Universitat de Lleida

# TESIS DOCTORAL

# Medicina de Precisión: Síndrome de Apnea Obstructiva del Sueño

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Dr. Manuel Sánchez de la Torre, professor associat de la Universitat de Lleida, Dr. Ferran Barbé Illa, professor catedràtic de la Universitat de Lleida, com a directors del treball de Tesi Doctoral "Medicina de Precisión: Síndrome de Apnea Obstructiva del Sueño", realitzat per Fernando Santamaría Martos.

CERTIFIQUEM

Que en el treball presentat per optar al Grau de Doctor de la Universitat de Lleida, s´han assolit els objectius fixats a l´inici de la Tesi els quals han estat realitzats en el Departament de Medicina de la Universitat de Lleida. La memòria que es presenta constitueix un treball compacte que dóna resposta a tota una sèrie d´interrogants plantejats a l´entorn de la aplicabilitat de la medicina de precisió en el maneig de la patologia del Síndrome d´Apnea del Son.

Per tant, considerem apte aquest treball per procedir a la seva lectura i defensa davant la comissió corresponent.

Per que així consti signem la present certificació a Lleida a 26 de Setembre del 2019.

Dr. Manuel Sánchez de la Torre — Dr. Ferran Barbé Illa

*"ESTA VIDA ES UN SUEÑO Y SOÑARÉ"*

Aquel Genio

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. 67/2014 de la Junta de Gobierno de 10 de abril del 2014 de la Universidad de Lleida.

Los estudios presentados en la presente tesis pertenecen a una misma línea de investigación iniciada en 2014 y dirigida a profundizar en el conocimiento de la medicina de precisión y su aplicabilidad para el manejo clínico del síndrome de apnea obstructiva del sueño. Los resultados obtenidos han aportado información relevante en este campo y han dado lugar a tres artículos publicados en revistas internacionales y a un trabajo cuyos resultados pretenden ser publicados próximamente.

1. **Fernando Santamaria-Martos**, Iván Benítez, Andrea Zapater, Cristina Girón, Lucía Pinilla, Jose Manuel Fernández-Real, Ferran Barbé, Francisco Jose Ortega and Manuel Sánchez-de-la-Torre. "*Identification and validation of circulating miRNAs as endogenous controls in obstructive sleep apnea*". PLoS ONE 14(3): e0213622. Factor de Impacto: 2.776

2. **Fernando Santamaria-Martos**, Iván Benítez, Francisco Ortega, Andrea Zapater, Cristina Girón, Lucía Pinilla, Lydia Pascual, Anunciación Cortijo, Mireia Dalmases, Jose M. Fernandez-Real, Ferran Barbé and Manuel Sánchez-de-la-Torre. "*Circulating microRNA profile as a potential biomarker for obstructive sleep apnea diagnosis*". Scientific Reports 9, Article number: 13456 (2019). Factor de Impacto: 4.525.

3. **Fernando Santamaria-Martos**, Iván Benítez, Lucía Pinilla, Francisco Ortega, Andrea Zapater, Cristina Girón, Olga Mínguez, Silvia Gómez, Rafaela Vaca, Jose M. Fernandez-Real, Ferran Barbé and Manuel Sánchez-de-la-Torre. "*MicroRNA profile of cardiovascular risk in patients with obstructive sleep apnea*". (Submitted).

4. **Fernando Santamaria-Martos**, Ivan Benítez, Cristina Girón, Ferran Barbé, Miguel-Angel Martínez-García, Luis Hernández, Josep M. Montserrat, Eduardo Nagore, Antonio Martorell, Francisco Campos-Rodriguez, Jaime Corral, Valentin Cabriada, Jorge Abad, Olga Mediano, Maria F. Troncoso, Irene Cano-Pumarega, Ana Maria Fortuna Gutierrez, Trinidad Diaz-Cambriles, Maria Somoza-Gonzalez, Isaac Almendros, Ramon Farre, David Gozal and Manuel Sánchez-de-la-Torre, on behalf of The Spanish Sleep Network. "*Biomarkers of carcinogenesis and tumour growth in*

*patients with cutaneous melanoma and obstructive sleep apnoea*". European Respiratory Journal. 2018 Mar 15;51(3). pii: 1701885. Factor de Impacto: 12.242

En los cuatro estudios, el doctorando ha sido responsable de todos los aspectos referentes a la metodología de investigación, análisis de los resultados, interpretación de los mismos y extracción de conclusiones, así como de la redacción de los manuscritos.

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# AVREBIATURAS

SAOS: síndrome de apnea obstructiva del sueño

IAH: índice de apnea-hipoapnea

ECG: electrocardiograma

EEG: electroencefalograma

EOG: electrooculograma

EMG: electromiograma

CPAP: continuous positive airway pressure

CV: cardiovascular

HTA: hipertensión arterial

ROS: reactive oxygen species

IL: interleucina

NF-kB: necrosis factor kB

HR: hazard ratio

IDR: ratio de densidad de incidencia

MAPA: monitorización ambulatoria de la presión arterial

AP-1: proteína activadora 1

HIF-1: hypoxia inducing factor 1

VEGF: factor de crecimiento endotelial vascular

TSat90: porcentaje de tiempo con saturación de oxígeno menor del 90%

OR: odds ratio

miRNAs: microRNAs

ECs: endogenous control

qPCR: quantitative PCR

TLDA: TaqMan-Low-Density-Array

BMI/IMC: body mass index

PSG: polisomnografía

Ct: thermal cycles

AUC: area under curve

CVD: cardiovascular disease

CVE: cardiovascular event

CM: cutaneous melanoma

ICAM-1: intercellular adhesion molecule 1

VCAM-1: vascular cell adhesion molecule 1

S100B: S100 calcium-binding protein B

MIA: melanoma inhibitory activity

ODI: oxygen desaturation index

ELISA: enzyme-linked immunosorbent assay

IQR: interquartile range

# RESUMEN

El síndrome de apnea-hipopnea del sueño (SAOS) afecta a más de un 10% de la población adulta, y dicha prevalencia aumenta con la edad. El SAOS se caracteriza por episodios repetidos de obstrucción de la vía aérea superior durante el sueño, causando hipoxemia intermitente y fragmentación del sueño. El SAOS se relaciona con un deterioro de la calidad de vida, somnolencia diurna excesiva, enfermedades cardiovasculares y cerebrovasculares, y además con un exceso de mortalidad, principalmente por enfermedad cardiovascular y cáncer. En cuanto a los factores de riesgo, la edad, el sexo masculino y el índice de masa corporal son los más importantes. Cada evento apnéico finaliza con un *arousal* (despertar electroencefalográfico). Los episodios de hipoxia intermitente y los *arousals* provocan un aumento de la actividad simpática y cambios repentinos de la presión arterial, que contribuyen al desarrollo de hipertrofia miocárdica, arritmias cardíacas y muerte por causa cardiovascular. Además, dichos episodios repetidos de hipoxia-reoxigenación, y el aumento de la actividad simpática pueden activar diferentes vías patogénicas que favorecen la carcinogénesis como son: estrés oxidativo, inflamación sistémica y activación del factor inducido por la hipoxia. Estas alteraciones fisiológicas explicarían el mayor riesgo de desarrollar enfermedad cardiovascular y cáncer en pacientes con SAOS. No obstante, el SAOS parece no contribuir de igual forma en todos los pacientes en el desarrollo de enfermedad cardiovascular y de cáncer. Por otro lado, el diagnóstico de SAOS es complejo y requiere de una prueba nocturna que ha de ser evaluada por expertos. Los cuestionarios que intentan diferenciar al paciente SAOS tienen una habilidad de discriminación baja, haciendo difícil el cribado y el diagnóstico rápido del SAOS.

El objetivo de la presente tesis consiste en la evaluación de la utilidad del uso de biomarcadores en el manejo del paciente con SAOS, tanto a nivel de diagnóstico, cómo en la estratificación del riesgo cardiovascular y carcinogénico.

En el primer estudio de la presente tesis se identificó un conjunto de microRNAs (miRNAs) circulantes, estables en pacientes con y sin SAOS, y que han permitido homogeneizar los resultados y reducir la variabilidad técnica en el análisis de estos biomarcadores.

En el segundo estudio se evaluó el perfil circulante de miRNAs para el diagnóstico del paciente con SAOS y se encontró una huella molecular basada en miRNAs que permite discriminar al paciente SAOS con mayor precisión que los cuestionarios actuales, basados exclusivamente en variables clínicas y/o antropométricas. Además, se identificaron una serie de miRNAs cuya concentración en plasma era modificada con el tratamiento con presión continua positiva de la vía aérea (CPAP).

En el tercer estudio no se encontró un perfil de miRNAs capaz de distinguir grupos con diferente riesgo cardiovascular dentro de los pacientes con SAOS. No obstante, se identificaron dos miRNAs asociados con distintos parámetros de presión arterial, especialmente aquellos relacinados con la presión arterial nocturna.

Por último, en el cuarto estudio se observó que, en pacientes con melanoma cutáneo, la presencia del SAOS se asocia con un incremento en los niveles sanguíneos de la molécula VCAM-1 que podría indicar la contribución del SAOS en la tumorigénesis vía adhesión por integrinas.

En resumen, en la presente tesis se ha avanzado en el uso de la medicina de precisión en los pacientes con SAOS. Y se ha observado que, mediante el análisis de biomarcadores circulantes específicos, a partir de una muestra de sangre periférica es posible determinar; i) si el paciente tiene alta probabilidad de padecer SAOS, ii) una elevada presión arterial nocturna, y iii) un mayor riesgo de carcinogénesis y crecimiento tumoral.

# RESUM

La síndrome d'apnea-hipopnea del son (SAOS) afecta més d'un 10% de la població adulta i aquesta prevalença va augmentant amb l'edat. La SAOS es caracteritza per episodis repetits d'obstrucció de la via aèria superior durant el son i causa hipoxèmia intermitent i interrupció del son. La SAOS es relaciona amb un deteriorament de la qualitat de vida, somnolència diürna excessiva, malalties cardiovasculars i cerebrovasculars, a més de contribuir a l'augment de la mortalitat deguda, principalment, per la malaltia cardiovascular i el càncer. Pel que fa als factors de risc, l'edat, el sexe masculí i l'índex de massa corporal en són els més importants. Cada esdeveniment apneic finalitza amb un arousal (despertar electroencefalogràfic). Els episodis d'hipòxia intermitent i els arousals provoquen un augment de l'activitat simpàtica i canvis sobtats de la pressió arterial que contribueixen al desenvolupament d'hipertròfia miocàrdica, arítmies cardíaques i mort per causa cardiovascular. A més, aquests episodis repetits d'hipòxia-reoxigenació, i l'augment de l'activitat simpàtica, poden activar diferents vies patogèniques, tals com l'estrès oxidatiu, la inflamació sistèmica o l'activació del factor induït per la hipòxia, que afavoreixen la carcinogènesi. Aquestes alteracions fisiològiques explicarien el major risc de desenvolupar malaltia cardiovascular i càncer en pacients amb SAOS. No obstant això, la SAOS sembla no contribuir de la mateixa manera en tots els pacients en el desenvolupament d'aquestes dues patologies. D'altra banda, el diagnòstic de la SAOS és complex i requereix d'una prova nocturna que ha de ser avaluada per experts. Els qüestionaris que intenten diferenciar al pacient amb SAOS tenen una habilitat de discriminació baixa i en fan difícil el cribratge i el diagnòstic ràpid.

L'objectiu de la present tesi és avaluar l'ús de biomarcadors en el maneig del pacient amb SAOS, tant a nivell de diagnòstic com per estratificar-ne el risc cardiovascular i carcinogènic.

En el primer estudi de la present tesi es van identificar microRNAs (miRNAs) circulants que fossin estables en pacients amb i sense SAOS i que permetessin homogeneïtzar els resultats i reduir-ne la variabilitat tècnica.

En el segon estudi es va avaluar el perfil dels miRNAs circulants per al diagnòstic del pacient amb SAOS i es va trobar una empremta que permetia discriminar al pacient amb SAOS amb més precisió que els qüestionaris actuals. A més, es van identificar una sèrie de miRNAs que canviaven amb el tractament de pressió contínua positiva de la via aèria (CPAP).

En el tercer estudi no es va trobar un perfil de miRNAs circulants capaç de distingir grups amb diferent risc cardiovascular dins dels pacients amb SAOS. Tot i això, es van identificar dos miRNAs que estaven associats amb paràmetres de pressió arterial en els pacients amb SAOS.

Finalment, en el quart estudi, es va observar que, en pacients amb melanoma cutani, la SAOS s'associa amb un increment en els nivells sanguinis de VCAM-1 que podria indicar la contribució de la SAOS en la tumorigènesi via adhesió per integrines.

En resum, en la present tesi s'ha avançat en l'ús de la medicina de precisió en els pacients amb SAOS. S'ha observat que mitjançant l'anàlisi de biomarcadors circulants a partir d'una única mostra sanguínia, es podria avaluar de forma massica si el pacient té altes probabilitat de tenir SAOS o no, si pot tenir elevada la pressió arterial nocturna i si té un major risc de carcinogènesi i creixement tumoral. L'obtenció d'na segona mostra sanguínia permetria valorar els canvis a nivell molecular dels pacients amb el tractament.

