hypertension, levels of this vitamin have been suspected to be involved in the regulation of blood pressure [16]. Elevated PTH secondary to a low level of vitamin D may also increase the risk of developing components of MS, including hypertension, diabetes and obesity [17, 13].

In this study, we hypothesized that vitamin D status may be associated with metabolic and cardiovascular complications in patients with sleep apnea.

To test this hypothesis we evaluated the associations of 25(OH)D status and PTH concentrations with the prevalence of diabetes, hypertension and MS among an OSAS population.

Methods

Subjects and Ethics

Our study sample comprised patients newly diagnosed with OSAS consecutively admitted to the sleep unit of our institution (Hospital Universitari Son Espases, Palma de Mallorca, Spain) between January 2008 and December 2010. During an interview conducted by physicians in the sleep medicine center, information was elicited about the patient's medical history, medication and lifestyle. No participant reported taking calcium or multivitamin supplements. No participant suffered from any other chronic disease (chronic obstructive pulmonary disease, liver cirrhosis, thyroid dysfunction, rheumatoid arthritis, osteoporosis, chronic renal failure and/or psychiatric disorders). The study was approved by the ethics committee of our institution, and all participants signed their consent after being fully informed of the goal and characteristics of the study.

Measurements and Definitions

The diagnosis of OSAS was established by full polysomnography (E-Series Compumedics, Abbotsford, Australia) that included the recording of oronasal flow, thoracoabdominal movements, electrocardiography, submental and pretibial electromyography, electrooculography, electroencephalography and the transcutaneous measurement of arterial oxygen saturation. Apnea was defined as the absence of airflow for more than 10 s. Hypopnea was defined as any airflow reduction that lasted more than 10 s and resulted in arousal or oxygen desaturation. A decrease in SaO_2 greater than 3% was considered to represent an oxygen desaturation. The apnea-hypopnea index (AHI) was defined as the sum of the number of apneas plus hypopneas per hour of sleep. Patients were classified into 2 groups according to their AHI as mild-to-moderate OSAS (AHI <30, n = 280) and severe OSAS (AHI ≥30, n = 546).

Excessive daytime sleepiness was quantified subjectively by the Epworth sleepiness scale.

The occurrence of the MS was analyzed according to the National Cholesterol Education Program Adult Treatment Panel III clinical criteria [18]: (1) waist circumference ≥102 cm in men and ≥88 cm in women, (2) fasting glucose ≥100 mg/dl, (3) triglycerides ≥150 mg/dl, (4) HDL cholesterol (HDLc) <40 mg/dl in men and <50 mg/dl in women, and (5) systolic blood pressure (SBP) ≥130 mm Hg or diastolic blood pressure (DBP) ≥85 mm Hg or a patient being on specific drug treatment. The MS was diagnosed if 3 of these 5 factors were present. Diabetes was defined by use of diabetes medications or a fasting glucose of ≥126 mg/ml. Hypertension was diagnosed if SBP was ≥140 mm Hg and/or DBP was ≥90 mm Hg or the individual was on specific treatment. Participants were considered obese when their body mass index (BMI) was higher than 30.

After fasting overnight, venous blood samples were obtained between 8 and 10 a.m. Blood was centrifuged and serum was immediately separated in aliquots and stored at –80°C until analysis.

Glucose, triglycerides, total cholesterol, HDLc, creatinine and uric acid were determined by standard enzymatic methods on a Hitachi Modular analyzer (Roche Diagnostics, Ind., USA). 25(OH)D levels were quantified in duplicate using a commercial enzyme immunosorbent assay kit (IDS, Boldon, UK) with a coefficient of variation of less than 10%. The lower limit of detection was 2 ng/ml. Vitamin D was stratified into 3 categories: normal >30 ng/ml, low (insufficient) 16–30 ng/ml and very low (deficient) ≤15 ng/ml. The PTH assay was performed using a chemiluminescence assay for the measurement of intact PTH on an Advia-Centaur analyzer (Siemens Medical Solutions Diagnostics, N.Y., USA).

Statistical Analysis

Results are presented as percentages, mean ± standard deviations (SD) or median (interquartile range). Skewed variables were logarithmically transformed before analysis. Patient characteristics were compared using independent sample Student t tests for continuous measures or repeated measures ANOVA when appropriate. Categorical variables were compared using the χ^2 or Fisher tests.

Correlations between the subjects' characteristics and vitamin D and PTH levels were explored using the Spearman rank test. Multiple regression analyses were used to confirm the significant

Table 1. Characteristics of the study sample grouped according to vitamin D status

	Categories of 25(OH)D, ng/ml			
	≤15 (n = 105)	16–30 (n = 377)	>30 (n = 344)	p_{trend}
Age, years[b]	54.0 ± 12.8	53.0 ± 12.4	50.9 ± 12.4	0.008
BMI[b]	31.9 ± 5.6	32.0 ± 6.7	30.4 ± 5.5	0.002
Waist circumference, cm[b]	109.6 ± 13.4	108.4 ± 14.5	106.3 ± 13.2	0.043
AHI, h^{-1}[b]	50.6 ± 30.4	47.3 ± 25.7	45.7 ± 24.4	0.092
Arousal index, h^{-1}[b]	56.6 ± 27.5	54.7 ± 22.4	52.2 ± 21.4	0.056
Mean SaO_2, %[b]	90.4 ± 4.9	91.8 ± 4.6	92.4 ± 3.9	<0.001
Minimal SaO_2, %[b]	77.9 ± 10.2	79.7 ± 9.6	80.6 ± 8.7	0.013
Epworth sleepiness scale[b]	9.8 ± 5.1	10.0 ± 5.1	10.2 ± 5.3	0.462
Current smoker, n (%)[a]	32 (30.5)	93 (24.7)	91 (26.5)	0.233
SBP, mm Hg[b]	130.6 ± 19.0	132.3 ± 18.3	131.0 ± 16.4	0.777
DBP, mm Hg[b]	81.6 ± 14.0	80.3 ± 12.6	80.2 ± 12.6	0.339
Hypertension, n (%)[a]	17 (16.2)	67 (17.7)	52 (15.5)	0.118
Obesity, n (%)[a]	63 (60.4)	221 (58.9)	167 (48.5)	0.006
Diabetes, n (%)[a]	29 (27.6)	63 (16.7)	54 (15.7)	0.026
MS, n (%)[a]	56 (53.8)	139 (37.3)	112 (32.7)	0.025
MS criteria, n[a]	2.8 ± 1.2	2.2 ± 1.2	2.1 ± 1.2	<0.001

[a] χ^2 linear trend test; [b] ANOVA linear trend test.

Table 2. Biochemical parameters of the study sample, grouped according to vitamin D status

	Categories of 25(OH)D, ng/ml			
	≤15 (n = 105)	16–30 (n = 377)	>30 (n = 344)	p_{trend}
Glucose, mg/dl[a]	116.4 ± 39.1	105.6 ± 24.4)	104.9 ± 21.8	0.002
Triglycerides, mg/dl[b]	214 (176–253)	157 (147–168)	147 ± (139–156)	<0.001
Cholesterol, mg/dl[a]	218 ± 52	205 ± 38	201 ± 38	0.001
HDLc, mg/dl[a]	50.7 ± 14.9	54.0 ± 18.8	53.6 ± 18.4	0.316
Creatinine, mg/dl[a]	0.89 ± 0.26	1.06 ± 0.2	0.95 ± 0.2	0.760
Uric acid, mg/dl[a]	6.2 ± 1.4	6.3 ± 3.1	6.5 ± 3.0	0.351
PTH[a, b]	60.9 (45.3–86.9)	53.0 (39.7–69.8)	47.8 (37.8–64.5)	<0.001

[a] ANOVA linear trend test; [b] logarithmically transformed for statistics.

associations detected with adjustment for age, gender, BMI, waist circumference and MS.

Logistic regression analysis was performed to estimate the odds ratios (ORs) and 95% confidence intervals (CIs) for obesity, diabetes, hypertension and MS across categories of 25(OH)D and tertiles of PTH after adjusting for age, sex and season of data collection: Winter (January–March), Spring (April–June), Summer (July–September) and Autumn (October–December). Models of MS components were also adjusted for the other components. The lowest group of either 25(OH)D or PTH served as the referent group.

A p value lower than 0.05 was considered significant.

Results

We studied 826 patients (635 men and 191 women) with newly diagnosed OSAS. The main clinical and biochemical characteristics are shown in tables 1 and 2.

Vitamin D levels were lower in the severe OSAS group (27.2 ± 15.6 ng/ml) than in the mild-to-moderate group (30.7 ± 14.2 ng/ml), but the difference did not reach statistical significance (p = 0.06). PTH levels were significantly higher in the severe OSAS group than in the mild-to-moderate group (58.5 ± 28.1 vs. 53.1 ± 23.6 pg/ml, p = 0.004).

Table 3. Adjusted OR and 95% CI for diabetes, obesity, hypertension and components of MS by categories of 25(OH)D among OSAS patients

	25(OH)D, ng/ml			
	≤15 (n = 105)	16–30 (n = 377)	>30 (n = 344)	p_{trend}
Diabetes[a]	1.00 (referent)	0.64 (0.37–1.01)	0.55 (0.33–0.94)	0.038
Obesity[a]	1.00 (referent)	1.24 (0.75–2.03)	0.92 (0.55–1.51)	0.314
Hypertension[a]	1.00 (referent)	1.03 (0.53–1.98)	0.87 (0.44–1.71)	0.630
MS[a]	1.00 (referent)	0.44 (0.27–0.67)	0.34 (0.21–0.56)	<0.001
High waist circumference[b]	1.00 (referent)	0.57 (0.31–1.06)	0.48 (0.25–0.89)	0.02
Hyperglycemia[b]	1.00 (referent)	0.74 (0.42–1.30)	0.75 (0.42–1.34)	0.324
Hypertriglyceridemia[b]	1.00 (referent)	0.38 (0.21–0.69)	0.37 (0.20–0.67)	<0.001
Low HDLc[b]	1.00 (referent)	0.62 (0.34–1.14)	0.47 (0.25–0.87)	0.018
High blood pressure[b]	1.00 (referent)	0.97 (0.51–1.85)	0.76 (0.39–1.49)	0.901

[a] Adjusted for age, season and sex; [b] adjusted for all variables in model 1 plus other components of MS.

Table 4. Adjusted OR and 95% CI for diabetes, obesity, hypertension and components of MS by tertiles of PTH among OSAS patients

	PTH, pg/ml			
	≤42.7 (n = 272)	42.8–62.5 (n = 275)	≥62.6 (n = 274)	p_{trend}
Diabetes[a]	1.00 (referent)	1.28 (0.87–1.86)	1.23 (0.83–1.81)	0.592
Obesity[a]	1.00 (referent)	1.62 (1.12–2.32)	2.05 (1.06–3.09)	<0.001
Hypertension[a]	1.00 (referent)	1.68 (1.02–2.78)	1.83 (1.01–3.05)	0.049
MS[a]	1.00 (referent)	0.94 (0.66–1.36)	1.30 (0.92–1.98)	0.136
High waist circumference[b]	1.00 (referent)	1.84 (1.21–2.80)	1.30 (0.84–1.99)	0.238
Hyperglycemia[b]	1.00 (referent)	1.26 (0.83–1.89)	1.27 (0.83–1.95)	0.218
Hypertriglyceridemia[b]	1.00 (referent)	0.85 (0.56–1.28)	0.88 (0.57–1.37)	0.599
Low HDLc[b]	1.00 (referent)	1.25 (0.80–1.81)	0.99 (0.97–1.01)	0.556
High blood pressure[b]	1.00 (referent)	1.26 (0.83–1.91)	1.91 (1.26–2.80)	0.002

[a] Adjusted for age, season and sex; [b] adjusted for all variables in model 1 plus other components of MS.

Spearman's correlation analysis showed that PTH levels correlated positively with AHI ($r = 0.210$, $p = 0.002$) and negatively with the mean and minimal nocturnal oxygenation saturation ($r = -0.378$, $p < 0.001$ and $r = -0.312$, $p < 0.001$). Significant associations between PTH levels and indices of nocturnal hypoxia were attenuated but not eliminated after adjustment for other variables in multivariate analysis (mean oxygenation saturation $p = 0.035$ and minimal oxygenation saturation $p = 0.043$).

Patients were divided into 3 categories based on their 25(OH)D levels. The median (range) 25(OH)D level was 27.8 (20.1–38.7) ng/ml. The prevalence of vitamin D insufficiency (vitamin D ≤30 ng/ml) was 58.3%. Although common, regardless of the season, vitamin D insufficiency was more prevalent during winter months (January–March 68.1%, April–June 63.3%, July–September 42.5% and October–December 59%; $p < 0.01$).

In 55.3% of the men and in 63.2% of the women, the serum 25(OH)D level was less than 30 ng/ml (insufficient status), and 10.5% of the men and 18.6% of the women had serum 25(OH)D levels below 15 ng/ml (very low status).

Patients with low 25(OH)D levels were older and had higher values of BMI, waist circumference, glucose, triglycerides, cholesterol and PTH than patients with sufficient 25(OH)D levels. Mean HDLc concentrations were

lower among those patients with low 25(OH)D levels (tables 1, 2).

After adjusting for age, sex and seasonality, there was a significant trend of decreasing odds for diabetes (OR 0.55, 95% CI 0.33–0.94, $p_{trend} = 0.038$) and MS (OR 0.34, 95% CI 0.21–0.56, $p_{trend} < 0.001$) with increasing vitamin D levels. Similarly, after additional adjustment for the other components of MS, there were inverse associations between 25(OH)D status and waist circumference (OR 0.48, 95% CI 0.25–0.89, $p_{trend} = 0.02$) hypertriglyceridemia (OR 0.37, 95% CI 0.20–0.67, $p_{trend} < 0.001$) and low HDLc (OR 0.47, 95% CI 0.25–0.87, $p_{trend} = 0.018$) (table 3). We also found no apparent modification of these relations according to smoking status (data not shown).

Table 4 displays metabolic variables according to tertiles of PTH levels. After adjusting for age, sex and seasonality, higher PTH levels were associated with a higher prevalence of obesity (OR 2.05, 95% CI 1.06–3.09, $p_{trend} < 0.001$) and hypertension (OR 1.83, 95% CI 1.01–3.05, $p_{trend} = 0.049$). In addition, there was an association between PTH and high blood pressure after adjusting for the other components of MS (OR 1.91, 95% CI 1.26–2.80, $p_{trend} = 0.002$).

Discussion

This study shows a high prevalence of low vitamin D levels among patients with OSAS. Low levels of 25(OH)D were associated with the presence of MS and diabetes. These associations were not explained by age, sex or season. We also found an increased prevalence of abdominal obesity, higher serum triglycerides and lower HDLc among those patients with lower 25(OH)D concentrations after adjustment for other components of MS. In addition, the odds for obesity and hypertension increased with increasing PTH levels. Furthermore, PTH levels were higher in patients with severe OSAS than in patients with mild-to-moderate OSAS, suggesting a potential role of OSAS in the metabolic and cardiovascular complications promoted by hypovitaminosis D.

Poor vitamin D status has been associated with the presence of MS and diabetes mellitus, suggesting that vitamin D could be an emerging association for these disorders [14, 19, 20]. A recent study reported an association of 25(OH)D concentrations with insulin resistance and β-cell dysfunction in subjects at risk for type 2 diabetes [20]. Our findings of an inverse association between 25(OH)D and diabetes among these patients suggest that lower concentrations of 25(OH)D may be a predictor of an increased likelihood of diabetes in patients with OSAS. On the other hand, Bozkurt et al. [21] showed that subjects with more severe OSAS indices tended to present lower vitamin D levels correlated to an increased prevalence of insulin resistance, prediabetes and diabetes. Nevertheless, compared with this previous study, the advantage of our study was that we were able to adjust for potential confounders for the analyses.

The search for additional factors that may contribute to a better understanding of the links between OSAS and the MS is highly desirable [22]. Evidence from clinical and experimental studies support an inverse relationship between 25(OH)D and components of the MS, including elevated blood pressure, dyslipidemia, high blood glucose concentrations and abdominal obesity [15, 23]. Several mechanisms have been proposed to explain these relations: (1) vitamin D has been independently associated with insulin sensitivity and β-cell function, (2) vitamin D negatively regulates the renin-angiotensin system to lower blood pressure, (3) vitamin D deficiency may cause an abnormal lipid profile by increasing peripheral insulin resistance [20, 24, 25].

Our findings that serum levels of 25(OH)D are inversely associated with triglyceride levels and positively associated with HDLc levels are in accordance with previous epidemiological studies [13, 26]. The role of vitamin D in lipid metabolism is currently unclear. Experimental evidence suggests that vitamin D deficiency may cause an abnormal lipid profile by increasing peripheral insulin resistance and contributing to MS [9]. Recently, Oh et al. [27] reported that 1,25(OH)2 vitamin D inhibits foam cell formation and suppresses macrophage cholesterol uptake in patients with type 2 diabetes, suggesting another mechanism of risk of vitamin D deficiency. Furthermore, it is possible that vitamin D can protect against cardiovascular risk by promoting formation of large HDL particles, affecting reverse cholesterol transport [28].

Obesity has been associated with vitamin D deficiency [29, 30]. Increased storage of 25(OH)D in adipose tissue is a plausible explanation for increased rates of vitamin D deficiency in obese individuals [31]. Alternatively, obesity may also be the consequence of low vitamin D levels [30]. Elevated PTH secondary to low vitamin D may promote fat accumulation and obesity through increases of calcium influx into adipocytes, which enhances lipogenesis and inhibits lipolysis [32]. In our study, patients with low levels of vitamin D were more obese than patients with sufficient levels. The high prevalence of vitamin D insufficiency in OSAS patients raises the possibility that low vitamin D status could promote weight gain and mag-

nify the adverse effects of obesity on metabolic variables and the cardiovascular system.

In this study, higher PTH levels were associated with a higher prevalence of obesity and hypertension. There was no evidence of associations of PTH with MS except for a positive association with high blood pressure after adjusting for the other components of MS. Previous studies found positive associations of PTH with SBP and DBP independent of 25(OH)D and potential confounding factors [13, 33]. It has been suggested that PTH affects blood pressure through a proliferate effect on vascular smooth-muscle cells, which may contribute to vessel-wall-thickening and consequently to higher blood pressure [34].

Taken together, our findings suggest that the associations of vitamin D and PTH with MS represent different underlying associations with individual components, with vitamin D likely acting through pathways involving glucose and lipid metabolism and PTH having a more important effect via blood pressure.

The main mechanisms of vascular damage in OSAS are intermittent hypoxia, intrathoracic pressure changes and arousals [5, 35]. On the other hand, OSAS is tightly linked with and may aggravate each component of MS [36]. In this study, PTH levels were higher in patients with severe OSAS and were independently associated with indices of nocturnal hypoxia, suggesting a possible additional mechanism by which OSAS may influence these levels. In addition, because PTH levels are partly dependent on vitamin D status, our results suggest that the common association of OSAS and low levels of vitamin D may have additive effects on metabolic and cardiovascular complications. Considering the high prevalence of a low vitamin D level among patients with OSAS and the possible role of vitamin D and PTH in the development of diabetes, MS and hypertension, it may be relevant to take these parameters into account when assessing metabolic and cardiovascular disorders in patients with OSAS. Continuous positive airway pressure treatment has been associated with improvements in vascular abnormalities, but alternative treatments are needed. Future prospective studies and randomized clinical trials are required to determine possible mechanisms of any preventive effect via vitamin D supplementation against metabolic and cardiovascular diseases in these patients.

This study has some limitations. The causative nature of the observed associations could not be established. Our observational design could not demonstrate the temporal relationship between low vitamin D status and risk of MS. Vitamin D levels may be a surrogate marker for healthy nutrition. In a recent cohort study carried out in a representative Spanish population, it was observed that one third (33.9%) of this population may be at risk of vitamin D deficiency [37]. In this sense, our study shows a high prevalence of low vitamin D levels among patients with OSAS (58.3%). In addition, OSAS patients may have reduced vitamin D production resulting from restricted mobility or lifestyle habits. An alternative explanation is that low vitamin D status is an indicator of chronic nonspecific illness rather than a direct contributor. Low levels of vitamin D may promote the acute-phase response and vitamin D analogs have been shown to inhibit the production of several proinflammatory cytokines [38]. Other potential confounding factors, such as the interaction between genetic variants, were not taken into account in our analysis.

Conclusions

This study shows that lower plasma 25(OH)D levels were associated with higher levels of glucose and a higher prevalence of MS. In addition, elevated PTH levels were associated with an increased prevalence of obesity and hypertension and were higher in patients with severe OSAS and independently associated with indices of nocturnal hypoxia, suggesting a possible additional mechanism by which OSAS may influence PTH levels.

These findings warrant further examination in prospective studies which should include clinical trials.

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All authors confirm that they have no conflicts of interest.

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8 ESTUDIO 4

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8.1 HIPÓTESIS

Los fenómenos que se producen durante las apneas obstructivas (episodios de hipoxia-reoxigenación, despertares, cambios en la presión intrapleural y aumento de la actividad neurovegetativa) pueden empeorar el pronóstico clínico de los pacientes con síndrome coronario agudo (SCA). El tratamiento con presión positiva continua en la vía aérea *(siglas en inglés de Continuous positive airway pressure)* (CPAP) revierte las repercusiones del síndrome apnea-hipopnea del sueño (SAHS) y mejora el pronóstico del paciente con esta enfermedad.

8.2 OBJETIVOS

- Diseñar detalladamente un estudio multicéntrico, aleatorizado, controlado y abierto en pacientes con SAHS que tiene como objetivo principal estudiar el impacto del SAHS y de su tratamiento con CPAP sobre la aparición de nuevos eventos cardiovasculares en pacientes con SAHS y SCA.

Este estudio cuyo investigador principal es el Dr. Barbé permitirá además:

- Determinar la prevalencia de SAHS en pacientes que han sufrido un episodio de SCA.
- Evaluar el efecto de la CPAP sobre: muerte cardiovascular, infarto de miocardio (IM) y accidente vascular cerebral isquémico, necesidad de revascularización, todas las muertes por cualquier causa, fibrilación auricular de nueva aparición y otras arritmias confirmadas por electrocardiograma, diabetes mellitus de nuevo diagnóstico y síntomas del SAHS (somnolencia diurna y calidad de vida).
- Identificar los marcadores biológicos que nos permitan establecer los mecanismos más importantes que intervienen en las complicaciones cardiovasculares en estos pacientes.
- Llevar a cabo un análisis de coste-efectividad del tratamiento con CPAP y del diagnóstico de SAHS de los pacientes con SCA.

8.3 METODOLOGÍA

El estudio ISAACC *(The impact of sleep apnea-hypopnea syndrome on the evolution of acute coronary syndrome. Effect of intervention with continuous positive airway pressure CPAP. A prospective randomized study)* es un estudio multicéntrico, prospectivo, aleatorizado, abierto y controlado. Los pacientes incluidos son aquéllos que de forma consecutiva son ingresados por SCA en la Unidad Coronaria de 15 hospitales universitarios nacionales y que no presentan somnolencia diurna. La Figura 8.1 muestra los centros colaboradores en el estudio ISAACC. El Institut de Recerca Biomèdica de Lleida (IRB Lleida) es el centro coordinador.

El SCA se define como una presentación aguda de la enfermedad coronaria (con o sin elevación del segmento T), angina inestable o IM tipo 1. La evaluación de la somnolencia diurna excesiva *(siglas en inglés de Excessive daytime sleepiness)* (EDS) se evaluará mediante el test de Epworth [89], en la que una puntuación ⩾10 puntos es indicativa de la presencia de somnolencia.

Figura 8.1: Centros colaboradores en el estudio ISAACC

Todos los pacientes que cumplen los criterios de selección Cuadro 8.1 y son incluidos en el proyecto, se someten a un estudio de sueño mediante poligrafía cardiorrespiratoria (PCR) entre las 24 y 72h tras el ingreso hospitalario. Se registra el flujo oronasal, movimientos toracoabdominales, electrocardiograma y oximetría de pulso. La apnea se define por la ausencia de flujo de aire que dura 10 segundos o más. Hipopnea se define como una reducción del flujo de aire que dura 10 segundos o más y se asocia con la desaturación de oxígeno. La desaturación de oxígeno se define como una disminución de la SaO2 arterial ⩾4 %. El índice de apnea-hipopnea (IAH) se define como el número de apneas y hipopneas por hora de sueño. Los 15 centros utilizan el mismo equipo de diagnóstico de SAHS (Embletta, Resmedic, España). El diagnóstico de SAHS se realiza a nivel local en cada centro y se basa en los resultados de la PCR de acuerdo con las normativas de la Sociedad Española de Neumología y Cirugía Torácica (SEPAR) [6]. Los pacientes con un IAH⩾15/h son asignados de manera aleatoria a tratamiento con CPAP (grupo 1: 632 pacientes) o a tratamiento conservador (grupo 2: 632 pacientes). Los pacientes con IAH<15/h (Grupo 3: 600 pacientes) son monitorizados como grupo de referencia Figura 8.2. Los tres grupos serán instruidos en medidas higiénico-dietéticas como se recomienda para todos los pacientes.

CRITERIOS DE INCLUSIÓN

1. Hombres y mujeres mayores de 18 años de edad.
2. Los pacientes ingresados por síntomas de SCA documentados con o sin elevación del segmento T y que presenta una evolución superior a 24 horas e inferior a 72 horas en el momento de la realización de la PR.
3. Los pacientes con sueño y la Escala de Epworth<10 (pacientes sin EDS).
4. Firma del consentimiento informado.

CRITERIOS DE EXCLUSIÓN

1. Tratamiento previo con CPAP para el diagnóstico de SAHS.
2. Incapacidad psicofísica para completar los cuestionarios.
3. Presencia de cualquier trastorno del sueño diagnosticado previamente: narcolepsia, insomnio, falta de sueño crónica, uso regular de medicamentos hipnóticos o sedantes y síndrome de piernas inquietas.
4. Los pacientes con >50% de las apneas centrales o la presencia de respiración de Cheyne-Stokes (CSResp).
5. Los pacientes con enfermedades crónicas: neoplasias, insuficiencia renal (filtración glomerular <15 ml/min), enfermedad pulmonar obstructiva crónica severa, depresión crónica y otras enfermedades crónicas muy limitantes.
6. Un historial médico que pueda interferir en los objetivos del estudio o, en opinión del investigador, en el compromiso de las conclusiones.
7. Cualquier factor médico, social o geográfico, que pueda poner en peligro el cumplimiento del paciente. (Por ejemplo, el consumo de alcohol (más de 80 gr/día en hombres y más de 60 gr/día en mujeres), desorientación, o un historial de incumplimiento).
8. Cualquier proceso, cardiovascular o de otro tipo, que limita la esperanza de vida a menos de un año.
9. Los pacientes en estado de shock cardiogénico que tienen pocas probabilidades de dar resultados a corto plazo.

Cuadro 8.1: Criterios de selección del estudio ISAACC

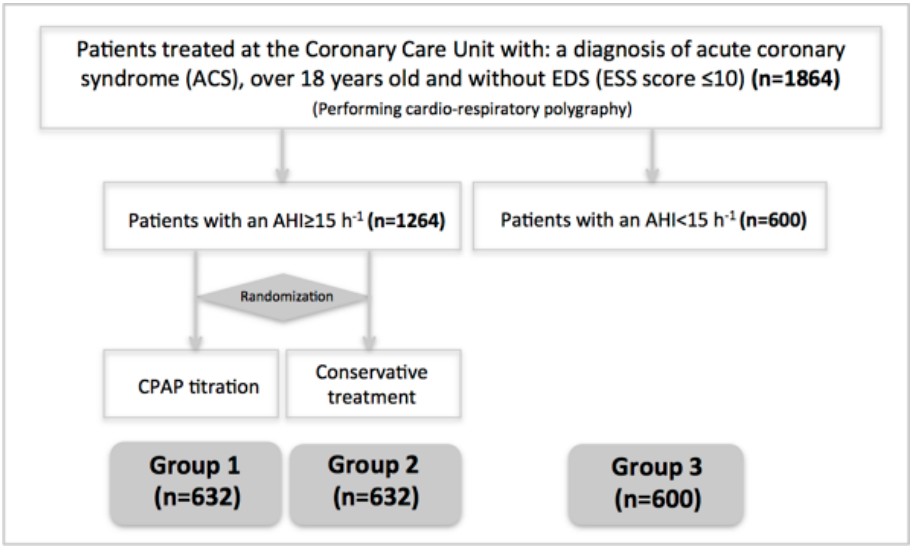

Figura 8.2: Diagrama del estudio ISAACC

La aleatorización del estudio se realiza mediante un sistema automático a través de una web especialmente diseñada para este estudio, disponible 24 h y protegida mediante clave de usuario y contraseña. La aleatorización de los pacientes a cada uno de los grupos se estratifica por centro. Dado que la prevalencia de pacientes con IAH<15/h es superior a la de los pacientes con IAH⩾15/h, el sistema selecciona de manera aleatoria los sujetos del grupo de referencia que finalmente van a ser incluidos en el estudio. De esta manera se garantiza que el ritmo de inclusión de todos los grupos sea similar.

Todos los pacientes aleatorizados a CPAP inician el tratamiento tan pronto como sea posible después de la aleatorización y continuan diariamente con el tratamiento durante un mínimo de un año o hasta que el estudio finalice. La titulación de presión a la CPAP se inicia la noche siguiente a la aleatorización y se llevará a cabo por los equipos automatizados. Se seguirá la metodología descrita por nuestro grupo [27]. Los pacientes que interrumpen el tratamiento continuarán las visitas clínicas programadas para registrar los eventos cardiovasculares durante el periodo de seguimiento.

Se realizan visitas de seguimiento al mes, y posteriormente a los 3, 6 y 12 meses tras la aleatorización, y cada 6 meses tras el primer año de seguimiento. Todos los pacientes realizan el mismo número de visitas de seguimiento y a todos se les realiza los mismos procedimientos. Se recogen variables sociodemográficas, antropométricas, clínicas, calidad de vida (Epworth y cuestionario EuroQol) y variables de sueño (resultado de la PCR). Se extraen muestras de sangre en la visita basal y al año para realizar determinaciones de marcadores de disfunción endotelial, de activación plaquetaria, de disfunción hemodinámica, de estrés oxidativo, del metabolismo óseo (vitamina D y hormona paratiroidea (PTH)) y otros marcadores de inflamación y de vitaminas antioxidantes. Las muestras se procesan y almacenan en los biobancos de cada uno de los centros (congeladas a -80 ºC). Al final del estudio se transportarán al Hospital Son Espases en donde se realizarán las determinaciones anteriormente descritas. Otras determinaciones bioquímicas se recogen a nivel local en cada centro. En el grupo CPAP se valora además el cumplimiento del tratamiento, definiéndose éste como la utilización de CPAP⩾4 h/dia, y otras variables asociadas al mismo. En este grupo de pacientes, y con el objetivo de aumentar la adherencia al tratamiento, se realiza un contacto telefónico o una visita presencial a los 10 días de haber iniciado la CPAP y se valoran soluciones para los mismos.

El estudio continuará hasta que un número mínimo, calculado estadísticamente, de episodios cardiovasculares se hayan observado o hayan participado todos los pacientes durante al menos una media de un año. Las variables principales del estudio son eventos cardiovasculares definidos como: muerte por causa cardiovascular, infarto no mortal, infarto cerebral no mortal u hospitalización por fallo cardíaco, angina inestable, accidente cerebrovascular transitorio o revascularización. Los pacientes son monitorizados un mínimo de un año o hasta que ocurra el primer evento desde la inclusión en el estudio. Los eventos cardiovasculares son certificados por un Comité de Eventos Clínicos (CEC) formado por tres profesionales externos.

El análisis estadístico se realizará en la población bajo el principio de análisis por intención de tratar (AIT). Se comparará el porcentaje de pacientes que han desarrollado un evento cardiovascular (variable principal del estudio) al año entre los grupos de estudio (grupo 1: CPAP y grupo 2: conservador). Se comparará el tiempo hasta el primer evento cardiovascular entre los grupos de tratamiento utilizando para ello un modelo de riesgos proporcionales de Cox. De igual manera se utilizará el método de Kaplan-Meier para la elaboración de las tablas de supervivencia y mediante la función estadística Log Rango se compararán las curvas de tiempo libre de evento entre todos los grupos. En segundo lugar se realizará un análisis multivariado mediante un modelo regresión de Poisson en que se incluirán las variables de interacción y/o confusión que se hayan identificado en el análisis descriptivo entre grupos (como pueden ser el nivel de cumplimiento al tratamiento, comorbilidades, edad, índice de masa corporal (IMC)). Un análisis secundario por protocolo (PP) se realizará teniendo en cuenta los pacientes cumplidores de la CPAP ($\geqslant 4$ h/dia de utilización). Se tratarán los valores perdidos durante el seguimiento mediante el sistema de múltiples imputaciones en todos los modelos. En el caso de los objetivos secundarios, se determinará la prevalencia de SAHS en la población con SCA que ingresan en la Unidad Coronaria. Se estudiarán las diferencias entre los grupos de estudio mediante un test de ANOVA en las diferentes variables de interés. Se realizará un estudio de colinealidad entre variables mediante correlaciones de Spearmen. En el caso de las variables cualitativas se evaluarán mediante el test de Chi-cuadrado (test exacto de Fisher si existe alguna frecuencia observada <5). Todos los test se realizarán a un nivel de significación del 5 % y los intervalo de confianza (IC) se calcularán al nivel de confianza del 95 %. En último lugar se realizará un análisis de coste-efectividad del tratamiento con CPAP en pacientes con SAHS y nuevo SCA mediante un modelo bayesiano. Para ello se incluirán los resultados obtenidos tras 12 meses de seguimiento en los pacientes con SAHS y nuevo SCA sin somnolencia diurna. En este análisis se comparará, por un lado, el tratamiento del SAHS mediante CPAP, y por otro la alternativa que consiste en dejar que los pacientes sigan su evolución natural sin tratamiento (tratamiento conservador). Se calculará la efectividad y el coste de ambas alternativas y se compararán mediante la razón coste-efectividad.

El estudio garantiza el cumplimiento de la Ley 15/1999 de Protección de Datos de Carácter Personal. La identidad de todos los participantes y todos los datos relativos al estudio se lleva a cabo en completa confidencialidad. El proyecto se ha registrado en la web de registro de ensayos clínicos ClinicalTrials.gov.

En el momento de presentar esta tesis (octubre de 2013) se han aleatorizado un total de 513 pacientes con IAH$\geqslant$15/h. Un total de 220 pacientes con IAH$<$15/h han sido incluidos en el grupo de referencia.

Trial Designs

Rationale and Methodology of the Impact of Continuous Positive Airway Pressure on Patients With ACS and Nonsleepy OSA: The ISAACC Trial

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ABSTRACT

Background: Obstructive sleep apnea (OSA) is common in acute coronary syndrome (ACS) and a possible cause of increased morbidity and mortality.

Objectives: The main objective is to determine in patients with ACS and OSA if CPAP treatment reduces the incidence of cardiovascular events (cardiovascular death, nonfatal myocardial infarction, nonfatal stroke, hospitalization for heart failure, and hospitalization for unstable angina or transient ischemic attack). The secondary objectives are to determine the prevalence of nonsleepy OSA in patients with ACS; assess the effect of CPAP on the incidence of newly diagnosed diabetes mellitus, symptoms, and quality of life; identify biomarkers of risk involved in cardiovascular complications in these patients; and conduct a cost-effectiveness analysis of diagnosis and treatment.

Population and Methodology: Multicenter, prospective, randomized and controlled study. Patients are admitted to the coronary care unit with diagnosis of ACS and without daytime sleepiness (Epworth Sleepiness Scale $\leq$10) at 15 teaching hospitals in Spain. All patients undergo a sleep study by cardiorespiratory polygraphy. Patients with an apnea-hypopnea index $\geq$15/hour will be randomized to treatment with CPAP (group 1, 632 patients) or conservative treatment (group 2, 632 patients). Patients with an apnea-hypopnea index $<$15/hour (group 3, 600 patients) will be followed as a reference group. Patients will be monitored at baseline (T0), 1 month (T1), 3 months (T2), 6 months (T3), 12 months (T4), and every 6 months thereafter (where applicable) during the follow-up period.

Conclusions: The ISAACC trial will contribute to evaluating the effect of CPAP treatment on cardiovascular events in patients with ACS and OSA.