# SUMMARY

Obstructive sleep apnea syndrome (OSA) affects more than 10% of the adult population, and this prevalence increases with age. OSA is characterized by repeated episodes of upper airway obstruction during sleep, causing intermittent hypoxemia and sleep fragmentation. OSA is related to a deterioration in life quality, excessive daytime sleepiness, cardiovascular and cerebrovascular diseases, and also with an excess of mortality, mainly due to cardiovascular disease and cancer. Regarding risk factors, age, male sex and body mass index are the most important ones. Each apneic event ends with an arousal (electroencephalographic awakening). Episodes of intermittent hypoxia and arousals cause an increase in sympathetic activity and sudden changes in blood pressure, which contribute to the development of myocardial hypertrophy, cardiac arrhythmias and cardiovascular death. In addition, these repeated episodes of hypoxia-reoxygenation, and the increase in sympathetic activity can activate different pathogenic pathways that lead to carcinogenesis, such as: oxidative stress, systemic inflammation and hypoxia-induced factor activation. These physiological alterations would explain the increased risk of developing cardiovascular disease and cancer observed in patients with OSA. However, OSA does not seem to contribute in the same way to every patient, regarding the development of cardiovascular disease and cancer. On the other hand, the diagnosis of OSA is complex and requires a night test that must be evaluated by experts. Questionnaires that attempt to differentiate OSA patients have a low discrimination ability, difficulting screening and fast diagnosis of OSA.

The objective of this thesis is to evaluate the utility of the use of biomarkers in the management of patients with OSA, both at the diagnostic level, and in the stratification of cardiovascular and carcinogenic risk.

In the first study of this thesis, a set of circulating microRNAs (miRNAs), stable in patients with and without OSA were identified, allowing the homogenization of the results and reducing the technical variability in the analysis of these biomarkers.

In the second study, the circulating miRNA profile for the diagnosis of the patient with OSA was evaluated. A molecular footprint based on miRNAs was found

allowing OSA patients to be discriminated more accurately than the current questionnaires, based exclusively on clinical and / or anthropometric variables. In addition, a set of miRNAs were identified whose plasma concentration was modified with the treatment with positive continuous airway pressure (CPAP).

In the third study, a profile of miRNAs was not able to distinguish groups with different cardiovascular risk among patients with OSA. However, two miRNAs associated with different blood pressure parameters were identified, specially those related to nocturnal blood pressure.

Finally, in the fourth study, it was observed that, in patients with cutaneous melanoma, the presence of OSA is associated with an increase in blood levels of the VCAM-1 molecule, that could indicate the contribution of OSA in tumorinegenesis via integrin adhesion.

In summary, this thesis provides a progression in the fild of precision medicine in patients with OSA. Additionally, it has been observed that, by analyzing specific circulating biomarkers, it is possible to determine from a peripheral blood sample; i) if the patient has a high probability of suffering OSA, ii) a high nocturnal blood pressure, and iii) an increased risk of carcinogenesis and tumor growth.

# 1. INTRODUCCIÓN

## 1.1. EL SÍNDROME DE LA APNEA OBSTRUCTIVA DEL SUEÑO

### 1.1.1. DEFINICIÓN Y PATOLOGÍA

El síndrome de la apnea obstructiva del sueño (SAOS) es un trastorno que deriva de la oclusión intermitente y repetitiva de la vía aérea superior durante el sueño (Figura 1). Este colapso ocurre por un tono motor inadecuado en lengua y/o de los músculos dilatadores de la vía aérea. La reducción de tamaño de la vía aérea y el incremento de la resistencia resulta en ronquidos por vibración del paladar blando, la úvula y/o las paredes laterales de la faringe [1, 2]. El colapso puede ser completo, lo que lleva a una apnea obstructiva (reducción del flujo aéreo de más del 90% asociado con movimientos respiratorios). La reducción parcial corresponde eventos a una hipopnea, que se define como la reducción de la ventilación de al menos un 30% y una desaturación de oxígeno mayor al 3% o un microdespertar (*arousal*) [3]. La gravedad de la enfermedad (Tabla 1) se define como el número de apneas e hipopneas por hora de sueño. La variable que se utiliza para medir la gravedad es el índice de apnea hipopnea (IAH), que corresponde al número medio de eventos por hora de sueño. Habitualmente, el SAOS se define con un IAH igual o mayor que 5 asociado a síntomas (sueño diurno excesivo, fatiga, alteraciones cognitivas…) o con un IAH igual o mayor de 15 independientemente de los síntomas asociados [4].

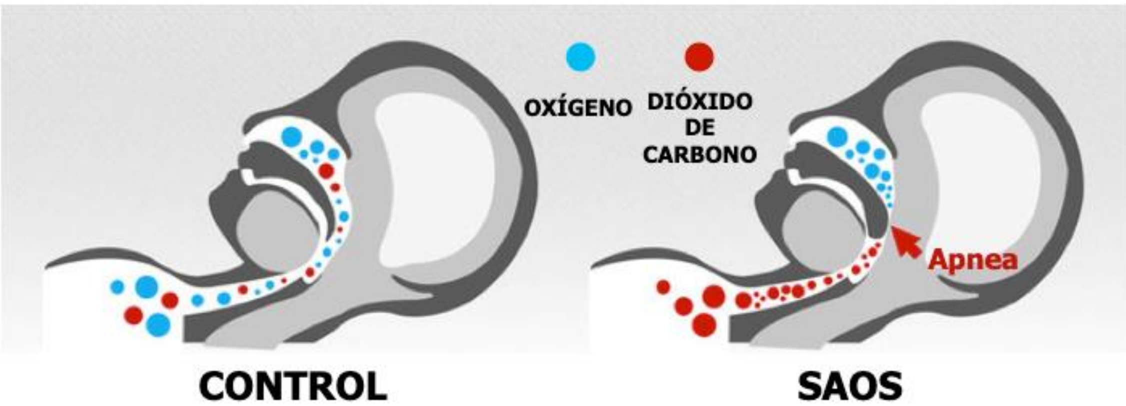


**Figura 1**: Obstrucción de la vía aérea superior en SAOS, comparativo intercambio gaseoso normal vs. apnea.

**Tabla 1**. Gravedad del SAOS en función del IAH.

| **IAH** | **Gravedad del SAOS** |
|---|---|
| IAH<5 eventos/h | No SAOS |
| 5≤IAH<15 eventos/h | SAOS leve |
| 15≤IAH<30 eventos/h | SAOS moderado |
| IAH≥30 eventos/h | SAOS grave |

Cada episodio de apnea tiene diversas consecuencias, la principal de ellas es la desaturación de oxígeno en sangre. Como respuesta a esta hipoxemia se produce un incremento progresivo de la presión intrapleural, cuyo objetivo es vencer la obstrucción faríngea. Finalmente, el restablecimiento del flujo aéreo provoca un microdespertar subconsciente y transitorio, cuya repetición durante la noche conlleva una desestructuración de la arquitectura normal del sueño. Esta fragmentación impide que el sueño alcance fases profundas y sea, por tanto, auténticamente reparador [5, 6].

El SAOS, en el momento actual, es un problema de salud pública de gran trascendencia. Por un lado, su manifestación clínica principal, la hipersomnia diurna, tiene un importante impacto familiar, laboral y social (deterioro de las relaciones personales, absentismo laboral, accidentes de tráfico, etc.). Por otra parte, es una enfermedad altamente prevalente que afecta a más del 10% de la población adulta [7, 8]. Uno de los mayores problemas es el infradiagnóstico, hasta un 93% de las mujeres y un 82% de los hombres que tienen SAOS moderado-grave no han sido diagnosticados [2].

### 1.1.2. DIAGNÓSTICO

Una historia médica detallada y la examinación clínica son muy importantes en la evaluación del paciente con sospecha de SAOS. Sin embargo, esto no es suficiente para diagnosticar la enfermedad. El sujeto con eventos respiratorios durante la noche en ausencia de síntomas clínicos no necesariamente padece SAOS. Por lo tanto, el diagnóstico de SAOS es una combinación de los síntomas clínicos y hallazgos objetivos del estudio de sueño [3, 4]. El estudio de sueño es la prueba más importante en el proceso de diagnóstico de SAOS. Existen, principalmente, dos tipos de estudio: polisomnografía completa y poligrafía respiratoria.

1) La polisomnografía convencional es una técnica en la que se analizan simultáneamente las variables neurofisiológicas y cardiorrespiratorias, y en la que la vigilancia del enfermo durante la noche es continua. Se trata de una técnica que permite evaluar la repercusión de las apneas y de las hipopneas sobre la función cardiorrespiratoria y la organización del sueño. En la polisomnografía se incluyen, como registros cardiorrespiratorios, el flujo aéreo nasobucal, los movimientos torácicos y abdominales (esfuerzo ventilatorio), la saturación transcutánea de oxígeno, el electrocardiograma (ECG) y la posición corporal. La arquitectura del sueño y su relación con las apneas, hipopneas y desaturaciones se estudia a través de los registros neurofisiológicos, en los que se integran el electroencefalograma (EEG), el electrooculograma (EOG) y el electromiograma (EMG) submentoniano. En la polisomnografía también pueden incluirse otros análisis, como el de los ruidos respiratorios (ronquido), los movimientos de las extremidades, la presión intraesofágica, que están indicados en algunas ocasiones y que pueden ser útiles en el diagnóstico diferencial de enfermos especialmente complejos.

2) La poligrafía cardiorrespiratoria es una técnica en la que se monitorizan el flujo aéreo nasobucal, los movimientos respiratorios toracoabdominales, la saturación de oxígeno, la posición corporal y el electrocardiograma. Al no registrarse las variables neurofisiológicas no es posible estudiar la arquitectura del sueño, la duración de sus distintas fases ni el tiempo durante el cual el enfermo ha estado dormido.

La polisomnografía es el método más fiable, pero también es más laborioso, complejo y caro. Por otro lado, requiere del ingreso del paciente durante una noche. Además, requiere la presencia de personal especializado tanto durante la noche, como en la interpretación de los resultados. En contraposición, la poligrafía cardiorrespiratoria es un método más sencillo, más económico, no supervisado y que el paciente tolera con mayor facilidad. Actualmente, la poligrafía se considera un método eficaz en el diagnóstico del paciente SAOS [3, 4, 9]. Este método diagnóstico debe ser, por tanto, el método elegido para el diagnóstico del SAOS en la mayoría de los pacientes con sospecha de SAOS.

Existen cuestionarios y modelos predictivos para el SAOS (entre ellos, el cuestionario Berlin, el Stop-Bang y el NoSAS), que se utilizan como screening para analizar que pacientes tienen SAOS con mayor probabilidad y descartar pacientes sin SAOS [10]. Sin embargo, hasta la fecha, no existe un cuestionario lo bastante fiable como para diagnosticar el SAOS. Asímismo, la contribución y el posible papel de biomarcadores específicos para el diagnóstico del SAOS no han sido claramente identificados.

### 1.1.3. TRATAMIENTO

Los objetivos del tratamiento son, por un lado, tener el control sobre los síntomas, especialmente la somnolencia diurna, y por otro minimizar el riesgo cardiovascular y de accidentes de tráfico y/o laborales asociados a la sintomatología asociada al SAOS. Existen diversas medidas y tratamientos para el paciente con SAOS entre las que destacaremos dos por su relevancia: medidas higiénico-dietéticas y tratamiento con presión positiva continua de la vía aérea (CPAP).

La adecuada higiene del sueño es importante. Las principales causas de somnolencia diurna excesiva son hábitos pobres de sueño, específicamente, dormir poco o dormir de forma irregular. Para una adecuada higiene del sueño hay que evitar la toma de alcohol o tabaco, de benzodiacepinas y dormir decúbito supino. La pérdida de peso en pacientes obesos puede ser suficiente para reducir el número de apneas hasta una condición no patológica. Las guías recomiendan dieta y cambios en el estilo de vida en pacientes con un índice de masa corporal (IMC)>25 $kg/m^2$.

Por otro lado, el tratamiento por excelencia del SAOS es la CPAP [11]. Este tratamiento *gold standard* para el SAOS impide el colapso inspiratorio del tracto respiratorio superior. Es un tratamiento crónico que consigue corregir los fenómenos obstructivos, el ronquido, las desaturaciones y los arousals secundarios a eventos respiratorios, la arquitectura de sueño, la capacidad de atención, entre otras variables cognitivas, reduce el riesgo de accidentes de tráfico y normaliza las cifras de tensión arterial en un porcentaje de enfermos hipertensos [12–15]. Se ha descrito que el uso de la CPAP debe ser regular y mayor a 4h/noche para que sea efectivo [12].

Mientras que la utilidad del tratamiento con CPAP para tratar los síntomas asociados al SAOS ha sido ampliamente demostrado, la utilidad de la CPAP para el tratamiento de la morbimortalidad cardiovascular asociada al SAOS aun no ha sido uniformemente demostrada.

## 1.2. ENFERMEDADES ASOCIADAS CON EL SAOS

### 1.2.1. ENFERMEDADES CARDIOVASCULARES

Las enfermedades cardiovasculares (CV) son la primera causa de mortalidad en el mundo. En España, representan el 32% del total de muertes en los varones y el 44% en las mujeres [16]. Desde hace décadas, se están incrementando los esfuerzos por identificar sus factores de riesgo, entre los cuales la hipertensión arterial (HTA) es probablemente el más importante [16, 17].

Las causas de HTA son múltiples, si bien en la mayoría de las ocasiones no se consigue descubrir su etiología a pesar de la realización de múltiples pruebas complementarias. Ello ha hecho que en los últimos tiempos se haya buscado con interés la presencia de nuevos factores etiológicos de la HTA, especialmente aquéllos modificables con el tratamiento, de entre los cuales, el SAOS ha sido considerado como uno de los más factibles [18, 19].

Las principales consecuencias del SAOS debidas al colapso de las vías respiratorias durante el sueño son los microdespertares, los cambios en la presión intratorácica y los episodios intermitentes de hipoxia-reoxigenación. Estos eventos ocurren de manera reiterativa durante el sueño e inducen la activación de vías intermedias que predisponen a aterosclerosis [5]. Tanto la investigación básica como clínica apoyan que el SAOS tiene un rol en el inicio y progresión de ciertas enfermedades cardiovasculares a través de diferentes mecanismos intermedios, que se describen a continuación (Figura 2).

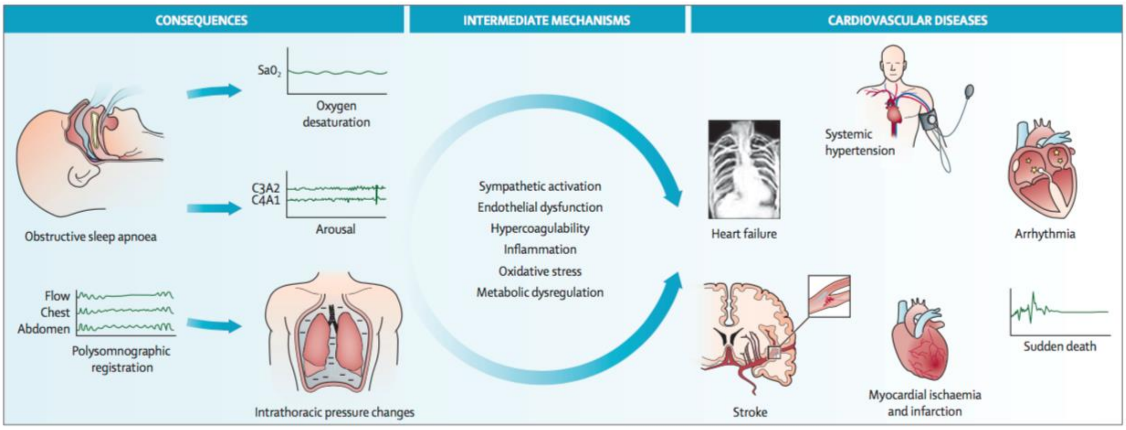


**Figura 2**. Consecuencias del SAOS y mecanismos intermedios que potencialmente contribuyen en el desarrollo de enfermedad cardiovascular (Sánchez-de-la-Torre *et. al.* Lancet Respir Med, 2012 [5]).

## MECANISMOS INTERMEDIOS

### ACTIVACIÓN SIMPÁTICA

Diversos estudios han reportado evidencias de la excitación simpática producida por el SAOS. En humanos, se ha demostrado que las apneas obstructivas inducen un incremento de los niveles de catecolaminas en suero y orina [20], así como de la actividad simpática obtenida directamente en el nervio simpático [21]. También diversos estudios aleatorizados han demostrado que el uso de CPAP disminuye los niveles. En modelos murinos, se ha demostrado que tanto la HI como la fragmentación de sueño son los principales iniciadores de la sobreactividad simpática [22]. El aumento de la actividad simpática produce vasoconstricción periférica y aumento del gasto cardiaco. Esto, a su vez, produce el incremento de la tensión arterial, lo que está relacionado con la aparición y el desarrollo de las ECV [23].

### ESTRÉS OXIDATIVO

El estrés oxidativo se produce cuando la generación de especies reactivas del oxígeno (ROS) supera la capacidad antioxidante de las células para eliminarlas. A concentraciones bajas, las ROS juegan un papel importante en la regulación de diversos procesos, mientras que a concentraciones elevadas pueden llevar a estrés oxidativo y daño celular. Esto provoca daños tisulares, envejecimiento de los tejidos, aparición de enfermedades cardiovasculares, diabetes e inflamación sistémica [5]. Se ha hipotetizado que la hipoxia intermitente es responsable del incremento en la producción ROS, ya que es un análogo de los periodos de daño reperfusión ampliamente conocidos en la producción de ROS y de daño celular. Diversos estudios en animales han demostrado que la hipóxia intermitente lleva a la producción de ROS y peroxidación lipídica y sugieren la contribución de estos procesos en ECV [24]. Sin embargo, los estudios en humanos reportan resultados contradictorios. Además, un estudio reciente relaciona la deprivación de sueño con un aumento en el estrés oxidativo [25]. Por otro lado, el efecto de la CPAP en los marcadores de estrés oxidativo es incierto y varios ensayos no han mostrado beneficio [26].

### INFLAMACIÓN SISTÉMICA

La inflamación sistémica se ha relacionado estrechamente con la formación y la progresión de la aterosclerosis. El SAOS, a través de la hipoxia intermitente ha emergido como un potente estímulo inflamatorio a nivel sistémico [5]. El desequilibrio entre los oxidantes y los antioxidantes y el incrementos de ROS se ha relacionado con el aumento de diversas moléculas proinflamatorias, como el factor de necrosis tumoral TNF-α, la interleucina 6 (IL-6) y la IL-8 [27, 28]. Por otro lado, diversos estudios en modelos animales y celulares han reportado una activación del factor nuclear kB (NF-kB), el principal regulador transcripcional de respuesta inflamatoria, inducida por la hipóxia intermitente [29, 30]. También se han encontrado aumentados los productos derivados de la activación de NF-kB en pacientes con SAOS respecto a controles. Estás moléculas circulantes reducen sus niveles con el tratamiento con CPAP [31].

### DISFUNCIÓN ENDOTELIAL

El endotelio vascular es el encargado de regular el tono muscular de las arterias y, por lo tanto, está implicado en el control de la tensión arterial. La inflamación sistémica, la activación simpática y el estrés oxidativo, que se producen en pacientes con apneas del sueño, pueden contribuir al desarrollo de disfunción endotelial. La disfunción endotelial precede y/o acelera el desarrollo de arteriosclerosis, que, a su vez, está relacionada con ECVs como la hipertensión arterial, el infarto de miocardio o el infarto cerebral. Diversos estudios muestran una evidencia indirecta de la reducción de disponibilidad del óxido nítrico y elevadas concentraciones de moléculas de adhesión, que sugieren que la inflamación y la disfunción endotelial contribuyen a las enfermedades cardiovascular en pacientes con SAOS. Además, la activación simpática y el estrés oxidativo propios del SAOS contribuirían a la disfunción endotelial [32, 33]. Todo esto activaría vías de inflamación que facilitan el reclutamiento y acumulación de células sanguíneas en los vasos endoteliales. El tratamiento efectivo con CPAP (> 4h/noche) en pacientes con SAOS revierte el daño endotelial y la inflamación, y mejora la capacidad de reparación endotelial [34].

### TRASTORNO METABÓLICO

La capacidad celular para percibir las fluctuaciones en los niveles de nutrientes es una habilidad esencial para la homeostasis celular. Se ha descrito, que el SAOS puede dar lugar a una desregulación en las vías de control de nutrientes, entre las que se encuentran el metabolismo de la glucosa y la insulina. La evidencia colectiva de los estudios transversales de las últimas dos décadas ha relacionado el SAOS con el síndrome metabólico, en concreto con la intolerancia a la glucosa, la resistencia a la insulina y la diabetes tipo 2 [35]. Aunque los factores de riesgo para el SAOS y la disfunción metabólica se superponen, existen datos observacionales que relacionan el SAOS con el metabolismo anormal de la insulina y la glucosa y el desarrollo de la diabetes tipo 2, independientemente de la obesidad [36]. Una hipótesis subyacente al impacto del SAOS en la desregulación del metabolismo es la reducción de la liberación de oxígeno a los tejidos, lo cual puede afectar directamente a la capacidad de los mismos para metabolizar glucosa, contribuyendo a una disfunción de las células β-pancreáticas y al desarrollo de resistencia a la insulina [36]. Por otro lado, el estrés oxidativo y la inflamación sistémica inducida por la hipoxia intermitente también representan mecanismos potenciales mediante los cuales el SAOS puede contribuir a esta desregulación. El efecto de la CPAP en el control del metabolismo es controvertido, existiendo resultados positivos para ciertas rutas metabólicas y negativos para otras [37–39].

### HIPERCOAGULABILIDAD

Existe una evidencia de hipercoagulabilidad en pacientes con SAOS, que contribuiría a un incremento del riesgo cardiovascular. Los pacientes con SAOS muestran un incremento de la activación plaquetaria, los niveles de los factores coaguladores, el fibrinógeno en plasma y otros marcadores que aumentan el riesgo trombótico [40]. La combinación de hipoxemia y microdespertares produce aumentos en las concentraciones de epinefrina y norepinefrina, y un elevado número de catecolaminas que causan la activación de las plaquetas [41]. Algunos estudios demuestran que el tratamiento con CPAP disminuye los niveles de algunos de los factores de coagulación [42, 43].

## EVIDENCIAS CLÍNICAS

Existen diversas evidencias clínicas que relacionan las enfermedades cardiovasculares y el SAOS. La HTA es muy prevalente en pacientes con SAOS, se estima que un 50% de los pacientes con SAOS padecen HTA. Por otro lado, uno de cada tres pacientes con HTA padece SAOS, esta relación es especialmente sólida dentro de los pacientes con HTA resistente, pacientes que no obtienen un buen control de la tensión arterial a pesar de estar tratados con 3 o más fármacos, donde un 80% padecen SAOS. Diversos estudios transversales y longitudinales han tratado de abordar esta relación, entre ellos cabe destacar el trabajo realizado por Nieto *et. al.*, un estudio transversal en el que se reclutaron 6132 sujetos de población general y se describió que las presiones sistólicas y diastólicas y la prevalencia de hipertensión incrementaba a medida que incrementaban los parámetros de gravedad del SAOS. Después de ajustar por diversas variables demográficas, esta relación se mantenía al comparar sujetos con SAOS grave respecto a pacientes sin SAOS (odds ratio (OR) 1.37 [95% IC, 1.03-1.83], p-valor de la tendencia= 0.005). Esta asociación se mantenía en los análisis estratificados [44]. Por el contrario, otro estudio longitudinal de población general de mediana edad, vio en 2148 sujetos (seguimiento 7.5 años) que los OR para incidencia de HTA no eran significativos después de ajustar por variables confusoras (p=0.051) [45].

A su vez, un número elevado de estudios han tratado de determinar el efecto de la CPAP en la tensión arterial. Un estudio prospectivo que incluyó a 1889 participantes sin hipertensión, determinó (media de seguimiento 12.2 años) que el riesgo relativo ajustado de incidencia de hipertensión era mayor en pacientes con SAOS que no podían ser tratados con CPAP (Hazard Ratio (HR), 1.33 [95% IC, 1.01-1.75]), en pacientes que declinaron el uso de CPAP (HR, 1.96 [95% IC, 1.44-2.66]), y en aquellos que no eran adherentes (HR, 1.78 [95% IC, 1.23-2.58]), respecto a pacientes sin SAOS. Mientras que el HR era menor en pacientes SAOS tratados con CPAP (HR, 0.71 [95%IC, 0.53-0.94]) [13].

Por otro lado, Barbé *et. al.* demostraron mediante un estudio aleatorizado que incluía 723 pacientes sin somnolencia diurna, que la prescripción de CPAP comparada con el cuidado habitual no reducía la incidencia de HTA o eventos cardiovasculares (Ratio de densidad de incidencia (IDR), 0.83 [95% IC, 0.63-1.1], p=0.20). Sin embargo, al realizar un análisis de subgrupos en pacientes que usaban la CPAP más de 4 horas por noche de media la CPAP se asociaba con una reducción de la incidencia de hipertensión (HR , 0.72 [95% IC, 0.52-0.98], p=0.04) [15]. Otro estudio aleatorizado incluyó 340 pacientes con nuevo diagnóstico de HTA y evaluó el efecto de la CPAP en la presión arterial respecto a CPAP placebo. El tratamiento con CPAP disminuyó la tensión arterial media de 24 h (medida por monitorización ambulatoria de la presión arterial (MAPA)) 2.1 mmHg (p=0.01) [46]. Un análisis post-hoc del mismo estudio demostró que la CPAP tenía un efecto diferencial en función del patrón circadiano de la presión arterial y solo los nondipper se beneficiaban de una bajada de la tensión arterial [47].