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Background

Obstructive Sleep Apnea and Acute Coronary Syndrome

Obstructive sleep apnea (OSA) is characterized by the presence of total or partial respiratory pauses (apneas or hypopneas, respectively) caused by the collapse of the upper airway during sleep. It is a common process that affects 2%–4% of the general population age 30–70 years and is characterized clinically by the presence of snoring, daytime sleepiness, and impaired quality of life.[1,2] Every apnea episode is accompanied by a decrease in arterial oxygen saturation

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that is rapidly normalized after subsequent ventilation (hypoxia-reoxygenation), sudden changes in intrapleural pressure, and increased sympathetic activity. Treatment for OSA involves the application of nasal pressure via continuous positive airway pressure (CPAP) during sleep. Continuous positive airway pressure is an effective treatment for obstructive respiratory events, as it corrects symptoms and improves the quality of life of patients with OSA.[3,4]

Acute coronary syndrome (ACS) affects 1% of the adult world population and is a major world cause of morbidity and mortality, as one-third of the world population dies from cardiovascular disease (CVD).[5] It is estimated that the prevalence of OSA in patients with ACS is higher than in the general population: 54%–69%.[6,7] However, the evaluation of respiratory disorders during sleep does not feature in guidelines for the diagnosis, treatment, and subsequent management of patients with ACS. It is reasonable to suggest that pathogenic alterations described in patients with OSA may have a deleterious effect on the evolution of a patient who has suffered from ACS. Repeated episodes of hypoxia-reoxygenation, increased sympathetic activity, and abrupt changes in intrathoracic pressure can adversely influence the progress of a patient who has suffered from ACS.[8] These changes can also activate several pathogenic pathways that promote atherogenesis, such as oxidative stress, endothelial dysfunction, hypercoagulability, and insulin resistance.[9] These physiological changes may explain the increased risk of CVD in patients with OSA.[10–13] Long-term observational studies show that OSA significantly increases the risk of cardiovascular (CV) events.[14] It is also accepted that patients with OSA have a higher risk of sudden death during sleep than people without OSA,[10,15,16] and that OSA is associated with poor prognosis of CVD.[17–19] A recent study in patients admitted for ACS showed that, after adjusting for various confounding variables, OSA is an independent predictor of the appearance of new CV events during hospitalization (hospital death, acute myocardial infarction [MI], or angina; odds ratio: 3.4, 95% confidence interval [CI]: 1.3-9.0, $P = 0.015$).[20]

Need for a Randomized Controlled Trial

Treatment with CPAP reverses obstructive apneas, avoids episodes of hypoxia-reoxygenation, prevents recurrent episodes of negative intrathoracic pressure, and reduces sympathetic activity and blood pressure.[21,22] Clinically, treatment with CPAP reverses excessive daytime sleepiness and improves patients' quality of life,[23] but the data on the impact of CPAP on vascular morbidity and mortality are largely based on observational studies.[24,25] Various studies have assessed the impact of CPAP on the incidence of hypertension and CV events in patients with OSA. A study by the Marin group showed that in men, OSA significantly increases the risk of CV events and death from this cause. This study also showed that CPAP reduces this risk, compared with patients not treated with CPAP (odds ratio: 0.35, $P = 0.008$).[14] Büchner et al reinforce these results and showed, in patients with moderate OSA, that CPAP treatment is an independent protective factor for CV events (hazard ratio: 0.36, 95% CI: 0.21-0.62, $P < 0.001$).[26] Along these lines, Barbé and colleagues recently published another study that included 725 patients without sleepiness (Epworth Sleepiness Scale [ESS] score ≤ 10) and found a lower incidence rate of hypertension and/or CV events in the group of OSA patients complying with CPAP (incidence density ratio: 0.69, 95% CI: 0.50–0.94, $P < 0.05$), compared with untreated OSA patients.[27]

There are currently insufficient data to support the use of CPAP treatment in the primary or secondary prevention of CVD.[28] The overall objective of our study is to evaluate the impact of sleep apnea and its treatment on the clinical course of patients with ACS. We hypothesize that ACS patients with sleep-disordered breathing have a worse prognosis than those without OSA, and that CPAP treatment improves the prognosis. Acceptance of this approach in clinical practice would require changes in coronary units, and the evaluation of night ventilation would become part of the routine explorations of ACS patients. In this article we describe the rationale and methodology of the study according to international guidelines.[29,30]

Main Objectives

The main objective of the study is to determine whether treatment with CPAP reduces the rate of new CV events (CV death, nonfatal MI, nonfatal stroke, hospitalization for heart failure, and hospitalization for unstable angina, transient ischemic attack [TIA], or revascularization procedures) in patients with ACS and OSA. The secondary objectives are to determine the prevalence of OSA (in nonsleepy patients) who have suffered an ACS episode; assess the effect of CPAP on the incidence of newly diagnosed diabetes mellitus (according to the usual standards), symptoms of OSA (ESS and the EuroQol EQ-5D Quality of Life test [EQ-5D]); identify biomarkers of risk related to CV complications in these patients; establish the relationship between CPAP compliance and the incidence of CV events; and conduct a cost-effectiveness analysis of diagnosis and treatment.

Methodology

This is a multicenter, open-label, parallel, prospective, randomized, controlled trial, which started in June 2011. The study will include patients consecutively admitted to each participating center's coronary care unit or cardiology hospitalization room with ACS during the period of the study. Acute coronary syndrome is defined as the acute presentation of coronary disease, with or without ST-elevation infarction (patients with a normal electrocardiogram [ECG] and ischemic symptoms but only a minor rise and fall in any biomarker will be included), unstable angina, or MI type 1.[31] The diagnosis of OSA will be based on the results of the sleep test, in accordance with the guidelines of the national consensus on the apnea-hypopnea syndrome.[32] Patients must meet the criteria for inclusion/exclusion (Table 1).

The estimated duration of the study is 3 years. It involves a total of 15 teaching hospitals in Spain, all with a sleep disorders unit and coronary care unit: Hospital Arnau de Vilanova (Lleida), Hospital Son Espases (Palma de Mallorca), Hospital Clínic (Barcelona), Hospital Germans Tries i Pujol (Barcelona), Hospital de Sant Pau (Barcelona), Txagorritxu Hospital (Vitoria), Hospital de Cruces (Bilbao),

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Table 1. Inclusion/Exclusion Criteria

Inclusion Criteria
Men and women age >18 years
Patients admitted for documented symptoms of ACS with or without T segment elevation and have an hospital stay between 48 h and 72 h in the moment to perfom polygraphy
Patients with an ESS score ≤10 (patients without excessive daytime sleepiness)
Signature of informed consent
Exclusion Criteria
Previous treatment with CPAP
Psychophysical inability to complete the questionnaires
Presence of any previously diagnosed sleep disorders: narcolepsy, insomnia, chronic sleep deprivation, regular use of hypnotics or sedative drugs, and restless legs syndrome
Patients with >50% of central apneas or the presence of Cheyne-Stokes respiration
Patients with chronic diseases: neoplasms, renal insufficiency (GFR <15 mL/min/1.73 m^2), severe COPD (FEV1 <50%), chronic depression, and other limiting chronic diseases
A medical history that could interfere with the objectives of the study or could, in the opinion of the investigator, jeopardize the findings
Any medical, social, or geographical factor that could jeopardize patient compliance; for example, alcohol consumption (>80 g/d for men and >60 g/d in women), disorientation, or a history of noncompliance
Any process, whether cardiovascular or otherwise, that reduces life expectancy to <1 year
Patients in cardiogenic shock (unlikely to yield results in the short term)

Abbreviations: ACS, acute coronary syndrome; COPD, chronic obstructive pulmonary disease; CPAP, continuous positive airway pressure; CRP, cardiorespiratory polygraphy; ESS, Epworth Sleepiness Scale; FEV1, forced expiratory volume in 1 second; GFR, glomerular filtration rate; OSA, obstructive sleep apnea.

Hospital San Pedro de Alcántara (Cáceres), Hospital Parc Taulí (Barcelona), Hospital de Guadalajara (Guadalajara), Yague Hospital (Burgos), Hospital San Juan (Alicante), Hospital Central de Asturias (Oviedo), Hospital Joan XXIII (Tarragona), and Hospital of Albacete, all members of the Spanish Sleep Network. The trial is led by the coordinator center (Hospital Arnau de Vilanova, Lleida), which has overall responsibility for the design and the study follow-up.

During the patient's hospital stay, the degree of daytime sleepiness (ESS score) will be assessed in patients treated in the coronary care unit with a diagnosis of ACS. Patients without daytime sleepiness (ESS ≤10) who meet the other selection criteria (Table 1) will undergo a cardiorespiratory polygraphy[27,33] after staying between 24 and 72 hours in the hospital. The definitions and diagnosis of the subjects' other disorders will also be carried out according to standard guidelines. All the participating hospitals will use

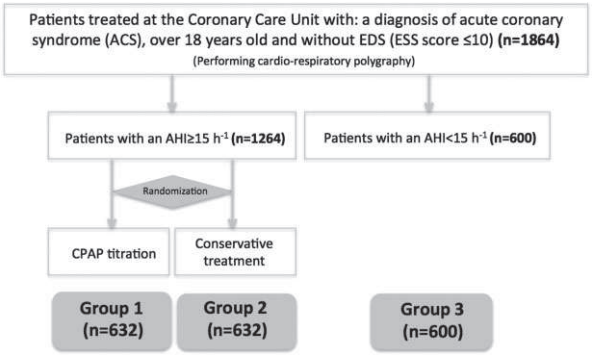

Figure 1. Study flow diagram.

the same model of polygraph (Embletta; ResMed, Spain). Oronasal flow, thoracoabdominal movements, ECG, and pulse oximetry will all be recorded. Apnea is defined by an absence of airflow lasting ≥10 seconds. Hypopnea is defined as a reduction in airflow lasting ≥10 seconds and is associated with oxygen desaturation. Oxygen desaturation is considered as a decrease in SaO_2 >4%. Apnea-hypopnea index (AHI) is defined as the number of apneas and hypopneas per hour of sleep. Excessive daytime sleepiness, as reported by the subject, will be defined as an ESS score >10, and no daytime sleepiness will be defined as an ESS score of ≤10. This distinction is important because, from an ethical viewpoint, a patient with OSA with an ESS score >10 should be treated with CPAP. The degree of self-reported sleepiness/drowsiness will be analyzed by the Spanish version of the ESS test.[34]

The study will consider 3 groups, with a total of 1864 patients, distributed as follows: patients with an AHI ≥15/hour will be randomized to treatment with CPAP (group 1, n = 632) or conservative treatment (group 2, n = 632); patients with an AHI <15/hour will be followed up according to the CV protocols and evaluated as a reference group (group 3, n = 600) (Figure 1). Patients with an ESS >10 will be excluded from the study and referred to the sleep unit of each participating center for evaluation. All participants will receive sleep-hygiene advice and dietary counseling for weight loss from the staff of the sleep units at each scheduled visit.

Randomization

The randomization in the ISAACC study will be carried out via an automated system available 24 hours a day, with totally secure access to the study Web site protected by password. The randomization will be stratified by center. Once a patient is included in the study, he or she will be registered and assigned to a randomly selected group (if he or she belongs to the group of patients with an AHI ≥15/hour).

As the frequency of patients with AHI <15/hour is higher than that of patients with AHI ≥15/hour and the rate of inclusion into the reference group must match that of the randomized patients, a probabilistic mechanism integrated into the Web site will select patients once the sleep-test results are known. Only patients selected by the Web site will be included in the study.

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Titration of Continuous Positive Airway Pressure

This will be conducted by means of automated equipment. It will follow the methodology described by our group.[35] After titration, patients will continue with fixed CPAP. This study group will also be instructed in the dietary-hygienic measures recommended for all patients.

Duration of Treatment

All the patients randomized to CPAP treatment will begin as soon as possible after randomization, and their treatment will continue on a daily basis for at least 1 year until the end of the study. Patients who interrupt the treatment will continue with their scheduled clinical visits so that their CV events during the follow-up period can be recorded.

Follow-up

All patients will be evaluated at baseline (T0), 1 month (T1), 3 months (T2), 6 months (T3), 12 months (T4), and every 6 months thereafter (where applicable) during the follow-up period (Table 2). The study will continue until all the patients have participated in it for at least 1 year. No relevant concomitant care or interventions will be prohibited during the trial.

Compliance

The aim of the visits at T1 and T2 is to facilitate adaptation and adherence to CPAP (group 1) and insist on dietary-hygienic modifications. All the collaborating centers have great experience in the management of CPAP treatment. All the patients will be given a phone number allowing them to contact the research team at any time. Telephone follow-up will be undertaken within 15 days of the start of treatment, and, if necessary, additional visits will be scheduled to try to immediately resolve any problems related to the adaptation to CPAP.

Study Variables

Sociodemographic and anthropometric variables will be recorded, along with those associated with the quality of

Table 2. Diagram of the Methodology at Each Visit

	Patient Inclusion (To)	1 Month (T1)	3 Months (T2)	6 Months (T3)	12 Months (T4)	From 18 to 36 Months (Each 6 Months)
Providing written consent to participate in the study	All groups					
Providing written consent for the storage of samples in the biobank	All groups					
CRP	All groups					
Assessment of sleepiness (ESS)	All groups	All groups	All groups	All groups	All groups	All groups
Randomization to treatment with CPAP or conservative	Groups 1 and 2					
Anthropometric and sociodemographic data collection	All groups				All groups	
BP measurement	All groups				All groups	
Performing specific questionnaires (EuroQol, HADS)	All groups				All groups	
Clinical follow-up		All groups	All groups	All groups	All groups	All groups
Monitoring and follow-up	All groups	All groups	All groups	All groups	All groups	All groups
Extraction, processing, and storage of blood samples	All groups				All groups	
Checking for compliance with CPAP treatment		Groups 1 and 2	Groups 1 and 2	Groups 1 and 2	Groups 1 and 2	Groups 1 and 2
Hemogram	All groups				All groups	
Coagulation fibrinolysis	All groups				All groups	
Basic biochemical profile	All groups				All groups	
Evaluation of markers of myocardial damage	All groups				All groups	
Evaluation of other specific biomarkers	All groups				All groups	

Abbreviations: BP, blood pressure; CPAP, continuous positive airway pressure; CRP, cardiorespiratory polygraphy; ESS, Epworth Sleepiness Scale; EuroQol, EuroQol EQ-5D Quality of Life Scale; HADS, Hospital Anxiety and Depression Scale.

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life (EuroQol EQ-5D) and degree of daytime sleepiness. The time of the day of the onset of ACS/MI symptoms will be recorded. The EQ-5D is a standardized instrument used to measure the quality of life, applicable to a wide range of health conditions and treatments; it provides a simple descriptive profile and a single index value for health status.

At baseline and after 1-year follow-up, blood samples will be obtained in fasting. These samples will be processed and sent to the Hospital Son Espases for the analysis of biomarkers related to CV function: markers of endothelial dysfunction, platelet activation, fibrinolytic system markers, markers of hemodynamic dysfunction, oxidative stress markers (isoprostanes), markers of inflammation (C-reactive protein, interleukin-6, interleukin-8, adiponectin), and markers of bone metabolism (vitamin D, intact parathyroid hormone) and antioxidant vitamins (A, E, and C). Aliquots of serum and plasma from patient blood samples will be immediately processed and stored at −80 °C prior to dispatch to the Hospital Son Espases. Additional biochemical biomarkers will be analyzed individually in each participant center.

Patients treated with CPAP will be monitored for compliance. The degree of compliance will be determined by dividing the number of hours of use (obtained from the internal clock of the CPAP device) by the number of days of treatment. Compliance is defined as CPAP use ≥4 hours/day.

Main dependent variables: Each follow-up visit will also include assessments of CV events, such as CV death, nonfatal MI, nonfatal stroke, and hospitalization for heart failure (with reduced or preserved ejection fraction) or unstable angina, TIA, or revascularization procedures. Hospitalization for heart failure is defined as hospital or emergency admission for the administration of intravenous diuretics and/or use of inotropes, for an acute illness with dyspnea and/or evidence of heart enlargement and/or pulmonary interstitial changes compatible with pulmonary edema.

All these events must be reported and documented via records provided by the patient or derived from the center's own records. Secondary dependent variables are biological risk markers implicated in CV complications, symptoms and quality of life, and the cost-effectiveness of the diagnosis and treatment with CPAP.

Criteria for Discontinuation

The follow-up for the main outcome will be terminated whenever a patient experiences a new CV event, withdraws informed consent, is unable to complete the follow-up, or moves from the conservative group to CPAP treatment. Exposure time was defined as the time between randomization and the first CV event, date of death, date of the last study visit, date of withdrawal, or loss to follow-up.

Data Collection and Analysis

The coordinating center (Hospital Arnau de Vilanova, Lleida) will prepare guidelines to standardize procedures in the protocol for all questionnaires and data collection and explain how to implement them. The center will provide instructions for entering data onto a database on a specific Web domain, taking into account the legal requirements for data protection. Informed consent will be obtained by the collaborating investigators. All the forms and questionnaires collected from patients will be processed in accordance with properly validated methods. The project will be completed by a cost study designed to quantify the costs of each process in each patient group according to the different results. The costs will be calculated on the basis of the direct cost of the data extracted from the medical records of each participating center. A cost-effectiveness study will also be developed, including an estimate of the quality-adjusted life-years gained. An external data safety monitoring committee will review the quality of the recorded data in each center. A blinded clinical events committee (CEC), comprising 3 external staff from the Hospital Arnau de Vilanova in Lleida, the Hospital Clínic in Barcelona, and the Hospital Son Espases in Palma de Mallorca, will assess the study's outcomes (CV events). All the reported primary and secondary outcomes will be centrally reviewed by the CEC, which will obtain data on the ECG and enzymatic changes and confirm any episode that meets all the diagnostic criteria for such an event. In case of doubt, the CEC will review the additional supporting documentation, such as computed tomography and magnetic resonance imaging, as well as neurological reports, enzyme readings, chest X-rays and blood tests, if available. Training sessions will be organized for the participating center to provide them with backup information and documentation to enhance the evaluation and diagnosis of CV events. Any event or serious adverse effect is defined by the World Health Organization criteria for International Drug Monitoring (1994).

Sample-Size Calculation

According to the data in the literature, the prevalence of OSA in people with CVD is 54%–69%.[6,7] An estimated 12%–20% of patients with ACS have a new CV event in the first year.[10,14] This percentage is higher in the population with concomitant OSA.[22] It is estimated that the number of patients required to detect a decrease of ≥25% in the likelihood of further CV events after starting treatment with CPAP in this group of patients is 632 subjects in each intervention group (groups 1 and 2). For comparisons between groups, it has been determined that a total of 600 subjects would be required in group 3, thus requiring the inclusion of 1864 subjects. This calculation assumes an α error of 0.05 and a statistical power of 80%. It has also been estimated that 10% of the patients will be lost in the follow-up. The sleep study should be performed in approximately 4214 patients with ACS. It is expected that, of the 4214 patients evaluated, approximately 1264 (40%) will have an AHI >15/hour.

Ethical Issues

The study will be conducted according to the guidelines and principles of the Declaration of Helsinki and standard ethical conduct for research involving humans. The study will also guarantee compliance at all times with Law 15/1999 on Protection of Personal Data (Spanish Government). The identity of all the participants and all the data pertaining to them will be held in complete confidentiality. The Ethics Committees for Clinical Research of all the participating centers approved this study. All the study subjects will provide written informed consent before participating. They

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must also give informed consent for the storage of biological samples in the biobanks of the participating centers. This study is registered with ClinicalTrials.gov (NCT01335087).

Statistical Analysis

All the study variables will be tabulated on a database accessible only to the study investigators. In the case of the qualitative variables, their frequency and valid percentage will be determined. Missing values will be taken into account for the results. The quantitative variables will include measures of central tendency and measures of dispersion and position.

Analysis of evolution variables (dependent variables): The statistical analysis will be based on the principle of intention to treat. In a first analysis, the time until a first CV event will be compared between treatment groups (groups 1 and 2) using a Cox proportional hazards model. Similarly, the Kaplan-Meier test will be used to prepare survival tables and the log-rank statistical function will be used to compare the event-free time between all groups. Secondly, a multivariate study will be undertaken, using a Poisson regression model; this will include interaction and/or confusion variables that identified in the descriptive analysis between groups (such as the level of compliance with treatment, comorbidities, age, and body mass index). A secondary per-protocol analysis will be performed, considering patients with CPAP compliance (use CPAP $\geq$4 hours/day). Multiple imputation will be used in all the models to include participants who were lost to the follow-up.

Secondary objectives: We will determine the prevalence of OSA in the population with ACS admitted to the coronary care units. We will study the differences between the study groups by analysis of variance for the different variables of interest. There will be a collinearity study between variables, using Spearmen correlations. The qualitative variables will be evaluated using the χ^2 test (or the Fisher exact test if any frequency is expected to be <5).

Cost-effectiveness analysis: There will be a study of the cost-effectiveness of treatment with CPAP in patients with OSA and new ACS, using the Bayesian model. This will include the results obtained after 12 months of follow-up in patients with OSA and new ACS without excessive daytime sleepiness. One group of patients will be treated for OSA with CPAP, and in another group the disease will be allowed to follow its natural course without treatment (conservative treatment). We shall calculate the effectiveness and cost of both alternatives and compare them by means of the cost-effectiveness ratio.

All the tests will be conducted at a significance level $\alpha = 0.05$ and the CIs will be calculated at the CI $(1\text{-}\alpha) = 0.95$. The SPSS version 19 software (IBM Corp., Armonk, NY) will be used for all analyses.

Progress During the Start-up Phase

Planning for the ISAACC study began in 2010. Fifteen centers are collaborating in the trial. The Hospital Arnau de Vilanova (Lleida) is the coordinating center. Recruitment began in June 2011, with the coordinating center as the first center; the last center was incorporated in December 2012. As of April 25, 2013, 437 patients with AHI $\geq$15/hour have been randomized into the trial and 220 patients have been included in the reference group. The impediments to recruitment have varied, but the most important point is that the prevalence of AHI $\geq$15/hour in patients with ACS is lower than expected.

Relevance of the Study

The results of this project will determine the impact of the sleep apnea syndrome and its treatment with CPAP on the evolution and prognosis of patients suffering from ACS. Acceptance of its relevance in clinical practice would require changes in coronary units, and the evaluation of night ventilation would become part of the routine explorations of ACS patients. The study also sought to identify clinical markers and/or the related biological clinical course of patients with ACS and OSA, making it possible to identify those individuals most susceptible to complications.

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9 DISCUSIÓN

La presente tesis doctoral tiene dos partes diferenciadas. Por un lado, analiza una serie de aspectos metabólicos que pudieran relacionar el síndrome apnea-hipopnea del sueño (SAHS) y la enfermedad cardiovascular recogidos en tres estudios. Por otro, a nivel clínico, expone detalladamente el diseño y el desarrollo de un protocolo de investigación que tiene como objetivo estudiar el impacto del SAHS y de su tratamiento con presión positiva continua en la vía aérea *(siglas en inglés de Continuous positive airway pressure)* (CPAP) sobre la aparición de nuevos eventos cardiovasculares en pacientes con SAHS y síndrome coronario agudo (SCA).

En el primer estudio se determinó la prevalencia de síndrome metabólico (SM) en una cohorte de 535 pacientes con diagnóstico de SAHS, y se evaluaron las relaciones entre las variables de sueño, la resistencia a la insulina (RI) y otras alteraciones metabólicas. Igualmente, se analizó la relación entre el SM y la presencia de somnolencia diurna. Nuestros datos mostraron que la prevalencia de SM, según la definición del III programa nacional de educación sobre el colesterol del panel de expertos en la detección, evaluación y tratamiento de la hipercolesterolemia en adultos (NCEP-ATP III), fue del 52 %. Usando la misma definición, otros estudios clínicos en pacientes con SAHS muestran una prevalencia de SM del 23-87 % [90, 91, 92, 93, 94, 95]. Por otro lado, estudios poblacionales han reportado una prevalencia inferior: 26-35 % [96, 97, 98]. La combinación más frecuente de los componentes de SM en el presente estudio incluía el perímetro de cintura elevado, hipertensión arterial (HTA) y niveles elevados de glucosa en ayunas en sangre. Estos hallazgos están en concordancia con los reportados por Kono et al. [99]. Por el contrario, en el estudio de Sasanabe et al. [91] se observó que la dislipemia, y no la glucosa en ayunas, era el tercer componente más frecuente.

La gravedad del SAHS se relacionó significativamente con la presencia de SM observándose que la presencia de SM era más frecuente en los pacientes con SAHS grave. Aproximadamente el 10 % de nuestros pacientes no presentaba ninguno de los componentes de SM. Este subconjunto de pacientes presentaban características diferentes del resto de la muestra, ya que los sujetos eran más jóvenes, menos obesos y tenían SAHS leve. Es posible que la ausencia de componentes de SM represente una etapa inicial de la historia natural del SAHS o que estos pacientes presenten un fenotipo diferente que deba ser estudiado en profundidad. Los marcadores de gravedad del SAHS (índice de apnea-hipopnea (IAH) y saturación de oxígeno media) mostraban relación con cada uno de los componentes de SM en los modelos crudos, pero tras ajustar por las variables de confusión (edad, consumo de tabaco, índice de masa corporal (IMC) y sexo) sólo la presión sistólica y diastólica se relacionaron con el IAH. En el caso de la saturación de oxígeno media, el perímetro de cintura y la presión sistólica mantenían la relación en el modelo ajustado.

El SM se considera como una manifestación clínica de la RI [62, 100, 101, 102]. Aunque existen datos que sugieren que la RI no se relaciona con el SM en pacientes con SAHS [103, 104], en nuestro estudio se observó una tendencia lineal entre el índice del modelo de evaluación homeostática *(siglas en inglés de Homestatic Model Assessment)* (HOMA) y el número de componentes de SM. Esta última variable, considerada como índice metabólico, podría ser un indicador de la carga metabólica en pacientes con SAHS. Las correlaciones entre mar-

cadores de gravedad del SAHS y el índice HOMA perdieron la significación tras ajustar por IMC, lo que indica un papel importante de la obesidad en la relación entre la SAHS y la RI.

Según nuestros datos, la prevalencia de somnolencia fue la misma en los pacientes con y sin SM. Por este motivo, ha demostrado ser un marcador poco sensible para SM y otras alteraciones del sueño a pesar de contemplarse en diversos trabajos como un marcador de gravedad del SAHS, especialmente para las alteraciones cardiovasculares y metabólicas [105, 106, 107, 108, 109]. El Epworth no evalúa la somnolencia diurna excesiva *(siglas en inglés de Excessive daytime sleepiness)* (EDS) de manera objetiva, y la falta de resultados de pruebas de latencia del sueño es uno de las principales limitaciones de nuestro estudio.

En resumen, los resultados obtenidos revelan que el SM, según la definición del NCEP-ATP III, se presenta en la mitad de los pacientes con SAHS y que el 38,2 % de los pacientes mostraban tener uno o dos componentes del SM. El número de componentes de SM correlacionó con el índice HOMA en pacientes con SAHS y puede ser un indicador clínico de RI. Sin embargo, la EDS no parece ser un marcador sensible de alteración metabólica.

El segundo estudio incluyó un total de 119 pacientes con SAHS y 119 controles similares en cuanto a edad, sexo e IMC con el objetivo de evaluar si la concentración plasmática de ácidos grasos (AG) libres era superior en el grupo de pacientes con SAHS y si estos niveles podían determinar la presencia de SM. Este estudio muestra diferentes hallazgos entre ellos que la prevalencia de SM es superior en los pacientes con SAHS que en los controles de la misma edad, sexo y el IMC, lo que sugiere que el SAHS es un factor de riesgo para el SM. También se muestra que la concentración plasmática de AG libres es elevada en pacientes con SAHS y por último se observa que el IAH se relaciona de manera independiente con la concentración plasmática de AG libres.

La prevalencia de SM en los pacientes con SAHS fue significativamente superior que en el grupo control (38 % vs. 21 %) y se asoció con un aumento de la presencia de factores de riesgo para la presencia de SM. El SM se asocia con el desarrollo de eventos cardiovasculares, diabetes y enfermedad del hígado graso de causa no alcohólica [110, 111, 112]. Nuestros datos muestran que el grupo SAHS presentaba también niveles alterados de glucosa, gamma glutamil transpeptidasa, proteína C reactiva y 8- isoprostanos. La concentración plasmática de AG fue significativamente superior en el grupo SAHS. Además, en los pacientes sin SM, la concentración plasmática de AG fue superior en los pacientes SAHS que en los controles. Por el contrario, al comparar pacientes y controles que presentaban SM, no se detectaron diferencias en los niveles de AG entre ambos grupos.

Nuestros datos han mostrado una correlación significativa entre los niveles de AG libres y el IAH e índice de *arousals*, lo que sugiere que la fragmentación del sueño y los despertares repetitivos pueden estar relacionados con la liberación de AG al torrente sanguíneo. El IAH se relaciona con la concentración plasmática de AG independientemente de la edad, el sexo, el IMC y la presencia de SM. Los pacientes con SAHS grave presentan niveles de AG superiores en comparación con el grupo de SAHS moderado o leve. Varias líneas de investigación apoyan una asociación independiente entre SAHS y la alteración del metabolismo de lípidos [113, 114]. En nuestro estudio se observó una relación entre la concentración de AG libres y la concentración de lipoproteína de alta densidad *(siglas en inglés de High density lipoprotein)* (HDL), lo que sugiere que el aumento del flujo de AG libres en el hígado puede ser un factor importante para el desarrollo de dislipemia en pacientes con SAHS. Es posible que los niveles elevados de AG libres en plasma puedan ser uno de los mecanismos involucrados en las complicaciones metabólicas y cardiovasculares de los pacientes con SAHS.

En nuestro análisis no se han tenido en cuenta algunas variables que pueden actuar como factores de confusión: el estado nutricional, la actividad física o algún componente genético. Tampoco se han ajustado los resultados por los niveles de albúmina.

En resumen, nuestro estudio muestra que la concentración plasmática de AG libres es elevada en los pacientes con SAHS y puede desempeñar un papel importante en la patogénesis del SM en pacientes con apnea del sueño. Son necesarios estudios de intervención para evaluar el impacto de la CPAP en la normalización de los niveles de AG ya que podría contribuir en la mejora de la RI y otras alteraciones metabólicas.

En el tercer estudio se estudió la relación entre la concentración plasmática de vitamina D y de hormona paratiroidea (PTH) con los factores de riesgo cardiovascular como la diabetes mellitus tipo 2 (DM 2), HTA y SM en pacientes con SAHS. En este estudio se incluyeron un total de 826 pacientes con nuevo diagnóstico de SAHS. Los resultados muestran una alta prevalencia de déficit de vitamina D en los pacientes con SAHS, siendo de un 55,3 % en varones y un 63,2 % en mujeres. El déficit de vitamina D se asoció con la presencia SM y DM 2 independientemente de la edad, el sexo o la estación del año. Un estudio reciente ha observado una asociación entre el déficit de vitamina D y un aumento en la RI y DM 2 [115]. Nuestros resultados muestran una asociación inversa entre los niveles de vitamina D y la prevalencia de DM 2. En estos pacientes el déficit de vitamina D podría ser un marcador de DM 2 en los pacientes con SAHS.

Por otro lado, la evidencia clínica y diversos estudios experimentales apoyan una relación inversa entre los niveles de vitamina D y los componentes del SM, incluyendo HTA, dislipemia, hiperglucemia y obesidad abdominal [79, 116]. Se han propuesto varios mecanismos para explicar estas relaciones. Según uno de ellos, la vitamina D regularía el sistema renina-angiotensina controlando las cifras de presión arterial. También se ha postulado que el déficit de vitamina D podría alterar el perfil lipídico (colesterol HDL) mediante el aumento de la resistencia periférica a la insulina [115, 117, 118]. En nuestro estudio, la obesidad se asoció de manera directa con el déficit de vitamina D [119, 120]. El aumento del depósito de vitamina D en el tejido adiposo podría ser la causa de la alta prevalencia de déficit de vitamina D observada en individuos obesos [121]. Alternativamente, la obesidad también puede ser la consecuencia del déficit de vitamina D [118]. En nuestro estudio también se encontró una mayor prevalencia de obesidad abdominal, un aumento de la concentración plasmática de triglicéridos y niveles más bajos de colesterol HDL en los pacientes con déficit de vitamina D tras ajustar por otros componentes del SM.

Por otra parte, una concentración plasmática elevada de PTH se asoció a una mayor prevalencia de obesidad y de HTA. La concentración plasmática de PTH fue también más alta en los pacientes con SAHS grave que en los pacientes con SAHS leve-moderado, y se asoció con los índices de hipoxia nocturna de manera independiente. Nuestros resultados coinciden con los de otros estudios previos en los que se observaron correlaciones positivas entre la concentración plasmática de PTH y las cifras de presión sistólica y diastólica independientemente de la concentración plasmática de vitamina D y de otros factores de confusión [77, 122]. Estos datos sugieren que la PTH ejerce un efecto sobre la presión arterial.

En conjunto los resultados obtenidos sugieren una asociación inversa entre la concentración plasmática de vitamina D y la prevalencia de DM 2 y SM, así como una asociación positiva entre la concentración plasmática de PTH y la prevalencia de obesidad y de HTA. Pese a ello, se necesitan más estudios clínicos prospectivos para poder establecer causalidad.

Los datos obtenidos en estos tres estudios han demostrado que los pacientes con SAHS presentan una alta prevalencia de factores de riesgo cardiovascular tales como el SM, la hipertrigliceridemia o el déficit de vitamina D. Estos datos ponen de manifiesto la necesidad de poner en marcha un estudio clínico que evalúe, por un lado, el impacto sobre el riesgo cardiovascular del SAHS en nuestro país, no sólo en lo que se refiere a la prevalencia de factores de riesgo, sino también a la evolución y el pronóstico de los pacientes una vez diagnosticada la enfermedad cardiovascular. Igualmente interesa cuantificar el impacto del tratamiento con CPAP en la evolución y pronóstico de los pacientes con SCA. Además de resaltar esta necesidad, los datos obtenidos permiten diseñar el estudio y la metodología más adecuada para dar respuesta a estas preguntas.

El cuarto artículo presentado detalla estos aspectos fundamentales para el éxito del estudio. En base a estos resultados, se ha programado un estudio multicéntrico nacional, prospectivo, aleatorizado, abierto y controlado, el Estudio ISAACC, con el objetivo de analizar el impacto del SAHS y de su tratamiento con CPAP en el pronóstico y la evolución de los pacientes con SCA.

Se eligió el diseño aleatorizado dado que éste permite que todos los factores ajenos al estudio se distribuyan por igual en los grupos a analizar y que la única diferencia entre ellos sea el tratamiento. Esto aumenta la validez interna y externa del estudio al luchar contra los sesgos de selección y confusión. Por otro lado, no se consideró el enmascaramiento del tratamiento (CPAP placebo) por el coste económico asociado a la distribución de 632 dispositivos (número de pacientes asignados al grupo conservador), aunque hubiera sido adecuado. En estos casos se puede cometer un sesgo de información. Para corregir este sesgo, la persona que evalúa el evento cardiovascular (variable principal) desconoce el grupo al que pertenece el paciente. Se creó un Comité de Eventos Clínicos (CEC) formado por 3 cardiólogos que durante todo el estudio, y de manera ciega, valora la documentación enviada por los centros y certifica que el evento reportado es realmente un evento cardiovascular. La persona a cargo de la monitorización del proyecto es la encargada de la comunicación entre los centros y el CEC. Una vez que se confirma el evento, el paciente finaliza el seguimiento.

Para el cálculo del tamaño de la muestra se tuvo en cuenta la prevalencia de SAHS en la población con enfermedad cardiovascular, estimada en un 54-69 % [123, 124]. Entre el 12 y el 20 % de los pacientes con SCA tiene un nuevo evento cardiovascular durante el primer año [41, 42, 125, 126, 127]. Este porcentaje es mayor en la población que presenta además SAHS [128]. Siendo conservadores, en esta aproximación hemos optado por estimar que el 20 % de los pacientes con SCA que presentan SAHS realizan un nuevo evento al año. Se estima que el número de pacientes con SAHS necesario para detectar una disminución de al menos el 25 % en la prevalencia de nuevos episodios cardiovasculares al año, después del inicio del tratamiento con CPAP, es de 632 sujetos en cada grupo de intervención (grupos 1 y 2, total de 1264 sujetos). Se ha considerado una ratio entre grupos de 1:1. Para las comparaciones entre los grupos de estudio, se determinó un total de 600 sujetos en el grupo 3, por lo que se requeriría la inclusión de 1864 pacientes. Este cálculo asume un error alfa de 0,05 y una potencia estadística del 80 %. Por otra parte, se ha tenido en cuenta una estimación de un 10 % de pérdidas durante el seguimiento. Se espera que un 15 % de los pacientes evaluados tengan un IAH$\geqslant$15/h. El cálculo de la muestra indica la aleatorización de 1264 pacientes, para ello es preciso realizar poligrafía cardiorrespiratoria (PCR) a 4214 pacientes con SCA. Un tamaño de muestra adecuado aumenta la validez interna del estudio y como consecuencia, la externa.

Otros factores que influyen en la validez externa del estudio son el ámbito de estudio y la selección de pacientes. En este caso se ha diseñado un estudio a nivel nacional que incluye

la colaboración de 15 centros distribuidos por toda el área geográfica, hecho que aumenta la generalización de los resultados. Otro punto importante es la selección de los pacientes ya que es una de las variables más influyentes en la validez externa. En este caso es muy importante la definición de los criterios de inclusión y exclusión. En nuestro estudio, sólo se incluyen aquellos pacientes con SCA, sin somnolencia. Los pacientes con SAHS y síntomas podrían ser candidatos de CPAP y en este caso no sería adecuada la aleatorización de los pacientes a CPAP o tratamiento conservador.