Sapiña-Beltrán & Santamaría-Martos *et. al.* también observaron un efecto de la CPAP en los parámetros de tensión arterial medida por MAPA en pacientes normotensos, que dependía de la presencia de hipertensión enmascarada y del patrón circadiano de la presión arterial [48]. Finalmente, un estudio evaluó el efecto de la CPAP en HTA resistente. Fue un estudio aleatorizado que incluyó 194 pacientes con HTA resistente y un IAH≥15 eventos/h. Los pacientes tratados con CPAP disminuían su TA media de 24-h 3.1 mmHg ([95% IC, 0.6-5.6], p=0.02) comparado con los no tratados [14].

Por otro lado el efecto del SAOS y del tratamiento con CPAP sobre eventos cardiovasculares mayores es controvertido [49, 50]. El SAOS es muy prevalente entre los pacientes con ictus y en pacientes con síndrome coronario agudo y se asocia con un riesgo aumentado de sufrirlos. En los últimos años varios estudios han tratado de arrojar luz sobre esta relación. En un estudio transversal con 6424 sujetos se demostró que existía un efecto moderado del SAOS en los eventos CV. Los OR entre el primer y el cuarto cuartil de IAH fueron 2.38 [95% IC, 1.22-4.62] para fallo cardíaco, 1.58 [95% IC, 1.02-2.46] para ictus y 1.27 [95% IC, 0.99-1.62] para síndrome coronario [51].

En otro estudio prospectivo realizado en hombres reclutados en unidad de sueño (377 roncadores, 403 SAOS leve-moderado no tratados, 235 SAOS grave no tratados y 376 SAOS tratados) y de población general (264 sanos) se observó que el SAOS grave incrementaba el riesgo de sufrir eventos cardiovasculares (seguimiento medio 10.1 años). El análisis multivariante mostró que el riesgo de los pacientes con SAOS grave no tratados respecto a participantes sanos era 2.87 [95% IC, 1.17-7.51 para eventos fatales y 3.17 [95% IC, 1.12-7.51] para no fatales. También se demostró que el tratamiento con CPAP reducía este riesgo [52]. En una cohorte consecutiva observacional se demostró que el SAOS incrementaba el riesgo de ictus o de muerte por cualquier causa (HR, 1.97 [95% IC, 1.12-3.48], p=0.01) [53]. Young *et. al.* observaron en la cohorte de Wisconsin (seguimiento de 18 años) que la mortalidad por causas cardiovasculares estaba aumentada en pacientes con SAOS grave respecto a pacientes sin SAOS (HR, 5.2 [IC 95%, 1.4-19.2]) [54].

A pesar de estos estudios observacionales, los ensayos aleatorizados que han intentado demostrar un efecto del tratamiento con CPAP en la prevención secundaria cardiovascular no han sido positivos. El estudio RICCADSA no observó un efecto beneficioso del tratamiento con CPAP en pacientes con síndrome coronario en cuanto a la reducción de eventos cardiovasculares a largo plazo. Se trata de un estudio aleatorizado que incluye a 244 pacientes con SAOS tratados CPAP o tratamiento conservador. Los autores observaron la incidencia con una media de seguimiento de 57 meses. La incidencia de eventos cardiovasculares no difería entre los grupos (18.1% CPAP vs. 22.1% no CPAP. HR, 0.80 [95% IC 0.46-1.41], p=0.449) [55].

El estudio SAVE constituye el estudio más importante hasta la fecha que evalúa el efecto de la CPAP en pacientes con síndrome coronario agudo. Este estudio no demostró un efecto beneficioso de la CPAP en la prevención de eventos cardiovasculares secundarios. Se aleatorizaron 2717 pacientes a CPAP o CPAP placebo y tras 3.7 años de seguimiento medio se observó un HR de 1.10 ([95% IC, 0.91-1.32], p=0.34) en los pacientes con CPAP respecto a placebo [56]. Un metaanálisis reciente concluyó que el uso de la CPAP comparado con CPAP placebo o con no tratamiento no se asociaba con un menor riesgo de eventos cardiovasculares o muerte en pacientes con SAOS [57]. Finalmente, el estudio más reciente hasta el momento (estudio ISAACC *in press*), realizado en nuestro grupo no demostró un efecto de la CPAP en la prevención secundaria de recurrencia de evento cardiovascular agudo en pacientes con SAOS, y además tampoco demostró un efecto del SAOS no tratado en la misma.

### 1.2.2. CÁNCER

A pesar de los avances médicos alcanzados durante las últimas décadas, el cáncer sigue siendo una de las mayores causas de mortalidad. Resulta extremadamente importante encontrar cuales son los factores de riesgo de esta enfermedad, especialmente aquellos que pueden ser tratados. En este contexto el SAOS podría tener un papel destacado. Tanto cáncer como SAOS son dos enfermedades que conparten vias fisiopatológicas, presentan una elevada prevalencia. Además, comparten ciertas características, como su asociación con la edad, un gran impacto sociosanitario y su potencial tratamiento [58].

#### ASPECTOS FISIOPATOLÓGICOS

Se han propuesto diversos mecanismos fisiopatológicos que podrían explicar la relación entre el SAOS y diferentes eventos tumorales (carcinogénesis, proliferación, invasión, agresividad, metástasis…). El SAOS podría estar produciendo este daño crónico a través de 3 mecanismos principales: estrés oxidativo, inflamación sistémica e hipoxia intermitente [59, 60] (Figura 3).

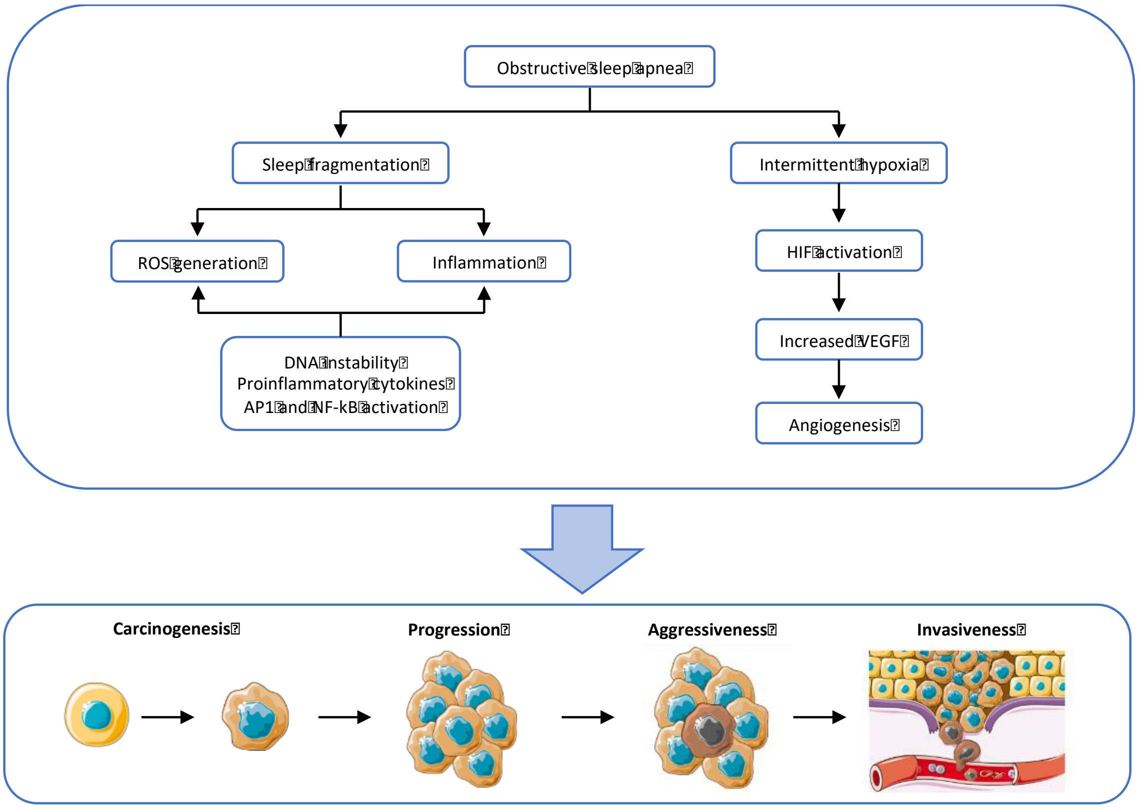


**Figura 3:** Mecanismos intermedios que relacionan el SAOS con procesos tumorales. (Adaptada de Mártinez-García *et al.* AJRCCM, 2019 [61]).

## ESTRÉS OXIDATIVO

El patrón de desaturación-reoxigenación que define la hipoxia intermitente, típico del SAOS, incrementa la producción de ROS, lo cual supone un importante incremente del estrés oxidativo. Esto lleva a un desequilibrio entre la producción de especies oxidantes y de productos antioxidantes que contrarrestarían esta oxidación. Este desequilibrio se ha relacionado con un incremento de la mutagénesis, cambios en la función y estructura celular, daño en el ADN, inestabilidad genómica que podría causar una mayor proliferación celular y transformación neoplásica [62, 63]. El estrés oxidativo también se ha relacionado con la activación de algunos factores de transcripción como la proteína activadora 1 (AP-1) y el NF-kB, que se asocian con una mayor propensión de desarrollar cáncer [64].

### FACTOR INDUCIDO POR LA HIPOXIA

Las células tienen diversos mecanismos para compensar situaciones de hipoxia. Uno de los principales es la producción del factor inducible por la hipoxia (HIF-1). HIF-1 orquesta la regulación de genes que codifican para mediadores que posibilitan la adaptación de la célula a la hipoxia. HIF-1 está compuesto de 2 subunidades: HIF-1 α y HIF-1β, de estas HIF-1α juega un papel más importante en la regulación de la hipoxia en tejido [65]. Compensa la hipoxia promoviendo una serie de mecanismos que activan la producción de moléculas angiogénicas. Entre ellas la más importante es el factor de crecimiento endotelial vascular (VEGF), que regula la formación de nuevos vasos sanguíneos colaterales que proveen de una mayor cantidad de oxígeno al área hipóxica [66, 67]. Este mecanismo compensatorio tiene beneficios a nivel cardiovascular, pero su efecto es deletéreo en pacientes con cáncer. Los tumores tienen grandes áreas hipóxicas que activan HIF-1 para compensar la falta de oxígeno. Estos sistemas compensatorios provocan una mayor neovascularización que provee a las células tumorales de un mecanismo de propagación. Por lo tanto, La hipoxia es un estímulo clave para la expresión génica de HIF-1 que activara VEGF, que juega un papel crucial en la angiogénesis tumoral, y es uno de los mayores mecanismos que favorecen la tumorigénesis y estimula la metástasis [68, 69].

### INFLAMACIÓN SISTÉMICA

El SAOS produce una inflamación tanto a nivel local como sistémico. Esta inflamación podría explicar parte de la asociación entre SAOS y cáncer. El desequilibrio entre los oxidantes y los antioxidantes y el incrementos de ROS se ha relacionado con el aumento de diversas moléculas proinflamatorias, como el factor de necrosis tumoral TNF-α, la IL-6 y la IL-8 [27, 28]. Estas moléculas activarían factores de transcripción como NF-kB y AP-1 que parecen esenciales en la transcripción de diversos genes asociados con inflamación y cáncer.

## EVIDENCIAS CLÍNICAS

Desde un punto de vista clínico, diversos estudios han observado que existe una asociación entre el SAOS (especialmente con la hipoxemia nocturna), la incidencia de cáncer y la mortalidad. A pesar de ello, la metodología retrospectiva de la mayoría de los estudios requiere un análisis más profundo antes de emitir un juicio definitivo entre la relación del SAOS y el cáncer [70, 71].

Uno de los primeros estudios poblacionales que analizaron la posible asociación de la presencia de SAOS con un mayor riesgo general de mortalidad por cáncer fue el de la cohorte de Wisconsin, que incluía a 1522 sujetos, con un seguimiento de 22 años [72]. Los autores observaron que la mortalidad se veía incrementada a medida que aumentaba la gravedad del SAOS. El SAOS grave fue un predictor independiente de la mortalidad por cáncer (HR, 4.8 [95% IC, 1.7-13.2]). Esta asociación era incluso mayor cuando se utilizaban las medidas oximétricas en vez del IAH. Cuando el porcentaje de tiempo de sueño con niveles de saturación de oxígeno menor del 90% (TSat 90%) era mayor del 11.2%, había un incremento en la mortalidad comparado con los que lo tenían <0.8% (percentil 97 y 3, respectivamente). Esta asociación era más fuerte en pacientes no obesos y no somnolientos.

El *Spanish Sleep Network* realizó uno de los primeros estudios clínicos que analizaban la incidencia de cáncer en pacientes con SAOS. Campos-Rodriguez *et al.* [73] realizaron un estudio de cohortes retrospectivo de 4910 pacientes sin historia previa de cáncer. Lo autores reportaron una mayor incidencia de cáncer cuando se medían las variables oximétricas, pero no cuando se medía el IAH. El SAOS grave, con un TSat 90% >12%, era un predictor independiente de incidencia de cáncer cuando se comparaba con TSat 90% <1.2% (HR, 2.33 [95% IC, 1.57-3.46]. Esta asociación se limitaba a hombres jóvenes, y en esta población, el IAH era también un predictor de la incidencia de cáncer, lo que sugiere que los pacientes jóvenes podrían ser más susceptibles de desarrollar cáncer en un contexto de SAOS. Contrariamente, un estudio que exploraba la asociación de los síntomas del SAOS y el riesgo de incidencia de cáncer, no encontró ninguna relación [74].