El elevado tamaño de la muestra prevista (n=1864) plantea la necesidad de una monitorización de los datos durante el periodo de seguimiento y de un control de calidad previo análisis de los mismos. En primer lugar, se plantea un análisis por intención de tratar (AIT). Según la definición más ampliamente aceptada, propuesta por la Asociación Estadística de los EEUU [129, 130], el AIT consiste en incluir a todos los pacientes en los grupos en los que fueron aleatoriamente asignados, independientemente de que cumplieran o no los criterios de inclusión, del tratamiento que recibieran, de que abandonaran el tratamiento o de que se desviaran del protocolo. Con el AIT se pretenden dos cosas: en primer lugar, se trata de conseguir que las condiciones iniciales de los dos grupos sean tan iguales como sea posible (esta es la razón de la asignación aleatoria y la esencia del diseño de un ensayo clínico) y esa igualdad no se pierda por acontecimientos no intencionados que puedan tener lugar tras la asignación aleatoria; en segundo lugar, que las condiciones del ensayo clínico sean más parecidas a las de la práctica clínica diaria, en la que también hay pacientes mal diagnosticados y que no cumplen o cumplen parcialmente el tratamiento. Es decir, el AIT permitiría obtener del ensayo clínico información sobre la efectividad del tratamiento (efecto en condiciones reales) en lugar de sobre la eficacia (efecto en condiciones ideales). Por el contrario, este tipo de análisis presenta la desventaja de tender a minimizar el efecto de la intervención, ya que en el mismo grupo se incluyen pacientes que no realizan la intervención correctamente o que la realizan parcialmente y que, por lo tanto, pueden obtener menos beneficios. Así, esta forma de análisis representa un enfoque conservador según el cual el efecto beneficioso de la intervención puede parecer menor que el efecto potencial si todos hubiesen realizado correctamente la intervención, pero cuando un resultado es significativo en este tipo de análisis, asegura que el efecto existe y, en general es más potente que el observado en el estudio.

Con el fin de tener información acerca de la eficacia del tratamiento, se realizará también un análisis por protocolo (PP). En este tipo de análisis se incluyen los pacientes que han utilizado correctamente la CPAP (cumplimiento $\geqslant 4$ h/dia de utilización) y que han finalizado el seguimiento. Con el objetivo de tener en cuenta las posibles variables que muestren discrepancias entre los grupos a pesar de la aleatorización, se realizarán modelos multivariados que tengan en cuenta las posibles variables de confusión.

El proyecto tiene un diseño experimental y se ha redactado acorde a las recomendaciones de las guías CONSORT y SPIRIT [131, 132]. El estudio se lleva a cabo de acuerdo con las directrices y los principios de la Declaración de Helsinki y la norma de conducta ética para la investigación con seres humanos, y garantiza el cumplimiento en todo momento con la Ley 15/1999, de Protección de Datos de Carácter Personal. La identidad de todos los participantes y todos los datos relativos al estudio se lleva a cabo en completa confidencialidad. Los Comités de Ética clínica de cada uno de los centros participantes han aprobado el proyecto. Todos los sujetos incluidos firman por escrito el consentimiento informado antes de participar. Los participantes también dan su consentimiento informado para el almacenamiento de muestras biológicas en los biobancos de los centros participantes. El proyecto se ha registrado en la web de registros de ensayos clínicos ClinicalTrials.gov.

Se han planteado otras alternativas para minimizar los posibles sesgos y limitaciones debidos al diseño abierto del estudio y garantizar su validez interna [133]. El centro coordinador ha centralizado todo el proceso, ha preparado una guía para la recolección de los datos y la estandarización de los procedimientos. Todas las escalas y cuestionarios utilizados en el estudio están validados al castellano. Se han realizado reuniones en cada centro colaborador al inicio del estudio y existe un contacto continuo entre el centro coordinador y el resto de centros. Esta comunicación permite la resolución de incidencias y dudas en el protocolo a tiempo real. Los datos se introducen en una base de datos a través de un aplicativo de Internet que cumple las normativas legales sobre protección de datos. En esta base de datos se realizan todos los procedimientos del estudio: inclusión de pacientes, aleatorización, registro de la toda la información recopilada, acceso a la documentación del estudio (cuestionarios, consentimientos, etc...). La aleatorización se realiza de manera automatizada en la base de datos. Un webmaster se encarga del mantenimiento y actualización de la base de datos y dominio web.

El estudio se inició en 2010. El Hospital Arnau de Vilanova (Lleida) es el centro de coordinador, donde se inició el reclutamiento en junio de 2011. El último centro se incorporó en diciembre de 2012. En la actualidad se han aleatorizado un total de 513 pacientes con IAH$\geqslant$15/h y 220 pacientes han sido incluidos en el grupo de referencia (IAH<15/h). Los obstáculos de reclutamiento han variado, pero el más importante es que la prevalencia de IAH$\geqslant$15/h en los pacientes con SCA incluidos es menor de lo esperado. Se prevé un total de 2 años para la finalización del reclutamiento.

En resumen, en estos momentos no hay datos suficientes para apoyar el uso del tratamiento con CPAP en la prevención primaria o secundaria de la enfermedad cardiovascular [134]. Los resultados del proyecto podrían determinar el impacto de la apnea del sueño y su tratamiento con CPAP sobre la evolución y el pronóstico de los pacientes con SCA. La aceptación de su relevancia en la práctica clínica requeriría cambios en las Unidades Coronarias y la evaluación de la ventilación nocturna pasaría a formar parte de las exploraciones de rutina de los pacientes con SCA. El estudio también tratará de identificar marcadores clínicos y/o biológicos relacionados con la evolución y el pronóstico de los pacientes con SCA, lo que hará posible identificar a los individuos más susceptibles a complicaciones cardiovasculares. Por todo ello se ha diseñado y puesto en marcha el estudio ISAACC.

10 CONCLUSIONES

Esta tesis doctoral se ha planteado para conocer mejor las bases fisiopatológicas y clínicas que relacionan el síndrome apnea-hipopnea del sueño (SAHS) y la enfermedad cardiovascular. Las conclusiones más relevantes de los estudios presentados son:

1. El síndrome metabólico (SM) según los criterios del III programa nacional de educación sobre el colesterol del panel de expertos en la detección, evaluación y tratamiento de la hipercolesterolemia en adultos (NCEP-ATP III) se detecta en alrededor de la mitad de los pacientes con SAHS en el momento del diagnóstico, presentando un 38,2 % de los pacientes restantes uno o dos componentes de SM.

2. El número de componentes del SM correlaciona de manera positiva con la presencia de resistencia a la insulina (RI) en pacientes de SAHS.

3. La presencia de somnolencia diurna excesiva *(siglas en inglés de Excessive daytime sleepiness)* (EDS), sin embargo, no resultó ser un marcador clínico sensible a un perfil metabólico alterado en pacientes con SAHS.

4. La concentración plasmática de ácidos grasos (AG) libres es elevada en los pacientes con SAHS.

5. Se observa una correlación positiva entre los índices de gravedad del SAHS y la concentración plasmática de AG libres. Una elevada concentración de AG libres puede desempeñar un papel en la patogénesis del SM.

6. En los pacientes con SAHS el déficit de vitamina D se asocia con niveles plasmáticos elevados de glucosa y una mayor prevalencia de SM.

7. En los pacientes con SAHS, una concentración plasmática elevada de hormona paratiroidea (PTH) se relaciona con un aumento de la prevalencia de obesidad e hipertensión. Estos resultados justifican la realización de más estudios.

8. Los resultados obtenidos en los tres estudios anteriores han servido de base para la realización de un estudio multicéntrico nacional, prospectivo, aleatorizado, abierto y controlado (Estudio ISAACC), cuyo objetivo es el de analizar el impacto del SAHS y de su tratamiento con presión positiva continua en la vía aérea *(siglas en inglés de Continuous positive airway pressure)* (CPAP) en el pronóstico y evolución de los pacientes con síndrome coronario agudo (SCA). Además de resaltar esta necesidad, los datos obtenidos permiten diseñar el estudio y la metodología más adecuada para dar respuesta a estas preguntas. El cuarto artículo presentado detalla estos aspectos fundamentales para el éxito del estudio.

11

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Parte II

APÉNDICES

A

OTROS TRABAJOS DE INVESTIGACIÓN RELEVANTES

La doctoranda Cristina Esquinas López es Diplomada en Enfermería (Universidad de Barcelona, 1999), obtuvo Diploma en Diseño y Estadística en Ciencias de la Salud (Universidad Autónoma de Barcelona, 2008), Máster en Salud Pública (Universidad Pompeu Fabra, 2008) y Máster en Metodología de la Investigación (Universidad Autónoma de Barcelona, 2010).

Lleva más de 10 años trabajando en investigación clínica centrada en el campo de la Neumología e impartiendo docencia en bioestadística y metodología de la investigación (profesora asociada en la Universidad de Barcelona, Universidad de Lleida y Universidad Internacional de Cataluña). Inicialmente inició su carrera investigadora en el campo de la Enfermedad Pulmonar Obstructiva Crónica (EPOC) y de la infección respiratoria, en cuya etapa colaboró en numerosos proyectos de investigación y obtuvo becas como investigadora principal (IP) de diversas sociedades científicas (Sociedad Española de Neumología y Cirugía Torácica, SEPAR; Societat Catalana de Pneumologia, SOCAP y Fundació Catalana de Pneumologia, FUCAP) e instituciones públicas (Instituto Carlos III, Fondo de Investigación Sanitaria (FIS)), ésta última en 2006 le concedió un contrato indefinido para personal de apoyo a la investigación y en 2008 el proyecto PI080472 (como IP).

Desde hace casi 4 años, forma parte del grupo de investigación de l'Institut de Recerca Biomèdica de Lleida (Fundació Dr Pifarré) liderado por el Dr. Barbé. Grupo investigador con alta experiencia científico-técnica en el campo de los trastornos respiratorios durante el sueño. La doctoranda en Enero de 2010 inició su trayectoria investigadora en este campo, centrándose en el estudio de la relación entre el síndrome apnea-hipopnea del sueño (SAHS) y los factores de riesgo cardiovascular así como el estudio del impacto de esta alteración y de su tratamiento con presión positiva continua en la vía aérea *(siglas en inglés de Continuous positive airway pressure)* (CPAP) en la evolución de la enfermedad cardiovascular. Los principales resultados de esta línea de investigación se han mostrado en la presente Tesis. Durante estos 4 años, la doctoranda ha colaborado en todos los proyectos de investigación que ha realizado el grupo financiados por las entidades públicas y privadas anteriormente mencionadas.

En esta trayectoria investigadora ha recibido becas como IP:

- Seguimiento del SAHS en las unidades especializadas de sueño y en atención primaria. Estudio comparativo aleatorizado de equivalencia sobre el cumplimiento y la respuesta clínica en pacientes con SAHS. Este estudio ha recibido financiación de diversas instituciones: Beca SEPAR 2011, Beca SOCAP 2012, FIS 2012 (PI12/01499).

Como colaboradora participa en diversos estudios, el más relevante:

- Impacto del SAHS en la evolución del síndrome coronario agudo (SCA). Efecto de la intervención con CPAP. Estudio prospectivo aleatorizado. ESTUDIO ISAACC". Proyecto financiado por: Societat Catalana de Cardiologia 2010, FIS 2010 (PI10/02763), SEPAR 2011.

El papel principal de la doctoranda en este proyecto es el de coordinadora nacional del proyecto. Fruto de este estudio, actualmente en fase de inclusión de pacientes, la doctoranda ha publicado un artículo que se desarrolla en la presente tesis. Durante este tiempo

ha presentado comunicaciones en congresos nacionales e internacionales. La producción científica de la doctoranda en la línea de trastornos del sueño, se va reflejada también por las siguientes publicaciones que no se han utilizado para esta tesis doctoral:

1. Zito A, Steiropoulos P, Barceló A, Marrone O, Esquinas C, Buttacavoli M, Barbé F, Bonsignore MR. Obstructive sleep apnoea and metabolic syndrome in Mediterranean countries. Eur Respir J. 2011;37:717-9.

2. Sánchez-de-la-Torre M, Barceló A, Piérola J, Esquinas C, de la Peña M, Durán-Cantolla J, Capote F, Masa JF, Marin JM, Vilá M, Cao G, Martinez M, de Lecea L, Gozal D, Montserrat JM, Barbé F. Plasma levels of neuropeptides and metabolic hormones, and sleepiness in obstructive sleep apnea. Respir Med. 2011;105:1954-60.

3. Piérola J, Alemany A, Yañez A, de-la-Peña M, Sánchez-de-la-Torre M, Esquinas C, Pérez-Gutierrez C, Burguera B, Barbé F, Barceló A. NADPH oxidase p22phox polymorphisms and oxidative stress in patients with obstructive sleep apnoea. Respir Med. 2011;105:1748-54.

4. Sánchez-de-la-Torre M, Pierola J, Vidal C, Barceló A, de la Peña M, Hussain Z, Capote F, Durán J, Agustí AG, de Lecea L, Torres G, Esquinas C, Martinez M, Barbé F. Non-synonymous polymorphism in the neuropeptide S precursor gene and sleep apnea. Sleep Breath. 2011;15:403-8.

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6. Sánchez-de-la-Torre M, Mediano O, Barceló A, Piérola J, de la Peña M, Esquinas C, Miro A, Durán-Cantolla J, Agustí AG, Capote F, Marin JM, Montserrat JM, García-Río F, Barbé F. The influence of obesity and obstructive sleep apnea on metabolic hormones. Sleep Breath. 2012;16:649-56.

7. Barbé F. et al. Spanish Sleep And Breathing Network. Effect of continuous positive airway pressure on the incidence of hypertension and cardiovascular events in nonsleepy patients with obstructive sleep apnea: a randomized controlled trial. JAMA. 2012; 307:2161-8.

8. Barceló A, Piérola J, Esquinas C, et al. Reduced plasma fetuin-A levels in patients with obstructive sleep apnoea. Eur Respir J. 2012;40:1046-8.

9. Mazzuca E, Battaglia S, Marrone O, Marotta AM, Castrogiovanni A, Barceló A, Esquinas C, Barbé F, Bonsignore M. Gender-specific anthropometric markers of adiposity, Metabolic Syndrome and visceral adiposity (VAI) in patients with obstructive sleep apnea. J Sleep Res. 2013. doi: 10.1111/jsr.12088. [Epub ahead of print]

A.1 ARTICULO 1

Zito A, Steiropoulos P, Barceló A, Marrone O, Esquinas C, Buttacavoli M, Barbé F, Bonsignore MR. Obstructive sleep apnoea and metabolic syndrome in Mediterranean countries. Eur Respir J. 2011;37:717-9.

and the prognosis of anti-PL7 ASS under treatment were heterogeneous.

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Obstructive sleep apnoea and metabolic syndrome in Mediterranean countries

To the Editors:

Obstructive sleep apnoea (OSA) is often associated with metabolic disturbances, including altered glucose metabolism and dyslipidaemia, which probably contribute to the increased cardiovascular risk in these patients [1]. The concept of the metabolic syndrome (MetS) as a cluster of cardiometabolic risk factors has gained popularity in recent years, and a much higher prevalence of the MetS has been found in OSA patients compared with the general population in several studies [1]. While the MetS largely reflects the effects of visceral obesity, environmental factors, *i.e.* the type of diet, could also play some role. The Mediterranean diet, rich in olive oil and fish, is protective against the MetS [2–4], but no study has examined the association of MetS and OSA in Mediterranean countries. We hypothesised that prevalence of the MetS might be lower in OSA patients living in the Mediterranean area compared with the prevalence values found in non-Mediterranean countries. Therefore, we retrospectively assessed the prevalence of the MetS according to the modified National Health and Nutrition Examination Survey Adult Treatment Panel (ATP) III criteria [5] in consecutive patients referred to sleep laboratories in Italy (n=107), Spain (n=138) and Greece (n=218).

Patients diagnosed with OSA in the period July 2007–September 2008 in Palermo, Italy (Respiratory Section, DIBIMIS, University of Palermo, and CNR Institute of Biomedicine and Molecular Immunology), Palma de Mallorca, Spain (Hospital Son Dureta) and Alexandroupolis, Greece (Sleep Unit, Medical School, Democritus University of Thrace), were evaluated in this study. All underwent clinical examination for clinical suspicion of OSA, and full polysomnography or nocturnal cardiorespiratory monitoring (eight channel) according to the American Academy of Sleep Medicine guidelines [6]. OSA was diagnosed when the apnoea/hypopnoea index (AHI) was >5 events·h^{-1}; mean lowest arterial oxygen saturation (S_{a,O_2}) was recorded. Daytime sleepiness was subjectively assessed by the Epworth Sleepiness Score questionnaire. Body mass index (BMI) was defined as kg·m^{-2}. Neck, waist and hip circumferences (cm) were measured. The MetS was diagnosed based on the presence of three or more of the following factors: waist circumference ⩾80 cm in females and ⩾94 cm in males; serum triglycerides ⩾150 mg·dL^{-1} or lipid-lowering treatment; high-density lipoprotein HDL cholesterol <40 mg·dL^{-1} in males and <50 mg·dL^{-1} in females or statin treatment; systemic hypertension (systolic blood pressure >135 mmHg and/or diastolic blood pressure >85 mmHg) or anti-hypertensive treatment; and fasting blood

glucose >100 mg·dL^{-1} or anti-diabetic treatment [5]. Plasma glucose and lipids were analysed by standard laboratory methods in venous blood withdrawn in the morning in a fasting state. Treatment for hypertension (*i.e.* diuretics, β-blockers and angiotensin-converting enzyme (ACE) inhibitors, *etc.*), diabetes (*i.e.* oral antidiabetic drugs and insulin), or dyslipidaemia (*i.e.* statins and clofibrate, *etc.*) was recorded in all patients.

Data are reported as mean±SD for continuous variables, and percentage of positive subjects for categorical variables. Continuous variables in the three patients' samples were compared by one-way ANOVA with Bonferroni correction for multiple comparisons. The Chi-squared test was used to assess differences in the frequency of categorical variables. Variables significantly associated with MetS were tested by multiple logistic regression. Statistical analysis was performed by using the SPSS version 17 software (SPSS Inc., Chicago, IL, USA) and significance was at $p<0.05$ for all tests.

A total of 463 patients were diagnosed with OSA in the study period (107 in Palermo, 218 in Alexandroupolis and 138 in Palma de Mallorca), but 127 had to be excluded due to missing information (20, 98 and nine, from the respective samples), leaving 336 patients for analysis. Excluded patients did not show any significant difference compared to included ones for sex distribution, age, BMI or OSA severity (data not shown).

Table 1 summarises the anthropometric and clinical features of the samples. The percentage of female patients was similar in Italy and Spain (between 30% and 35%), and lowest in the Greek sample (19%). Age and AHI were similar in the three groups. Greek patients showed a significantly lower S_{a,O_2} during sleep. Mean BMI and waist circumference were highest in the Greek patients, who also showed significantly higher treatment rates for hypertension (ACE inhibitors 34.2%, β-blockers 11.7% and diuretics 19.2%), diabetes (oral hypoglycemic drugs 15.8%), or dyslipidaemia (29.2%) compared to Italian patients (ACE inhibitors 17.2%, β-blockers 8.0%, diuretics 6.9%, oral hypoglycemic drugs 3.4% and dyslipidaemia treatment 9.2%), or Spanish patients (ACE inhibitors 14.2%, β-blockers 7.4%, diuretics 16.8%, oral hypoglycemic drugs 7.4% and dyslipidaemia treatment 11.9%). Waist-to-hip ratio was lowest in Italian patients, intermediate in Greek patients and highest in Spanish patients.

According to the National Cholesterol Education Program (NCEP)-ATP III definition, MetS was present in 67.3% of the patients of the entire cohort (fig. 1 and table 1), with the lowest prevalence in the Italian sample ($p<0.0001$ by Chi-squared test). Significant differences in the prevalence of single MetS components were found among the samples for waist circumference, triglycerides and fasting blood glucose, while prevalence of hypertension or low HDL-cholesterol was similar in the three samples. Logistic regression analysis in the entire sample identified age, BMI, male sex, lowest S_{a,O_2} and presence of daytime sleepiness as significantly associated with MetS.

The results of this retrospective study do not support the hypothesis that patients with OSA from Mediterranean countries may be protected against MetS. Indeed, the prevalence rates observed were similar to those reported by other clinical studies in Western countries ranging between 30% and 87% [1]. Prevalence of MetS in Mediterranean OSA patients was at least twice as high as in the general population of the respective countries, which is rising and currently estimated to be between 22% and 25% [7–9]. The Greek sample showed the highest prevalence of markers of abdominal obesity, accounting

TABLE 1 Characteristics of the patients

	All patients	Palermo, Italy	Alexandroupolis, Greece	Palma de Mallorca, Spain	p-value
Patients n	336	87	120	129	
Males %	72.3	65.5	80.8	69.0	<0.05
Age yrs	55.3±12.3	54.3±11.1	57.3±12.8	54.2±12.4	NS
BMI kg·m^{-2}	33.0±7.2	33.0±7.5	34.4±6.4	31.7±7.6	<0.01$^{\#}$
Neck circumference cm	41.7±4.6	40.0±4.7	43.4±3.8	40.9±4.8	<0.0001¶
AHI events·h^{-1}	41.8±28.1	44.2±28.9	39.9±27.2	42.1±28.4	NS
Lowest nocturnal S_{a,O_2} %	78.4±11.7	81.9±11.5	73.5±12.6	80.3±9.2	<0.001¶
Epworth Sleepiness Scale score	9.6±5.4	9.9±5.4	9.7±5.7	9.4±5.1	NS
Waist-to-hip ratio	1.00±0.09	0.95±0.07	1.00±0.07	1.03±0.10	<0.001^{+}
Waist circumference cm	112.0±14.9	108.3±16.2	117.6±14.7	108.7±12.7	<0.0001¶
SBP mmHg	130.9±17.8	131.5±18.6	129.7±19.1	131.5±16.4	NS
DBP mmHg	81.9±10.6	84.7±12.3	81.4±8.2	81.1±11.2	NS
Cholesterol HDL mg·dL^{-1}	47.7±12.5	45.8±12.2	47.9±11.3	48.9±13.6	NS
Triglycerides mg·dL^{1}	165.0±102.1	140.9±78.4	168.7±94.1	177.0±119.6	<0.05§
Fasting blood glucose mg·dL^{-1}	109.7±32.2	103.6±30.3	112.2±30.4	111.5±34.5	NS
Prevalence of MetS %	67.3	50.6	76.7	69.8	<0.0001

Data are presented as mean±SD, unless otherwise stated. BMI: body mass index; AHI: apnoea/hypopnoea index; S_{a,O_2}: arterial oxygen saturation; SBP: systolic blood pressure; DBP: diastolic blood pressure; HDL: high-density lipoprotein; MetS: metabolic syndrome; NS: nonsignificant. Results of multiple comparison testing: $^{\#}$: Greece > Spain; ¶: Greece > Italy and Spain; $^{+}$: Spain > Greece > Italy; §: Spain > Italy.

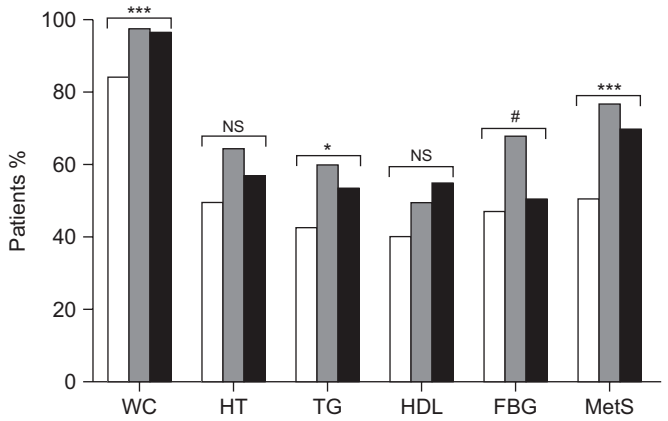

FIGURE 1. Metabolic syndrome (MetS) and its components. Significant differences in the prevalence of MetS were found among the samples for waist circumference (WC), triglycerides (TG) and fasting blood glucose (FBG). Hypertension (HT) and low high-density lipoprotein (HDL)-cholesterol were similar in the three samples. □: Italy; ■: Greece; ■: Spain. NS: nonsignificant. *: $p<0.05$; ***: $p<0.001$; #: $p<0.005$.

for the highest prevalence of MetS. The lowest prevalence of MetS was found in Southern Italy, together with the highest percentage of female patients and a relatively low waist-to-hip ratio. The Italian sample was quite similar to the Spanish one for percentage of females and waist circumference, but prevalence of MetS was almost 20% higher in Spain than in Italy. This finding leaves the possibility that some differences in diet may play a role, but we could not check for dietary composition in this retrospective study. Epidemiological data from Spain recently showed that prevalence of MetS in the general population by NCEP-ATP III criteria increased from 18% to 25% in a decade, especially in young subjects, in conjunction with a major shift from the Mediterranean diet towards the Western diet [10]. Our study suggests that such a change might have similarly occurred in patients with OSA, although significance of severity of hypoxaemia and sleepiness by logistic regression analysis confirms a possible role of intermittent hypoxia in the pathogenesis of metabolic disorders. In conclusion, prevalence of the MetS in patients with OSA was similar in Mediterranean and non-Mediterranean countries, suggesting that exposure to Mediterranean diet was either absent in these patients or insufficient to exert any protective effect. Studies on the dietary habits of OSA patients are warranted, especially regarding diet composition, as they may be useful in weight control and cardiovascular prevention programmes.

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A.2 ARTICULO 2

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Plasma levels of neuropeptides and metabolic hormones, and sleepiness in obstructive sleep apnea

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KEYWORDS
OSA;
Sleep apnea;

Summary
Background: Obstructive sleep apnea (OSA) is related to obesity and metabolic disorders. The main clinical symptoms are excessive daytime sleepiness (EDS) and snoring. However, not all

Abbreviation: AHI, Apnea—hypopnea index; BMI, Body mass index; CO_2, Carbon dioxide; CPAP, Continuous positive airway pressure; CRP, C-reactive protein; CSF, Cerebrospinal fluid; EDS, Excessive daytime sleepiness; EDTA, Ethylenediamine tetra-acetic acid; EIA, Enzyme immunoassay; ESS, Epworth Sleepiness Scale; OSA, Obstructive sleep apnea; RIA, Radio immunoassay; SaO_2, Oxygen saturation; SD, Standard deviation; VIP, Vasoactive intestinal peptide.

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EDS;
Metabolism

patients with OSA manifest EDS. Hypocretin-1, neuropeptide Y, leptin, ghrelin and adiponectin are implicated in both metabolic and sleep regulation, two conditions affected by OSA. We hypothesized that levels of these peptides may be related to EDS in OSA patients.
Methods: We included 132 patients with EDS, as defined by an Epworth Sleepiness Scale (ESS) score $\geq$13 (mean $\pm$ SD, 15.7 $\pm$ 2.3) and 132 patients without EDS as defined by an ESS score $\leq$9 (6.5 $\pm$ 1.9). All patients had an apnea–hypopnea index (AHI) $\geq$ 20 h^{-1}. Both groups were matched for gender (males; 83.3% vs. 85.6%), age (50.15 $\pm$ 11.2 yrs vs. 50.7 $\pm$ 9.9 yrs), body mass index (BMI) (31.8 $\pm$ 5.6 kg m^{-2} vs. 32.1 $\pm$ 4.8 kg m^{-2}), and apnea–hypopnea index (AHI) (45.5 $\pm$ 19.1 h^{-1} vs. 43 $\pm$ 19.2 h^{-1}).
Results: OSA patients with EDS showed significantly higher plasma hypocretin-1 levels ($p < 0.001$) and lower plasma ghrelin levels ($p < 0.001$) than OSA patients without EDS. There were no statistically significant differences in neuropeptide Y ($p = 0.08$), leptin ($p = 0.07$) and adiponectin ($p = 0.72$) between the two groups. In the multiple linear regression model ESS score was associated with plasma levels of hypocretin-1, ghrelin and total sleep time.
Conclusion: Our study shows that EDS in patients with OSA is associated with increased circulating hypocretin-1 and decreased circulating ghrelin levels, two peptides involved in the regulation of body weight, energy balance, sympathetic tone and sleep–wake cycle. This relationship is independent of AHI and obesity (two key phenotypic features of OSA).

Introduction

Obstructive sleep apnea (OSA), a disease that affects about 4% of the general population, is a chronic condition characterized by repetitive collapse of the upper airway during sleep leading to significant hypoxemia, sleep fragmentation, and excessive daytime sleepiness (EDS).[1,2] The existing definition of EDS in the International Classification of Sleep Disorders[3] is based on the behavior of unintentionally falling asleep and difficulty maintaining alertness. EDS is considered an important health problem, leading to road accidents, psychosocial morbidity and poor quality of life.[4] EDS has been associated with cardiac dysfunction. Patients with EDS manifest significantly lower baroreflex sensitivity and significantly higher low-to-high frequency spectral power ratios of heart rate variability during the various stages of nocturnal sleep.[5] Although EDS is one of the main symptoms of sleep apnea, for unclear reasons not all patients with OSA complain of EDS. In fact, about 15% of patients with OSA do not report EDS.[6,7] The pathogenesis of EDS in OSA patients appears to be related to underlying intermittent hypoxia and disrupted sleep. However, EDS is also related to metabolic and sympathetic activities. In a previous study, we have shown that EDS in OSA is associated with insulin resistance, independently of obesity.[8]

Hypocretin-1 (also known as orexin-A) is a neuropeptide that influences arousal and the sleep–wake cycle, cardiovascular function, temperature, metabolic rate and locomotor activity.[9,10] Neuropeptide Y is a neurotransmitter that stimulates appetite and is involved in the regulation of sympathetic activity, body weight, and energy balance.[11,12] Leptin is a protein produced in adipose tissue that interacts with receptors in the hypothalamus to inhibit eating and control body weight and fat distribution. Ghrelin is a stomach-derived peptide that has been related to the regulation of body weight through stimulation of the appetite. Finally, adiponectin, a hormone that is closely related to metabolism, is secreted by adipocytes and may have both anti-atherogenic and anti-inflammatory properties.[13]

The aim of this study was to address determinants of sleepiness in patients with OSA. It evaluated plasma levels of neuropeptides (hypocretin-1 and neuropeptide Y) and metabolic hormones (ghrelin, leptin and adiponectin) in OSA patients and their relationship with EDS in two cohorts of patients, one with OSA and another either with or without EDS, matched for age, gender, apnea–hypopnea index (AHI) and body mass index (BMI). These markers are related to energy metabolism, arousal and sympathetic activity, pathways that are altered in OSA and that may be associated with manifestations of EDS in OSA patients.

Materials and methods

Patients

We included 132 patients with EDS and 132 patients without EDS. All patients had an AHI $\geq$ 20 h^{-1}. Participants were recruited from subjects who were seen at the sleep units of four teaching hospitals in Spain. Both groups of patients were matched for gender, age, BMI and AHI. Exclusion criteria were the presence of chronic obstructive pulmonary disease, chronic inflammatory intestinal diseases, liver cirrhosis, diabetes mellitus, depression, thyroid dysfunction, rheumatoid arthritis, chronic renal failure, psychiatric disorders, malignant tumors (particularly gastric tumors), gastric surgery and/or the use of drugs that could affect sleep. None of the patients were being treated with continuous positive airway pressure (CPAP) at the time they were enrolled in the study.

Daytime sleepiness was assessed using the Spanish version of the Epworth Sleepiness Scale (ESS).[14] ESS scores $\geq$13 were considered indicative of EDS, while ESS scores $\leq$9 were considered to represent an absence of EDS. Patients with an ESS score higher than 9 and lower than 13 were excluded from the study, because such ESS scores do not allow for an accurate delineation of EDS status.

All patients included in the study provided written consent after being fully informed of the goals, methods and potential risks of the study. The Ethics Committee of the participating institutions approved this project.

Measurements

OSA diagnoses were established by overnight polysomnography, which included recordings of oronasal airflow, thoracoabdominal movements, electrocardiography, electroculography, electroencephalography, Chin electromyography, and arterial oxygen saturation measurements. Apnea was defined as the absence of airflow for more than 10 s. Hypopnea was defined as an airflow reduction greater than 50% that lasted for more than 10 s and resulted in arousal or oxygen desaturation greater than 4%.[15] Oronasal flow was measured with both a nasal pressure transducer and an oronasal thermistor. Polysomnography screening was done according to international guidelines. AHI was defined as the number of apneas plus hypopneas per hour of sleep.

Due to the known variations in hormonal levels within the sleep/wake cycle[16] blood samples were collected in all patients immediately after morning awakening. Venous blood samples were obtained from an antecubital vein using a collecting tube with ethylenediamine tetraacetic acid (EDTA). Blood samples were centrifuged, and the plasma fraction was immediately separated into aliquots and stored at −80 °C until analysis. Blood in EDTA tubes and aliquots of plasma were transported in dry ice from participating hospitals to the Arnau de Vilanova Hospital, and stored at −80 °C pending centralized analysis.

Plasma hypocretin-1 levels were measured using a previously validated[17] Enzyme immunoassay (EIA) system designed for measuring hypocretin-1 in plasma (Phoenix Pharmaceuticals, California, USA). The assay sensitivity for hypocretin-1 was 0.22 ng/ml with intra- and inter-assay coefficients of variation of <5% and <14%, respectively. Specificity of the hypocretin-1 assay was 100% with no cross-reactivity (0%) with orexin-A 16−33, agouti-related protein 83−132-amide, neuropeptide Y, α-melanocyte stimulating hormone or leptin. Plasma neuropeptide Y levels were measured using an EIA system (Phoenix Pharmaceuticals, California, USA). The assay sensitivity for neuropeptide Y was 0.09 ng/ml. Specificity of the assay for neuropeptide Y was 100% with no cross-reactivity (0%) with peptide YY, pancreatic polypeptide, vasoactive intestinal peptide (VIP), insulin, amylin or somatostatin. Ghrelin, leptin and adiponectin plasma levels were measured using commercially available ELISA kits (Phoenix Pharmaceuticals Inc., Burlingame, CA; DRG Instruments GmbH, Germany; and Mediadiagnost, Reutlingen, Germany, respectively). Intra- and inter-assay coefficients of variation for ghrelin were less than 5% and less than 9%, for leptin they were 6.43% and 10.1%, and for adiponectin were 3.37% and 6.05%, respectively. Measurements were always done in duplicate, and mean values were used for analysis. Glucose and triglyceride concentrations were determined by standard enzymatic methods using a Hitachi 917 biochemical analyzer (Roche Diagnostics, Indianapolis, USA). Plasma C-reactive protein (CRP) was measured using a chemoluminescent immunometric assay (Inmunolite 2000 High Sensitivity CRP).

Statistical analysis

Results are shown as means ± standard deviations (SD). Differences between groups were assessed by the Mann−Whitney non-parametric test for quantitative variables and the Fisher's exact test for dichotomous categorical variables. Correlations between variables of interest were assessed using the non-parametric Spearman test.

A multivariate linear regression model was used to assess the association of hypocretin-1, neuropeptide Y, ghrelin, leptin and adiponectin plasma levels with ESS values, adjusting by potential confounding factors (both known confounding factors as well as variables differently distributed in both study groups). Only statistically significant variables or confounding factors were kept in the model. All data analyses were performed using the SPSS (version 16) statistical software. *P* values lower than 0.05 were considered statistically significant.

Results

Table 1 shows pertinent anthropometric and clinical data for the two study groups. OSA was severe in both groups, as demonstrated by their high AHI scores. Percentage of time with $SaO_2 < 90\%$ and F1 sleep was lower in patients with excessive daytime somnolence, and total sleep time was higher in this group.

Plasma hypocretin-1 levels were higher in patients with EDS than in those without EDS (2.52 ± 0.25 ng ml^{-1} vs. 1.64 ± 0.17 ng ml^{-1}, $p < 0.001$). Plasma neuropeptide Y levels show a tendency (non-significant) to be lower in patients with EDS (0.91 ± 0.07 ng ml^{-1} vs. 0.97 ± 0.06 ng ml^{-1}, $p = 0.087$) (Fig. 1). Plasma ghrelin levels were lower in patients with EDS than in those without EDS (6.34 ± 3.52 ng ml^{-1} vs. 8.94 ± 5.74 ng ml^{-1}; $p < 0.001$; Fig. 2). Plasma leptin levels were similar in patients with and without EDS (11.45 ± 10.6 ng ml^{-1} vs. 10.12 ± 13.27 ng ml^{-1}; $p = 0.071$), and this was also true for plasma adiponectin levels (5.42 ± 2.98 μg ml^{-1} vs. 5.47 ± 2.78 μg ml^{-1}; $p = 0.728$; Fig. 2).

We assessed the association of ESS, BMI and AHI with neuropeptides and metabolic hormones and found that ESS score was related to hypocretin-1 and ghrelin, and BMI was related to leptin and ghrelin (Table 2).

The adjusted multivariate linear regression model used to explain ESS score showed a statistically significant relationship with ghrelin and hypocretin-1 levels, together with the total sleep time. ESS score was not associated with AHI and BMI.