Otro estudio poblacional prospectivo [75] alcanzó conclusiones similares, que indicaban la asociación de SAOS moderado y grave con una mayor mortalidad por cáncer (HR, 3.4 [95% IC, 1.1-10.2]) e incidencia (HR, 2.5 [95% IC, 1.2-5.0]) en una cohorte de 397 sujetos.

En dos cohortes del Instituto Nacional de Salud Taiwanés, el riesgo de desarrollar cáncer del sistema central nervioso (HR, 1.54 [95% IC, 1.57-3.46]) [76] y cáncer de mama (HR, 2.09 [95% IC, 1.06-4.12]) [77] se observó mayor en pacientes SAOS que en controles no SAOS. Este estudio también encontró que cuando se dividían los pacientes en subtipos de cáncer, los pacientes SAOS presentaban un mayor riesgo de desarrollar un tumor cerebral pero no de médula espinal. Ambos estudios presentan limitaciones ya que no se incluye la gravedad del SAOS en el análisis y los *hazard ratio* no estaban ajustados por variables confusoras básicas.

Dos estudios recientes conducidos por Martinez-García *et al.* evaluaron la relación entre cáncer y SAOS. En el primero, un estudio retrospectivo de 5421 sujetos [78] mostró que existe una asociación entre la mortalidad por cáncer y el TSat 90% ($p$=0.019). Además, si se estratificaba por gravedad, para un TSat 90% >13%, no se observaba una mayor mortalidad que para un TSat 90% <1.2% (HR, 2.06 [95% IC, 1.72-4.58]). Otra vez, la asociación era más fuerte en pacientes jóvenes (menores de 65 años). El segundo estudio y quizás uno de los más importantes hasta la época [79], mostró en 443 pacientes que la gravedad del trastornos respiratorios estaba asociada con una mayor agresividad del melanoma cutáneo especialmente en pacientes jóvenes. Los pacientes con los terciles altos para IAH o índice de desaturaciones al 4% tenían mayor probabilidad de presentar un melanoma agresivo (índice Breslow >1mm) (HR, 1.94 [95% IC, 1.14-3.32]) que aquellos en el tercil inferior. Por el contrario, Kendzerska *et. al.* [80] no encontraron ninguna relación entre el IAH y la prevalencia o incidencia de cáncer en 10149 sujetos seguidos durante 7.8 años.

En los últimos años, cuatro estudios diferentes de casos-control intentaron hallar la relación entre SAOS y cáncer. En una cohorte de 68422 pacientes con cáncer apareados por edad, género y años desde el diagnóstico de cáncer con 136844 controles se mostró un mayor riesgo de desarrollar cáncer de mama (HR, 2.10 [95% IC, 1.16-3.80]), nasal (HR, 5.96 [95% IC, 2.96-11.99]), y de próstata (HR, 3.69 [95% IC, 1.98-6.89]) [81]. Lee *et. al.* [82] evaluaron 163 pacientes a los que se les realizó una polisomnografía (PSG) completa y seguidamente una colonoscopia y encontraron que el SAOS se asociaba con un riesgo incrementado de neoplasia colorrectal avanzada (OR, 14.12 [95% IC, 1.52-131.25], cuando se comparaba SAOS moderado-grave respecto a controles). Otro estudio de casos control encontró que los pacientes SAOS tenían mayor probabilidad de tener tumores de nódulo linfático negativo que pacientes sin SAOS (37.7 vs. 21.8%, p=0.004), y el diagnóstico de SAOS era un predictor independiente del estado del nódulo linfático [83]. Finalmente, Vilaseca *et. al.* [84] observaron que el SAOS se asociaba con una mayor agresividad del carcinoma de células renales de células claras (OR, 1.41 [95% IC, 1.00-1.99]) medido usando la escala de Fuhrman en 2579 pacientes con nefrectomía. En otro estudio poblacional en el que se buscaban comorbilidades urológicas, Chung *et. al.* [85] encontraron que el SAOS se asociaba con una mayor prevalencia de cáncer de próstata (OR, 2.14 [95% IC, 1.03-4.43]).

Usando una cohorte poblacional a escala nacional de aproximadamente 5.6 millones de individuos, que incluía 1.7 millones de pacientes SAOS, Gozal *et. al.* [86] mostraron que el diagnóstico, metástasis o muerte por cualquier tipo de cáncer era similar en pacientes SAOS que en controles con comorbilidades similares. Cuando focalizaban en algunos tipos específicos de cáncer, encontraron que la incidencia de cáncer de riñón (HR, 1.30 [95% IC, 1.23-1.37]) y pancreático (HR, 1.14 [95% IC, 1.06-1.23]) y melanoma (HR, 1.13 [95% IC, 1.09-1.18]) estaba aumentada. Por otro lado, el riesgo de cáncer colorrectal, de mama y de próstata parecía disminuir.

Dos metaanálisis recientes alcanzaron conclusiones contradictorias. Palamer *et. al.* [87] compararon datos de 34848 pacientes con SAOS respecto a 77380 sin SAOS y sugirieron que los pacientes con SAOS tienen cerca de un 40% más de riesgo general de cáncer (HR, 1.40 [95% IC, 1.01-1.95]). Sin embargo y a pesar del gran número de pacientes, inferir una asociación independiente no es posible en este análisis considerando el diseño retrospectivo de las cohortes y una elevada heterogeneidad entre estudios. Por el contrario, otro meta-análisis [88] con 114105 participantes que incluía seis estudios indicaba que el SAOS no estaba independientemente asociado con la incidencia y la mortalidad por cáncer.

Finalmente, uno de los estudios más recientes realizado por Campos-Rodriguez *et. al.*, observó que en 83 mujeres jóvenes con diagnóstico de cáncer de mama no existía una relación entre la gravedad del SAOS y diferentes biomarcadores de gravedad del tumor [89].

## 1.3. MEDICINA DE PRECISIÓN EN PACIENTES CON SAOS

La medicina de precisión se define como la prevención o el tratamiento aplicado, en función de las necesidades individuales de los pacientes, determinadas a partir de las características genéticas, de biomarcadores, fenotípicas o psicosociales, que distinguen a unos enfermos de otros, a pesar de tener una presentación clínica similar [90]. Este concepto proporciona una nueva dimensión en el diagnóstico y tratamiento de las enfermedades. Por un lado, ello implica nuevas y más complejas clasificaciones diagnósticas basadas en los factores anteriormente mencionados que diferencias subgrupos de pacientes dentro de una enfermedad específica. Por otro lado, implica el desarrollo de nuevos tratamientos personalizados, aplicables solo a grupos específicos de pacientes entre los que sufren la enfermedad. Estos tratamientos serían más específicos, más efectivos y menos nocivos. Por lo tanto, se evitarían indicaciones de tratamientos en pacientes no respondedores, generando una reducción significativa en los costes del sistema de salud y mejorando los resultados del paciente [58].

Dentro del SAOS existen diferentes retos que deben ser abordados en los próximos años, y en los que la medicina de precisión podría jugar un papel principal [91]:

1) Es necesario desarrollar una estrategia masiva y coste-efectiva para diagnóstico del SAOS, que permita reducir el infradiagnóstico actual y reducir las listas de espera y costes actuales.

2) El SAOS se asocia a diferentes patologías, tal y como se he comentado ampliamente, pero la heterogeneidad en la manifestación de estas enfermedades en pacientes con SAOS es muy elevada. Es imprescindible establecer herramientas que permitan estratificar el riesgo que tienen los pacientes con SAOS de desarrollar distintas patologías asociadas al SAOS, principalemente las enfermedades cardiovasculares y el cáncer.

3) Asimismo, la respuesta al tratamiento con CPAP también resulta heterogénea, existiendo perfiles de pacientes en los que la CPAP reduce el riesgo cardiovascular y pacientes que no muestran dicho beneficio. Encontrar los perfiles asociados a una

mayor respuesta el tratamiento permitirá elegir la mejor estrategia terapéutica en cada caso.

Para afrontar todos estos retos, el uso de nuevos marcadores moleculares puede ser de gran ayuda. Un biomarcador debe poder ser rápida y fácilmente medible, resistente a la degradación y debe poder ser recogido de una forma mínimamente invasiva [92]. En estas condiciones destacan los microRNAs (miRNAs) y las proteínas [93] como biomarcadores de gran utilidad. Ambos pueden ser fácilmente medibles a partir de una muestra de sangre. Los miRNAs son una clase de pequeños RNAs no codificantes que regulan negativamente la expresión génica postranscripcionalmente uniéndose al mRNA diana, y degradándolo o reprimiendo la síntesis de proteínas [94]. Juegan un papel central en diversos procesos biológicos, como la respuesta a estrés, apoptosis, proliferación y diferenciación, y diversos estudios sugieren que se encuentran desregulados en diversas patologías, incluyendo ECV, desregulaciones metabólicas y cáncer [95]. Por otro lado, los marcadores proteicos han sido ampliamente utilizados para el cribado, diagnóstico, prognosis y monitorización de la respuesta terapéutica en diversas enfermedades [93].

Existen pocos estudios que utilicen biomarcadores para establecer diferentes fenotipos en SAOS. Entre ellos destaca el trabajo realizado por Sánchez-de-la-Torre *et. al.* en el que identificaron un perfil molecular de miRNAs capaz de identificar al paciente SAOS con hipertensión resistente que se beneficiará del tratamiento con CPAP reduciendo su presión arterial [96]. Por otro lado, un estudio intentó evaluar las consecuencias del tratamiento con CPAP en el transcriptoma leucocitario. Observaron que el tratamiento con CPAP se asociaba con alteraciones en la expresión génica de leucocitos, y que estos cambios se relacionaban con vías relacionadas con el cáncer [97]. En cualquier caso estudios adicionales a estos son necesarios [98]. Finalmente, recientemente se han publicado varias revisiones que tratan de evaluar el proceso de medicina de precisión en pacientes con SAOS y otras comorbilidades (Figura 4) [58].

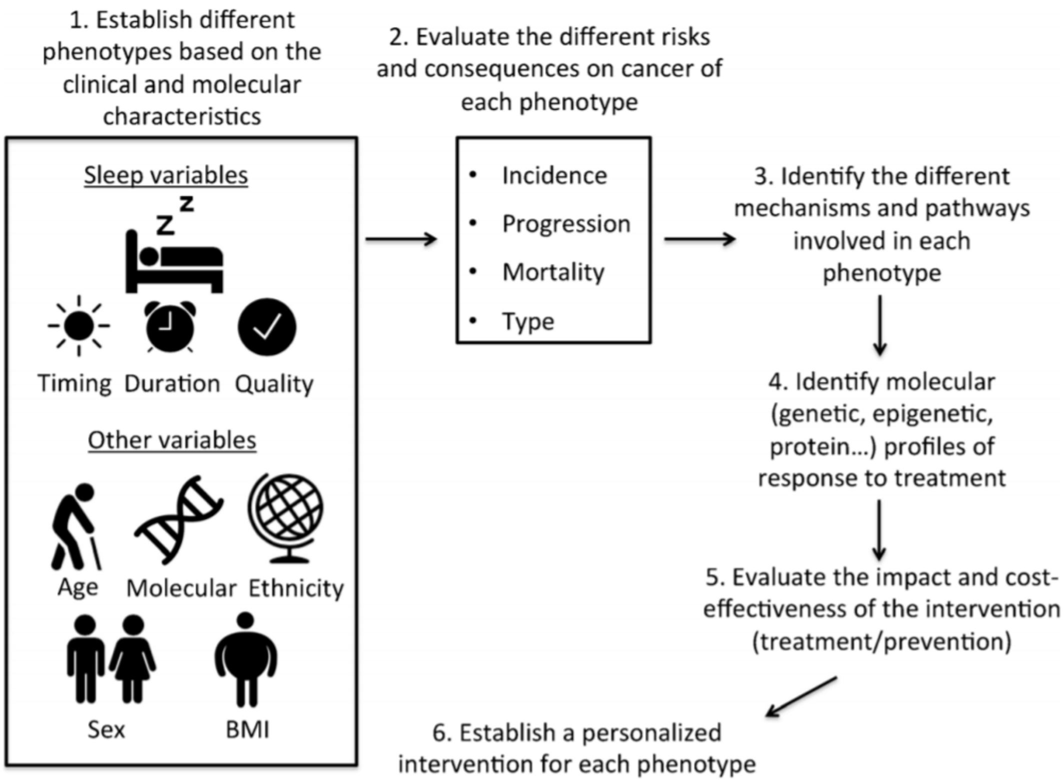


**Figure 4:** Diagrama de flujo para el uso de medicina de precisión en sueño y cáncer (Santamaria-Martos *et. al.* Curr Sleep Med Rep, 2017 [58]).

# 2. HIPÓTESIS Y OBJETIVOS

La hipótesis del presente trabajo de tesis: "El uso de biomarcadores constituye una herramienta de utilidad para el manejo del paciente con apnea obstructiva del sueño, tanto a nivel de diagnóstico, cómo para la estratificación del riesgo cardiovascular y carcinogénico de los mismos".

Para intentar dar respuesta a la hipótesis del presente trabajo, en esta tesis doctoral se han planteado los siguientes objetivos específicos:

1. Identificar miRNAs adecuados como controles endógenos en la normalización de miRNAs circulantes en pacientes con SAOS.

2. Establecer un perfil de miRNAs diferencial entre los pacientes con y sin SAOS y explorar su relevancia clínica y contribución en el diagnóstico de la enfermedad. Además, evaluar los cambios de patrones de miRNAs después del tratamiento con CPAP.

3. Establecer el perfil específico de miRNAs en fenotipos clínicos relevantes de riesgo cardiovascular en pacientes con SAOS.

4. Evaluar la relación entre biomarcadores de carcinogénesis y crecimiento tumoral y SAOS en pacientes con melanoma cutáneo.

## ESTUDIO 1

**Identificación y validación de miRNAs circulantes como controles endógenos en apnea obstructiva del sueño**

Este estudio corresponde con el siguiente artículo:

**Fernando Santamaria-Martos**, Iván Benítez, Andrea Zapater, Cristina Girón, Lucía Pinilla, Jose Manuel Fernández-Real, Ferran Barbé, Francisco Jose Ortega and Manuel Sánchez-de-la-Torre.

"*Identification and validation of circulating miRNAs as endogenous controls in obstructive sleep apnea*". PLoS ONE 14(3): e0213622.

Factor de Impacto: 2.776

### HIPÓTESIS

Existe un conjunto de miRNAs circulantes que son estables y pueden ser utilizados como controles endógenos en pacientes con SAOS.

### OBJETIVO

Identificar controles endógenos para la normalización de los miRNAs circulantes en SAOS y así estandarizar los análisis de estos biomarcadores en SAOS.

## ESTUDIO 2

**Perfil circulante de miRNAs como potencial biomarcador para el diagnóstico de apnea obstructiva del sueño**

Este estudio corresponde con el siguiente artículo:

**Fernando Santamaria-Martos**, Iván Benítez, Francisco Ortega, Andrea Zapater, Cristina Girón, Lucía Pinilla, Lydia Pascual, Anunciación Cortijo, Mireia Dalmases, Jose M. Fernandez-Real, Ferran Barbé and Manuel Sánchez-de-la-Torre.

"*Circulating microRNA profile as a potential biomarker for obstructive sleep apnea diagnosis*". Scientific Reports 9, Article number: 13456 (2019).

Factor de Impacto: 4.525

### HIPÓTESIS

Existe un conjunto de miRNAs circulantes capaz de diferenciar al pacientes con SAOS de aquel que no lo tiene. El tratamiento con CPAP altera este perfil de miRNAs.

### OBJETIVO

Examinar perfiles circulantes de miRNAs para establecer diferencias entre los pacientes con y sin SAOS y explorar su relevancia clínica y contribución en el diagnóstico de la enfermedad. Además, evaluar los cambios en los patrones de miRNAs después de 6 meses de tratamiento con CPAP.

## ESTUDIO 3

**Perfil de miRNAs de riesgo cardiovascular en pacientes con apnea obstructiva del sueño**

Este estudio corresponde con el siguiente artículo:

**Fernando Santamaria-Martos**, Iván Benítez, Lucía Pinilla, Francisco Ortega, Andrea Zapater, Cristina Girón, Olga Mínguez, Silvia Gómez, Rafaela Vaca, Jose M. Fernandez-Real, Ferran Barbé and Manuel Sánchez-de-la-Torre.

"*MicroRNA profile of cardiovascular risk in patients with obstructive sleep apnea*". (Submitted in Respirology).

Factor de Impacto:

### HIPÓTESIS

Existe un conjunto de miRNAs circulantes capaz de diferenciar los diferentes fenotipos de riesgo cardiovascular en pacientes con SAOS.

### OBJETIVO

Examinar los miRNAs circulantes para establecer el potencial perfil específico de miRNAs en los fenotipos clínicos relevantes de riesgo cardiovascular en pacientes con SAOS.

## ESTUDIO 4

**Biomarcadores de carcinogénesis y crecimiento tumoral en pacientes con melanoma cutáneo y apnea obstructiva del sueño**

Este estudio corresponde con el siguiente artículo:

**Fernando Santamaria-Martos**, Ivan Benítez, Cristina Girón, Ferran Barbé, Miguel-Angel Martínez-García, Luis Hernández, Josep M. Montserrat, Eduardo Nagore, Antonio Martorell, Francisco Campos-Rodriguez, Jaime Corral, Valentin Cabriada, Jorge Abad, Olga Mediano, Maria F. Troncoso, Irene Cano-Pumarega, Ana Maria Fortuna Gutierrez, Trinidad Diaz-Cambriles, Maria Somoza-Gonzalez, Isaac Almendros, Ramon Farre, David Gozal and Manuel Sánchez-de-la-Torre, on behalf of The Spanish Sleep Network.

"*Biomarkers of carcinogenesis and tumour growth in patients with cutaneous melanoma and obstructive sleep apnoea*". European Respiratory Journal. 2018 Mar 15;51(3). pii: 1701885.

Factor de Impacto: 12.242

### HIPÓTESIS

El SAOS altera las concentraciones circulantes de biomarcadores de carcinogénesis y crecimiento tumoral en pacientes con melanoma cutáneo.

### OBJETIVO

Evaluar la relación entre biomarcadores de carcinogénesis y crecimiento tumoral y SAOS.

# 3. ARTÍCULOS

## ARTÍCULO 1

"*Identification and validation of circulating miRNAs as endogenous controls in obstructive sleep apnea*". **Fernando Santamaria-Martos**, Iván Benítez, Andrea Zapater, Cristina Girón, Lucía Pinilla, Jose Manuel Fernández-Real, Ferran Barbé, Francisco Jose Ortega and Manuel Sánchez-de-la-Torre. PLoS ONE 14(3): e0213622.

RESEARCH ARTICLE

# Identification and validation of circulating miRNAs as endogenous controls in obstructive sleep apnea

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## Abstract

microRNAs (miRNAs) are non-coding RNAs highly relevant as biomarkers for disease. A seminal study that explored the role of miRNAs in obstructive sleep apnea syndrome (OSA) demonstrated their usefulness in clinical management. Nevertheless, the miRNAs that may act as endogenous controls (ECs) have not yet been established. The identification of ECs would contribute to the standardization of these biomarkers in OSA. The objective of the study is to identify miRNAs that can be used as ECs in OSA. We evaluated 100 patients divided into two different cohorts: a learning cohort of 10 non-OSA and 30 OSA patients, and a validation cohort (20 non-OSA and 40 OSA patients). In the learning cohort, a profile of 188 miRNAs was determined in plasma by TaqMan Low Density Array. The best EC candidates were identified by mean center+SD normalization and concordance correlation restricted normalization. The results were validated using NormFinder and geNorm to assess the stability of those ECs. Eight miRNAs were identified as EC candidates. The combination miRNA-106a/miRNA-186 was identified as the most stable among all candidates. We identified a set of ECs to be used in the determination of circulating miRNA in OSA that may contribute to the homogeneity of results.

## Introduction

Obstructive sleep apnea (OSA) is a prevalent disease that affects approximately 10% of adults [1–3]. It is caused by intermittent collapse of the upper airway during sleep, which leads to oxygen desaturation, arousals and intrathoracic pressure changes. OSA is associated with hypertension, cardiovascular and cerebrovascular diseases, diurnal somnolence and decreased quality of life [4].

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**Competing interests:** The authors have declared that no competing interests exist.

Understanding the causes and consequences of OSA and identifying actions to perform personalized medicine are research priorities in the field [5,6]. One of the greatest challenges is identifying accurate and feasible biomarkers useful in diagnosis, prognosis and response to treatment. This challenge has stimulated an interest in novel biomarkers to improve OSA diagnosis and comprehensive management [7], through which micro-ribonucleic acids (miRNAs) have emerged as a new opportunity [8].

microRNAs (miRNAs) are small non-coding RNA molecules that represent an important class of regulatory epigenetic mechanisms [9]. miRNAs influence important cellular functions, such as development and differentiation, and play a pivotal role in many biological processes related to health and disease [10–12]. miRNAs can be rapidly released from tissue into circulation in several pathologies and exist in a high-stability, cell-free form in plasma [13]. Therefore, circulating miRNAs have great potential as non-invasive biomarkers for molecular diagnostics and prognostics and as therapeutic targets [14].

The role of miRNAs in OSA has been little studied. A seminal study demonstrated the utility of these molecules as biomarkers of adequate response to the treatment of OSA with continuous positive airway pressure (CPAP) [8]. However, prior to any analysis of circulating miRNAs, normalization is crucial due to the variability that occurs in plasma RNA isolation and analysis [14,15].

Normalization is a process aimed at differentiating biological variation from experimental artefacts. Normalization reduces the variability due to technical error and contributes to standardization and homogeneity in the analysis of miRNAs [16]. Currently, mean-center normalization is the most accurate method to normalize the results when a high number of miRNAs are being analysed [17]. However, a small number of miRNAs are often analysed, in which case mean-center normalization is not a valid method; instead, endogenous control (EC) normalization is the best option [14,17]. Therefore, suitable ECs (i.e., those that are disease- and tissue-specific, highly detected in all samples, and stable both within and between groups) are needed. The use of unsuitable normalizers can lead to misleading results [18–20].

To the best of our knowledge, there is no current consensus on EC miRNAs for quantitative polymerase chain reaction (qPCR) analysis of plasma miRNAs in OSA. Therefore, the aim of this study was to identify suitable ECs for normalization of circulating miRNAs in OSA studies with the objective of standardizing the analysis of these biomarkers in OSA.

## Methods

### Study cohort and sample collection

One hundred patients aged between 18 and 60 years and referred because of suspected OSA were enrolled at the Sleep Unit of Santa Maria Hospital (Lleida, Spain). OSA was diagnosed via a conventional polysomnographic sleep study. The results from all sleep studies were analysed by trained personnel, using standard criteria. Apnea was defined as an interruption or reduction of oronasal airflow $\geq$ 90% for at least 10 seconds. Hypopnea was defined as a 30–90% reduction in oronasal airflow for at least 10 seconds associated with an oxygen desaturation of at least 3% or an arousal on the electroencephalogram. The apnea-hypopnea index (AHI) was calculated based on the average number of apnea plus hypopnea episodes per hour of sleep. The cohort was split into two cohorts: i) TaqMan Low Density Array (TLDA) cohort: an initial sample of 40 male patients (10 patients without OSA and 30 patients with OSA) matched by age and body mass index (BMI). A general miRNA profile was performed. ii) Validation cohort: the remaining 60 patients (20 patients without OSA and 40 with OSA) were used to validate the candidate ECs (Fig 1). The initial exclusion criteria discarded patients with previous use of CPAP and any condition that, in the opinion of the responsible physician