Discussion

These results show that, compared to OSA patients without EDS, plasma levels of hypocretin-1 were elevated in OSA patients with EDS, whereas plasma levels of ghrelin were reduced. These relationships were independent of obesity

Table 1 Anthropometric and clinical characteristics (mean ± SD) of OSA patients with and without EDS.

	OSAS patients (n = 264)		p-value
	With EDS (n = 132)	Without EDS (n = 132)	
Sex, m [n (%)]	110 (83.3)	113 (85.6)	0.61
Age, years	50.15 (11.27)	50.72 (9.96)	0.797
BMI*, Kg/m^2	31.85 (5.68)	32.18 (4.84)	0.191
Epworth Scale	15.77 (2.32)	6.55 (1.97)	*by design*
AHI*, (events/h^{-1}	45.53 (19.11)	43.05 (19.23)	0.251
TST*, (min)	351.26 (50.53)	314.83 (72.65)	**<0.001**
% Time with SaO_2 <90%	11.95 (21.6)	15.02 (21.05)	**0.03**
F1 sleep, (%) of sleep	6.26 (9.76)	10.92 (8.65)	**<0.001**
F2 sleep, (%) of sleep	64.95 (13.5)	66.19 (9.71)	0.789
F3 + F4 sleep, (%) of sleep	14.25 (10.8)	11 (7.84)	0.08
REM sleep, (%) of sleep	15.37 (9.91)	14.33 (6.59)	0.55
Glucose, (mg/dl)	101.74 (18.07)	102.3 (16.96)	0.854
Triglycerides$^+$, (mg/dl)	134 (98.75)	125.5 (98.75)	0.521
SBP*, (mm Hg)	131.72 (16.88)	131.08 (15.06)	0.434
DBP*, (mm Hg)	82 (14.05)	82.33 (10)	0.542
Nocturnal heart rate, (bpm)	65.18 (11.07)	64.79 (9.2)	0.743
Cigarette smokers, [n (%)]	40 (15.06)	30 (11.7)	0.257
CRP*, (mg/l)	3.64 (4.84)	3.62 (4.01)	0.473

*EDS; Excessive Daytime Sleepiness, BMI; Body Mass Index, AHI; Apnea—hypopnea index, TST; Total sleep time, SBP; Systolic blood pressure, DBP; Diastolic blood pressure, CRP; C-reactive protein. $^+$Median (IQR).
The statistically significant p values are denoted by bold.

and the number of apneas during sleep (two key phenotypic features of OSA). No differences were found in the plasma levels of neuropeptide Y, leptin and adiponectin between these two groups. The present study has the largest number of patients of any to address this issue so far, and the two groups included in the study were well characterized for the presence or absence of daytime sleepiness. These results could indicate a relationship between EDS in patients with OSA and plasma levels of hypocretin-1 and ghrelin, two peptides that participate in metabolic and sleep regulation.

The origin and function of hypocretin-1 in plasma is unknown, but it is postulated that plasma hypocretin-1 may originate from leaks in the blood—brain barrier and from peripheral synthesis.[18,19] Some authors have reported lower plasma hypocretin-1 levels in patients with OSA than controls.[20—22] Nevertheless, others reported otherwise.[23,24] These discrepant findings could be related to the different populations studied and to methodological issues with hypocretin-1 assays. These studies also analyzed the correlation of certain clinical variables associated with OSA and plasma hypocretin-1 levels. Nishijima et al.[21] showed significant negative correlations with ESS. Nevertheless others authors did not find a significant correlation with ESS score.[20,23] We found a statistically significant linear correlation between hypocretin-1 and ESS score (Table 2). Hypocretin-1 is a neuropeptide that influences the sleep—wake cycle, arousal regulation and the maintenance of the alert state.[9] It could be hypothesized that the increased plasma hypocretin-1 observed in OSA patients with EDS could indicate a mechanism that facilitate wakefulness and counteracts the effects of sleepiness.

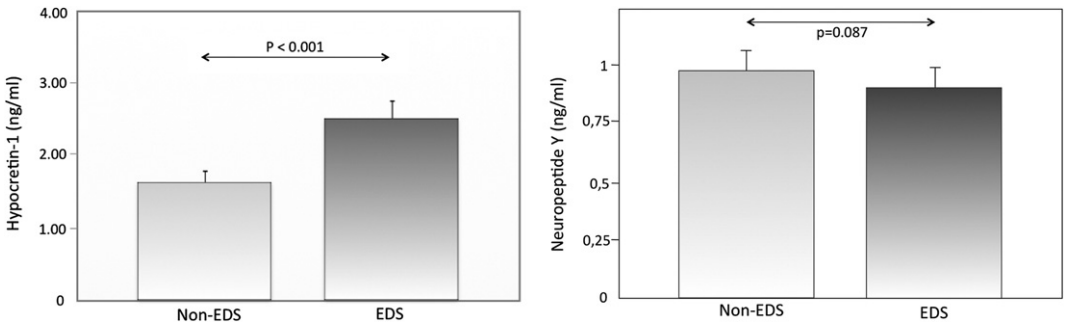

Figure 1 Plasma levels of neuropeptides in OSA patients with and without EDS (mean ± C.I. 95%).

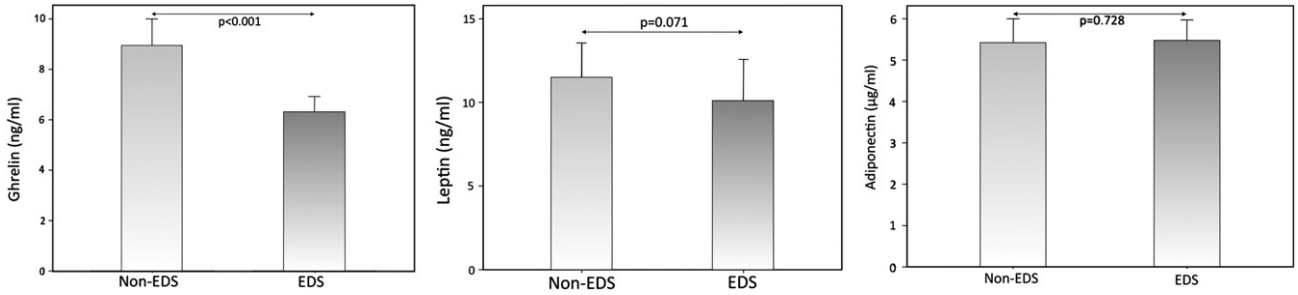

Figure 2 Plasma levels of metabolic hormones in OSA patients with and without EDS (mean ± C.I. 95%).

Patients with sleep apnea and without EDS would not be able to activate these mechanisms and therefore OSA patients would be less susceptible to the effects of sleep apnea and would not manifest EDS.

Previous studies have analyzed neuropeptide Y plasma levels in OSA patients. Barceló et al.[25] described increased levels of neuropeptide Y in OSA patients compared with control subjects. Although plasma neuropeptide Y values were lower in our patients with EDS than in patients without EDS, the differences were small and did not achieve statistical significance (Fig. 2). Plasma neuropeptide Y levels did not appear to be correlated with clinical factors, such as BMI, AHI and ESS.

In the present study we also analyzed the relationship between EDS and plasma levels of three metabolic hormones: ghrelin, leptin and adiponectin. We found that plasma ghrelin levels were lower in OSA patients with EDS than in patients without EDS. Previous studies have shown that plasma ghrelin levels are higher in patients with OSA compared to controls.[26] Plasma ghrelin levels could be modulated by, or conversely, modulate EDS in the context of OSA. Indeed, synthesis of ghrelin hormone is affected by sleep duration, as shown by Taheri et al., such that short sleep duration was associated with elevated plasma ghrelin levels.[27] In our study, as might be expected, patients without EDS, who had higher plasma ghrelin levels than patients with EDS, were also those with shorter sleep duration, assessed by total sleep time. After adjustment for confounding variables, the relationship between ESS score and plasma ghrelin levels remained statistically significant. There was no difference between OSA patients with and without EDS in plasma levels of leptin. This hormone has been positively correlated with BMI, suggesting the presence of leptin resistance in the context of obesity.[28] As expected, we found a significant positive correlation between BMI and plasma leptin levels in our study (Table 2). We did not find any significant differences in plasma leptin levels between OSA patients with and without EDS. We also did not find any differences in plasma adiponectin levels between these two groups.

In the present study ESS score was not associated with AHI and BMI. The possible correlation between daytime sleepiness and AHI is unclear.[1,29–31] Previous studies have also indicated an association between BMI and EDS, but we did not find this association in our study. Bixler et al.[32] described an association between EDS and BMI in the general population with a BMI between 18 and 69 kg m^{-2}. Nevertheless, our study population of OSA patients may be different from the general population. Also, in Bixler et al. the presence of EDS was established based on a moderate or severe rating on either of two questions. Nevertheless, in our study we performed a more accurate daytime sleepiness characterization using the Spanish validated version of the Epworth Sleepiness Scale (ESS).[14] In the present study, we also found a statistically significant relationship between ghrelin and hypocretin-1 levels with the total

Table 2 Linear correlation values for the analysis of neuropeptides/metabolic hormones, and clinical and anthropometric variables.

	BMI	ESS	AHI
Hypocretin-1	$r = 0.03$	$r = 0.3$	$r = 0.07$
	$p = 0.69$	$\mathbf{p < 0.0001}$	$p = 0.23$
Neuropeptide Y	$r = 0.01$	$r = -0.08$	$r = 0.06$
	$p = 0.92$	$p = 0.18$	$p = 0.29$
Ghrelin	$r = 0.155$	$r = -0.236$	$r = 0.083$
	$\mathbf{p = 0.01}$	$\mathbf{p < 0.001}$	$p = 0.17$
Leptin	$r = 0.368$	$r = 0.03$	$r = 0.001$
	$\mathbf{p < 0.001}$	$p = 0.73$	$p = 0.81$
Adiponectin	$r = 0.1$	$r = 0.03$	$r = 0.02$
	$p = 0.11$	$p = 0.93$	$p = 0.82$

*BMI; Body mass index, ESS; Epworth sleep scale, AHI; Apnea—hypopnea index.
The statistically significant *p* values are denoted by bold.

sleep time. Nevertheless, we can observe the existence of an association but we cannot indicate causality.

The present study has several strengths: its multicentric design, close matching of study subjects for gender, age, BMI and AHI, and large sample size. In addition, the exclusion of patients with ESS scores in the range where substantial overlap occurs allowed for better demarcation of those patients with EDS and those without EDS. However, our study has several limitations that deserve comment: First, sleepiness was assessed using subjective methods, rather than using a multiple sleep latency test. As mentioned, we did not include patients in the study with ESS scores between 10 and 12, in order to improve the discriminatory value of the Epworth score. Second, in the present study, the associations between metabolic hormones and neuropeptides were analyzed from a single morning sample, rather than from multiple samples within the circadian cycle. Third, we did not include the treatment effect. An interventional study would enable better discrimination of the strength of the associations described herein and would enable confirmation of their validity. Indeed, if improvements in sleepiness were predicted by changes in any of these plasma markers or combinations thereof, the intrinsic roles and clinical value of such assays would be greatly enhanced.

Conclusions

Our study shows that EDS in patients with OSA is associated with increased circulating hypocretin-1 and decreased circulating ghrelin levels, two peptides involved in the regulation of body weight, energy balance, sympathetic tone and sleep—wake cycle. This relationship is independent of AHI and obesity (two key phenotypic features of OSA).

Author contributions

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Analysis and interpretation of the data: Sánchez de la Torre M., Barceló A., de la Peña M., Durán J., Capote F., Masa J.F., Marin J.M., Martinez M., de Lecea L., Barbé F.

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Administrative, technical, or logistic support: Sánchez de la Torre M., Barceló A., Pierola J., de la Peña M., Durán J., Capote F., Masa J.F., Marin J.M., Cao G., Barbé F.

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Conflict of interest disclosures

None declared.

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A.3 ARTICULO 3

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NADPH oxidase p22phox polymorphisms and oxidative stress in patients with obstructive sleep apnoea

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Summary
Background: Obstructive Sleep Apnoea (OSA) is associated with increased oxidative stress. NADPH oxidases are the main source of Reactive Oxygen Species (ROS) in the vasculature. Several polymorphisms related to NADPH oxidase expression or activity have been identified. We compared the distribution of the allelic frequencies of A-930G and C242T polymorphisms and their possible relationship with the levels of 8-isoprostanes as a marker of oxidative stress in patients with OSA and in a control group without OSA.
Methods: This is a case-control study. We determined the A-930G and C242T p22phox genotypes in 427 patients with OSA and in 139 healthy subjects recruited from the Sleep Unit of Son Dureta University Hospital, (Palma de Mallorca, Spain). 8-Isoprostane was measured as an oxidative stress marker.
Results: The distribution of the p22phox genotypes in OSA and in control subjects was different. The risk of OSA was associated with the presence of the G allele in the A-930G p22phox independently of age, gender, Body Mass Index (BMI), hypertension, dyslipemia and

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diabetes, but no association was found with the C242T polymorphism. The median level of 8-isoprostane was significantly higher in OSA patients. Synergic effect in 8-Isoprostane levels was observed when these two polymorphisms were analysed together.
Conclusion: the A-930G polymorphism of the p22phox gene may play an important role in genetic susceptibility to OSA. Furthermore, the C242T and A-930G polymorphisms of the p22phox gene have a synergic effect on the 8-isoprostane levels, suggesting that they may be involved in the development of oxidative stress in these patients.

Introduction

Obstructive sleep apnoea syndrome (OSA) is a common disorder characterised by the occurrence of numerous episodes of absence of respiratory flow (apnoea) during sleep.[1] These episodes cause repetitive cycles of hypoxia/re-oxygenation. The treatment for OSA is the Continuous Positive Airway Pressure (CPAP) that corrects the apnoeas and consequently these episodes.

Experimental and clinical data have provided support for an association between OSA and oxidative stress.[2–8] Oxidative stress is caused by an imbalance between the production of Reactive Oxygen Species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage.

The major source of ROS in the vasculature are the NADPH oxidases or *noxes*, the only enzyme family whose sole function is to generate ROS.[9,10] Vascular isoforms generate superoxide anion (O_2^-) constitutively at a low rate for physiological purposes. A variety of stimuli induce an increased superoxide production. O_2^- and its metabolite H_2O_2 serves as a second messenger to activate multiple intracellular signalling pathways.[9–11] The signalling pathways leading to the expression redox-sensitive genes are the most affected ones, resulting in the production of adhesion molecules, chemotactic factors, inflammatory mediators and vasoactive substances.[2,12–14] NADPH oxidases are multicomponent enzymes consisting of cytosolic accessory proteins (Rac, p47phox, p67phox) which after stimulation associate with the membrane catalytic subunits (Nox, p22phox) to facilitate superoxide generation.[11] The p22phox subunit has a central role to the normal functioning of the oxidases because it stabilises the large subunit and serves as docking site to the cytosolic factors. The p22phox subunit is functionally critical for the enzymatic activity,[15] and all vascular NADPH oxidases appear to have an obligatory need for it.[16]

Several polymorphisms of CYBA, the gene encoding p22phox, have been identified: I)The A-930G polymorphism: located in the promoter, the G allele is associated with a higher promoter activity[17,18] and an increased level of oxidative stress.[19,20] II) The C242T polymorphism (rs4673) is located in the exon 4 of the CYBA gene, leading to a his72-to-tyr (H72Y) substitution. The T allele is associated with significantly lower basal and stimulated vascular superoxide production in human blood vessels from patients with atherosclerosis, and hypertension.[21] Furthermore, in healthy volunteers, neutrophil oxidative bursts increase from the TT to the CC genotype.[15]

We hypothesised an existing association between OSA and the two polymorphisms A-930G and C242T. These two polymorphisms might play a relevant role in the oxidative stress in OSA patients. To asses this hypothesis, we compared the distribution of the allelic frequencies of A-930G and C242T polymorphisms and their possible relationship with the levels of 8-isoprostanes as a marker of oxidative stress in one group of patients with OSA and in a control group without OSA.

Methods

Subjects and ethics

This is a case—control study. Participants were recruited between January 2007 and June 2009. A total of 427 patients with OSA were included in the study along with 139 subjects without OSA. Both cases and controls were recruited from all individuals suspected of having OSA referred to the Sleep Unit from primary care services of Palma de Mallorca. Exclusion criteria (for both cases and controls) were presence of chronic obstructive pulmonary disease, liver cirrhosis, thyroid dysfunction, rheumatoid arthritis, chronic renal failure and/or psychiatric disorders. Participants were afterwards studied at the Sleep Unit of Son Dureta University Hospital, (Palma de Mallorca, Spain). The diagnosis of OSA was established by full polysomnography (E-Series; Compumedics, Abbotsford, Australia), and included recording of oronasal flow and thoracoabdominal movements, ECG, submental and pretibial electromyography, electrooculography, electroencephalography and transcutaneous measurement of arterial oxygen saturation. Apnoea was defined by the absence of airflow for more than 10 s. Hypopnoea was defined as any airflow reduction that lasted more than 10 s and resulted in arousal or oxygen desaturation. We considered desaturation a decrease in SaO_2 greater than 4%.The apnoea/hypopnoea index (AHI) was defined as the number of apnoeas plus hypopnoeas per hour of sleep. OSA was diagnosed when the AHI was over 10 events h^{-1}. Individuals were considered as controls if the AHI was lower than 10 events h^{-1}. Arterial hypertension was diagnosed if systolic blood pressure (SBP) was $\geq$140 mmHg or diastolic blood pressure (DBP) $\geq$ 90 mmHg. Similarly, the threshold for diabetes was a glucose level of 126 mg dL^{-1}; for insulin resistance, a Homeostasis Model Assessment (HOMA)[22] index of 4; for dyslipemia, a total cholesterol level of 200 mg dL^{-1} or triglycerides levels of 200 mg dL^{-1}; and for obesity, a BMI of 30 kg m^{-2}.

The present study was approved by the ethics committees of the participating institutions, and all participants signed their informed consent after being fully explained about its

goal and characteristics. This project has been developed according to the STREGA statement recommendations.[23]

Measurements

Blood samples (10 mL) were obtained at 08:00 AM after an overnight fast, and collected in tubes containing ethylenediamine tetra-acetic acid (EDTA) and in tubes without anticoagulant for biochemical determinations. After centrifugation, serum and plasma were immediately separated into aliquots and stored at −80 °C. From the EDTA tubes, buffy coat was taken to isolate DNA of the leucocytes.

Biochemical analysis

Glucose, triglyceride, total cholesterol and HDL cholesterol (HDLc) concentrations were determined by standard enzymatic methods using a Hitachi 917 biochemical analyser (Roche Diagnostics, Manheim, Germany). The plasma concentration of insulin and high sensitivity C- reactive protein (CRP) were measured by commercial chemiluminescent assays using an Immulite 2000 analyser (Siemens Diagnostics, Llamberis, United Kingdom) in serum. Insulin resistance was calculated using the HOMA index. 8-Isoprostanes were measured with an 8-isoprostane EIA kit (Cayman Chemicals Company, USA) in plasma samples.

DNA extraction and genotyping

DNA isolation of each blood sample was performed from EDTA tubes using a DNA extraction Kit (Wizard Genomic; Promega Corporation, Madison, WI, USA). Each polymorphism was genotyped by a specifically designed Restriction Fragments Length Polymorphism (RFLP) method and validated by sequencing the codifying region for both polymorphisms. The Polymerase Chain Reaction (PCR) primers were 5′GGAAACCACCAAGTGCCTCGGATGG3′ and 5TCTGCACCCTGATGCTACCAAGGAC3′ for p22phox A-930G polymorphism and 5′CTCTGTTTGTCTTCAGTAAAGG3′ and 5′ACTCACAGGAGATGCAGGACG3′ for p22phox C242T. PCRs were carried out in a volume of 30 μl, containing 30 ng genomic DNA, 2.0 mM, $MgCl_2$, 200 mM deoxyribonucleoside triphosphates, 300 nM primers, 0.025 U EuroTaq DNA polymerase (Euroclone, Pero, Italy) and 1*x* reaction buffer. Amplified products were digested with 3 U of *RsaI* restriction enzyme for 1 h at 37 °C (New England Biolabs, Beverly, MA, USA) for the C242T amplification product and with 3 U of *BbvI* restriction enzyme for 1 h at 37 °C (New England Biolabs, Beverly, MA, USA) for the A-930G amplification product. The resulting fragments were separated on 1.5% agarose (Invitrogen) and visualised with ethidium bromide staining under ultraviolet illumination (Syngene Gen Genius; Synoptics Group, Cambridge, UK) (Fig. 1).

Statistical analysis

Results are presented as mean ± standard deviation, median, frequency or percentage as required. Continuous variables were analysed using the Student's *t*-test and ANOVA, whereas categorical variables were analysed using the χ^2 test. Non-parametric tests (Mann—Whitney *U* test, Kruskal—Wallis) were used for variables that were not normally distributed.

The Hardy—Weinberg equilibrium for allelic distribution was tested using the formula $1 = p^2 + 2pq + q^2$, where *p* and *q* are the allelic frequencies of C and T in C242T polymorphism and A and G in the A-930G, respectively. In both polymorphisms the allelic frequencies were in Hardy—Weinberg equilibrium. This is a logistic regression and it has already been explained below. Note that, at least in our population, the C242T polymorphism is not in linkage disequilibrium with the A-930G polymorphism, as has been published in the Caucasian population.[24]

The adjusted association between genotypes and OSA was examined using binomial logistic regression. A *p*-value < 0.05 was considered significant.

Results

Subject characteristics, allelic, genotype frequencies

Demographic and clinical characteristics are summarised in Table 1. The allelic and genotypic distribution of the A-930G and C242T polymorphisms is represented in Tables 2 and 3 respectively. In the A-930G polymorphism the

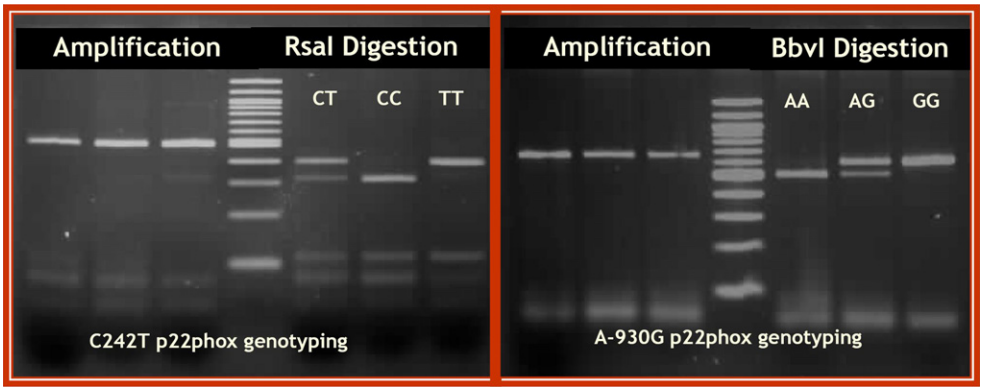

Figure 1 Gentyping p22Phox polymorphisms by RFLP's. Representative agarose gel (1.5%) showing the genotyping by PCR reaction and a digestion of different genotypes for p22phox C242T and A-930G polymorphisms.

Table 1 Demographic and clinical characteristics.

Sample characterisation	OSA Mean ± SD	CONTROL Mean ± SD
N	427	139
Age (years)**	51 ± 12	45 ± 12
Men n (%)**	352 (82%)	89 (74%)
Body Mass Index (kg m^{-2})**	31.4 ± 6	27.9 ± 5
Obesity n (%)**	237 (56%)	42 (31%)
Epworth Scale**	10 ± 5	8 ± 4
AHI (events h^{-1})**	48 ± 24	3 ± 2
SBP (mmHg)**	131 ± 17	121 ± 16
DBP (mmHg)**	80 ± 12	76 ± 11
Hypertension n (%)**	204 (49%)	33 (24%)
Glucose (mg/dl)**	106 ± 23	93 ± 15
Diabetes n (%)*	77 (18%)	13 (9%)
Dyslipemia n (%)**	272 (64%)	66 (55%)
Cholesterol(mg/dL)	207 ± 40	206 ± 40
Triglycerides (mg/dL)**	159 ± 101	120 ± 50
CRP (mg/l)**	3.01 ± 2.36	2.14 ± 2.15
Insulin (mlU/ml)	14.2 ± 9.8	16 ± 21
HOMA index	3.9 ± 3	3.8 ± 5.5

*$p < 0.05$; **$p < 0.01$.

prevalence of the G allele was not significantly different between our groups (0.64 for OSA and 0.55 for control group, $p = 0.091$) and in the C242T polymorphism, the prevalence of the C allele was also not significantly different between the groups (0.66 for OSA and 0.60 for the control group, $p = 0.337$). However, the prevalence of the genotypes was different between cases and controls in A-930G polymorphism (38.9% for GG, 49.9% for AG and 11.2% for AA in OSA patients and 29.6% for GG, 47.4% for AG and 23% for controls, $p = 0.002$) and also in C242T polymorphism (10.3% for TT, 48% for CT and 41.7% for CC in OSA patients and 18.7% for TT, 42.4% for CT and 38.8% for controls, $p = 0.033$).

Genetic association analysis

We genotyped the A-930G and C242T p22Phox polymorphisms in DNA isolated from the leukocytes of the participants. When we analysed the relationship of the p22pPhox polymorphisms with OSA, we detected that the G allele of the A-930G polymorphism is associated with OSA following a dominant model independently of age, obesity, gender, hypertension and diabetes (Table 4, $p = 0.012$ for AGvs.AA and $p = 0.006$ for GGvs.AA). The C242T was associated with OSA in the crude analysis, but this association disappears when we adjusted for age, obesity, gender and hypertension (Table 4). Interaction between both polymorphisms was not significant.

Differences in 8-isoprostane levels between OSA and control group

The median level of 8-isoprostane was significantly higher ($p = 0.001$) in OSA patients (12.10 ng dL^{-1} (IQR: 6.23—23.98)) than in the control group (5.07 ng dL^{-1} (IQR: 1.41—11.56)).

Patients with the GG genotype for the A-930G polymorphism had significantly higher 8-Isoprostane levels compared to those with the AG or AA genotype ($p = 0.001$). Patients with the CC genotype for the C242T polymorphism had significantly higher 8-Isoprostane levels than those subjects with the CT or TT genotype ($p = 0.009$). Furthermore, synergic effect was observed when these two polymorphisms were analysed together (Fig. 2).

Discussion

This study provides evidence of a relationship between the p22phox A-930G polymorphism and OSA independently of age, obesity, gender, hypertension, dyslipemia and diabetes.

OSA is thought to be a complex disorder that involves multiple genes,environmental influences and

Table 2 Allelic and genotype frequencies for p22phox A-930G.

	OSA patients n (%)	Controls n (%)	*p* Value
Genotype GG	163 (38.9)	40 (29.6)	0.002
Genotype GA	209 (49.9)	64 (47.4)	
Genotype AA	47 (11.2)	31 (23.0)	
G Allelic frequency	0.64	0.55	0.091
A Allelic frequency	0.36	0.45	

Table 3 Allelic and genotype frequencies for p22phox C242T.

	OSA Patients n (%)	Controls n (%)	*p*-value
Genotype TT	43 (10.3)	26 (18.7)	0.033
Genotype CT	200 (48.0)	59 (42.4)	
Genotype CC	174 (41.7)	54 (38.8)	
T Allelic frequency	0.34	0.40	0.337
C Allelic frequency	0.66	0.60	

developmental factors.[25]The role of specific genes that influence the development of OSA is not clear. Previous studies have reported that several single nucleotide polymorphisms (SNPs) such as serotonin transporter (5-HTT)[26] or ApoE[27,28] might be involved in the pathogenesis of OSA. Our results show different allelic frequencies of the A-930G polymorphism between OSA patients and controls. The G allele of this polymorphism was associated with sleep apnoea independently of age, obesity, gender, hypertension, and diabetes and C242T polymorphism. Furthermore, carriers of the AG and GG genotype had also a higher risk of developing OSA (odds ratio 2.209 and 2.536 respectively, Table 4). These results suggest that the A-930G polymorphism might be a genetic maker for OSA. In our results the role of the p22phox C242T polymorphism is not significant because it depends on the factors cited before.

We analysed the relationship of these polymorphisms with the severity of OSA and EDS without significance for AHI or Epworth index in OSA patients (data not shown).

A different rate of p22phox expression due to different promoter activity[17,18] has been described for the A-930G polymorphism. G allele is associated with higher p22phox expression and an increased level of oxidative stress.[19,20] Oxidative stress is not only a characteristic of OSA but also an important component in the associated conditions and comorbidities that aggregate with it, such as sympathetic activation, obesity, hypertension, hyperlipidemia and diabetes mellitus.[2,3,8] Since in our results A-930G polymorphism is associated with sleep apnoea independently of these comorbidities, we conclude that an increased tendency to oxidative stress might be a risk factor for OSA.

Table 4 Association between the P22phox polymorphisms and OSA adjusted for clinical characteristics. Logistic regression.

Characteristics	Odds ratio (CI 95%)
Age**	1.03 (1.01–1.05)
Obesity**	2.89 (1.80–4.63)
Sex (Male vs. Female)**	3.45 (2.07–5.74)
Hypertension*	1.70 (1.01–2.86)
Diabetes	1.86 (0.85–4.05)
242T poymorphism	
Genotype CT (vs. CC)	1.25 (0.77–2.03)
Genotype TT (vs. CC)	0.67 (0.34–1.31)
A-930G polymorphism	
Genotype AG (vs. AA)*	2.21 (1.19–4.10)
Genotype GG (vs. AA)**	2.54 (1.31–4.90)

*$p < 0.05$; **$p < 0.01$.

For the C242T polymorphism a different rate of O2-production has been demonstrated depending on the genotype. The T allele is associated with a reduced NADPH oxidase, in a basal or stimulated activity.[15,29] The presence of this allele has been suggested to be protective to several pathologies such as atherosclerosis[29,30] and hypertension.[21] A recent meta-analysis showed that the T allele seems to have a protective effect against Coronary Artery Disease (CAD) in Asian population.[31] However, high heterogeneity has been demonstrated within ethnic groups showing controversial results.

In our study different allelic frequencies of p22phox C242T between OSA and the control group are shown (Table 3). However, the significance in the relationship between OSA and C242T polymorphism depends on the confounding factors included in the analysis (age, obesity, gender, hypertension, diabetes and the A-930G polymorphism. The relationship of C242T polymorphism with OSA has been studied in the Chinese Han population.[32] In contrast to our results, this study suggested that the T allele contributes to the susceptibility to OSA. The different results of the Han study could be due to the high heterogeneity within ethnic groups or to the fact that confounding factors were not included in the analysis. Similar differences between Asian and Caucasian population in the allelic frequencies were found in the analysis of this polymorphism in coronary artery disease.[31]

In order to analyse the role of oxidative stress as the link of the relationship OSA-p22phox polymorphisms, we measured the 8-isoprostane levels. 8-Isoprostane levels were significantly higher in the OSA group than in the control group. These data confirmed previous reporting of increased oxidative stress in OSA patients.

In our results carriers of the G allele presented higher 8-isoprostane levels than those with the A allele. In the case of the C242T polymorphism the presence of the C allele was related to higher 8-isoprostane levels. Furthermore, an interesting finding of our study was the already described synergic effect of the A-930G and the C242T polymorphisms in hypertensive patients[21] but on the 8-Isoprostane levels (Fig. 2) in OSA patients. This means that the concurrence of the −930 GG and 242CC genotypes associates with greater 8-Isoprostane levels in OSA patients and also the concurrence of the −930AA and 242 TT genotypes associates with lower 8-Isoprostane levels in OSA patients.

In interpreting the results of our study several limitations should be considered. First, although we have measured 8-Isoprostane as oxidative stress indicator we have not directly quantified the NADPH oxidase activity.

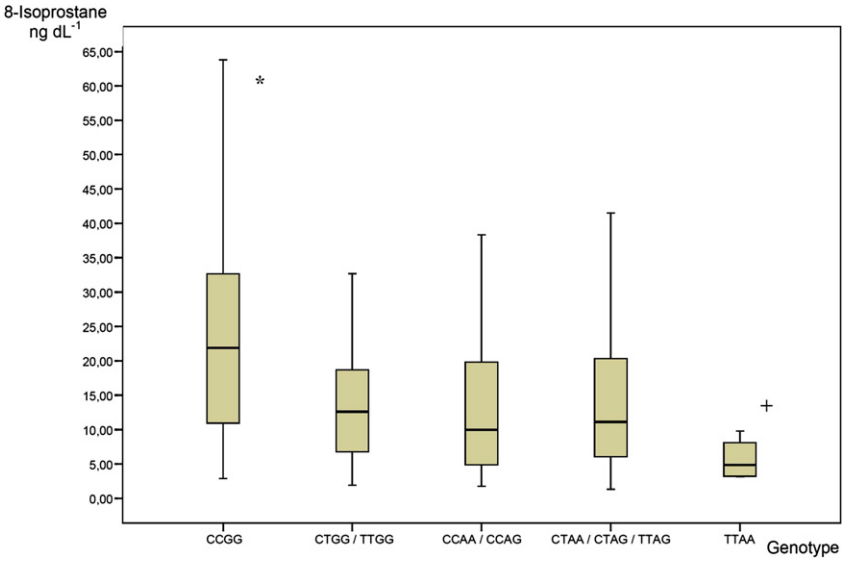

Figure 2 Synergic effect of the polymorphisms on the 8-Isoprostane level. Box plot of the distribution of 8-Isoprostane levels among combined genotypes of C242T and A-930G p22phox gene in patients with OSA. The median is shown as a line across the-box, the bottom and the top of the box indicate from 25th to 75th percentiles. Bars indicate the 1.5-fold of the whole box length. * $p < 0.005$ compared with other combinations. + $p < 0.005$ compared with other combinations.

Second, we have not analysed the NADPH oxidase expression in relationship with both polymorphisms, but it has been already described in previous studies.[15,18,24,29] Moreover, no replication study in another group has been done.

Conclusions

In conclusion, the present results support our hypothesis that the A-930G polymorphism of the p22phox gene may play an important role in genetic susceptibility to OSA. Furthermore, the C242T and A-930G polymorphisms of the p22phox gene have a synergic effect on the 8-isoprostane levels, suggesting that they may be involved in the development of oxidative stress in these patients. Further studies are required to describe the exact role of p22phox gene and NADPH oxidase in the pathogenesis of obstructive sleep apnoea.

Conflicts of interest statement

Neither the authors nor the author's institutions have any conflicts of interest to declare.

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A.4 ARTICULO 4

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ORIGINAL ARTICLE

Non-synonymous polymorphism in the neuropeptide S precursor gene and sleep apnea

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Abstract
Background Obstructive sleep apnea syndrome (OSAS) is a complex disease with a strong genetic basis. One of the primary molecular domains affected by OSAS is sympathetic activity. Neuropeptide S (NPS) plays an important role in the regulation of the sleep–wakefulness cycle, anxiety states, and daytime sleepiness. It is important to study neuropeptides related to sympathetic activity regulation and how their function could be modified by genetic variants affecting the expression of these molecules.
Objectives We investigated the association of the non-synonymous polymorphism rs4751440 in the NPS precursor gene with OSAS and certain variables related to OSAS (daytime sleepiness, body mass index (BMI), insulin resistance, and blood pressure). This polymorphism causes an amino acid substitution in exon 3 of the human NPS precursor gene.
Patients and methods We included 253 OSAS patients and 70 healthy subjects. Genotyping was done by polymerase chain reaction using specific flanking primers and agarose gel electrophoresis. Daytime sleepiness, BMI, plasma levels of high-density lipoprotein, glucose, total cholesterol, insulin, triglycerides, and the homeostasis model assessment index were also determined.

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Results A similar genotypic and allelic distribution was found in OSAS patients and controls. The risk of OSAS was not associated with the rs4751440 polymorphism. There was no significant interaction between daytime sleepiness or metabolic variables and the rs4751440 polymorphism.
Conclusion Genotypic and allelic frequency distribution of the rs4751440 polymorphism was similar in OSAS patients and controls. In this population-based study, we could not show a significant association between rs4751440 polymorphism and susceptibility to OSAS or certain phenotypes related to OSAS (daytime sleepiness, BMI, systolic blood pressure, and insulin resistance) with the exception of diastolic blood pressure.

Keywords Neuropeptide S · Obstructive sleep apnea syndrome · Genetic association studies

Introduction

Obstructive sleep apnea syndrome (OSAS) is a common disorder characterized by excessive daytime sleepiness (EDS), repeated episodes of upper airway obstruction during sleep, and nocturnal hypoxemia [1]. EDS occurs in about 16% of the general population [2] and can lead to substantial impairments in quality of life [3]. In fact, daytime sleepiness associated with OSAS is a reported predictor for mortality and cardiovascular disease in adults [4].

Obstructive sleep apnea is a complex disease that is affected by multiple factors, which interact to determine the overall phenotype. In addition to environmental risk factors, OSAS has a strong genetic basis that is likely to be the sum of small to moderate effects originating in a large number of genetic variants [5, 6]. One of the primary molecular domains affected by OSAS is sympathetic activity. The pathogenesis of the clinical features of OSAS as well as the neurobiology of sleep and wakefulness are incompletely understood. Therefore, it is important to study neuropeptides related to sympathetic activity regulation and how their function could be modified by genetic factors affecting the expression of these molecules. Changes in the function of these neuropeptides could affect clinical manifestation of OSAS.

The work on orphan G-protein coupled receptors (GPCRs) during the last decade has increased our knowledge in the neurobiological mechanisms underlying the modulation of sleep–wakefulness. The first step was the discovery that the neuropeptide and GPCR ligand hypocretin (also known as orexin) could potentially induce wakefulness, and its absence, or a null mutation in one of its receptors, was associated with narcolepsy [7–9]. The most recent example of involvement in sleep–wakefulness is neuropeptide S (NPS) [10]. NPS is a peptide transmitter that modulates the arousal and fear responses and is highly conserved in mammals [11]. NPS precursor mRNA is expressed only in several discrete regions located mainly in the brainstem [12]. Central administration of NPS promotes behavioral arousal and suppresses all stages of sleep in rodents. NPS was shown to induce transient increases in intracellular Ca^{2+}, indicating that it might have excitatory effects at the cellular level [10, 11].

Recently, a number of polymorphisms in the human NPS precursor gene were identified [13–15]. The presence of polymorphic variants in the gene coding for the NPS precursor could create differences in the expression pattern of this gene, and therefore the activity of this neuropeptide in individuals with these variants. Changes in the normal function of NPS protein, as result of changes in the sequence of the NPS precursor gene, could induce differences in the clinical manifestations of sleep apnea. In the present study, we have explored the association between a non-synonymous single-nucleotide polymorphism (SNP) (rs4751440) and OSAS, as well as with several intermediate phenotypes of OSAS. The SNP rs4751440 is located in the coding sequence of the NPS precursor gene, on chromosome 10, and induces a change in the protein sequence (rs4751440-G/C, Val→Leu).

Patients and methods

Participants were recruited from consecutive subjects who attended the sleep units of the three tertiary university hospitals in Spain (Palma de Mallorca, Sevilla, and Vitoria) to evaluate sleep disorder breathing. Subjects with an apnea–hypopnea index (AHI) greater than 10 h^{-1} were included as patients and subjects with AHI lower than 10 h^{-1} as controls. A total of 253 OSAS patients and 70 controls were enrolled for the study. All subjects included in the study were Caucasian and were from the same geographic region.

Apnea was defined by the absence of airflow for more than 10 s. Hypopnea was defined as any airflow reduction that lasted for more than 10 s and resulted in arousal or oxygen desaturation (SaO_2 >4%) [6]. The apnea–hypopnea index was defined as the sum of the number of apneas plus hypopneas per hour of sleep. The polysomnographic study included recordings of oronasal flow, thoracoabdominal movements, electrocardiography, chin and pretibial electromyography, electroculography, electroencephalography, and transcutaneous measurements of arterial oxygen saturation. Daytime sleepiness was assessed using the Spanish version of the Epworth sleep scale [15]. The threshold for diabetes was a fasting glucose >126 mg/dl; for insulin resistance it was a homeostasis model assessment (HOMA) index >4; for hyperlipidemia it was a total cholesterol >200 mg/dl; and for obesity it was a body mass index (BMI) >30 kg/m^2. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured in the two groups following international

guidelines [16]. Exclusion criteria (for both cases and controls) were the presence of chronic obstructive pulmonary disease, liver cirrhosis, thyroid dysfunction, rheumatoid arthritis, chronic renal failure, and/or psychiatric disorders.

The study was approved by the Ethics Committees of the participating institutions, and all participants signed consent forms after being fully informed of its goals and methods.

Measurements

Blood samples (10 ml) were obtained for biochemical determinations between 8:00 and 10:00 A.M. after an overnight fast, drawn in tubes containing EDTA and without anticoagulant. After centrifugation, serum and plasma were immediately separated into aliquots and stored at −80°C. Blood in EDTA tubes and aliquots of serum and plasma were transported in dry ice from Txagorritxu Hospital (Vitoria) and Virgen del Rocio Hospital (Sevilla) to Son Dureta Hospital (Palma de Mallorca) and stored at −80°C until analysis.

Biochemical analysis

Glucose, triglycerides, and total cholesterol concentrations were determined using standard enzymatic methods on a Hitachi 917 biochemical analyzer (Roche Diagnostics, Indianapolis, USA). High-density lipoprotein (HDL) was measured by a homogeneous, enzymatic colorimetric method using a commercial reagent set (Roche Diagnostics). The plasma concentration of insulin was measured by a commercial chemoluminescent assay on an Immulite 2000 analyser (DPC, Los Angeles, USA). Insulin resistance was calculated using the HOMA.

DNA extraction and polymerase chain reaction

DNA extraction from each blood sample was done using a DNA extraction Kit (Wizard Genomic, UK). The coding sequence region of neuropeptide S is located in human chromosome 10q26.2. A region located in exon 3 containing the SNP of interest, rs4751440, was amplified with specific primers. This SNP is located at the chromosome position 129,240,846 and induces two allelic variants: allele C and allele G. This SNP is a non-synonymous polymorphism that induces a change in the sequence of the protein (Val→Leu). The amplification was done by polymerase chain reaction (PCR) using PCR primers as indicated: HNPS-F: 5′-TTTCTCCTCACCCATCTGAATTGCCAGGTG-3′, HNPS-R: 5′-TAGATTAATTCCCCGAGTCCTTTGCACAC-3′.

PCR was carried out in a volume of 30 μl containing 30 ng of genomic DNA, 2.0 mM $MgCl_2$, 200 μM dNTPs, 300 nM of each primer, 0.025 U EuroTaq DNA polymerase (Euroclone Pero, Italy), and a 1× reaction buffer. PCR began with an initial denaturation at 95°C for 3 min, followed by 35 amplification cycles (95°C for 15 s, 53°C for 15 s, and 72°C for 45 s) and final extension of 10 min at 72°C. Amplified products of 292 bp were separated on 3% agarose gels and visualized with ethidium bromide staining under an ultraviolet transilluminator (Syngene Gen Genius, Synotopics Group, USA)

Genotyping

The nucleotide sequence of the amplified region, located in exon 3 of the NPS precursor gene, was determined by sequencing analysis. Sequencing was done with an ABI Prism 310 Genetic Analyzer (ABI, Foster City, CA). Sequencing analysis was performed using the ClustalW software package (Professor J. Felsenstein, Department of Genetics, University of Washington, Washington, DC).

Statistical analysis

All data analyses were performed using SPSS (version 11) statistical software. Fixing a confidence level of 95% and assuming a 70% of rs4751440-GG genotype in the control group, the sample size was calculated to obtain a statistical power of 80% to detect differences with the rs4751440-GG percentage in the OSAS group greater than 15%. This sample size allows the detection of odds ratios of 2.5 or higher as statistically significant. Hardy–Weinberg equilibrium was determined in each group with the chi-square test. The relationships between the polymorphisms and other variables in these two independent samples were analyzed by using the *t* test for quantitative, normally distributed variables, the nonparametric Mann–Whitney test for other quantitative variables, and the Fisher's exact test for dichotomous categorical variables. Linear regressions were performed to examine the relationship of the Epworth scale, SBP, DBP, BMI, HOMA, and glucose levels (log-transformed if necessary) to the different alleles. The independent variables in these linear regression models were genotype, group (defined as patients with OSAS or controls), and their interaction.

Results

The main demographic and clinical characteristics, as well as the biochemical profiles of all subjects, are shown in Table 1. Patients with OSAS had significantly higher Epworth scores, AHI, SBP, DBP, BMI, glucose plasma levels, insulin, and HOMA index scores than controls ($p<0.05$). Differences in age, sex, triglycerides, cholesterol, and HDL cholesterol levels were not significant.

Table 1 Anthropometric and clinical characteristics (mean (SD)) of patients with obstructive sleep apnea syndrome (OSAS) and controls

	OSAS (n=253)	Controls (n=70)	p value
Age (years)	50.04 (10.88)	47.6 (9.47)	0.2
Male (%)	89.7	85.7	0.45
Epworth scale	11 (5)	7 (4)	**<0.001**
AHI (events/h)	50.76 (24.57)	2.15 (1.56)	**<0.001**
SBP (mmHg)	133.27 (16.05)	124.56 (15.1)	**<0.001**
DBP (mmHg)	83.96 (12.74)	78.97 (10.8)	**0.006**
BMI (kg/m^2)	32.12 (5.58)	28.26 (4.660)	**<0.001**
Glucose (mg/dl)	106.96 (20.64)	96.37 (16.37)	**<0.001**
Triglycerides (mg/dl)	163.61 (114.18)	137.34 (78.33)	0.053
Cholesterol (mg/dl)	211.26 (37.57)	217.82 (3867)	0.267
HDL (mg/dl)	50.27 (12.04)	50.36 (10.79)	0.86
Insulin (μU/ml)	17.10 (15.5)	14.17 (15.76)	**<0.001**
HOMA index	4.64 (4.23)	3.63 (4.72)	**<0.001**

Significant p values are denoted in bold

AHI apnea–hypopnea index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *BMI* body mass index, *HDL* high-density lipoprotein

The genotypic and allelic frequencies of polymorphism rs4751440, located on chromosome 10 (position 129240346), were determined. This rs4751440 polymorphism presents two alleles (rs4751440-C/G) and generates three possible genotypes (rs4751440-GG, rs4751440-GC, and rs4751440-CC). Genotype distribution was compared between the two groups included in the study. Genotypic and allelic frequencies in healthy controls and OSAS patients are presented in Table 2. The genotypic distribution in the OSAS group was rs4751440-GG, 183 (73.1%); rs4751440-GC, 63 (24.9); and rs4751440-CC, 5 (2%). In the control group was rs4751440-GG, 50 (71.4%); rs4751440-GC, 18 (25.7%); and rs4751440-CC, 2 (2.9%). There were no significant differences in the distribution of genotypes between the two groups analyzed (p=0.79). The allelic distribution in the OSAS group was rs4751440-G, 433 (85.5%) and rs4751440-C, 73 (14.5%). In the control group, it was rs4751440-G, 118 (84.3%) and rs4751440-C, 22 (15.7%). There were no significant differences in the distribution of alleles between the two groups analyzed (p=0.68). No differences were found for the distribution of alleles (p=0.26) and genotypes (p=0.55) among controls of the three participant hospitals. The same result was obtained when we analyze the distribution of alleles (p=0.79) and genotypes (p=0.8) among patients. The control and OSAS patient groups were in Hardy–Weinberg equilibrium with respect to the rs4751440. The association of the alleles and genotypes, in terms of odds ratio for OSAS, was studied. We compared CC vs. GG (odds ratio [OR]=0.676; 95% confidence interval [CI], 0.12–3.58; p=0.645) and CG vs. GG (OR=0.946; 95% CI, 0.51–1.74; p=0.858). No association was found between the presence of OSAS and the different allelic variants of the SNP rs4751440. In addition, no relationship between this polymorphism and phenotypes related to OSAS (HOMA, Epworth, BMI, glucose plasma levels, and SBP) was found. Only DBP were related to genotype for NPS. The mean DBP levels in OSAS group were 82.85 mmHg for rs4751440-GG, 87.28 mmHg for rs4751440-GC, and 83 mmHg for rs4751440-CC. In the control group, mean DBP levels were 77.08 mmHg for rs4751440-GG, 84.17 mmHg for rs4751440-GC, and 77.5 mmHg for rs4751440-CC; DBP mean levels were statistically different between rs4751440-GG and rs4751440-GC in patients (p=0.02) and control group (p=0.018).

We performed a multivariate model to test the effects of the interaction between genotype for NPS and group on the variables analyzed (HOMA, Epworth, BMI, glucose plasma levels, SBP, and DBP). Age and sex effects were included whenever they had a significant contribution in explaining the variability of the modeled quantity. It was the case for glucose plasma levels and also for blood pressure. In the

Table 2 Distribution of genotypic and allelic frequencies in healthy controls and OSAS patients (n(%))

SNP rs # NPS precursor gene	Genotypes/alelles	OSAS (n=253)	Controls (n=70)	p value
rs4751440	GG	185 (73.1)	50 (71.4)	0.79
	GC	63 (24.9)	18 (25.7)	
	CC	5 (2)	2 (2.9)	
	G	433 (85.5)	118 (84.3)	0.68
	C	73 (14.5)	22 (15.7)	

multivariate model, group–genotype interaction was not found for any variable analyzed (Table 3).

Discussion

The present study describes a similar distribution of NPS genotypes in obstructive sleep apnea syndrome patients and healthy controls and shows that NPS gene polymorphisms were not correlated with OSAS phenotype. Therefore, we could not find a genetic association between the non-synonymous SNP rs4751440 and OSAS.

The study of the prevalence of a given single-nucleotide polymorphism and the allelic frequency are the most common ways to describe the association of a SNP and a phenotype [17]. Our study aimed to describe the prevalence in OSAS patients, and in subjects without this syndrome, of a non-synonymous SNP located in the coding sequence of the NPS precursor gene. This study also aimed to analyze the association of this polymorphic variation and susceptibility to the disease that could be explained by changes in the function of the NPS due to this polymorphic change that alters the protein sequence. We could not show a contribution of rs4751440 polymorphism in our study that could contribute to the pathogenesis of OSAS, despite the fact that this polymorphism induces a change in the protein sequence. This result could be due to our sample size limitation, but it could also be possible that this change does not compromise the function of the protein and therefore does not have an effect on clinical sleep apnea or susceptibility to the disease. Another possible explanation could be that this polymorphism induces changes in the function of NPS, but these changes are not significant enough to induce increased susceptibility to the disease or alter clinical variables related to it.

Obstructive sleep apnea is a complex disease that is affected by multiple factors, which interact to determine the overall phenotype. Due to sympathetic activity being one of the primary molecular domains affected by OSAS, an analysis of factors potentially affecting sympathetic activity regulation in OSAS patients is crucial. Neuropeptide S has been recently recognized as the endogenous ligand for the orphan G-protein coupled receptor. The NPS regulates important biological functions such as arousal response, sleeping/waking regulation [18, 19], locomotion, anxiety, and food intake [10]. NPS is an example of the impact of orphan receptor research on neuroscience and our understanding of sympathetic activity regulation [10, 11].

In patients with OSAS, sympathetic activity has been related to blood pressure [20–22], daytime sleepiness, BMI, and insulin resistance [23–25]. We have examined the association between NPS genetic forms and different phenotypes that are related to sympathetic activity in patients with obstructive sleep apnea [2, 23–26]. Our sample was unable to detect a relationship between genotype and all these indirect markers of sympathetic activity except for DBP. This last observation reinforces the relationship between NPS and sympathetic activity.

Some limitations in the present study deserve comments: (1) The sample size of this study has a power of 80% to detect differences in rs4751440-GG percentages between groups of 15% or more (or odds ratios of 2.5 or higher). As a consequence, we cannot discard lower differences, which could be of clinical relevance. (2) We have not measured directly sympathetic activity. Nevertheless, there were included variables that have been related with sympathetic activity (blood pressure, daytime sleepiness, BMI, and insulin resistance).

In conclusion, our results showed that genotypic and allelic frequency distributions of the rs4751440 polymorphism were similar in OSAS patients and controls. In this population-based study, we could not show a significant association between rs4751440 polymorphism and susceptibility to OSAS or a significant association between rs4751440 polymorphism and certain phenotypes related to OSAS (daytime sleepiness, BMI, systolic blood pressure, and insulin resistance) with the exception of DBP.

Table 3 Relationship of genotype and patient group with metabolic variables

	p value					
	BMI[a]	HOMA[a]	Glucose[a]	Epworth	DBP	SBP
Group	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Genotype	0.368	0.202	0.02198	0.811	**0.006**	0.251
Interaction	0.734	0.424	0.2955	0.617	0.957	0.375

p values for the variables group, genotype and their interaction, from a multivariate model. Age and sex effects were included whenever they had a significant contribution in explaining the variability of the modeled quantity. It was the case for glucose and also for both blood pressures

DBP diastolic blood pressure, *SBP* systolic blood pressure

[a] Variables were log-transformed due to a lack of normality of the data. Significant *p* values denoted in bold

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A.5 ARTICULO 5

Barceló A, Piérola J, de la Peña M, Esquinas C, Sanchez-de la Torre M, Ayllón O, Alonso A, Agusti AG, Barbè F. Day- night variations in endothelial dysfunction markers and haemostatic factors in sleep apnoea. Eur Respir J. 2012; 39:913-8.

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Day–night variations in endothelial dysfunction markers and haemostatic factors in sleep apnoea

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ABSTRACT: Patients with sleep apnoea have a significant alteration in the day–night pattern of myocardial infarction and sudden cardiac death observed in the general population. The aim of this study was to investigate the influence of sleep apnoea on the diurnal variations in various haemostatic parameters (factor VII, von Willebrand factor and plasminogen activator inhibitor (PAI)-1) and markers of endothelial dysfunction (asymmetric dimethylarginine (ADMA) and soluble CD40 ligand (sCD40L)).

We studied 26 male patients with obstructive sleep apnoea syndrome (OSAS; 13 patients with severe OSAS (apnoea/hypopnoea index (AHI) >30 events·h^{-1}) and 13 patients with mild-to-moderate OSAS (AHI <30 events·h^{-1})) and 12 controls of similar body mass index (BMI) and waist circumference. In each subject, six different samples were obtained over 24 h.

Although all the markers values tended to be higher in patients with severe OSAS, differences did not reach statistical significance at any time. PAI-1 levels were significantly related to BMI ($p<0.001$), mean ($p<0.001$) and minimal ($p=0.047$) nocturnal oxygenation saturation. ADMA levels were significantly related to arousal index ($p=0.046$).

The results of this study suggest that day–night variations in factor VII:antigen (Ag), von Willebrand factor:Ag, PAI-1, sCD40L and ADMA levels may be dependent on either the obesity index or metabolic dysfunction rather than on sleep apnoea alone.

KEYWORDS: Endothelial, sleep apnoea

There is evidence that patients with obstructive sleep apnoea syndrome (OSAS) have an increased risk for cardiovascular diseases, including premature death from vascular events [1–5]. In the general population, cardiovascular (CV) events predominantly occur in the first few hours after waking [6–8]. This has been explained on the basis of the circadian variations of heart rate, blood pressure, platelet aggregation and fibrinolytic activity [9–12]. In contrast, patients with OSAS show a marked variation of the day–night pattern of myocardial infarction and sudden cardiac death observed in the general population such that CV events occur preferentially in the middle of the night, while patients are sleeping [13, 14].

It appears that several factors involved in the development of cardiovascular disease are temporally regulated [15–19]. Vascular tone and endothelial function vary according to the time of day and there are differences between healthy individuals and patients with atherosclerotic disease with respect to the rhythm of nitric oxide (NO) production [20–22].

Circadian periodicity has been observed in components of the haemostatic system (plasminogen activator inhibitor (PAI)-1, factor VII and von Willebrand factor) [19, 23, 24] and markers of endothelial damage (soluble CD40 ligand (sCD40L) and asymmetric dimethylarginine (ADMA)) [21, 25]. Whether an abnormal circadian pattern of endothelial function and thrombotic potential contributes to the altered distribution of CV events in OSAS is poorly understood. Circadian rhythms are likely to be affected by established CV risk factors, including obesity, ageing, diabetes and hypertension, that occur frequently in OSAS [21, 26–29]. A recent study shows that differences in the day–night rhythm of PAI-1 between OSAS patients and non-OSAS controls may not be independent of metabolic factors [30]. The present study was designed to investigate whether the circadian changes in haemostatic and endothelial markers observed in healthy subjects occur in patients with sleep

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apnoea. To address this issue, we compared the diurnal variations in the concentration of various haemostatic parameters (factor VII, von Willebrand factor and PAI-1) and markers of endothelial dysfunction (ADMA and sCD40L) in patients with OSAS and in a control group of similar body mass index (BMI), waist circumference and metabolic profile.

METHODS

Subjects and ethics

We studied 26 male patients with OSAS (13 patients with severe OSAS (apnoea/hypopnoea index (AHI) >30 events·h^{-1}) and 13 patients with mild-to-moderate OSAS (AHI <30 events·h^{-1}). As a reference group, we included 12 controls without OSAS. They had all been referred to the sleep laboratory for snoring or suspected OSAS. Each participant was interviewed and was informed in detail of the purpose of this study. They were matched for BMI ($\pm$3 kg·m^{-2}) and waist circumference ($\pm$5 cm). The diagnosis of OSAS was established by full polysomnography (E-Series Compumedics, Abbotsford, Australia), including recording of oronasal flow, thoracoabdominal movements, ECG, submental and pre-tibial electromyography, electro-oculography, electroencephalography and transcutaneous measurement of arterial oxygen saturation. Apnoea was defined by the absence of airflow for >10 s. Hypopnoea was defined as any airflow reduction that lasted >10 s and resulted in arousal or oxygen desaturation. We considered desaturation a decrease in arterial oxygen saturation >4%. The AHI was defined as the sum of the number of apnoeas plus hypopnoeas per hour of sleep.

No participant suffered from any chronic disease (diabetes, systemic hypertension, chronic obstructive pulmonary disease, liver cirrhosis, thyroid dysfunction, rheumatoid arthritis, chronic renal failure and/or psychiatric disorders), or was taking any type of medication. The study was approved by the Ethics Committee of our institution, and all the participants signed their consent after being fully informed of its goal and characteristics.

Protocol

Participants arrived at the sleep unit of our institution (Hospital Universitario Son Espases CIBERES, Palma de Mallorca, Spain) at 21:00 h, after fasting for at least 6 h. A heparinised venous catheter (Introcan Safety®; Braun, Melsungen, Germany) was inserted into an antecubital vein to allow serial blood sampling throughout the night without disturbing sleep [31, 32]. From this catheter, six different samples (20 mL each) were obtained during the next 24 h (22:00, 02:00, 06:00, 10:00, 14:00 and 18:00 h). Blood was collected into tubes containing EDTA (10 mL) and into tubes containing sodium citrate (10 mL). The sample obtained at 10.00 h was followed by an additional one (10 mL) collected into tubes without any anticoagulant for general biochemical assessment. Blood samples were immediately processed and centrifuged during 15 min at 2,500 × *g* (model CR4 22; Jouan S.A., Saint-Herblain, France). Serum and plasma were frozen at -80°C until analysis.

During the study, participants remained in the hospital. Arterial blood pressure was measured between 08:00 and 10:00 h using a mercury sphygmomanometer after they had been seated for >5 min, with their arm resting on a standard support. During the day, they were allowed to rest or to just perform low-activity tasks and they ate a standardised three-meal diet.

Haematological and biochemical analysis

Blood cell counting was performed on fresh samples using automatic electronic cell counter (XE 2100, Sysmex Corp, Kobe, Japan). Measurements of glucose, cholesterol, triglycerides, uric acid, creatinine and liver enzymes (alanine transaminase (ALT), aspartate transaminase (AST) and γ-glutamyl transpeptidase (GGT)) were performed using a standard automated enzymatic methods on a Hitachi 917 biochemical analyser (Roche Diagnostics, Indianapolis, IN, USA). High-density lipoprotein cholesterol (HDLc) was measured by a homogeneous, enzymatic colorimetric method using a commercial reagent set (Roche Diagnostics). Low-density lipoprotein cholesterol (LDLc) was calculated using the Friedewald equation.

ELISA assays

Factor VII:antigen (Ag), von Willebrand:Ag, PAI-1, sCD40L and ADMA levels were determined by ELISA using commercially available kits (Diagnostica Stago, Asnieres, France (factor VII:Ag, von Willebrand:Ag and PAI-1); R&D Systems Inc., Minneapolis, MN, USA (scD40L); and BioMedica Diagnostic systems, Vienna, Austria (ADMA)). Measurements were always performed in duplicate, and mean values were used for analysis. The intra-assay coefficients of variation were 3.5% for factor VII:Ag, 6.1 % for von Willebrand:Ag, 4.4% for PAI-1, 5.1% for sCD40L and 6.3% for ADMA.

Statistical analysis

Results are presented as percentages, median (interquartile range) or mean$\pm$SD. Between-group comparisons were performed using the Kruskal–Wallis test. The Chi-squared test was used to compare categorical variables.

The distributions of endothelial and haemostatic variables were skewed and therefore the log transformation of these measures was applied before statistical analyses. ANOVA test for repeated measures was performed to compare with and within-group measurements of these variables at different times (22:00, 02:00, 06:00, 10:00, 14:00 and 18:00 h).

Comparison between groups for the concentration during follow-up of each marker was performed by the analysis of the areas under the curves (AUCs) using the Kruskal–Wallis test [33]. Separate intergroup comparisons for night (02:00 and 06:00 h), ''morning'' (10:00 and 14:00 h) or evening (18:00 and 22:00 h) AUCs were also calculated.

Correlations between the subjects' characteristics and haemostatic variables were explored using the Spearman rank test. Multiple regression analyses were used to confirm the significant associations detected with adjustment for age, BMI, waist circumference and smoking status.

Statistical significance was defined as $p<0.05$. All statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Tables 1 and 2 show the main clinical characteristics and haematological and biochemical parameters of the two groups of patients and controls studied. By definition, patients with OSAS showed abnormal sleep parameters, whereas these variables were normal in controls. BMI, systolic and diastolic pressure, and glucose, triglycerides, cholesterol, creatinine, AST, ALT and GGT levels were similar between both groups of patients and controls, although the latter were slightly younger (table 1).

The number of circulating erythrocytes, leukocytes and platelets was similar in all the groups (table 2).

Figure 1 shows the circadian pattern of these variables observed in each group. Although all the markers values tended to be higher in patients with severe OSAS, these differences did not reach statistical significance at any time.

No differences between groups were detected between the AUC for PAI-1 (p=0.30), for factor VII:Ag (p=0.695), for von Willebrand factor:Ag (p=0.534), for sCD40L (p=0.990) and for ADMA (p=0.395). Moreover, we did not find any differences between the AUC for these variables at three different intervals (morning, afternoon and night) during a 24-h period.

Spearman's correlation analysis showed that PAI-1 levels correlated positively with BMI (r=0.667, p<0.001) and negatively with the mean (r= -0.596, p<0.001) and minimal nocturnal oxygenation saturation (r= -0.333, p=0.047) (fig. 2). The dependency of the association between PAI-1 and oxygenation saturation on measures of adiposity and smoking status was explored in a multiple regression analysis. Significant associations were diminished, but not eliminated, after adjustment for age, BMI, waist circumference and smoking status (mean oxygenation saturation, p=0.031).

ADMA levels were also significantly related to the arousal index (p=0.046) (fig. 2).

TABLE 1 Characteristics of subjects studied

	Severe OSAS	Mild-to-moderate OSAS	Controls
Subjects n	13	13	12
Age yrs	43±2	39±2	37±2
AHI events·h^{-1}	59±6	20±3	6±1**
Mean S_{a,O_2} %	93±1	95±1	97±1**
Minimum S_{a,O_2} %	80±2	85±1	90±1**
Arousal index	51±7	22±3	8±2**
Epworth scale	9±1	9±1	8±1
TC 90 %	22.3±7.2	2.3±1.2*	1.0±0.5*
TST min	385.5±40.5	335.5±70.5	322.7±50.5
REM sleep %	14.7±6.2	14.3±8.3	16.9±7.7
BMI kg·m^{-2}	28±1	27±1	27±1
Waist circumference cm	100±2	98±3	96±3
Neck circumference cm	41±1	40±1	39±1
SBP mmHg	131±3	123±3	124±4
DBP mmHg	84±2	72±3	74±2
Smokers	3 (23)	4 (31)	5 (41)

Data are presented as mean±SD or n (%), unless otherwise stated. OSAS: obstructive sleep apnoea syndrome; AHI: apnoea/hypopnoea index; S_{a,O_2}: arterial oxygen stauration; TC 90: % time with S_{a,O_2} <90%; TST: total sleep time; REM: rapid eye movement; BMI: body mass index; SBP: systolic blood pressure, DBP: diastolic blood pressure. *: p<0.05 *versus* severe OSAS; **: p<0.01 *versus* OSAS.

TABLE 2 Haematological and biochemical parameters

	Severe OSAS	Mild-to-moderate OSAS	Controls
Subjects n	13	13	12
Glucose mg·dL^{-1}	97±2	97±3	98±3
Triglycerides mg·dL^{-1}	155±22	136±21	158±37
Cholesterol mg·dL^{-1}	200±6	183±11	170±7*
HDLc mg·dL^{-1}	45±3	46±4	40±4
LDLc mg·dL^{-1}	128±6	111±11	98±3*
Uric acid mg·dL^{-1}	6.1±0.4	6.0±0.4	6.0±0.3
Creatinine mg·dL^{-1}	0.94±0.04	0.93±0.02	0.91±0.02
AST U·L^{-1}	23±2	23±3	24±2
ALT U·L^{-1}	28±3	27±2	27±2
GGT U·L^{-1}	32±3	30±2	30±2
Erythrocytes ×10^6 cells·μL^{-1}	5.1±0.1	5.0±0.1	5.0±0.1
Leukocytes ×10^3 cells·μL^{-1}	8.0±0.6	7.3±0.3	7.8±0.4
Platelets ×10^3 cells·μL^{-1}	222±12	228±13	237±19

Data are presented as mean±SD, unless otherwise stated. OSAS: obstructive sleep apnoea syndrome; HDLc: high-density lipoprotein cholesterol; LDLc: low-density lipoprotein cholesterol; AST: aspartate transaminase; ALT: alanine transaminase; GGT: γ-glutamyl transpeptidase. *: p<0.05 *versus* severe OSAS.

DISCUSSION

This study shows that day–night variations in factor VII:Ag, von Willebrand factor:Ag, PAI-1, sCD40L and ADMA levels are not significantly different between patients with OSAS and controls without OSAS of similar adiposity and metabolic profile. Our results suggest that sleep apnoea does not have any direct effect on the oscillations of these haemostatic substances. Nevertheless, it is possible that the procoagulant consequences of sleep apnoea may become apparent in the presence of comorbidities, such as the metabolic syndrome.

Coagulation and fibrinolysis may influence cardiovascular risk in OSAS, but the relationship of adiposity with these processes is unclear [34]. Factor VII, von Willebrand factor, representatives of the haemostatic system and PAI-1, as the most important inhibitor of the fibrinolytic system, have been associated with visceral obesity [35–37]. In addition, adipose tissue has emerged as a key secretory organ that may regulate CD40L expression, suggesting a novel mechanism that accounts for the prothrombotic state of obese individuals and patients with metabolic syndrome [38, 39].

In the general population, acute CV events occur frequently in the early morning hours, when there is a marked rise in neural

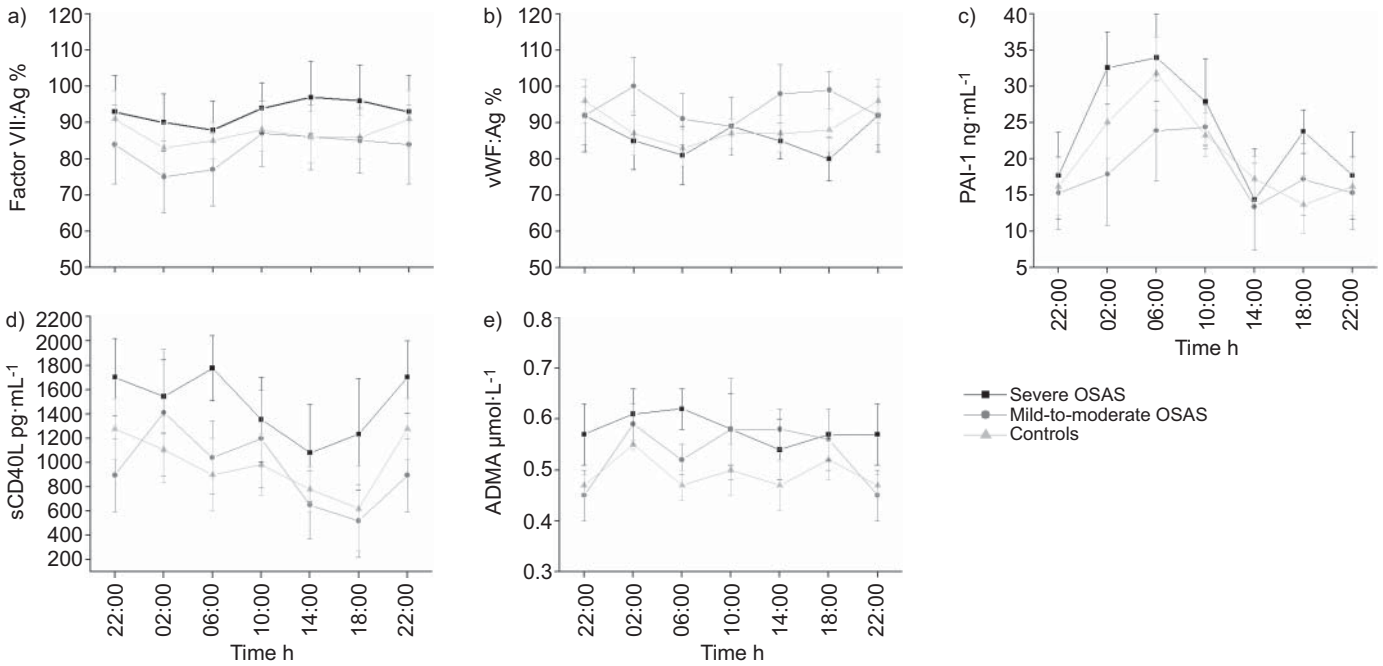

FIGURE 1. Mean values of a) factor VII: antigen (Ag), b) von Willebrand factor (vWF):Ag, c) plasminogen activator inhibitor (PAI)-1, d) soluble CD40 ligand (sCD40L) and e) asymmetric dimethylarginine (ADMA) at different times during the day in both groups of patients and controls. OSAS: obstructive sleep apnoea syndrome.

and hormonal sympathetic activity, increased platelet activity and hypercoagulability [6, 8, 10, 40]. By contrast, in patients with OSAS, the timing of myocardial infarction and sudden death shifts from the morning hours to the night, while the patient is actually sleeping [13, 14]. The mechanisms by which this occurs are unclear. There is an intimate relationship between the circadian clock, metabolism and obesity [41, 42], and several studies have shown that diabetic patients exhibit a blunted circadian variation in haemostatic and fibrinolytic factors potentially associated with morning peaks of cardiovascular events [26, 29]. The current study assesses the circadian behaviour of these biomarkers in two groups of patients with OSAS and in a control group with a similar degree of obesity. There were no significant differences in the median values of these markers at different intervals between the three groups. Despite the fact that all markers values tended to be higher in patients with severe OSAS, these differences not reached statistical significance at any time. Nevertheless, the fact that our results suggest that the changes in these patterns may be dependent on the obesity index or metabolic dysfunction rather than on sleep apnoea alone does not mean that they are irrelevant in the pathogenesis of cardiovascular complications in these patients. Metabolic and hormonal aspects should be considered in future studies to test this hypothesis.

Several studies that have assessed haemostasis parameters in healthy subjects and patients with a history of coronary artery disease have shown that, despite the higher activity of PAI-1 in patients, the periodicity of changes was maintained in both groups. In our study, the circadian pattern of PAI-1 found in the controls was still present in the patients, albeit at a higher level. PAI-1 was associated with BMI and mean and minimum nocturnal oxygenation saturation. In a recent study, VON KÄNEL

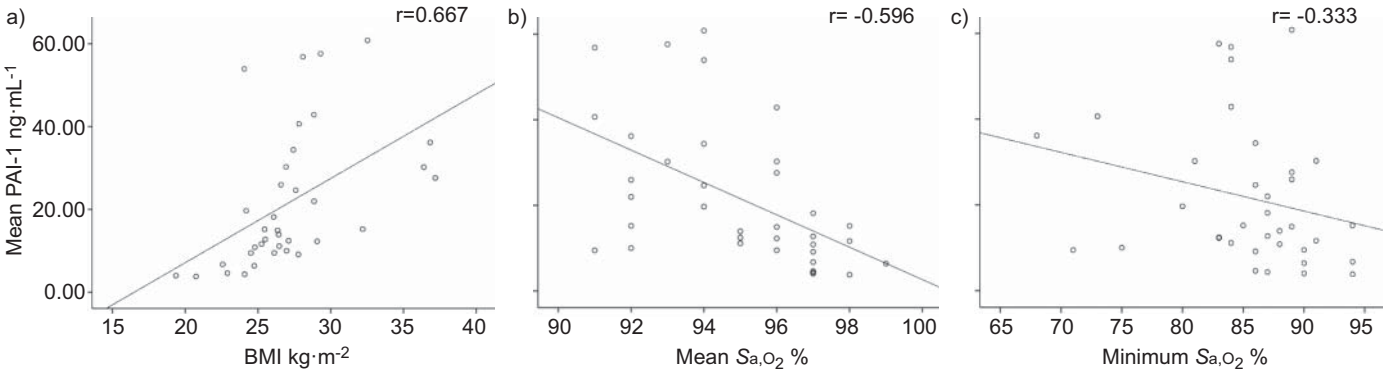

FIGURE 2. Relationship between plasminogen activator inhibitor (PAI)-1 and a) body mass index (BMI), and b) minimum and c) mean nocturnal arterial oxygen saturation (S_{a,O_2}).

et al. [30] found that the relationship between OSAS and PAI-1 was attenuated after controlling for BMI and mean arterial pressure. We did not include hypertensive subjects and, even though most of this association was explained by central obesity, PAI-1 levels were independently associated with indices of nocturnal hypoxia, even after adjusting for confounders. Furthermore, recent results of the Cleveland Family Study provide evidence for a positive relationship between OSA and PAI-1 levels [43]. These observations suggest a potential role of PAI-1 in the link between obesity, OSAS and CV risk.