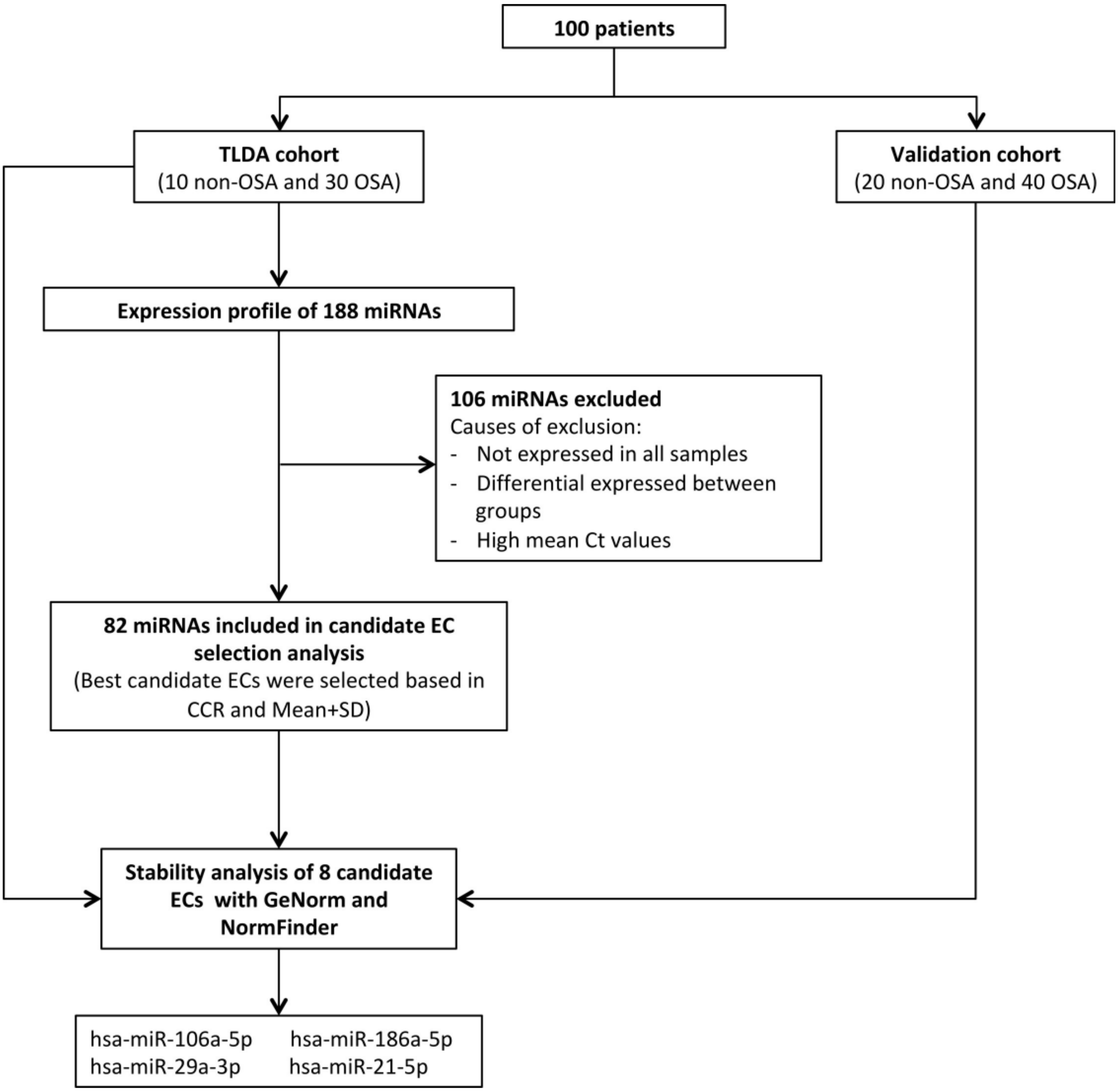


**Fig 1. Flowchart of the study.** Patients who were referred because of suspected OSA were divided into the TLDA cohort (general profile of miRNAs) and the validation cohort and further divided for study on the basis of non-OSA and OSA. Ten non-OSA and 30 OSA patients were used as the TLDA cohort. miRNAs that do not follow the characteristics of a good EC were excluded. Two different strategies (mean-center+SD and CCR) were used in order to select candidate ECs. Stability analysis by GeNorm and NorFinder was performed in the TLDA cohort and in an independent validation cohort (20 non-OSA and 40 OSA patients).

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investigator, made the person unsuitable for the study (e.g., pregnancy, drug and alcohol consumption or less than one year of life expectancy). TLDA cohort patients were selected who had no other comorbidities apart from those typically associated with OSA (e.g., hypertension, dyslipidaemia, cardiovascular events). All recruited patients signed an informed consent form in accordance with the Helsinki Declaration of 1964 and the ethics committee of the centre (Clinical Research Ethics Committee (CEIC) of the Arnau de Vilanova University Hospital) approved the study. All methods were performed in accordance with current clinical practice guidelines and regulations. A venous fasting blood sample was obtained from each patient between 08.00 and 09.00 a.m. Plasma was obtained by standard venepuncture and centrifugation in EDTA-coated tubes (Vacuette, Greiner Bio-One, Kremsmünster, Austria). Plasma was separated via centrifugation at 1500 g for 10 min at 4˚C. All specimens were immediately aliquoted, frozen, and stored in a dedicated -80˚C freezer. No freeze-thaw cycles were performed during the experiment.

## Circulating RNA extraction and purification

RNA extraction was performed from 300 μL of plasma using a mirVana PARIS isolation kit (Applied Biosystems, Vilnius, Lithuania) according to the manufacturer's instructions. Non-human cel-miR-39 was spiked into the plasma immediately before extraction. RNA concentration and integrity were examined by RT-qPCR quantification of cel-miR-39. RNU6 was selected as the plasma quality indicator because it is highly expressed in different cell types but not well detected in plasma. High detection of RNU6 in plasma indicates cellular contamination [19].

## TaqMan low density array

A general profile with 188 miRNAs reported in previous experiments as the most prevalent in plasma were selected for profiling [21–23]. qRT-PCR was performed on the RNA of 40 patients using TaqMan Low Density Array (TLDA) human microRNA custom TLDAs (Life Technologies, Foster City, CA, USA). Briefly, a fixed volume of 3 μL of RNA solution from the 40 μL of RNA isolation eluate was used as the input for retrotranscription using a TaqMan MicroRNA Reverse Transcription Kit and the TaqMan MicroRNA Multiplex RT Assays, which are customised to run the TLDAs. Preamplification was performed using TaqMan PreAmp Master Mix and Megaplex PreAmp Primers for our custom selection. RT-PCR was carried out by means of an Applied Biosystems 7900HT thermocycler.

## Candidate EC selection

ECs are miRNAs that are highly represented in all study samples and show similar abundances between groups. Two different approaches were used to choose the best candidates in OSA: i) the miRNA with the values most similar to the global mean expression [24], which was determined by normalizing the data set with the global mean to select the miRNA with the smallest standard deviation (mean-center+SD); and ii) concordance correlation restricted (CCR) normalization procedures, which use a concordance correlation coefficient to select miRNAs that are concordant with the global mean of the fully detected miRNAs [17]. Numerous strategies have been proposed to select the best EC from miRNA arrays [25]. The latest proposals indicate that the similarity between the values of an EC and the global mean (gold standard) is one of the best approximations. Based on these studies, we decided to select the five miRNAs with the lowest variability after normalization by the global mean [23] (mean center+SD) and the five with the highest concordance correlation coefficient of agreement with the global mean of the miRNAs detected in all samples [16] (CCR) as candidates for EC.

### RT-PCR validation

Two-step validation was performed. First, the miRNAs selected as candidate ECs were validated internally to check the variability of determination when changing from an RT-PCR miRNA array to individual RT-PCR. Then, external validation of the EC candidates was performed with an external cohort using individual miRNA TaqMan hydrolysis probes.

### Candidate EC stability analysis

To determine the miRNAs with the best properties to be ECs, we estimated the stability of the candidate ECs. Stability of each candidate EC was performed using the geNorm [26] and NormFinder [27] algorithms. The ranking resulting from each method and its concordance were studied. Furthermore, we evaluated the best combination with the NormFinder algorithm based on the pair that reduced variability the most.

All the analyses were performed using R-project version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria).

## Results

### Patient characteristics

The 100 patients were middle-aged (less than 60 years old), overweight-obese and mainly males. The clinical and demographic characteristics of the patients are shown in Table 1.

### Selection of the most suitable EC candidates by RT-PCR miRNA array

Among the general profiles, 8 candidate ECs were selected based on the two different methods (Table 2). The eight EC candidates exhibited the desirable characteristics of a good normalizer in that they were highly detected in all samples and were not significantly different between the OSA and non-OSA patients (Fig 2).

To identify the most stable candidate ECs, the GeNorm and NormFinder algorithms were applied. Both methods reported miR-106a and miR-29a as the most stable. In contrast, miR-27a and miR-145 were the most variable (Table 3). Moreover, NormFinder identified miR-106a and miR-186 as the combination that best reduced the variability among profiles. To verify the ECs' stability in other OSA classifications, patients were also divided into non-OSA, moderate OSA and severe OSA groups. A similar analysis was performed, and miR-106a/miR-186 remained the most stable pair, followed by miR-29a and miR21 (data not shown).

Different strategies of normalization (mean-center, exogenous control and EC selection) were evaluated by comparing their reduction of variability (Fig 3). The variability reduction obtained with mean center (gold standard) was also achieved with EC selection. Exogenous control showed less capacity to reduce variability. A cumulative distribution plot of the coefficient of variation of all miRNA Ct values was examined before and after normalization, confirming the suitability of the ECs as normalizers.

### Internal validation by individual probes

The variability between miRNA arrays and individual probes was evaluated by internal validation in the TLDA cohort. Then, the GeNorm and NormFinder algorithms were used to rank the EC candidates. Similar results were obtained from the TLDA and individual probes (data not shown).

**Table 1. Baseline characteristics of the patients.**

| | | TLDA cohort | | | Validation cohort | | |
|---|---|---|---|---|---|---|---|
| | | Non-OSA (AHI<15 events/h) | OSA (AHI≥15 events/h) | p-value | Non-OSA (AHI<15 events/h) | OSA (AHI≥15 events/h) | p-value |
| | | N = 10 | N = 30 | | N = 20 | N = 40 | |
| Gender: Male n (%) | | 10 (100%) | 30 (100%) | | 12 (60.0%) | 30 (75.0%) | 0.37 |
| Age (years), median [IQR] | | 48.5 [41.8;55.5] | 52.0 [44.2;55.8] | 0.743 | 46.0 [41.5;50.2] | 51.0 [43.8;55.2] | 0.033 |
| BMI (kg/m$^2$), median [IQR] | | 26.1 [23.3;26.9] | 27.1 [25.9;30.0] | 0.089 | 29.8 [26.2;36.3] | 33.1 [28.7;36.0] | 0.279 |
| Hip perimeter (cm), mean (SD) | | 94.6 (10.8) | 101 (8.44) | 0.104 | 106 (15.5) | 109 (13.1) | 0.391 |
| Waist perimeter (cm), median [IQR] | | 102 [98.0;104] | 104 [100;108] | 0.301 | 111 [102;118] | 111 [106;118] | 0.849 |
| Physical activity n (%) | | | | 0.385 | | | 0.356 |
| | Sedentary | 4 (40%) | 15 (50%) | | 9 (45.0%) | 17 (43.6%) | |
| | Moderate | 3 (30%) | 12 (40%) | | 6 (30.0%) | 17 (43.6%) | |
| | Active | 3 (30%) | 3 (10%) | | 5 (25.0%) | 5 (12.8%) | |
| AHI (events/h), median [IQR] | | 8.45 [6.18;10.8] | 32.3 [27.7;49.6] | <0.001 | 7.52 [4.99;9.01] | 38.9 [24.8;70.0] | <0.001 |
| TSat90 (%), median [IQR] | | 0.08 [0.00;0.14] | 2.34 [0.31;6.90] | <0.001 | 0.12 [0.00;0.32] | 4.23 [1.73;18.8] | <0.001 |
| TSat90 (%), median [IQR] | | 0.08 [0.00;0.14] | 2.34 [0.31;6.90] | <0.001 | 0.12 [0.00;0.32] | 4.23 [1.73;18.8] | <0.001 |

IQR: interquartile range; SD: standard deviation; BMI: body mass index; AHI: apnea-hypopnea index (number of events·h-1); TSat90: percentage of time spent with oxygen saturation less than 90%.

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## Confirmation of ECs by individual probes in an external cohort

Low Ct values of EC candidates were observed in all patients. Furthermore, when comparing differences between groups, none of the miRNAs showed significant differences. The GeNorm and NormFinder algorithms reported miR-186, miR-106a, miR-21 and miR-29a as the miRNAs with the highest stability (see Table 3 and Fig 4). In contrast, miR-145, miR-103 and miR-27a showed the lowest stability (see Table 3 and Fig 4). Both methods showed high concordance with respect to their stability values. The results showed that miR-186 is the best EC, followed by miR-106a, miR-21 and miR-29, which have similar values of stability for both methods (see Fig 4).

## Discussion

miRNAs are new, reliable, non-invasive biomarkers. Because they play a major role in the development and prognosis of different diseases, their use could go beyond being a simple biomarker. Their utility has been demonstrated in the management of several diseases [28] as well as in the personalized management of OSA [8]. Plasma miRNAs could help in the identification of new OSA phenotypes for personalized management. In the present study, we identified a set of miRNAs that act as ECs for the use of these biomarkers in sleep apnea.