Vascular tone and the concentration of NO metabolites in plasma exhibit a circadian variation [21]. Plasma concentrations of ADMA are elevated in several clinical syndromes associated with increased cardiovascular risk [44]. We have previously shown that plasma ADMA levels are elevated in patients with OSAS [45]. However, there is also evidence that ADMA levels are higher in obese and insulin-resistant individuals [46]. In the current study, the ADMA mean values tended to be higher in patients with severe OSAS but these differences did not reached any statistical significance. These results suggest that the concentration of ADMA may vary in OSAS according to the degree of obesity and metabolic disturbances. There was a significant correlation, however, between ADMA levels and the arousal index, suggesting a possible additional mechanism by which OSA may influence ADMA levels and lead to endothelial dysfunction.

One potential limitation of our study is that it included only male subjects. Furthermore, none of the participants described symptoms associated with excessive daytime sleepiness. As a consequence, our data cannot automatically be extrapolated to female patients or patients with excessive daytime sleepiness.

Compared with controls, patients with OSAS showed a higher variability in mean levels of PAI-1 at different times. This implies that other factors, not studied here, may be involved in these changes. Obesity may influence the circadian rhythms of cardiovascular and metabolic markers. The fact that other factors were not related to BMI, in either the patients or the controls, might be explained by the narrow range of BMI in the subjects studied herein. Nevertheless, hormonal aspects should be considered in the future.

Furthermore, although the control group had a very similar metabolic profile to that of the OSAS groups, the sample size limits the conclusions, and large studies should be carried out to evaluate the role of these haemostatic factors in the process of cardiovascular complications in patients with sleep apnoea.

In conclusion, the results of this study indicate that the day–night variations in the levels of several endothelial markers and haemostatic factors are not different between patients with sleep apnoea and controls of a similar weight. It is becoming increasingly clear that the circadian clock and metabolism directly influence one another. The search for additional factors that may contribute to better understanding the links between OSAS, metabolism and cardiovascular disease is highly desirable.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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A.6 ARTICULO 6

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ORIGINAL ARTICLE

The influence of obesity and obstructive sleep apnea on metabolic hormones

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Abstract
Background Obstructive sleep apnea syndrome (OSAS) is a common disorder characterized by excessive daytime sleepiness and repetitive upper airway obstruction episodes during sleep. Clinically, obesity is a major risk factor for developing OSAS. However, OSAS has been associated with hormonal and metabolic alterations that could predispose patients to obesity. The aim of this study was to investigate the independent role of apneas and obesity on plasma levels of metabolic hormones (adiponectin, ghrelin, and leptin) in patients with OSAS.
Methods We have studied patients with OSAS and controls with and without obesity. All patients were male, had an apnea–hypopnea index of 20/h or greater, and were eligible for nasal continuous positive airway pressure (nCPAP) treatment. Patients were considered obese (n=28) when their BMI was higher than 30 kg/m^2 and non-obese (n=21) when it was lower than 27 kg/m^2. Non-obese control subjects (n=20) were non-snorers with a normal cardiorespiratory sleep study, while obese control subjects (n=10) were recruited from those obese subjects who were visited in our sleep unit and for whom OSAS was excluded by full polysomnography. A single blood sample was obtained from an antecubital vein in all participants after the completion of the nocturnal sleep laboratory recording. Plasma leptin, adiponectin, and ghrelin levels were determined by radioimmunoassay.
Results The adiponectin, ghrelin, and leptin plasma levels were similar in both patients and controls. There were

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differences in leptin and adiponectin plasma levels between the obese and non-obese in both patient and control groups. In the case of ghrelin, differences between obese and non-obese subjects were only seen in patients. There were no significant differences in hormone levels between the obese controls and obese patients or between non-obese controls and non-obese patients. After 3 months of nCPAP treatment, adiponectin levels decreased significantly both in obese and non-obese patients, and leptin levels decreased in obese patients. Finally, nCPAP did not reduce ghrelin in either obese or non-obese patients.

Conclusions The basal levels of leptin, adiponectin, and ghrelin were mostly associated with obesity. We found that sleep apnea was not a determinant factor in leptin, adiponectin, and ghrelin hormonal levels. Interestingly, nCPAP treatment diminishes leptin in obese OSA patients and adiponectin levels in obese and non-obese patients with OSAS.

Keywords OSAS · Metabolism · CPAP

Introduction

Obstructive sleep apnea syndrome (OSAS) is characterized by repetitive upper airway obstruction episodes during sleep [1]. These obstructions may cause important reductions in oxygen saturation and produce multiple arousals during the night, which provoke the disruption of sleep structure and excessive daytime sleepiness. Sleep apnea is present in 4% of middle-aged men and 2% of middle-aged women [2, 3].

The pathogenesis of the syndrome is unknown, but there exist multiple etiological factors. One of the most important factors is obesity [4, 5]. Visceral fat accumulation and neck circumference are both important indicators for OSA [6, 7]. Fat accumulation around the upper airway could decrease airway diameter and predispose it to collapse. In fact, weight loss may lead to a marked improvement in sleep apnea severity [8]. Recent evidence has shown that changes in the physiological sleep architecture and time needed to fall asleep in healthy people are followed by changes in the production of different hormones related to metabolic control and the potential development of obesity [9, 10].

To investigate the independent contributions of sleep apnea and obesity to plasma levels of leptin, ghrelin, and adiponectin, we compared their concentrations in obese [body mass index (BMI) > 30 kg/m^2] and non-obese patients with OSAS (BMI <27 kg/m^2), as well as in a control group of subjects without OSAS (with and without obesity). To assess the biological effects of the therapeutic suppression of apneic events, measurements were repeated in patients with OSAS 3 months after treatment with continuous positive airway pressure (CPAP) was initiated.

Methods

Subjects and ethics

Patients were recruited prospectively from those who attended the sleep units of the three participating university hospitals in Spain (Palma de Mallorca, Seville, and Vitoria). All patients were male, had an apnea–hypopnea index (AHI) of 20/h or greater, and were eligible for nasal CPAP (nCPAP) treatment [11]. To maximize the possibility of finding clear-cut results, we decided a priori not to include patients with obstructive sleep apnea who had moderate obesity. Patients were considered obese (n=28) when their BMI was higher than 30 kg/m^2 and non-obese (n=21) when it was lower than 27 kg/m^2 [12, 13]. Patients were studied twice: at diagnosis and after having been treated effectively with nCPAP (REM Star; Respironics, Murrysville, PA, USA) for 3 months. Compliance with treatment was checked by the timer incorporated into the nCPAP device.

Non-obese control subjects (n=20) were non-snorers recruited from the nonmedical staff of our hospital. In these subjects, the diagnosis of OSAS was excluded by a cardiorespiratory sleep study that recorded nasal flow, thoracic movements, heart rate, snoring, body position, and transcutaneous hemoglobin saturation (EdenTec, Eden Prairie, MN, USA). Obese control subjects (n=10) were recruited from those obese subjects visited at our sleep unit and in whom OSAS was excluded by full polysomnography.

We excluded patients who suffered from any chronic disease (chronic obstructive pulmonary disease, chronic inflammatory intestinal diseases, diabetes mellitus, liver cirrhosis, thyroid dysfunction, rheumatoid arthritis, chronic renal failure, and/or psychiatric disorders); malignant tumors (particularly gastric tumors); gastric surgery; or who were being treated with any type of medication. This information was based on the physician-adjudicated condition. The study was approved by the ethics committee of the participating institutions, and all participants provided written consent after being fully informed of its goal and characteristics.

Polysomnographic evaluation

The diagnosis of OSAS was established with a full polysomnography (E-Series Compumedics, Abbotsford, Australia) in all groups, except the non-obese control group where the diagnosis of OSAS was excluded by a cardiorespiratory sleep study. The diagnosis of OSAS with a full polysomnography included the recording of the oronasal flow by a nasal cannula, thoracoabdominal movements, electrocardiography, submental and pretibial electromyography, elec-

trooculography, electroencephalography (C3-A2, C4-A1), and transcutaneous measurement of arterial oxygen saturation. Apneas were defined as the absence of airflow for more than 10 s. Hypopneas were defined as any airflow reduction >50% that lasted more than 10 s and resulted in arousal or oxygen desaturation. The AHI was defined as the sum of the number of apneas plus hypopneas per hour of sleep. A decrease in SaO_2 >4% was considered desaturation. The nocturnal sleep and sleep-disordered breathing score was standardized using Rechtschsffen and Kale's criteria [14].

Plasma levels of hormones

A single blood sample was obtained from an antecubital vein in all participants after the completion of the nocturnal sleep laboratory recording (7:00 A.M.). Blood samples were collected in vacutainers with EDTA and placed on ice immediately. Plasma was obtained by centrifugation (3,000 rpm for 20 min at 4°C) no later than 3 h after the sample was drawn. Samples were frozen immediately at −80°C until analysis. Plasma leptin (DPC, Texas, USA), adiponectin (Linco Research, Inc., St. Charles, MO, USA), and ghrelin (Peninsula Laboratories, Inc., San Carlos, CA, USA) levels were determined by radioimmunoassay following the instructions of the manufacturer. Intra- and inter-assay coefficients of variation were 5.7% and <9.9% for ghrelin, 3.7% and 5.3% for leptin, and 5.7% and 5.9% for adiponectin, respectively. All samples were processed in the same manner. Measurements were always done in duplicate, and mean values were used for analysis.

Statistical analysis

Differences in categorical variables were assessed with the χ^2 test. The Kruskal–Wallis analysis of variance by ranks was used to compare differences in socio-demographic, clinical, and sleep variables between groups. A second analysis using the Mann–Whitney post hoc test was done to compare the same variables by groups corrected using the Holm–Bonferroni test for multiple comparisons. All p values reported are two-tailed; case-wise deletion of missing data was chosen. The results are shown as the mean ± standard deviation (SD). To test correlations between BMI, Epworth sleep scale (ESS), AHI, and levels of hormones, a nonparametric Spearman test was used. A multivariate linear regression model was used to test the influence of OSAS and obesity on ghrelin, leptin, and adiponectin plasma levels. A previous analysis of confounding and interaction factors was performed. As a result, systolic blood pressure (SBP) was included in the final model. No interaction terms were statistically significant. The difference in the plasma levels of hormones before and after 3 months of CPAP treatment was also studied using a nonparametric test for related samples (Mann–Whitney).

Results

Clinical data

The main clinical characteristics of the study subjects are shown in Table 1. Age was similar in patients and controls. There was no significant difference in the BMI values between obese patients and controls after adjustment for multiple testing, nor between non-obese patients and non-obese controls. Obese patients showed higher systolic blood pressure than non-obese control subjects, a finding which remained significant after adjustment for multiple testing. Disease severity (as assessed by the AHI) was similar between obese and non-obese patients, as shown in Table 1.

Effects of obesity on hormonal levels

To investigate the effects of obesity on the plasma level of hormones, we compared the levels of ghrelin, leptin, and adiponectin in obese and non-obese control subjects. As seen in Table 2, obese controls showed significantly higher leptin and lower adiponectin plasma levels than non-obese controls. No differences were found in ghrelin plasma levels between obese and non-obese control subjects. The previous results were obtained considering an adjustment for multiple comparisons. Finally, a positive correlation between BMI and leptin plasma levels and a negative correlation between BMI and ghrelin plasma levels were found (Table 3).

Effects of OSAS on hormonal levels

To investigate the effects of OSAS on hormonal levels, we compared the results obtained in non-obese patients and non-obese controls. For this comparison, no differences were found for the three hormones analyzed (Table 2). Finally, OSAS, assessed by the AHI, shows a trend (non-significant) to be associated with leptin plasma levels. We were not able to find any correlation between the Epworth sleep scale score, a clinical marker related to OSAS, and any of the three hormones analyzed (Table 3).

Combined effects of OSAS and obesity on hormonal levels

To assess the effects of OSAS when combined with obesity, we compared the results in obese patients and those obtained in obese control subjects. No differences were found for the three hormones analyzed (Table 2). A

Table 1 Clinical characteristics of patients with OSAS (obese and non-obese) and control subjects (obese and non-obese)

	OSAS patients (n=49)		Controls (n=30)		Kruskal–Wallis	p value	Mann–Whitney post hoc
	Obese (1), (n=28)	Non-obese (2), (n=21)	Obese (3), (n=10)	Non-obese (4), (n=20)			
Sex, M (n, %)	28 (100)	21 (100)	10 (100)	20 (100)			
Age (years)	46.61 (11.03)	49.33 (10.71)	48.7 (9.28)	42.9 (9.16)	4.92	0.177	
BMI (kg/m^2)	34.34 (3.49)	25.02 (1.22)	32.01 (1.61)	24.71 (2.39)	NA	NA	(1) vs. (3): p=0.025 (2) vs. (4): p=0.855
Epworth scale	10.25 (3.86)	8.1 (4.2)	10 (4.76)	8.67 (2.49)	5	0.171	
AHI (h^{-1})	48.92 (17.52)	41.45 (18.3)	2.87 (1.51)	3.06 (1.52)	NA	NA	(1) vs. (2): p=0.115 (3) vs. (4): p=0.907
Mean SaC2	93.24 (2.1)	93.91 (2.18)	93.46 (1.65)	94.16 (1.56)	2.96	0.397	
TST (men)	348.2 (55.3)	339.86 (47.89)	326 (165.08)	366.5 (109.18)[b]	1.28	0.734	
SBP (mmHg)	132.78 (13.79)	123.43 (14.64)	133.13 (20.16)	119.5 (12.34)	12.37	**0.006**	(1) vs. (2): p=0.016 (1) vs. (3): p=0.954 (1) vs. (4): **p=0.002** (2) vs. (3): p=0.114 (2) vs. (4): p=0.304 (3) vs. (4): p=0.079
DBP (mmHg)	82.3 (12.06)	79.14 (10.3)	83.75 (10.6)	76 (9.81)	3.86	0.277	
Glucose (mg/dL)	103.07 (17.54)	100.42 (9.5)	93.87 (10.09)	92.4 (20.93)	5.56	0.135	
Cholesterol (mg/dL)	206 (28.92)	213.71 (44.16)	220.25 (33.31)	201.94 (34.49)	2.31	0.509	
HDL cholesterol (mg/dL)	47.92 (12.02)	54.19 (11.24)	43 (1.41)	50.76 (11.37)	5.76	0.124	
Triglycerides[a] (mg/dL)	129 (82)	104 (56)	150 (126)	115 (84)	5.95	0.114	

Statistically significant p values are shown in bold

BMI body mass index, *AHI* apnea–hypopnea index, *TST* total sleep time, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *NA* not applicable

[a] Median (IQR)

[b] Nocturnal recording time p values adjusted for multiple comparisons by Holm–Bonferroni method

multivariate linear regression model was also performed to determine the effect of obesity and OSAS on the synthesis of metabolic hormones. The only association found was between obesity and the plasma levels of ghrelin, leptin, and adiponectin (data not shown).

Effects of CPAP treatment

Sixteen non-obese and 22 obese patients followed nCPAP treatment for more than 4 h/night (mean usage, 5.7±1.4 h/night). BMI values did not change in any patients. After 3 months of nCPAP treatment, adiponectin levels decreased significantly in obese and non-obese patients, reaching similar values to those in the control group (Fig. 1a). There were no differences in the nCPAP treatment effect between obese patients and non-obese patients (p=0.804). CPAP treatment reduced the plasma levels of leptin in obese OSA patients (Fig. 1b). There were no changes in ghrelin plasma levels, as shown in Fig. 1c. Changes in adiponectin and leptin plasma levels after treatment with nCPAP were not related to age, weight, blood pressure, or the levels of these peptides determined at baseline.

Discussion

This study aimed to determine the effect of OSA and obesity on the plasma levels of ghrelin, leptin, and adiponectin, three hormones related to metabolic control, body composition, energy homeostasis, and eating behavior. We did not find any relevant effect of OSAS on the plasma levels of ghrelin, leptin, and adiponectin. In contrast, we found that the levels of these hormones were mainly associated with obesity. We observed a change in adiponectin and leptin plasma levels after nCPAP treatment in some of the groups of patients with OSAS. It is plausible that OSAS has a small effect on plasma hormone levels, as suggested by the changes in some levels after nCPAP treatment. However, this interpretation should be consid-

Table 2 Hormonal profile(s) of patients with OSAS (obese and non-obese) and controls (obese and non-obese)

	OSAS patients (n=49)		Controls (n=30)		Post-CPAP treatment (3 months)		p value
	Obese (1), (n=28)	Non-obese (2), (n=21)	Obese (3), (n=10)	Non-obese (4), (n=20)	OSAS obese (1b), (n=22)	OSAS non-obese (2b), (n=16)	
Length (ng/ml)	19.27 (11.35)	7.02 (3.24)	16.06 (7.22)	7.85 (3.79)	15.48 (8.4)	7.35 (2.97)	(1) vs. (2): ***p*<0.001** (1) vs. (3): p=0.848 (2) vs. (4): p=0.393 (3) vs. (4): ***p*=0.004** (1b) vs. (1): ***p*=0.016** (2b) vs. (2): p=0.6
Ghrelin (pg/ml)	699.61 (272.55)	1,024.69 (533.5)	733.3 (198.13)	906.77 (313.5)	719.52 (318.9)	938.85 (601.15)	(1) vs. (2): ***p*=0.01** (1) vs. (3): p=0.54 (2) vs. (4): p=0.838 (3) vs. (4): p=0.148 (1b) vs. (1): p=0.375 (2b) vs. (2): p=0.101
Adiponectin (μg/ml)	24.83 (18.13)	36.94 (21.42)	18.58 (17.22)	29.47 (15.88)	18.3 (11.6)	24.51 (9)	(1) vs. (2): ***p*=0.024** (1) vs. (3): p=0.182 (2) vs. (4): p=0.297 (3) vs. (4): ***p*=0.022** (1b) vs. (1): ***p*=0.036** (2b) vs. (2): ***p*=0.044**

p values were adjusted for multiple comparisons by the Holm–Bonferroni method. Statistically significant p values are shown in bold

ered with caution because this study is not a randomized trial and the effects of CPAP on the plasma levels could be related to other factors not controlled for in the present study.

Leptin is a circulating hormone produced by adipocytes. It is a key physiologic regulator of both energy intake and expenditure (thus, body weight) and sympathetic activity [15, 16]. We found that leptin levels in non-obese patients with OSAS were significantly lower than in obese ones. This observation is consistent with the situation seen in control subjects (Fig. 1b). We also observed that leptin levels in obese patients with OSAS were not different from those in obese control subjects (Fig. 1b), which is at odds with some [17–20], but not all, previous studies [21, 22]. In our own previous research, we found that OSA only had an effect on leptin plasma levels in non-obese subjects, but leptin plasma levels were primarily associated with obesity [21]. We only found an association between leptin plasma levels and obesity in the present study. These different findings could be explained by the differences in the clinical and anthropometric characteristics of the populations analyzed. We have previously seen a small decrease in leptin plasma levels in obese OSAS patients after 3 months of nCPAP treatment, as described elsewhere [20, 23]. Harsch et al. [19] found that leptin levels changed significantly after 8 weeks of treatment with nCPAP, but not after 2 days. We also found that after 3 months of treatment, leptin plasma levels in obese OSAS patients reached similar levels to those seen in the obese control subjects (Fig. 1b).

Table 3 Linear correlation values for the analysis of metabolic hormones, and clinical and anthropometric variables

	BMI	ESS	AHI
Leptin	r=0.71	r=0.13	r=0.27
	***p*<0.001**	p=0.38	p=0.07
Ghrelin	r=−0.42	r=−0.21	r=−0.14
	***p*=0.005**	p=0.17	p=0.36
Adiponectin	r=−0.22	r=−0.22	r=−0.06
	p=0.11	p=0.13	p=068

Statistically significant p values are shown in bold

BMI body mass index, *ESS* Epworth sleep scale, *AHI* apnea–hypopnea index

Ghrelin is a peptide secreted by the stomach with a strong effect on appetite, food utilization, body weight, and body composition in both animals and humans [24]. In the present study, we showed that non-obese patients with OSAS presented significantly higher ghrelin plasma levels than obese patients. We have found a negative correlation between ghrelin plasma levels and BMI, as previously reported [10]. Harsch et al. [19] showed that ghrelin plasma levels were significantly higher in obese OSA patients than

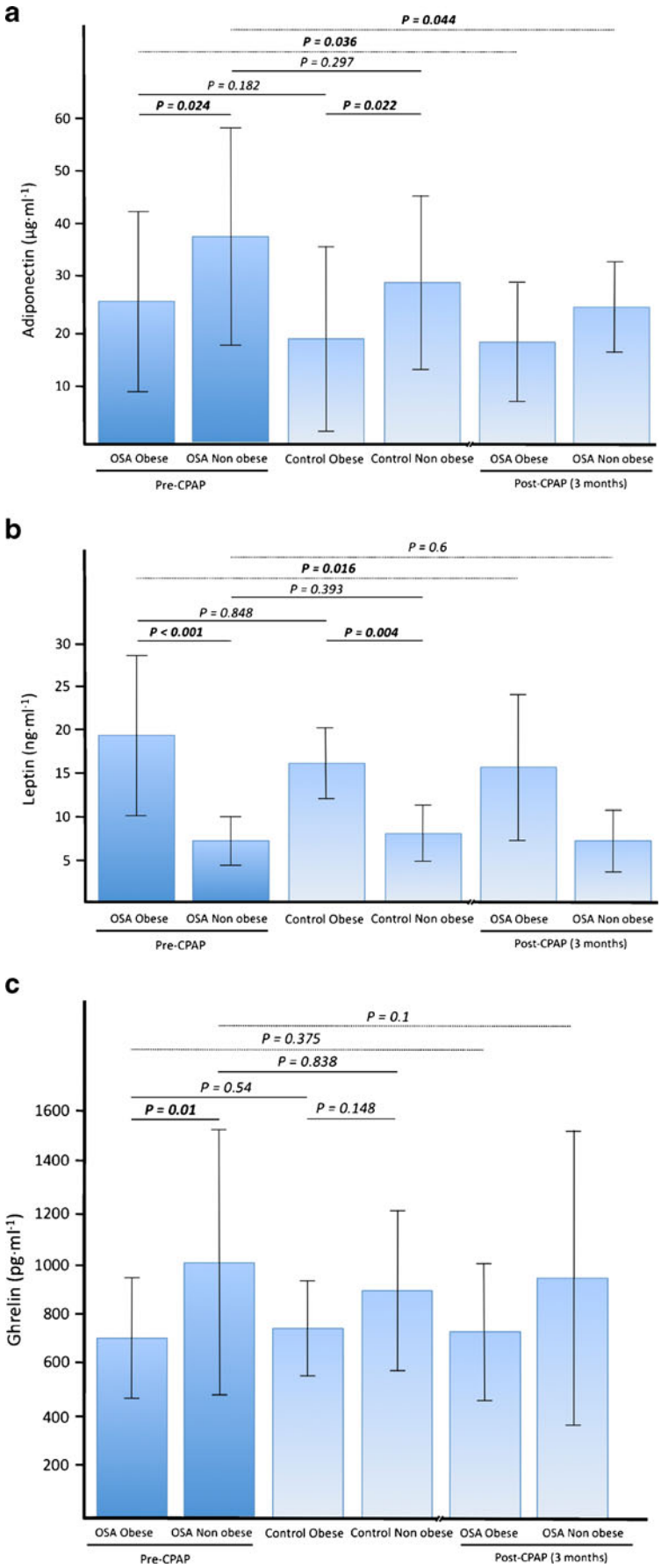

Fig. 1 **a** Adiponectin plasma levels (mean±SD) in the OSA group before and after 3 months of nCPAP treatment and the control group. **b** Leptin plasma levels (mean±SD) in the OSA group before and after 3 months of nCPAP treatment and the control group. **c** Ghrelin plasma levels (mean±SD) in the OSA group before and after 3 months of nCPAP treatment and the control group

in obese controls. They did not measure peptide levels in non-obese patients and controls, and then they could not measure the independent effect of obesity and sleep apnea on hormonal profiles. Harsch et al. [19] analyzed the short-term effect of nCPAP on ghrelin levels. After 2 days of treatment, plasma ghrelin levels decreased in all patients except one. Nevertheless, other authors did not find that CPAP had any effect on ghrelin levels after 1 month of CPAP treatment [25]. We did not find any changes in ghrelin plasma levels after 3 months of nCPAP treatment in our study (Fig. 1c).

Adiponectin is a hormone secreted by adipocytes which is closely linked to metabolism and may have both anti-atherogenic and anti-inflammatory properties [26]. Reduced plasma adiponectin levels have been associated with endothelial inflammatory responses, the presence of coronary heart disease, dyslipidemia, insulin resistance, and type 2 diabetes in humans [27]. Previous studies have described obese subjects that presented lower adiponectin plasma levels than non-obese subjects. Our study found this to be true as well (Table 2). Nevertheless, this relationship in OSAS patients and the influence of OSAS on adiponectin plasma levels are not clear. Some authors have shown increased adiponectin plasma levels in OSAS patients [28], whereas others have shown the opposite [29]. We did not find any differences in adiponectin plasma levels between patients and controls; we only detected differences between obese and non-obese subjects (Table 2). However, we did find a CPAP treatment effect in adiponectin plasma levels. After nCPAP treatment, adiponectin plasma levels decreased in both obese and non-obese patients, and the decrease was similar between these two groups. This result is partially consistent with other research [30]. The decrease in adiponectin plasma levels after nCPAP treatment is contrary to what one would expect. CPAP treatment is clearly related to a decrease in cardiovascular morbidity and mortality [31, 32]. This protective effect should be associated with an increase in adiponectin plasma levels [24]. It could be postulated that the reduction in the risk of cardiovascular disease after CPAP treatment could result from a complex mechanism where a number of factors that are not controlled for in this study intervene. Finally, we did not find a correlation between ESS and AHI, two clinical variables related to OSAS, and these metabolic hormones (Table 3).

Some potential limitations of our study deserve comment. First, in the group of non-obese control subjects, OSAS was excluded based on a cardiorespiratory sleep study. This could have potentially misclassified these subjects. Nevertheless, we believe that it is highly unlikely that these non-obese, non-snorer subjects without daytime sleepiness and without evidence of respiratory disturbances during sleep suffered from OSAS. Second, patients with OSAS and control subjects were matched for BMI. This did not, however, exclude potential differences in body fat distribution. Finally, we cannot exclude a type 2 error due to the relatively low number of obese control subjects included in the study. The main strengths of the present study revolve around the multicenter design. In addition, the exclusion of patients with BMI values in the range where substantial overlap occurs allowed for better a differentiation of those patients with obesity and those without obesity.

In conclusion, we found that obesity is the major contributor to the plasma levels of leptin, adiponectin, and ghrelin in OSAS patients in the present study. Obstructive sleep apnea is not a determinant factor in the plasma levels of these hormones. Although CPAP treatment was associated with changes in adiponectin and leptin in some groups of patients with OSAS, randomized controlled trials should be conducted to clarify the relationship between OSAS and metabolic regulation.

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Competing interests None declared.

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A.7 ARTICULO 7

Barbé F. et al. Spanish Sleep And Breathing Network. Effect of continuous positive airway pressure on the incidence of hypertension and cardiovascular events in nonsleepy patients with obstructive sleep apnea: a randomized controlled trial. JAMA. 2012; 307:2161-8.

Scan for Author Video Interview

Effect of Continuous Positive Airway Pressure on the Incidence of Hypertension and Cardiovascular Events in Nonsleepy Patients With Obstructive Sleep Apnea

A Randomized Controlled Trial

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Context Continuous positive airway pressure (CPAP) is the first-line treatment for patients with symptomatic obstructive sleep apnea (OSA). However, its indication for all patients with sleep-disordered breathing, regardless of daytime symptoms, is unclear.

Objective To evaluate the effect of CPAP treatment on the incidence of hypertension or cardiovascular events in a cohort of nonsleepy patients with OSA.

Design, Setting, and Patients Multicenter, parallel-group, randomized controlled trial in 14 teaching hospitals in Spain. Between May 2004 and May 2006, 725 consecutive patients were enrolled who had an apnea-hypopnea index of 20 h^{-1} or greater and an Epworth Sleepiness Scale score of 10 or less (scores range from 0-24, with values <10 suggesting no daytime sleepiness). Exclusion criteria were previous cardiovascular event, physical or psychological incapacity, chronic disease, or drug or alcohol addiction. Follow-up ended in May 2009.

Intervention Patients were allocated to receive CPAP treatment or no active intervention. All participants received dietary counseling and sleep hygiene advice.

Main Outcome Measures Incidence of either systemic hypertension (taking antihypertensive medication or blood pressure greater than 140/90 mm Hg) or cardiovascular event (nonfatal myocardial infarction, nonfatal stroke, transient ischemic attack, hospitalization for unstable angina or arrhythmia, heart failure, or cardiovascular death).

Results Seven hundred twenty-three patients underwent follow-up for a median of 4 (interquartile range, 2.7-4.4) years (1 patient from each group did not receive allocated treatment); 357 in the CPAP group and 366 in the control group were included in the analysis. In the CPAP group there were 68 patients with new hypertension and 28 cardiovascular events (17 unstable angina or arrhythmia, 3 nonfatal stroke, 3 heart failure, 2 nonfatal myocardial infarction, 2 transient ischemic attack, 1 cardiovascular death). In the control group there were 79 patients with new hypertension and 31 cardiovascular events (11 unstable angina or arrhythmia, 8 nonfatal myocardial infarction, 5 transient ischemic attack, 5 heart failure, 2 nonfatal stroke). The hypertension or cardiovascular event incidence density rate was 9.20 per 100 person-years (95% CI, 7.36-11.04) in the CPAP group and 11.02 per 100 person-years (95% CI, 8.96-13.08) in the control group. The incidence density ratio was 0.83 (95% CI, 0.63-1.1; $P=.20$).

Conclusions In patients with OSA without daytime sleepiness, the prescription of CPAP compared with usual care did not result in a statistically significant reduction in the incidence of hypertension or cardiovascular events. However, the study may have had limited power to detect a significant difference.

Trial Registration clinicaltrials.gov Identifier: NCT00127348

JAMA. 2012;307(20):2161-2168 www.jama.com

Obstructive sleep apnea (OSA) is a common disease that affects 3% to 7% of the general population.[1,2] Sleep apnea is caused by the collapse of the upper airway during sleep, which leads to transient asphyxia. These events lead to brain arousal, intermittent hypoxemia (which induces hypersomnolence), poor quality of life, and metabolic disturbances. Cross-sectional and longitudinal studies have shown an association between OSA and hyperten-

See also pp 2169 and 2197.

Author Video Interview available at www.jama.com.

Author Affiliations and a List of the Members of the Spanish Sleep and Breathing Network appear at the end of this article.
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sion,[3,4] and OSA has been associated with cardiovascular diseases,[5-7] although this association is attenuated in some studies when adjusted for age and obesity.[8]

Continuous positive airway pressure (CPAP) acts as a pneumatic splint to the upper airway during sleep and corrects the obstruction, improving daytime sleepiness and quality of life in patients with OSA.[9] Also, CPAP treatment can reduce blood pressure in these patients.[10] Observational studies suggest that CPAP treatment reduces the incidence of fatal and nonfatal cardiovascular events in patients with severe[11] and moderate[12] OSA. However, none of these studies were randomized. To perform such studies in symptomatic patients would be unethical because available evidence of the effectiveness of CPAP on symptom control and accident risk precludes the possibility of withholding treatment in a long-term study. The indication for CPAP treatment in nonsleepy patients is under debate. Two short-term randomized controlled trials have shown no effect of CPAP on blood pressure and clinical variables in nonsleepy patients with OSA.[13,14] Nonetheless, because of the high incidence of cardiovascular complications in patients with OSA, some authors advocate the long-term use of CPAP treatment for all patients with sleep-disordered breathing, regardless of daytime symptoms.[15]

The primary objective of this study was to evaluate the effect of CPAP treatment on the incidence of hypertension or cardiovascular events in a cohort of nonsleepy patients with OSA. We studied this composite outcome because hypertension and cardiovascular events have been associated with OSA, they share related pathological pathways, and their clinical relevance has been established.[16] The secondary objective was to evaluate the relationship between OSA severity and the incidence of hypertension or cardiovascular events.

METHODS

Setting and Participants

Recruitment took place in 14 Spanish teaching hospitals between May 2004 and May 2006. Follow-up ended in May 2009. Patients were referred to sleep units for evaluation of observed apneas or snoring. The study was approved by the ethics committee of each participating center, and patients provided written informed consent.

Patients were eligible if they were between 18 and 70 years old, showed 20 or more apneas plus hypopneas per hour (apnea-hypopnea index [AHI]) in an overnight sleep study, and had no daytime hypersomnolence, defined as an Epworth Sleepiness Scale (ESS) score of 10 or lower (ESS scores have a range of 0-24). The exclusion criteria were physical or psychological incapacity, any previous cardiovascular event, chronic disease, drug or alcohol addiction, chronic intake of hypnotics, or refusal to participate in the study. Patients with a history of hypertension were not excluded.

The OSA diagnosis was made using a conventional polysomnographic or cardiorespiratory sleep study. The cardiorespiratory study included, at minimum, continuous recording from nasal cannulae, thoracic-abdominal motion, oxygen saturation, and body position. Results from all sleep studies were analyzed by trained personnel at each participating center, using standard criteria. An apnea was defined as an absence of airflow of 10 seconds or longer and a hypopnea as an airflow reduction (>50%) lasting 10 seconds or longer with a greater than 4% decrease in oxygen saturation. Obstructive apneas were defined as the absence of airflow in the presence of chest or abdominal wall motion. The AHI was calculated based on the average number of apnea plus hypopnea episodes per hour of sleep or recording time.

Randomization, Blinding, and Intervention

Eligible patients were randomly assigned in a 1:1 ratio to receive CPAP treatment or no active intervention. Randomization was performed using a computer-generated list of random numbers in the coordinating center and was stratified by center. The results were mailed in numbered opaque envelopes. The coordinating center saved a sealed copy of the randomization list sent to each center. Blood pressures and all cardiovascular events were assessed objectively by personnel not involved in the study and blinded to patient allocation. Patients, researchers, and the statistician were not blinded to patient allocation.

CPAP titration was performed using conventional polysomnography or an autoCPAP device following a validated protocol.[17] CPAP adherence was measured using the machines' internal clocks. All participants received sleep hygiene advice and dietary counseling for weight loss from the staff of the sleep units. There was no specific weight loss program, and patients were referred to their general practitioner to monitor weight loss.

Outcomes and Follow-up

The primary outcome was the incidence of either systemic hypertension (among participants who were normotensive at baseline) or cardiovascular events (among all participants). The secondary outcome was the association between the incidence of hypertension or cardiovascular events and the severity of OSA assessed by the AHI and oxygen saturation.

Information regarding medication use, OSA severity, sleepiness (ESS score), blood pressure, a basic biochemical assessment, and results of electrocardiography were recorded for each patient at inclusion. Cardiovascular events included nonfatal myocardial infarction, nonfatal stroke, transient ischemic attack, hospitalization for unstable angina or arrhythmia, heart failure, and cardiovascular death. All cardiovascular events were identified by a cardiologist or neurologist not involved in the study and blinded to the patients' randomization status. Hypertension was defined as taking antihypertensive medication or blood pressure greater than 140/90 mm Hg. Blood pressure measurements were taken by experienced nurses, following international guidelines.[18]

Patients were evaluated at 3, 6, and 12 months and annually thereafter. The

study was designed to follow up patients for at least 3 years. However, follow-up for the main outcome was stopped when the patients developed new-onset hypertension or experienced a cardiovascular event, withdrew informed consent, were unable to complete follow-up, or when there was a change from control group to CPAP treatment. Exposure time was defined as the time between randomization and first cardiovascular event or new-onset hypertension, date of death, date of the last study visit, date of withdrawal, or loss to follow-up.

At each visit a physician recorded cardiovascular events, blood pressure, ESS score, weight, CPAP adherence, medications, alcohol and tobacco consumption, and other clinically relevant events.

Sample Size

The sample size was calculated assuming that the incidence of hypertension or new cardiovascular event in this population over a period of 3 years would be 10% annually. This estimation was based on published studies[19] and data provided by the Spanish National Epidemiology Center. We estimated that CPAP would reduce the incidence of new hypertension or cardiovascular events by 60% based on a cohort study by Marin et al[11] that showed a cardiovascular event rate of 2.13 per 100 person-years in an untreated group vs 0.64 per 100 person-years in a group of patients treated with CPAP, a 70% relative rate reduction. We assumed $\alpha = .05$, $\beta = .10$, 2-sided significance testing, and 10% study dropout. Under these assumptions, the study would need to enroll a total of 345 patients per group.

Statistical Analysis

Data were stored in a web-based application. R version 2.10.1 was used for all analyses. Estimated means for treatment adherence, body mass index (BMI, calculated as weight in kilograms divided by height in meters squared), and ESS scores during the follow-up period were computed individually for each patient as the area under the curve obtained from the values reported in follow-up visits, taking into account the time between visits and the last available information.

Mann-Whitney tests (or *t* tests if data were normally distributed) and Pearson χ^2 tests with a Yates continuity correction (or Fisher exact tests if expected frequencies were less than 5) were used for continuous and categorical variables, respectively, when comparing the CPAP and control groups. The effect of the CPAP intervention was assessed as an incidence density ratio (IDR) of hypertension or cardiovascular events, and significance was assessed using the Wald test. A modified intention-to-treat approach was used, in which all patients receiving the allocated intervention were included in the analysis of the primary outcome.

For modeling incidence, a Poisson regression model was used and adjusted for intervention group. An additional analysis including random hospital (site) effect was performed because of the hospital imbalances in baseline variables. A goodness-of-fit test for the assumption of a Poisson distribution was performed in every adjusted model to determine if a negative binomial model should be used instead. The use of a Cox proportional hazards regression model was not possible because the data did not satisfy the proportional hazards assumption.[20] Available information was used with no need for imputation.

The cumulative incidence, CI(t), of events at time t was estimated using the incidence density at that time, ID(t), in the equation $CI(t) = 1 - \exp\{-ID(t)*t\}$. Therefore, each point of the graph shows the cumulative incidence after t years of follow-up in the study, expressed as a percentage. A post hoc analysis was performed to assess a possible dose-response relationship. For this analysis, the CPAP group was partitioned based on whether the mean CPAP adherence during follow-up was less than 4 hours.[11] The outcomes in each of these CPAP subgroups were compared with those in the control group. The Kruskal-Wallis rank-sum test was used to analyze the dose-response relationship as well as differences between hospitals in quantitative baseline variables.

The secondary outcome of the study was to assess the association between the incidence of cardiovascular events or hypertension and disease severity. To assess this association, we tested the interaction of severity variables (AHI and time with arterial oxygen saturation [Sa_{O_2}] <90%, dichotomized into 2 groups based on their median values) with the intervention in a Poisson regression model, with the outcome variable being the incidence of hypertension or cardiovascular events.

Differences between baseline values and the mean area under the curve for BMI and ESS score in follow-up visits were calculated for each patient. These differences were compared between intervention groups using the Mann-Whitney nonparametric test.

RESULTS

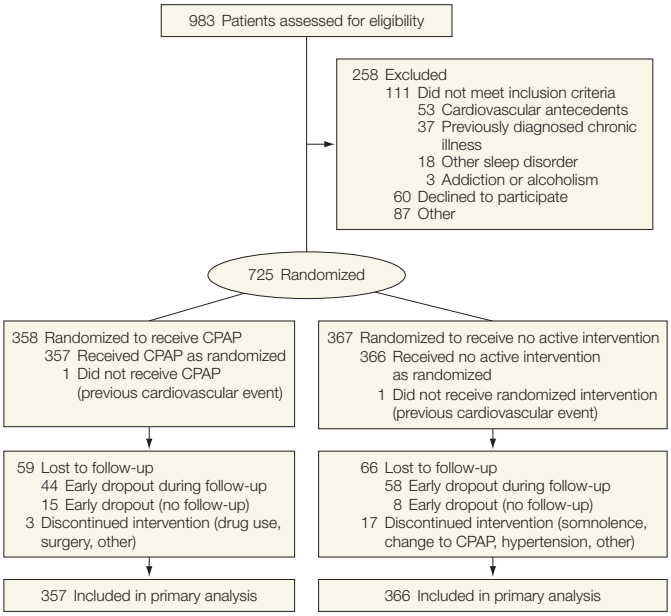
A total of 725 patients were randomized, with 357 in the CPAP group and 366 in the control group analyzed (FIGURE 1). Baseline variables were comparable between the CPAP and control groups, with the exception of AHI and time with Sa_{O_2} less than 90%. Fifty percent of the sample was hypertensive at inclusion (TABLE 1). Many of the baseline variables had site-specific differences (eTable 1, available at http://www.jama.com).

Follow-up time until a cardiovascular event, loss to follow-up, or the end of the study ranged from 0 to 5.38 years, with a median of 4.0 years (interquartile range [IQR], 2.7-4.4 years). There were no significant differences in median follow-up between groups using the Mann-Whitney test (4.01 years [IQR, 3.01-4.37] for the CPAP group vs 3.96 years [IQR, 2.19-4.38] for the control group; $P = .13$).

Primary Outcome: Effects of CPAP on the Incidence of Hypertension or Cardiovascular Events

A total of 147 patients with new hypertension and 59 cardiovascular events were identified. In the CPAP group, there were 68 patients with new hyperten-

Figure 1. Participant Flow



Only those patients excluded in the allocation process were excluded from the analysis. Discontinuation of intervention was more frequent in the control group, in which patients were excluded because of somnolence (n=7), change to continuous positive airway pressure (CPAP) (n=5), hypertension (n=2), drug abuse (n=1), pregnancy (n=1), or other cause (n=1).

sion and 28 cardiovascular events (17 hospitalizations for unstable angina or arrhythmia, 3 nonfatal stroke, 3 heart failure, 2 nonfatal myocardial infarction, 2 transient ischemic attack, 1 cardiovascular death). In the control group, there were 79 patients with new hypertension and 31 cardiovascular events (11 hospitalizations for unstable angina or arrhythmia, 8 nonfatal myocardial infarction, 5 transient ischemic attack, 5 heart failure, 2 nonfatal stroke). The incidence density rate for hypertension or cardiovascular events in the CPAP group was 11.02 per 100 person-years and in the control group was 9.20 per 100 person-years. The IDR was 0.83 (95% CI, 0.63-1.1; $P=.20$) (TABLE 2 and FIGURE 2A). Adjustment for random hospital (site) effect did not substantially modify these results (TABLE 3)

Secondary Outcomes: Relationship Between Incidence of Events and OSA Severity

Disease severity was assessed using AHI and time with SaO_2 less than 90%. The effect of CPAP on the incidence of hypertension or cardiovascular events with reference to the control group showed no significant differences across subgroups of AHI ($P=.48$) or the percentage of time with SaO_2 less than 90% ($P=.23$). No significant interaction was found between treatment group and each of the disease severity variables.

Subgroup Analyses

The mean CPAP adherence ranged from 0 to 8.76 h/night, with a median of 5.00 h/night (IQR, 2.18-6.25). In a post hoc analysis, we partitioned the patients in the CPAP group based on a mean adherence of 4 h/night.[11] There were 127 patients (36%) with a mean adherence less than 4 h/night (median, 1.00 [IQR, 0.00-2.75]) and 230 with adherence of 4h/night or longer (median, 5.96 [IQR, 5.05-6.70]). There were significant differences between these subgroups in AHI, time with SaO_2 less than 90%, neck circumference, BMI, and hypertension when compared at baseline (TABLE 4).

Primary Outcome by Adherence Subgroups

Patients who used CPAP for less than 4 h/night had an IDR of 1.13 (95% CI, 0.78-1.64; $P=.51$), compared with the control group. In contrast, patients who used CPAP for 4 h/night or longer had an IDR of 0.72 (95% CI. 0.52-0.98; $P=.04$) (Table 2 and Figure 2B). Adjustment for random hospital (site) effect did not substantially modify these results (Table 3).

Secondary Outcome by Adherence Subgroups

In post hoc analyses the effect of CPAP on the incidence of hypertension or cardiovascular events with reference to the control group showed a significant interaction with time with SaO_2 less than 90% when CPAP was partitioned based on a threshold of 4 hours of adherence ($P=.03$), while interaction with AHI remained nonsignificant ($P=.57$). Thus, the effect of CPAP was significantly different when taking into account adherence, depending on whether the value of the percentage of time with SaO_2 less than 90% was greater than the overall median of 6.8%.

Another stratified analysis was conducted, adjusting for AHI. The sample was first divided into subgroups based on the percentage of time with SaO_2 less than 90% (with a cutpoint of 6.8% of the time). These subgroups were then divided again based on CPAP adherence (with a cutpoint of 4 h/night). For 6.8% of time or less with SaO_2 less than 90%, the IDR was 0.71 (95% CI, 0.40-1.27; $P=.25$) for CPAP adherence less than 4 h/night and 0.72 (95% CI, 0.45-1.15; $P=.17$) for CPAP adherence of 4 h/night or longer. For greater than 6.8% of time with SaO_2 less than 90%, the IDR was 1.89 (95% CI, 1.14-3.13; $P=.01$) for CPAP adherence less than 4 h/night and 0.71 (95% CI, 0.45-1.13; $P=.15$) for CPAP adherence of 4 h/night or longer. Thus, IDR differed significantly from the control group only in the subgroup with greater than 6.8% of time with SaO_2 less than 90% and CPAP adherence less than 4 h/night.

Additional Results

The separate incidence of cardiovascular events or new-onset hypertension

is reported in the supplementary online content. When assessed separately, CPAP did not show any statistically significant effect on the incidence of new cardiovascular events (eTable 2, eTable 3, and eFigure 1) or on new-onset hypertension (eTable 4, eTable 5, and eFigure 2).

There were 8 deaths in the CPAP group and 3 in the control group. The IDR for death was 2.6 (95% CI, 0.70-11.8; $P=.16$). Of the patients assigned to CPAP, 5 died of cancer, 1 of cardiovascular causes, 1 of trauma, and another of unknown causes. Among controls, the causes of death were cancer for 2 and unknown for 1.

Decreases in BMI were significantly greater in the control group than in the CPAP group, with a significant difference of 0.25 (95% CI, 0.04-0.46; $P=.02$) (eTable 6). Both groups had changes in ESS scores, but these changes were significantly less in the control group than in the CPAP group (0.2 vs 1.3; $P<.001$), with a significant difference of –1.1 (95% CI, –1.4 to –0.7; $P<.001$) (eTable 7).

COMMENT

This study suggests that in patients with OSA and without daytime sleepiness, the prescription of CPAP compared with usual care did not result in a statistically significant reduction in the incidence of hypertension or cardiovascular events. However, a larger study or longer follow-up might have been able to identify a significant association between treatment and outcome. A post hoc analysis suggested that CPAP treatment may reduce the incidence of hypertension or cardiovascular events in patients with CPAP adherence of 4 h/night or longer. The disease severity assessed by the AHI and time with SaO_2 less than 90% was not related to the incidence of hypertension or cardiovascular events. However, patients with worse oxygen saturation at night and with CPAP adherence of less than 4 h/night showed a higher rate of hypertension or cardiovascular events than the control group.

Compelling evidence indicates that severe OSA is associated with an increased incidence of hypertension, cardiovascular events, or sudden death during sleep.[4,6,7,21] Three systematic reviews,[22-24] 4 meta-analyses including 21 randomized controlled trials,[10,25-27] and 3 recent studies[28-30] have evaluated the effect of CPAP therapy on blood pressure in patients with OSA. Results showed significant blood pressure reductions, especially in patients with more severe OSA.[25,26] However, evidence for an effect of CPAP treatment

Table 1. Patient Characteristics at Baseline

Characteristic	Control (n = 366)	CPAP (n = 357)	*P* Value[a]
Age, mean (SD), y	51.8 (11.01)	52.0 (10.90)	.75[b]
Men, No. (%)	306 (83.6)	313 (87.7)	.12
Body mass index, mean (SD)[c]	31.1 (4.98)	31.3 (4.86)	.45[b]
Neck circumference, mean (SD), cm	42.0 (3.70)	42.4 (3.64)	.19
Current smoking, No. (%)	94 (25.7)	113 (31.7)	.09
Any alcohol use, No. (%)	129 (46.7)	131 (48.3)	.77
Blood pressure, mean (SD), mm Hg			
Systolic	130.9 (16.89)	131.6 (16.56)	.58[b]
Diastolic	79.9 (11.46)	80.0 (11.41)	.99[b]
Hypertension, No. (%)	183 (50.0)	190 (53.2)	.43
Any antihypertensive drugs, No. (%)	82 (22.4)	95 (26.6)	.22
ACE inhibitors, No. (%)	48 (13.1)	48 (13.4)	.98
Angiotensin II receptor blockers, No. (%)	8 (2.2)	11 (3.1)	.60
Calcium channel blockers, No. (%)	11 (3.0)	19 (5.3)	.17
β-Blockers, No. (%)	17 (4.6)	21 (5.9)	.56
Diuretics, No. (%)	29 (7.9)	30 (8.4)	.92
≥1 Antihypertensive drug treatment, No. (%)	26 (7.1)	30 (8.4)	.61
Epworth Sleepiness Scale score	6.5 (2.24)	6.5 (2.27)	.95[b]
Apnea-hypopnea index, median (IQR), h^{-1}	35 (26-49)	42 (29-59)	<.001[d]
Time with SaO_2 <90%, median (IQR), %	6 (1.6-15.0)	8 (2.0-22.8)	.04[d]
Diagnosis by polysomnography, No. (%)	186 (50.8)	198 (55.5)	.24
Glucose, median (IQR), mg/dL	97 (90-109)	98 (90-108)	.80[d]
Lipids, mg/dL			
Total cholesterol, mean (SD)	213.0 (41.78)	212.6 (43.28)	.89[b]
Triglycerides, median (IQR)	116.5 (84.3-169.0)	122.5 (86.3-175.0)	.26[d]
Altered electrocardiogram, No. (%)	20 (5.5)	20 (5.6%)	.93

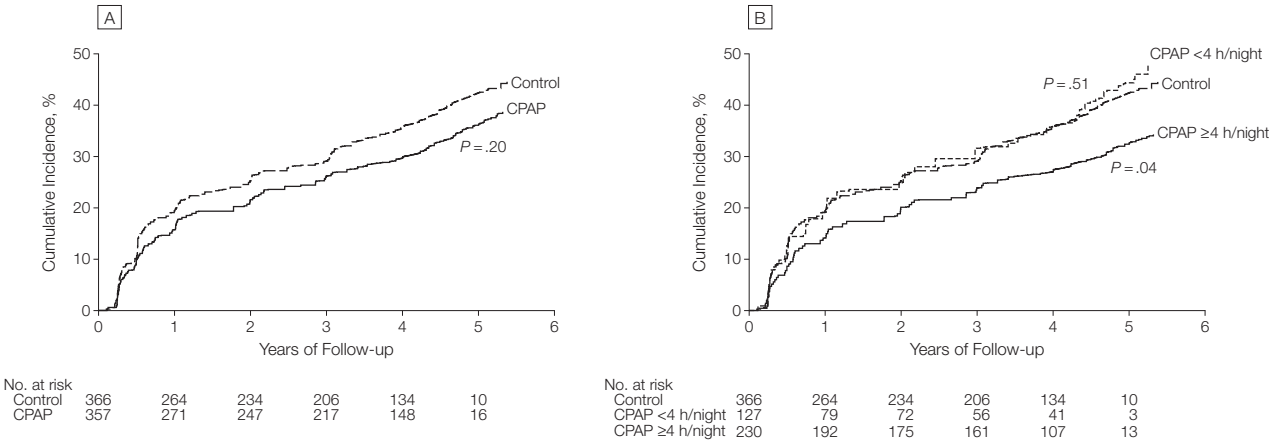
Abbreviations: ACE, angiotensin-converting enzyme; CPAP, continuous positive airway pressure; IQR, interquartile range; SaO_2, arterial oxygen saturation.
SI conversion factors: To convert glucose values to mmol/L, multiply by 0.0555; total cholesterol values to mmol/L, multiply by 0.0259; triglycerides values to mmol/L, multiply by 0.0113.
[a] Pearson χ^2 test *P* value except as noted.
[b] From *t* test.
[c] Calculated as weight in kilograms divided by height in meters squared.
[d] From Mann-Whitney test.

Table 2. Risk for New Hypertension or Cardiovascular Event

	Intention-to-Treat Analysis		Analysis by Adherence to CPAP Use	
	Control (n = 366)	CPAP (n = 357)	CPAP <4 h/Night (n = 127)	CPAP ≥4 h/Night (n = 230)
Events	110	96	37	59
Person-years	997.837	1043.436	296.4271	747.0089
Events per 100 person-years (95% CI)	11.02 (8.96-13.08)	9.20 (7.36-11.04)	12.48 (8.46-16.50)	7.90 (5.88-9.91)
IDR (95% CI)	1 [Reference]	0.83 (0.63-1.10)	1.13 (0.78-1.64)	0.72 (0.52-0.98)
P value[a]		.20	.51	.04

Abbreviations: CPAP, continuous positive airway pressure; IDR, incidence density ratio.
[a] From Wald test.

Figure 2. Cumulative Incidence of Hypertension or Cardiovascular Events During Follow-up



A, Cumulative incidence of hypertension or cardiovascular events for the intervention groups during follow-up and the *P* value for the incidence density ratio of continuous positive airway pressure (CPAP) vs control (Wald test). B, Panel A with CPAP group stratified according to adherence (<4 vs ≥4 h/night) and the *P* values for their incidence density ratios in reference to the control group.

Table 3. Poisson Regression Predicting Hypertension or Cardiovascular Events, With Hospital as a Random Effect, Intention-to-Treat Analysis

	Adjusted IDR (95% CI)	*P* Value	Variance, Random Hospital Effect
CPAP vs control group	0.81 (0.61-1.06)	.13	0.1141
CPAP adherence subgroup analysis			
CPAP <4 h/night group vs control group	1.12 (0.77-1.64)	.55	0.1183
CPAP ≥4 h/night group vs control group	0.69 (0.50-0.94)	.02	

Abbreviations: CPAP, continuous positive airway pressure; IDR, incidence density ratio.

on cardiovascular events is less definitive. Peker et al[31] reported a significant increase in the incidence of cardiovascular disease among incompletely treated patients with OSA compared to those who were efficiently treated. Several authors[11,12,32,33] have reported an improvement in long-term survival associated with treatment in different cohorts of patients with OSA. Marin et al[11] followed up a male cohort of treated and untreated patients with OSA, snorers, and the general population for 10 years in an observational study. They showed that CPAP treatment was associated with a lower risk of fatal and nonfatal cardiovascular events in patients with severe OSA when compared with patients who refused CPAP treatment. The incidence of cardiovascular events in patients receiving CPAP treatment was not different from that in the general population or in nonapneic snorers. Overall, observational studies suggest a protective effect of CPAP treatment on cardiovascular events in patients with OSA.

In a post hoc analysis in our study, CPAP was effective in patients who used the treatment for more than 4 h/night. Even in these patients, the magnitude of the effect was less than in previous observational studies.[11] In the study by Marin et al,[11] the group treated with CPAP had an event rate of 0.64 per 100 person-years, whereas the event rate in our treatment group was 2.08 per 100 person-years (eTable 2). These results are not unexpected, because randomized trials often show smaller positive effects than observational studies.

Another possible explanation is that CPAP is less effective in nonsleepy patients. Short-term studies focusing on nonsleepy patients have shown no effect of CPAP on clinical symptoms or blood pressure,[13,14] and a long-term study showed a mild beneficial effect of CPAP on blood pressure only in patients who used CPAP for 5.6 h/night or longer.[28] In our study, the protective association of CPAP use with the incidence of hypertension or cardiovascular events in the subgroup of patients with adherence of 4 h/night or longer was seen despite that subgroup being more obese and having a higher prevalence of hypertension. However, the results of post hoc analyses should be interpreted with caution and considered hypothesis generating. In these analyses, patients were not randomized to the subgroups, and findings may have resulted from a confounding bias, such as the healthy-user effect (eg, adherent CPAP users may be more likely to be adherent with other medications, treatments, exercise, nutrition, or other health maintenance).

It has been postulated that nonsleepy patients treated with CPAP will probably have poor adherence. Our results do not support this statement and show that CPAP treatment is feasible in this population, even in the absence of any specific reinforcement. An unexpected finding was the relatively high number of deaths during follow-

up. The study sample size was not large enough to reach a conclusion about any relationship to treatment, but this issue deserves further research.

The strengths of our study include its multicentric design and the generalizability of the trial findings. Baseline cardiovascular risk factors were not different between groups, and median follow-up time was similar. Nevertheless, this study has several potential limitations.

First, the lack of effect of CPAP on hypertension or cardiovascular events could be related to an inadequately powered estimation of the sample size. The lack of previous studies suitable for sample size calculation contributed to the difficulty of sample size estimation. The potential protective effect of CPAP was probably overestimated, and a larger study or longer follow-up might have been able to identify a significant association between treatment and outcome. Including patients with hypertension also might have contributed to a lack of study power, because these patients could not develop 1 component of the combined primary end point, incident hypertension. Incident hypertension accounted for more than two-thirds of the primary end point events. Second, medical research personnel were not blinded to patient allocation. However, cardiovascular events and blood pressure were assessed objectively, by personnel not involved in the study and blinded to patient allocation.

Third, at baseline the CPAP group had higher AHI scores and slightly greater time with Sa_{O_2} less than 90%. These differences, although statistically significant, were likely to be of little clinical relevance, because both groups had severe OSA, and all other characteristics and cardiovascular risk factors were similar between the groups at baseline. Fourth, blood pressure values were measured at clinic visits, an approach that can be affected by the white coat effect, observer bias, limited reproducibility, and the intrinsic inaccuracy of the examiner's auscultation technique. However, measurements were taken by experienced nurses following international guidelines. Fifth, one possible explanation for the smaller effect size of CPAP in this trial compared with prior studies is that the titration protocols may have been less effective, resulting in an elevated residual AHI on CPAP treatment. Although we do not know if this occurred, our CPAP titration protocol has been validated in previous studies performed by our research group.[17]

In conclusion, in this study of patients with OSA without daytime sleepiness, the prescription of CPAP compared with usual care did not result in a statistically significant reduction in the incidence of hypertension or cardiovascular events. However, the study may have had limited power to detect a significant difference. A post hoc analysis suggested that CPAP treatment may reduce the incidence of hypertension or cardiovascular events in patients with CPAP adherence of 4 h/night or longer.

Table 4. Comparison of Subgroups of CPAP Adherence at Baseline

	CPAP <4h/Night (n = 127)	CPAP ≥4h/Night (n = 230)	*P* Value[a]
Age, mean (SD), y	51.9 (10.16)	52.1 (11.31)	.85
Men, No. (%)	112 (88.2)	201 (87.4)	.96
Body mass index, mean (SD)[b]	30.4 (4.85)	31.8 (4.81)	.009
Neck circumference, mean (SD), cm	41.7 (3.59)	42.8 (3.62)	.01
Current smoking, No. (%)	42 (33.1)	71 (30.9)	.76
Any alcohol use, No. (%)	43 (47.3)	88 (48.9)	.90
Blood pressure, mean (SD), mm Hg			
Systolic	129.3 (16.84)	132.9 (16.30)	.053
Diastolic	78.6 (11.86)	80.7 (11.11)	.11
Hypertension, No. (%)	58 (45.7)	132 (57.4)	.04
Any antihypertensive drugs, No. (%)	28 (22.0)	67 (29.1)	.19
ACE inhibitors, mean (SD)	17 (13.4)	31 (13.5)	.89
Angiotensin II receptor blockers, No. (%)	6 (4.7)	5 (2.2)	.31
Calcium channel blockers, No. (%)	4 (3.1)	15 (6.5)	.27
β-Blockers, No. (%)	4 (3.1)	17 (7.4)	.16
Diuretics, No. (%)	8 (6.3)	22 (9.6)	.39
≥1 Antihypertensive drug treatment, No. (%)	9 (7.1)	21 (9.1)	.64
Epworth Sleepiness Scale score	6.6 (2.18)	6.5 (2.33)	.74
Apnea-hypopnea index, median (IQR), h^{-1}	33 (27-53)	47 (33-63)	<.001[c]
Time with Sa_{O_2} <90%, median (IQR)	5 (1.1-15.0)	10 (2.2-27.0)	.005[c]
Diagnosis by polysomnography, No. (%)	74 (58.3)	124 (53.9)	.50
Glucose, median (IQR), mg/dL	99 (91-105)	98 (90-110)	.97[c]
Lipids, mg/dL			
Total cholesterol, mean (SD)	216.2 (46.63)	210.6 (41.38)	.28
Triglycerides, median (IQR)	117 (84.0-177.3)	125 (87.0-171.3)	.40[c]
Altered electrocardiogram, No. (%)	4 (3.1)	16 (7.0)	.21
Automatic CPAP titration, No. (%)	101 (79.5)	178 (77.4)	.74
CPAP pressure, median (IQR), cm H_2O	8 (7-9)	9 (7-10)	.07[c]

Abbreviations: ACE, angiotensin-converting enzyme; CPAP, continuous positive airway pressure; IQR, interquartile range; Sa_{O_2} arterial oxygen saturation.
SI conversion factors: To convert glucose values to mmol/L, multiply by 0.0555; total cholesterol values to mmol/L, multiply by 0.0259; triglycerides values to mmol/L, multiply by 0.0113.
[a] From *t* test except as noted.
[b] Calculated as weight in kilograms divided by height in meters squared.
[c] From Mann-Whitney test

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Online-Only Material: The eAppendix, eFigures 1 and 2, eTables 1-7, and the Author Video Interview are available at http://www.jama.com.

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A.8 ARTICULO 8

Barceló A, Piérola J, Esquinas C, et al. Reduced plasma fetuin-A levels in patients with obstructive sleep apnoea. Eur Respir J. 2012;40:1046-8.

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LETTERS

Reduced plasma fetuin-A levels in patients with obstructive sleep apnoea

To the Editors:

Obstructive sleep apnoea syndrome (OSAS) has been increasingly linked to cardiovascular disease. fetuin-A is an inhibitor of vascular calcification and an anti-inflammatory cytokine. We tested the hypothesis that plasma levels of fetuin-A are decreased in patients with OSAS.

We studied 119 patients with OSAS and 35 controls. Participants were recruited and studied at the sleep unit at Hospital Universitari Son Espases, Palma de Mallorca, Spain. Serum levels of fetuin-A, glucose, triglycerides, cholesterol, high-density lipoprotein (HDL) cholesterol, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyltransferase (GGT) were determined.

Plasma fetuin-A levels were significantly lower in patients with OSAS than in controls (mean $\pm$ SD 368 $\pm$ 66 *versus* 445 $\pm$ 53 ng·mL^{-1}, p=0.015). In multivariate analysis, fetuin-A levels were independently associated with OSAS (p=0.034). OSAS is associated with reduced levels of fetuin-A and fetuin-A could be one of the contributing factors for the development of cardiovascular complications in OSAS patients.

There is evidence that patients with OSAS have an increased risk for cardiovascular diseases [1]. The mechanisms underlying this association are unclear but candidate mechanisms include endothelial dysfunction, oxidative stress, systemic inflammation and metabolic dysregulation [2]. Vascular calcification has recently received much attention because of its relationship with cardiovascular disease [3, 4]. Chronic inflammation may promote vascular calcification and recent studies have demonstrated a relationship between vascular calcification and endothelial dysfunction [5, 6].

Fetuin-A is a circulating protein mostly synthesised in the liver [7, 8] that is known to be an important inhibitor of vascular calcification and a potent anti-inflammatory cytokine [8–10]. Fetuin-A is considered to be a mediator that links chronic inflammation to cardiovascular diseases [11]. However, previous studies investigating the role of fetuin-A in patients with cardiovascular disease have published contradictory results [12]. Furthermore, several studies have proposed that serum fetuin-A levels are associated with the presence of the metabolic syndrome, suggesting fetuin-A as a risk factor for this condition [13].

Therefore, the aim of this study was to determine serum levels of fetuin-A in patients with OSAS and to examine the associations between fetuin-A levels and a number of conventional cardiovascular and metabolic risk factors.

The study population included 154 subjects admitted to the Hospital Universitari Son Espases sleep unit from January 2010 to December 2010. They had all been referred to the sleep laboratory for snoring or suspected OSAS. The case or control status was defined by the apnoea–hypopnoea index (AHI) threshold of $\geqslant$10.

No participant suffered from any other chronic disease (chronic obstructive pulmonary disease, liver cirrhosis, thyroid dysfunction, rheumatoid arthritis, chronic renal failure and/or psychiatric disorders). The study was approved by the Hospital Universitari Son Espases Ethics Committee and all participants signed a consent form after being fully informed of the study goal and characteristics.

The diagnosis of OSAS was established by full polysomnography (E-Series; Compumedics Ltd, Abbotsford, Australia) that included recording of oronasal flow, thoracoabdominal movements, electrocardiography, submental and pretibial electromyography, electrooculography, electroencephalography and trancutaneous measurement of arterial oxygen saturation (S_{a,O_2}). Apnoea was defined by the absence of airflow for >10 s. Hypopnoea was defined as any airflow reduction that lasted >10 s and resulted in arousal or oxygen desaturation. We considered desaturation a decrease in S_{a,O_2} >3%. The AHI was defined as the sum of the number of apnoeas plus hypopneas per hour of sleep. Excessive daytime sleepiness was quantified subjectively by the Epworth sleepiness scale. The occurrence of the metabolic syndrome was analysed according to the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) clinical criteria [14]. The diagnosis of cardiovascular disease (myocardial infarction, unstable angina, coronary bypass or coronary angioplasty, stroke or transient ischaemic attack) was recorded according to the clinical history.

After fasting overnight, venous blood samples were obtained between 08:00 h and 10:00 h. Blood was centrifuged and serum was immediately separated into aliquots and stored at -80°C until analysis. Glucose, triglycerides, total cholesterol, HDL cholesterol, creatinine, AST, ALT and GGT were determined using standard enzymatic methods on a Hitachi Modular analyser (Roche Diagnostics, Indianapolis, IN, USA). The plasma levels of fetuin-A were determined by ELISA using a commercial kit (BioVender laboratory Medicine, GmbH, Heidelberg, Germany). Measurements were always performed in duplicate and mean values were used for analysis.

Results are presented as percentages, median or mean $\pm$ SD. Parametric (unpaired t-test) and nonparametric tests (Wilcoxon test) were performed to assess the statistical significance of differences between groups. Correlations between variables were explored using the Spearman-rank test. To determine the independent effect of sleep apnoea on fetuin-A levels we used stepwise multiple regression analyses with study group (OSAS

versus non-OSAS), AHI, arousal index, age, sex, body mass index (BMI), metabolic and cardiovascular risk factors as the independent variables and fetuin-A as the dependent variable. A p-value <0.05 was considered significant.

Characteristics of the study population are summarised in table 1. No differences were found for age, sex, BMI and waist circumference between patients with OSAS and controls. The prevalence of diabetes and metabolic syndrome was higher in the OSAS group than in the control group (p=0.006 and p=0.005).

Compared with controls subjects, OSAS patients showed abnormal plasma levels of glucose and GGT. Fetuin-A levels were significantly lower in patients with OSAS than in subjects without OSAS (368±66 *versus* 445±53 ng·mL^{-1}, p=0.015). Fetuin-A levels were significantly related to AHI (r= -0.226, p=0.006) and to the arousal index (r= -0.236, p=0.010). In multivariate analysis, study group and AHI were significantly associated with fetuin-A levels (p=0.034 and p=0.041, respectively).

We categorised all subjects into fetuin-A tertiles: 1) <332 ng·dL^{-1}, 2) 332–431 ng·dL^{-1}, and 3) >431 ng·dL^{-1}. Fetuin-A levels were inversely associated with the prevalence of OSAS: first tertile, 88%; second tertile, 81%; and third tertile, 60% (p=0.012). Patients with OSAS and a history of cardiovascular disease revealed a trend towards lower levels of fetuin-A compared with patients without prevalent cardiovascular disease (364±97 *versus* 402±159 ng·mL^{-1}, p=0.07). Fetuin-A levels did not differ between patients with and without diabetes (383±93 *versus* 387±147 ng·mL^{-1}, p=0.892) or metabolic syndrome (373±131 *versus* 394±147 ng·mL^{-1}, p=0.483).

TABLE 1 Baseline characteristics and sleep profiles in controls and obstructive sleep apnoea syndrome (OSAS) patients

	Controls	OSAS
Subjects n	35	119
Age yrs	47±13	47±12
Males	24 (70)	88 (74)
BMI kg·m^{-2}	28±6	28±4
Waist circumference cm	100±13	101±11
Hypertension	8 (23)	26 (22)
Diabetes	1 (3)	14 (12)*
Metabolic syndrome	4 (12)	41 (35)*
Cardiovascular disease	6 (17)	28 (24)
AHI events·h^{-1}	5.0±2.1	40.1±20*
Arousal index events·h^{-1}	22.2±4.5	46.9±18.3*
Mean S_{a,O_2} %	97±2.3	94±2.6*
Minimal S_{a,O_2} %	90±3.6	83.3±3
Glucose mg·dL^{-1}	94±4	103±22*
Triglycerides mg·dL^{-1}	124±51	137±64
Cholesterol mg·dL^{-1}	207±41	212±39
HDLc mg·dL^{-1}	56±15	55±16
Creatinine mg·dL^{-1}	0.88±0.2	0.96±0.3
AST U·L^{-1}	22±7	21±7
ALT U·L^{-1}	27±15	27±13
GGT U·L^{-1}	32±27	37±29*

Data are presented as mean±SD or n (%), unless otherwise stated. BMI: body mass index; AHI: apnoea–hypopnoea index; S_{a,O_2}: arterial oxygen saturation; HDLc: high-density lipoprotein cholesterol; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma glutamyltransferase. *: p<0.05.

This study shows that circulating fetuin-A levels are decreased in patients with OSAS and that they are associated with OSAS severity. These observations suggest that fetuin-A could be one of the contributing factors for the development of cardiovascular complications in OSAS patients.

The high cardiovascular mortality and morbidity rates in patients with OSAS are only partially explained by the high prevalence of traditional cardiovascular risk factors [2]. Accumulating evidence suggests that inflammatory balance and especially anti-inflammatory factors are determinants for the prognosis of atherosclerotic disease [15, 16]. However, recent studies have demonstrated that the prevalence and extent of vascular calcification is a strong predictor of cardiovascular events [17–19].

Fetuin-A deficiency is a potential missing link between a state of chronic inflammation and high incidence of cardiovascular events and mortality [20]. Fetuin-A is an anti-inflammatory mediator that participates in macrophage deactivation and inhibition of apoptosis of vascular smooth muscle cells [9]. In addition, fetuin-A complexes with calcium and phosphorus in the circulation and protects against vascular calcification [8, 9]. In this study, fetuin-A levels were significantly lower in OSAS patients than in subjects without OSAS. The mechanisms that support a relationship between OSAS and a decrease in the levels of fetuin-A are not known. We found a significant correlation between fetuin-A levels and AHI and arousal index suggesting that intermittent hypoxia and sleep fragmentation may influence the synthesis and the rate of fetuin-A release into the circulation. However, low fetuin-A levels in OSAS patients could be a consequence of the chronic inflammation that characterises OSAS. Patients with OSAS tend to be in a state of microinflammation in which downregulation of proteins such as fetuin-A may be expected. As chronic inflammation may contribute to fetuin-A depletion, it is plausible that serum fetuin-A levels may represent a useful marker for the prediction of clinical outcome in OSAS patients.

Functions and regulatory mechanisms of fetuin-A seem to differ according to the pathophysiological characteristics of the population studied. Fetuin-A was shown to act as a natural inhibitor of the insulin receptor tyrosine kinase in liver and skeletal muscle and different studies have suggested that high fetuin-A levels are associated with the presence or development of metabolic syndrome [13].

In this study, we controlled for most potential confounding factors and found no attenuation of the inverse association of fetuin-A with OSAS. Furthermore, patients with a history of cardiovascular disease showed lower levels of fetuin-A compared with patients without prevalent cardiovascular disease but differences just failed to reach the statistical level of significance. These observations suggest that the effects of fetuin-A may be of greater importance in the cardiovascular risk of these patients independent of metabolic factors.

Taken together our results suggest a mechanistic relationship between changes in fetuin A production or release and OSAS.

It is also possible that lower fetuin-A levels might reflect the presence of advanced atherosclerosis with calcification in patients with OSAS and could imply a novel link between OSAS and increased cardiovascular risk. Additional studies are needed to clarify the significance of these findings.

Several limitations should be considered. First, we did not evaluate biomarkers of inflammation so we could not investigate potential relationships between them and fetuin-A. Secondly, we did not measure the plasma levels of fetuin-A after continuous positive airway pressure and this may be a limitation in the assessment of the independent effects of OSAS on this marker. Thirdly, the number of subjects was limited, thus studies in larger populations are needed to confirm our data.

This study shows that plasma fetuin-A levels are decreased in OSAS. Future studies are needed to determine if low fetuin-A levels are related to the pathogenesis of cardiovascular risk in OSAS.

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A.9 ARTICULO 9

Mazzuca E, Battaglia S, Marrone O, Marotta AM, Castrogiovanni A, Barceló A, Esquinas C, Barbé F, Bonsignore M. Gender-specific anthropometric markers of adiposity, Metabolic Syndrome and visceral adiposity (VAI) in patients with obstructive sleep apnea. J Sleep Res. 2013. doi: 10.1111/jsr.12088. [Epub ahead of print]

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Gender-specific anthropometric markers of adiposity, metabolic syndrome and visceral adiposity index (VAI) in patients with obstructive sleep apnea

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SUMMARY

Obstructive sleep apnea often coexists with visceral adiposity and metabolic syndrome. In this study, we analysed gender-related differences in anthropometrics according to sleep apnea severity and metabolic abnormalities. In addition, the visceral adiposity index, a recently introduced marker of cardiometabolic risk, was analysed. Consecutive subjects with suspected obstructive sleep apnea (n = 528, 423 males, mean age ± standard deviation: 51.3 ± 12.8 years, body mass index: 31.0 ± 6.2 kg m^{-2}) were studied by full polysomnography (apnea–hypopnoea index 43.4 ± 27.6 h^{-1}). Variables of general and visceral adiposity were measured (body mass index, neck, waist and hip circumferences, waist-to-hip ratio). The visceral adiposity index was calculated, and metabolic syndrome was assessed (NCEP-ATP III criteria). The sample included controls (apnea–hypopnoea index <10 h^{-1}, n = 55), and patients with mild–moderate (apnea–hypopnoea index 10–30 h^{-1}, n = 144) and severe sleep apnea (apnea–hypopnoea index >30 h^{-1}, n = 329). When anthropometric variables were entered in stepwise multiple regression, body mass index, waist circumference and diagnosis of metabolic syndrome were associated with the apnea–hypopnoea index in men (adjusted R^2 = 0.308); by contrast, only hip circumference and height-normalized neck circumference were associated with sleep apnea severity in women (adjusted R^2 = 0.339). These results changed little in patients without metabolic syndrome; conversely, waist circumference was the only correlate of apnea–hypopnoea index in men and women with metabolic syndrome. The visceral adiposity index increased with insulin resistance, but did not predict sleep apnea severity. These data suggest gender-related interactions between obstructive sleep apnea, obesity and metabolic abnormalities. The visceral adiposity index was a good marker of metabolic syndrome, but not of obstructive sleep apnea.

INTRODUCTION

Obesity is a major risk factor for obstructive sleep apnea (OSA), and OSA is more prevalent among obese than non-obese patients. The first studies, mostly conducted in men, identified body mass index (BMI), and neck and waist circumference as major predictors of OSA. Women with OSA were usually older and more obese than men, but had smaller neck circumference and less centrally distributed fat (Kapsimalis and Kryger, 2002a,b).

More recent literature re-examined anthropometrics in patients with OSA by assessing gender-related differences in adipose tissue distribution, suggesting the need to take into account the new information in order to develop new models possibly indicative of gender-specific OSA phenotypes. Some studies highlighted the close relationship of OSA severity with neck circumference normalized by height (NC/h) and visceral fat in both genders (Kawaguchi *et al.*, 2011; Simpson *et al.*, 2010). Conversely, a recent study found that visceral abdominal fat was increased in men but not in women with OSA; women with OSA showed increased total and subcutaneous fat with a normal ratio between visceral and subcutaneous fat (Kritikou *et al.*, 2013). Anthropometric measurements are readily available and cheap to obtain, and different anthropometric markers of OSA according to gender and metabolic status might be useful in assessing the clinical probability of the disease.

According to some authors, the metabolic abnormalities of visceral obesity could predispose to OSA (Vgontzas *et al.*, 2005). Metabolic syndrome (MetS) is frequently used in clinical practice to identify a cluster of risk factors associated with visceral obesity. The diagnosis of MetS according to the NHANES-ATP III definition is based on the presence of at least three out of five criteria according to predefined cut-off values (Alberti *et al.*, 2009). MetS was found in about half of patients with OSA (Bonsignore *et al.*, 2012), with increased waist circumference and blood pressure being the most common abnormalities associated with upper airway obstruction during sleep (Bonsignore *et al.*, 2012; Kono *et al.*, 2007). In this study, we hypothesized that analysis of anthropometrics may show gender-related differences between patients with and without MetS.

Recently, the visceral adiposity index (VAI) has been proposed as a new marker of cardiometabolic risk. VAI provides a gender-specific estimate of risk based on continuous values of BMI, waist circumference, serum triglycerides (TG) and high-density lipoprotein (HDL) cholesterol (Amato *et al.*, 2010), but does not include cardiovascular variables. Age-specific cut-off values to identify high-risk subjects have been published (Amato *et al.*, 2011a). VAI has been used in different patient populations, including non-alcoholic fatty liver disease (Petta *et al.*, 2012; Vongsuvanh *et al.*, 2012), polycystic ovary syndrome (Amato *et al.*, 2011b) and acromegaly (Ciresi *et al.*, 2012), all of these conditions being often associated with OSA.

The main aim of this study was to assess gender-related distribution of adipose tissue by common anthropometric measurements, and their association with a diagnosis of OSA, in a large sample of patients with suspected OSA, in order to identify clinically useful indicators of OSA in male and female subjects with and without metabolic abnormalities. An additional goal was to analyse the relationship between VAI and OSA severity; for this analysis, patients who had been diagnosed with non-insulin-dependent diabetes or were on pharmacological treatment for dyslipidaemia were excluded.

PATIENTS AND METHODS

We analysed data collected in a cohort of 529 consecutive subjects referred for suspected OSA that has been described elsewhere (Bonsignore *et al.*, 2012). The study protocol was approved by the local Institutional Review Board (approval number IB741/09PI), and all participants gave their informed written consent.

Complete data were available in 528 patients (423 males). All underwent full polysomnography because of clinical suspicion of OSA.

Anthropometrics

As a general measure of obesity, BMI was defined as weight/height^2 (kg m^{-2}). Anthropometric measurements were obtained with the patient standing erect. Neck circumference was measured at the level of the cricothyroid membrane. Waist circumference was measured at the midpoint between the costal margin and the iliac crest at the end of normal expiration. Hip circumference was measured at the level of the greater trochanter. The waist-to hip ratio was also calculated.

Sleep study

Full polysomnography (E-Series Compumedics, Abbotsford, Australia) included recording of oronasal flow, thoraco-abdominal movements, electrocardiogram, submental and pretibial electromyography, electrooculogram, electroencephalogram and pulse oximetry. Apnea was defined by absence of airflow lasting 10 s or longer. Hypopnoea was defined as any airflow reduction $\geq$30 or $\geq$50% lasting 10 s or longer, associated, respectively, with either oxygen desaturation $\geq$4% or arousal (Iber, 2007). The apnea–hypopnoea index (AHI) was defined as the number of apneas and hypopnoeas per hour of sleep. OSA was diagnosed if AHI was $\geq$10 events h^{-1}. Subjective daytime sleepiness, quantified by the Epworth Sleepiness Scale (ESS; Johns, 1991), was defined as an ESS score $\geq$10.

Metabolic measurements

Fasting venous blood samples were obtained in the morning after polysomnography. Glucose, triglycerides, total cholesterol and HDL-cholesterol were determined by standard enzymatic methods on a Hitachi Modular analyser (Roche Diagnostics, Indianapolis, IN, USA). In 256 patients, plasma insulin concentration was measured by chemiluminescent assays on an Immulite 2000 analyser (Siemens Medical Solutions Diagnostics, New York, NY, USA). Insulin resistance was calculated using the homeostasis model assessment (HOMA) index (Matthews *et al.*, 1985). C-reactive protein (CRP) was determined by an immunoturbidimetric method (Tina-quant CRP detection method, Roche Diagnostics).

MetS

Metabolic syndrome was diagnosed based on the presence of three or more of the following factors: waist circumference ≥80 cm in women and ≥94 cm in men (all patients were Caucasian); TG ≥ 150 mg dL^{-1}, or lipid-lowering treatment; HDL-cholesterol <40 mg dL^{-1} in men, <50 mg dL^{-1} in women, or lipid-lowering treatment; increased blood pressure (see below), or anti-hypertensive treatment; and fasting blood glucose >100 mg dL^{-1} or anti-diabetic treatment (Alberti *et al.*, 2009). The metabolic index was defined as the number of MetS components occurring in each patient.

Office blood pressure was measured by a standard mercury sphygmomanometer while the subject was quietly seated after at least 5 min of rest. Increased blood pressure was recorded if systolic blood pressure was >130 mm Hg or diastolic pressure was >85 mm Hg, or the patient was on anti-hypertensive treatment.

Analysis of VAI

This analysis was obtained after exclusion of patients with diabetes and/or using drugs to treat dyslipidaemia (n = 398 subjects, 73 women). VAI was calculated as follows (WC, waist circumference; TG and HDL-cholesterol levels were expressed in mM; Amato *et al.*, 2010):

$$\text{Maels: VAI} = \left(\frac{\text{WC}}{39.68 + (1.88 \times \text{BMI})}\right) \times \left(\frac{\text{TG}}{1.03}\right) \times \left(\frac{1.31}{\text{HDL}}\right)$$

$$\text{Females: VAI} = \left(\frac{\text{WC}}{36.58 + (1.89 \times \text{BMI})}\right) \times \left(\frac{\text{TG}}{0.81}\right) \times \left(\frac{1.52}{\text{HDL}}\right)$$

Statistics

Data are expressed as means ± standard deviation, or percentages for nominal variables. Non-normally distributed variables were analysed after logarithmic transformation. Two-way ANOVA was used to assess differences in variables according to OSA severity and gender, and their interaction. Chi-square analysis was used to analyse nominal variables (i.e. prevalence of systemic hypertension or MetS according to OSA severity). Unpaired *t*-test was used to compare data between genders for variables showing a significant interaction by two-way ANOVA, and between pre- and post-menopausal women.

Univariate relationships were assessed by simple linear regression. The distribution of AHI was normalized by square root transformation ($AHI^{0.5}$). Neck circumference was also analysed after normalization for height (NC/h). Stepwise multiple regression was used to identify variables correlated with AHI in both genders. Finally, receiving-operator curves (ROCs) were used to identify clinically useful cut-off values. Significance was at $P < 0.05$ for all tests. The statistical programs Statview 5.0.1 for Mac and SPSS version 13.0 for Windows were used for analysis.

RESULTS

The sample included 423 men and 105 women. According to OSA severity, there were 55 controls (AHI < 10 h^{-1}), 144 mild–moderate OSA (>10–<30 events h^{-1}) and 329 patients with severe OSA (AHI ≥ 30 h^{-1}). About one-third of the patients were hypertensive (28% of men, 32% of women); use of anti-hypertensive drugs was reported as follows: angiotensin-converting enzyme inhibitors (n = 83), diuretics (n = 47), calcium-antagonists (n = 3) and beta-blockers (n = 2). Current smokers accounted for 29% of the subjects. Some patients reported co-morbidities: non-insulin-dependent diabetes (n = 50); ischaemic heart disease (n = 24); chronic heart failure (n =12); chronic obstructive pulmonary disease (n = 35). As for treatment, use of insulin and/or oral anti-diabetic drugs was reported by 44 patients; 54 patients were treated for dyslipidaemia.

Anthropometric and metabolic measurements in men and women

Table 1 shows anthropometrics and sleep data for both genders. Increasing OSA severity was associated with increasing age, markers of obesity (BMI, neck, waist and hip circumference) and blood pressure. A significant interaction between OSA severity and gender was only found for hip circumference, with higher values in women compared with men in the severe OSA group ($P < 0.0001$ by unpaired *t*-test). Compared with men, women were older and showed higher BMI, lower neck and waist circumference, and lower waist to hip (W/H) ratio. Both blood pressure and prevalence of hypertension increased with OSA severity, without gender differences. Prevalence of subjective daytime sleepiness did not differ between genders or in relation to OSA severity.

The results of metabolic assessment are reported in Table 2. Fasting blood glucose, insulin and HOMA index increased significantly with OSA severity without gender differences. Total cholesterol and triglyceride levels showed significant differences for both OSA severity and gender, while HDL-cholesterol was higher in women and unaffected by OSA severity. A significant trend was observed for CRP to increase with OSA severity, with higher values in women compared with men. No significant interaction between gender and OSA severity was found for any metabolic variable tested.

The prevalence of MetS was comparable in men and women (44.4% versus 43.4%), and increased with OSA severity similarly in both genders.

Relationships between anthropometrics and AHI in men and women

Fig. 1 illustrates the univariate relationships between $AHI^{0.5}$ and anthropometric markers in men and women. Table 3

Table 1 Anthropometrics and sleep data

	Controls (M 35/F 20)	*Mild–moderate OSA (M 112/F 32)*	*Severe OSA (M 276/F 53)*	P *OSA severity*	P *Gender*	P *Gender* OSA severity*
Age (years)						
M	44.4 ± 16.0	48.6 ± 11.7	52.6 ± 12.7	**<0.0001**	**0.040**	0.895
F	48.6 ± 14.2	50.8 ± 11.2	55.9 ± 11.0			
BMI (kgm^{-2})						
M	26.4 ± 3.8	28.0 ± 4.0	32.2 ± 5.6	**<0.0001**	**0.011**	0.272
F	27.0 ± 5.8	29.7 ± 7.5	35.5 ± 8.3			
Neck circumference (cm)						
M	39.8 ± 3.2	41.3 ± 3.0	43.6 ± 3.3	**<0.0001**	**<0.0001**	0.898
F	35.0 ± 3.2	36.2 ± 2.9	38.9 ± 3.9			
Neck/height						
M	0.23 ± 0.02	0.24 ± 0.02	0.26 ± 0.02	**<0.0001**	**<0.0001**	0.611
F	0.22 ± 0.02	0.23 ± 0.02	0.25 ± 0.03			
Waist circumference (cm)						
M	97.4 ± 11.9	102.3 ± 9.8	112.3 ± 12.5	**<0.0001**	**0.005**	0.470
F	92.3 ± 15.7	95.9 ± 15.8	109.9 ± 16.3			
Hip circumference (cm)						
M	104.0 ± 7.4	107.0 ± 8.0	114.4 ± 11.3	**<0.0001**	**0.041**	**0.039**
F	104.3 ± 10.8	107.7 ± 14.7	121.4 ± 14.9			
Waist to hip ratio						
M	0.94 ± 0.08	0.96 ± 0.07	0.98 ± 0.07	**0.002**	**<0.0001**	0.491
F	0.89 ± 0.13	0.89 ± 0.06	0.90 ± 0.06			
AHI						
M	5.7 ± 2.7	21.7 ± 6.0	59.6 ± 21.3	**<0.0001**	0.820	0.533
F	4.9 ± 2.4	18.9 ± 5.1	61.4 ± 25.6			
Lowest SaO_2%						
M	89.1 ± 3.6	85.2 ± 4.7	77.4 ± 9.8	**<0.0001**	0.974	0.633
F	90.6 ± 4.4	84.9 ± 5.7	76.5 ± 9.3			
Mean SaO_2%						
M	95.1 ± 1.8	94.2 ± 1.7	90.9 ± 4.4	**<0.0001**	0.425	0.624
F	96.2 ± 1.7	93.9 ± 3.4	91.3 ± 4.0			
ESS						
M	8.9 ± 5.0	8.9 ± 5.0	10.2 ± 4.9	0.534	0.813	0.153
F	8.3 ± 5.7	10.3 ± 5.5	9.2 ± 5.4			
EDS*	24 (43.6%)	72 (50%)	178 (54.3%)	NS		
Systolic blood pressure (torr)						
M	124.5 ± 18.2	126.6 ± 17.6	135.0 ± 15.8	**<0.0001**	0.169	0.085
F	117.7 ± 12.8	121.9 ± 15.7	137.2 ± 17.0			
Diastolic blood pressure (torr)						
M	74.2 ± 10.9	78.5 ± 12.8	81.7 ± 12.8	**<0.0001**	0.099	0.064
F	68.5 ± 6.8	74.3 ± 8.8	83.2 ± 13.1			
Diagnosis of hypertension*	5 (9.1%)	24 (16.7%)	124 (37.8%)	**<0.0001**		

AHI, apnea–hypopnoea index; BMI, body mass index; EDS: excessive daytime sleepiness (defined as ESS ≥ 10); ESS, Epworth Sleepiness Score; OSA, obstructive sleep apnea; SaO_2: oxyhaemoglobin saturation.
Numerical data shown as means ± SD and analysed by two-way ANOVA; the *P*-values refer to significance of group and gender comparisons, and their interaction; Significant values shown in bold.
*Categorical variables were analysed by chi-square, and results are reported for the whole sample given the lack of significant difference between genders in the corresponding numerical variables.

summarizes univariate regression coefficients for all variables tested according to gender. In both men and women, univariate analysis showed that BMI, waist and hip circumferences, and NC/h explained between 23 and 29% of $AHI^{0.5}$ variability, with the exception of NC/h in men (19%).

Analysis of the ROC curves showed in women that hip circumference could predict severe OSA (AHI > 30 h^{-1}) with an AUC of 0.80 (Fig. 2, upper panel); a cut-off value of hip circumference in women of 107.5 cm showed a sensitivity of 86.8% and a specificity of 67.3%. In men, waist circumference predicted severe OSA with an AUC of 0.75 (Fig. 2, lower panel); a cut-off value of waist circumference in men of 104.5 cm showed a sensitivity of 73.6% and a specificity of 62.6%.

Anthropometric variables were entered in stepwise multiple regression with $AHI^{0.5}$ as dependent variable (Table 4). In men, the model including BMI, waist and hip circumferences and NC/h, explained 30.2% of $AHI^{0.5}$ variability, with BMI and waist circumference as significant variables; in women, the model explained 33.9% of the

Table 2 Metabolic data

	Controls (M 35/F 20)	*Mild–moderate OSA (M 112/F 32)*	*Severe OSA (M 276/F 53)*	P *OSA severity*	P *Gender*	P *Gender* OSA severity*
FBG (mg dL^{-1})						
M	94.1 ± 9.9	101.2 ± 21.0	108.5 ± 25.3	**<0.0001**	0.308	0.780
F	94.9 ± 21.4	104.1 ± 33.9	114.4 ± 32.6			
Insulin (mUL^{-1})†						
M (*n* = 260)	9.7 ± 6.3	11.6 ± 8.3	15.5 ± 10.6	**<0.004**	0.658	0.470
F (*n* = 62)	13.0 ± 9.0	9.7 ± 10.2	14.8 ± 12.2			
HOMA-IR†						
M (*n* = 260)	2.22 ± 1.44	3.03 ± 2.42	4.31 ± 3.34	**<0.0003**	0.658	0.583
F (*n* = 62)	2.79 ± 1.95	2.47 ± 2.24	4.15 ± 3.09			
Total cholesterol ($mgdL^{-1}$)						
M	189 ± 36	208 ± 43	201 ± 38	**<0.002**	**0.012**	0.749
F	198 ± 36	225 ± 40	211 ± 35			
HDL-cholesterol ($mgdL^{-1}$)						
M	51 ± 12	52 ± 20	51 ± 15	0.271	**<0.0001**	0.538
F	58 ± 13	66 ± 21	62 ± 23			
Triglycerides ($mgdL^{-1}$)						
M	128 ± 51	156 ± 85	163 ± 98	**0.048**	**0.049**	0.922
F	111 ± 55	128 ± 66	143 ± 64			
CRP (mgL^{-1})†						
M	1.8 ± 3.2	3.0 ± 3.7	3.9 ± 5.0	**<0.001**	**0.045**	0.456
F	3.8 ± 4.2	2.4 ± 1.7	5.6 ± 5.7			
Metabolic index						
M	1.4 ± 1.3	1.9 ± 1.3	2.6 ± 1.3	**<0.0001**	0.870	0.635
F	1.3 ± 1.3	1.8 ± 1.2	2.8 ± 1.3			
MetS diagnosis*	20.0%	32.6%	53.2%	**<0.0001**		
Visceral adiposity index†						
M (*n* = 325)	1.78 ± 1.10	1.95 ± 1.33	2.26 ± 1.72	0.154	0.698	0.696
F (*n* = 73)	1.41 ± 0.61	1.84 ± 1.26	2.11 ± 1.18			

CRP, C-reactive protein; FBG, fasting blood glucose; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment-insulin resistance; MetS, metabolic syndrome; OSA, obstructive sleep apnea.
Numerical data shown as means ± SD and analysed by two-way ANOVA; the *P*-values refer to significance of group and gender comparisons and their interaction; Significant values shown in bold.
*Categorical variables analysed by chi-square; results reported for the whole sample given the lack of significant difference between genders in the corresponding numerical variables.
†Analysis on log-transformed values.

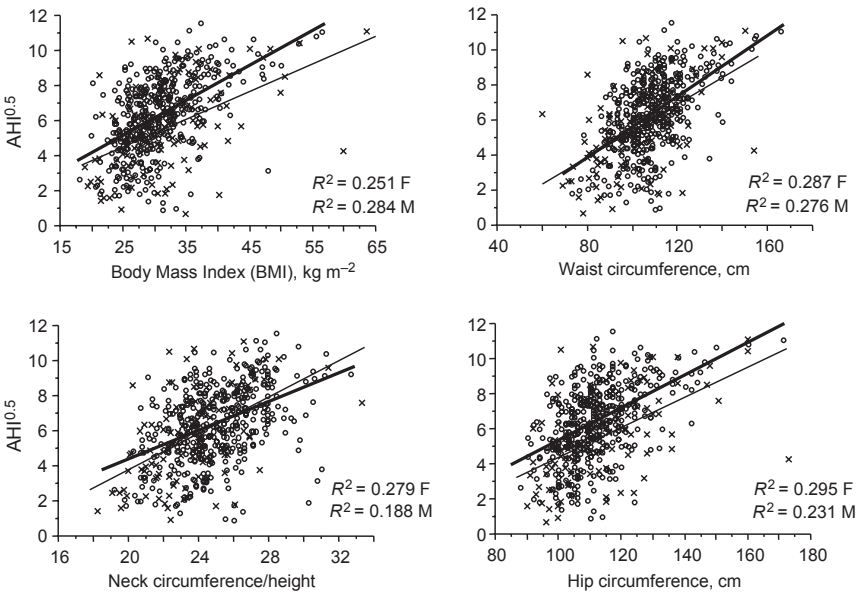

Figure 1. Relationship between anthropometric variables and $AHI^{0.5}$ in men (open circles, thick line) and women (crosses, thin line).

Table 3 Summary of univariate regression coefficients in men and women, dependent variable: $AHI^{0.5}$

	Men (n = *423)*	*Women* (n = *105)*
Age	0.158 $P < 0.005$	0.260 $P < 0.012$
BMI	0.533 $P < 0.0001$	0.501 $P < 0.0001$
Neck/height	0.433 $P < 0.0001$	0.528 $P < 0.0001$
Waist circumference	0.525 $P < 0.0001$	0.536 $P < 0.0001$
Hip circumference	0.481 $P < 0.0001$	0.543 $P < 0.0001$
Waist to hip ratio	0.232 $P < 0.0001$	NS

BMI, body mass index.

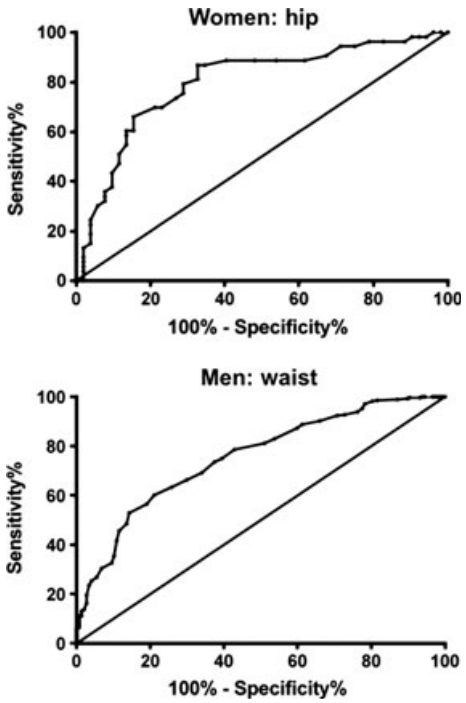

Figure 2. ROC curves for prediction of severe OSA (AHI > 30 h^{-1}). Upper panel: hip circumference in women (AUC: 0.80; 95% CI: 0.71–0.89). Lower panel: waist circumference in men (AUC: 0.75; 95% CI: 0.71–0.80).

$AHI^{0.5}$ variability, with hip circumference and NC/h as significant variables.

Analysis of anthropometrics and AHI according to MetS diagnosis

We then assessed whether a diagnosis of MetS might help predict OSA. First, adding MetS diagnosis as an independent variable to previous stepwise multiple regression slightly improved the results in men but did not affect results in women (Table 4).

Second, we analysed anthropometric variables in men and women according to the presence/absence of MetS (Table 4). In patients with a MetS diagnosis, waist circumference was the only variable significantly associated with $AHI^{0.5}$ irrespective of gender. In patients without a MetS diagnosis, BMI and waist circumference were significant in men, while hip circumference was the only significant variable in women.

Effects of menopause

Menopausal status was also analysed by taking age 50 years as cut-off for pre-menopausal (n = 69) and menopausal (n = 36) status. BMI, waist circumference or daytime sleepiness did not differ between pre-menopausal and menopausal groups, but the latter showed significantly higher AHI, NC/h, W/H ratio, triglyceride levels and metabolic index, and lower SaO_2 during sleep compared with pre-menopausal women (data not shown).

VAI

In patients without diabetes or treatment for dyslipidaemia (n = 398), VAI progressively increased with the metabolic index in both men and women (two-way ANOVA: $P < 0.0001$ for metabolic index category; $P = 0.253$ for gender; $P = 0.470$ for metabolic index–gender interaction; Fig. 3, upper panel). In addition, VAI and HOMA index were positively correlated in both genders (analysis on log-transformed values; R^2: 0.06, $P < 0.0005$ in men; R^2: 0.24, $P < 0.001$ in women). No significant differences in VAI were shown between controls and patients with mild–moderate or severe OSA (two-way ANOVA: $P = 0.205$ for OSA severity; $P = 0.725$ for gender; $P = 0.791$ for OSA severity–gender interaction; Fig. 3, lower panel).

DISCUSSION

Similar to previous studies, this study explored the relationships between OSA severity and anthropometric measurements according to gender; to the best of our knowledge, no previous study took the metabolic profile of patients with suspected OSA into account together with anthropometrics. In addition, the relationships between OSA severity and VAI, a recently proposed marker of cardiometabolic risk, were explored.

The results confirmed major gender-related differences, as in men BMI and waist circumference explained 30.2% of $AHI^{0.5}$ variability, while in women hip circumference and NC/h explained 33.9% of $AHI^{0.5}$ variability. Similar results were obtained in the subgroup of patients without MetS. Conversely, in patients with MetS, waist circumference was the only predictive marker of $AHI^{0.5}$ irrespective of gender. Severe OSA was predicted by hip circumference in women and by waist circumference in men. VAI correlated well with the metabolic index, but was a poor marker of OSA severity.

Anthropometrics and gender

Our findings confirm a close relationship between markers of visceral adiposity and OSA in men, but suggest a profoundly different relationship in women. In men, both waist circumference and W/H ratio progressively increased with OSA

Table 4 Summary of stepwise multiple regression analyses: dependent variable $AHI^{0.5}$

Independent variables	*Men*		*Women*	
	R^2 = 0.302		R^2 = 0.339	
BMI	β = 0.305 (CI: 0.058–0.174)		NS	
Waist circumference	β = 0.270 (CI: 0.019–0.069)		NS	
Hip circumference	NS		β = 0.349 (CI: 0.023–0.088)	
Neck circumference/height	NS		β = 0.306 (CI: 0.098–0.510)	
	R^2 = 0.308		R^2 = 0.339	
BMI	β = 0.291 (CI: 0.052–0.168)		NS	
Waist circumference	β = 0.238 (CI: 0.013–0.064)		NS	
Hip circumference	NS		β = 0.349 (CI: 0.023–0.088)	
Neck circumference/Height	NS		β = 0.306 (CI: 0.098–0.510)	
Metabolic Syndrome	β = 0.097 (CI: 0.024–0.804)		NS	
	MetS+	MetS−	MetS+	MetS−
	R^2 = 0.207	R^2 = 0.232	R^2 = 0.216	R^2 = 0.258
BMI	NS	β = 0.328 (CI: 0.061–0.216)	NS	NS
Waist circumference	β = 0.459 (CI: 0.056–0.101)	β = 0.186 (CI: 0.0–0.064)	β = 0.484 (CI: 0.039–0.136)	NS
Hip circumference	NS	NS	NS	β = 0.521 (CI: 0.044–0.112)
Neck circumference/Height	NS	NS	NS	NS

BMI, body mass index; CI, confidence interval; NS, not significant.

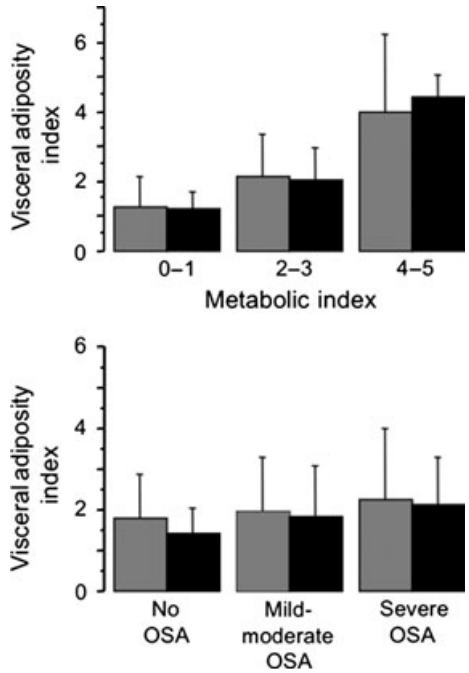

Figure 3. VAI increased with the metabolic index in patients with suspected OSA (men: grey bars; women: black bars; two-way ANOVA: $P < 0.0001$ for metabolic index category; $P = 0.253$ for gender; $P = 0.470$ for metabolic index–gender interaction; upper panel), but did not show any difference in relation to OSA severity in either gender (two-way ANOVA: $P = 0.205$ for OSA severity; $P = 0.725$ for gender; $P = 0.791$ for OSA severity–gender interaction; lower panel).

severity (Table 3). Moreover, in a multivariate model, a MetS diagnosis was a significant predictor of OSA in men. Therefore, waist circumference and BMI are the most useful anthropometric markers in clinical practice to assess the risk of having OSA in men.

In the female gender, univariate relationships between anthropometrics and $AHI^{0.5}$ were similar to those found in males (Table 3); however, only hip circumference and NC/h remained significant in multiple regression (Table 4). MetS was not a significant predictor of OSA in women. Similar gender-related differences had been reported in a large sample of Greek patients with suspected OSA (Bouloukaki *et al.*, 2011). Moreover, in a small study in women, AHI did not correlate with BMI or abdominal fat, while for each 1% increase in total body fat (assessed by dual-energy X-ray absorptiometry), the probability of OSA increased by 12.8% (Bezerra *et al.*, 2013). These data are in line with the recent report of increased visceral adipose tissue and visceral–subcutaneous fat ratio only in men with OSA (Kritikou *et al.*, 2013).

The high predictive power of hip circumference in women was a surprising finding. Other studies had reported a significant univariate relationship between OSA severity and hip circumference in both genders (Bouloukaki *et al.*, 2011; Martinez-Rivera *et al.*, 2008; Simpson *et al.*, 2010), but used W/H ratio or neck circumference in their further analyses. W/H ratio cut-off values of >1.00 and >0.85 were reported for an increased risk of OSA in obese men and women, respectively (Martinez-Rivera *et al.*, 2008). In our sample, the mean W/H ratio in women was much higher than the normal value of 0.78 ± 0.06 reported by Taylor *et al.* (1998), but did not show any progression with OSA severity. Conversely, for hip circumference, a significant interaction was shown between gender and OSA severity, with higher values in women compared with men with severe OSA. Our

data, therefore, suggest that hip circumference might be a useful marker of severe OSA in women.

Multiple regression did not confirm a high value of other anthropometric indices to predict $AHI^{0.5}$. Several studies suggested that NC/h is a valuable measurement to predict OSA (Ahbab *et al.*, 2013; Dancey *et al.*, 2003; Davies *et al.*, 1992; Sharkey *et al.*, 2010; Simpson *et al.*, 2010; Soylu *et al.*, 2012). A study in Japanese patients reported that NC/h was a marker of visceral fat in obese patients, and of OSA severity in non-obese patients, independent of gender (Kawaguchi *et al.*, 2011). Other studies, however, did not confirm a relationship between neck size and AHI in obese patients with OSA (Martinez-Rivera *et al.*, 2008), or found a positive relationship only in men (Onat *et al.*, 2009). NC/h has been proposed as an additional marker of cardiometabolic risk besides waist circumference (Onat *et al.*, 2009; Preis *et al.*, 2010). In our analysis, NC/h correlated with $AHI^{0.5}$ in univariate analysis in both genders, but remained significant in multivariate regression only in women. Increased fat deposition in the neck has been found in obese and non-obese male patients with OSA compared with controls (Mortimore *et al.*, 1998), but other studies have questioned that deposition of fat in the neck is involved in the pathogenesis of OSA (Hora *et al.*, 2007). Recent work has underlined major gender-related differences in active responses to upper airway obstruction (Chin *et al.*, 2012). Additional studies are therefore needed to assess the role of adipose tissue deposition in the neck in upper airway patency in both genders.

Anthropometrics, MetS and gender

In the regression model with anthropometrics and MetS as predictors of $AHI^{0.5}$, MetS was significantly associated with OSA severity only in men. The role of the metabolic status was then further explored by comparing patients with and without MetS. In patients with MetS, waist circumference was the only variable associated with OSA in both men and women. Instead, in patients without MetS, previous gender-related differences were confirmed, with significant risk for OSA associated with BMI and waist circumference in men, and hip circumference in women (Table 4). Therefore, in clinical practice, waist circumference should be used when patients show a clear dysmetabolic pattern indicative of visceral obesity. A different paradigm should be used in the assessment of patients without MetS, as BMI and waist circumference in men, and hip circumference in women, are the anthropometric measures most suggestive of OSA. ROC curve analysis showed that a hip circumference >107.5 cm had a sensitivity of 86.8% and a specificity of 67.3% for a diagnosis of severe OSA in women.

VAI

In patients with OSA, VAI increased with increasing metabolic abnormalities, and was positively correlated with the HOMA index, in agreement with data by Amato *et al.* (2010). Use of VAI, a continuous marker of visceral obesity, showed poor correlation with OSA severity. These results are at variance with the previously documented increase in the metabolic index with increasing OSA severity (Bonsignore *et al.*, 2012).

The different results obtained with VAI and metabolic index deserve comment. It is possible that the poor relationship between AHI and VAI is at least in part secondary to the absence of the blood pressure factor in the calculation of VAI. Our previous analysis of MetS in patients with OSA showed significant correlations between AHI or nocturnal hypoxaemia and blood pressure, but not other components of MetS after correction for age, smoking, BMI and sex (Bonsignore *et al.*, 2012). Another reason why VAI performs poorly in patients with OSA is that, unlike MetS, VAI does not take into account treatment for dyslipidaemia; however, we avoided this possible bias by excluding patients on treatment. Finally, lack of significant differences in HDL-cholesterol with increasing OSA severity may further contribute to the low sensitivity of VAI in patients with OSA.

The literature on VAI reports some controversial results on its usefulness in clinical samples with different diseases (Amato *et al.*, 2011b; Ciresi *et al.*, 2012; Petta *et al.*, 2012; Vongsuvanh *et al.*, 2012). Based on our results, MetS performs better than VAI in patients with OSA.

Strengths and limitations

The points of strength of our study are the large clinic sample studied by polysomnography, and the quite large number of women allowing to perform statistical analysis. A limitation of the study is that a consecutive series of patients with suspected OSA resulted in an unbalanced gender distribution (men to women ratio 4 : 1), reflecting the usual male predominance in referral to the sleep laboratory. The major limitation was that the amount and distribution of adipose tissue were not objectively assessed in our patients. However, our results suggest that analysis of anthropometrics may yield clinically useful information to develop gender-specific predictive equations for OSA. In addition, we did not assess the menopausal status, but the chosen age cut-off is commonly employed in clinical studies. Finally, our observation was cross-sectional and does not allow to assess whether VAI might be useful to predict cardiovascular events.

CONCLUSIONS

Our data indicate that BMI and waist circumference are the most useful anthropometric variables associated with OSA in men, whereas hip circumference seems the best marker of OSA in women. In patients with overt MetS, waist circumference was the only significant variable associated with OSA. The VAI was a good marker of MetS, but did not show any clinical utility as a marker of OSA. Overall, our results indicate complex gender–obesity–OSA interactions, which deserve further study.

AUTHOR CONTRIBUTIONS

MRB, FB and AB conceived the research project and data analysis, and participated in the final draft of the paper. Data collection was performed in the sleep laboratory, Palma de Mallorca (AB). EM, OM, AMM and AC analysed the data and prepared the manuscript, including tables and figures. SB and CE performed statistical analyses.

CONFLICT OF INTEREST

No conflicts of interest declared.

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