The use of circulating miRNAs represents an advance in the field of minimally invasive biomarkers [29]. Due to their simple and rapid quantification, hundreds of miRNAs can be

**Table 2. Candidate ECs.**

| MiRNA Name | Molecule type | Accesion Number* | Mature Sequence | Selection method |
|---|---|---|---|---|
| **hsa-miR-103a-3p** | miRNA | MIMAT0000101 | AGCAGCAUUGUACAGGGCUAUGA | CCR and MC+SD |
| **hsa-miR-106a-5p** | miRNA | MIMAT0000103 | AAAAGUGCUUACAGUGCAGGUAG | MC+SD |
| **hsa-miR-140-3p** | miRNA | MIMAT0004597 | UACCACAGGGUAGAACCACGG | CCR |
| **hsa-miR-145-5p** | miRNA | MIMAT0000437 | GUCCAGUUUUCCCAGGAAUCCCU | CCR |
| **hsa-miR-186-5p** | miRNA | MIMAT0000456 | CAAAGAAUUCUCCUUUUGGGCU | CCR and MC+SD |
| **hsa-miR-21-5p** | miRNA | MIMAT0000076 | UAGCUUAUCAGACUGAUGUUGA | MC+SD |
| **hsa-miR-27a-3p** | miRNA | MIMAT0000084 | UUCACAGUGGCUAAGUUCCGC | CRR |
| **hsa-miR-29a-3p** | miRNA | MIMAT0000086 | UAGCACCAUCUGAAAUCGGUUA | MC+SD |
| **cel-miR-39-3p** | miRNA | MIMAT0000010 | UCACCGGGUGUAAAUCAGCUUG | Spike-in control |
| **RNU6** | snoRNA | NR_004394** | GTGCTCGCTTCGGCAGCACATATACT AAAATTGGAACGATACAGAGAAGATT AGCATGGCCCCTGCGCAAGGATGACA CGCAAATTCGTGAAGCGTTCCATATTTT | Plasma quality indicator |

*mirBase database accession number

**NCBI Gene ID

https://doi.org/10.1371/journal.pone.0213622.t002

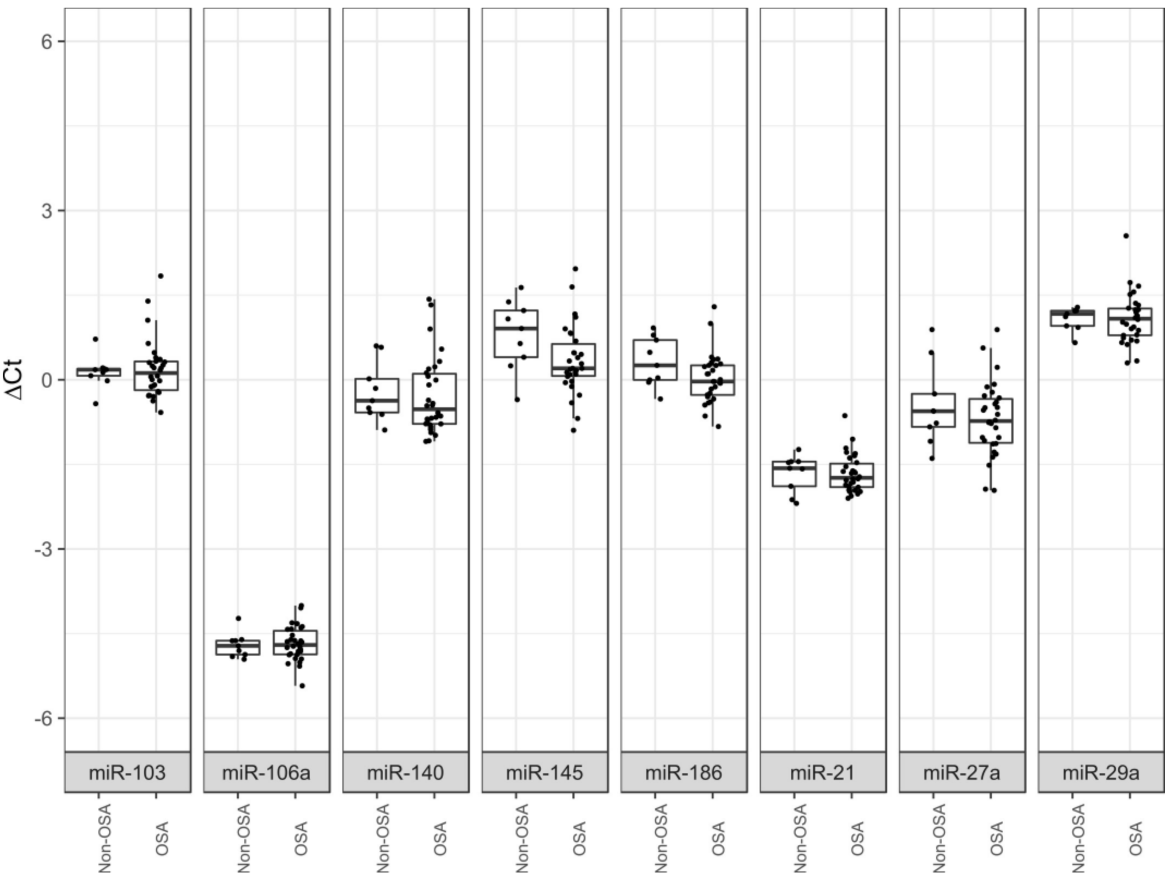


**Fig 2. Boxplot comparing OSA and control Ct values.** The eight miRNAs were highly detected, and non-significant differences were found between groups.

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evaluated simultaneously in several patients, making them one of the most important tools in precision medicine [30].

There are several methods for quantifying miRNAs [31]. Among them, RT-PCR is currently the most commonly available and reliable method. In a couple of hours, this technique allows the levels of miRNAs to be evaluated to perform relative quantification analysis [31,32]. The main problem that currently exists in the relative quantification of miRNAs is technical variability. Currently, there is not a universal method that eliminates such variability. Different techniques have been used, such as spike-in normalization, gene normalization, and small nucleolar RNA normalization, leading to non-replicable results [14,33]. The gold standard—normalization—is based on mean-center methods and is very useful when high-throughput miRNA approximations are being analysed, but it is not an option for the analysis of a few miRNAs. In such cases, the use of ECs is the best option [17,24]. ECs are specific and should be identified for each disease and tissue. The identification and validation of specific suitable ECs is perhaps the most critical step in the analysis of miRNAs to avoid inaccurate interpretation of the data that may lead to biased results. An ideal EC gene will be detected at a constant level across all samples, exhibit relatively stable levels between samples and groups and have no

**Table 3. Stability ranking of ECs.**

| | TaqMan Low Density Array | | | | External validation | | | |
|---|---|---|---|---|---|---|---|---|
| | GeNorm | | NormFinder | | GeNorm | | NormFinder | |
| | Ranking | Stability value* | Ranking | Stability value | Ranking | Stability value | Ranking | Stability value |
| 1 | miR-106a | 0.500 | miR-106a | 0.108 | miR-186 | 0.656 | miR-186 | 0.051 |
| 2 | miR-29a | 0.590 | miR-29a | 0.121 | miR-106a | 0.686 | miR-21 | 0.071 |
| 3 | miR-103 | 0.600 | miR-186 | 0.126 | miR-21 | 0.695 | miR-106a | 0.072 |
| 4 | miR-186 | 0.602 | miR-21 | 0.127 | miR-29a | 0.716 | miR-29a | 0.080 |
| 5 | miR-21 | 0.611 | miR-140 | 0.139 | miR-140 | 0.757 | miR-140 | 0.107 |
| 6 | miR-140 | 0.704 | miR-103 | 0.144 | miR-27a | 0.782 | miR-27a | 0.109 |
| 7 | miR-27a | 0.734 | miR-145 | 0.180 | miR-103 | 0.882 | miR-103 | 0.137 |
| 8 | miR-145 | 0.751 | miR-27a | 0.187 | miR-145 | 1.078 | miR-145 | 0.168 |
| Best combination | | | miR-106a and miR-186 | | | | miR-106a and miR-186 | |

*Stability values are not comparable between methods.

https://doi.org/10.1371/journal.pone.0213622.t003

known association with the disease under investigation [16,34]. To the best of our knowledge, this is the first study that aims to identify and validate suitable ECs in OSA.

In the present study, we performed different bioinformatics analyses to determine the most reliable ECs among OSA patients. We evaluated 188 miRNAs and selected eight EC candidates (miR-106a, miR-186, miR-29a, miR-21, miR-103, miR-27a, miR-140 and miR-145), one spike-in synthetic miRNA (cel-miR-39) and one commonly use normalizer (RNU6). Although RNU6 and cel-miR-39 are commonly used in the normalization of miRNAs [14], we used them only as quality indicators. RNU6 is not highly detected in plasma; low Ct values are obtained when haemolyses occurs. Cel-miR-39 has been used only to assess the efficiency of the process, which requires repeating all samples with low efficiency.

The lack of a comprehensive analysis of normalizers for miRNAs in OSA patients could compromise miRNA results. For this reason, we performed miRNA array screening including 188 miRNAs as potential ECs. The data were analysed using mean-center normalization methods to identify the best candidates to serve as ECs. The best normalization strategy for miRNA analysis in OSA patients was identified as the combined use of miR-186 and miR-106a to normalize RT-PCR results. The combined use of 2 or more miRNAs leads to more reliable results than using either one alone. miR-29a and miR-21 are also suitable as ECs for miRNA analysis in OSA.

Different studies previously used miR-186 [35], miR-106a [14,36,37], miR-29a [38] and miR-21 [39] as ECs in other diseases.

The present study has several limitations that deserve comment. First, only patients between 18 and 60 years old have been studied, and larger studies should be performed to determine the validity of these ECs in patients of other ages. Although a large quantity (188) of miRNAs have been studied, not all of them have been analysed; it is possible that other miRNAs would be better ECs, but this does not invalidate the results, because the stability (when compared with other diseases) is sufficiently high. No other comorbidities apart from those typically associated with OSA (e.g., cardiovascular disease, diabetes, and obesity) have been included in the analysis. However, the use of TLDA did reduce the technical variability of the process, in turn providing reliable results. Despite a limited number of non-OSA patients

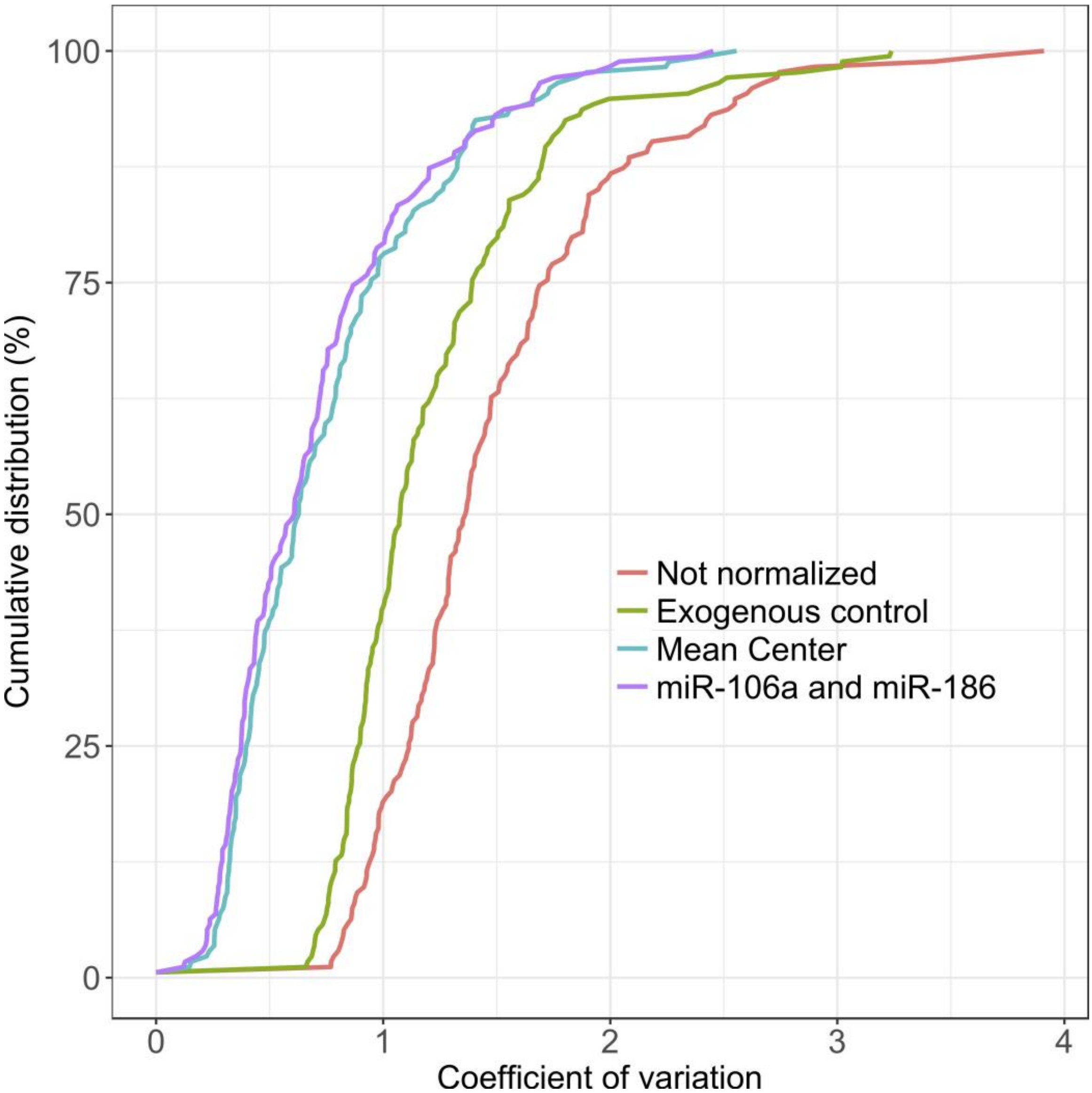


**Fig 3. Cumulative distribution plot of the different normalization strategies.**

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being included in the learning phase (10 non-OSA subjects in the TLDA cohort), the results were validated in a large patient cohort, and the stability values were assessed using two different methods.

## Conclusions

The present study represents the first step in the standardization of the analysis of miRNAs as biomarkers in OSA. In the era of precision medicine, miRNA analysis could open a new field

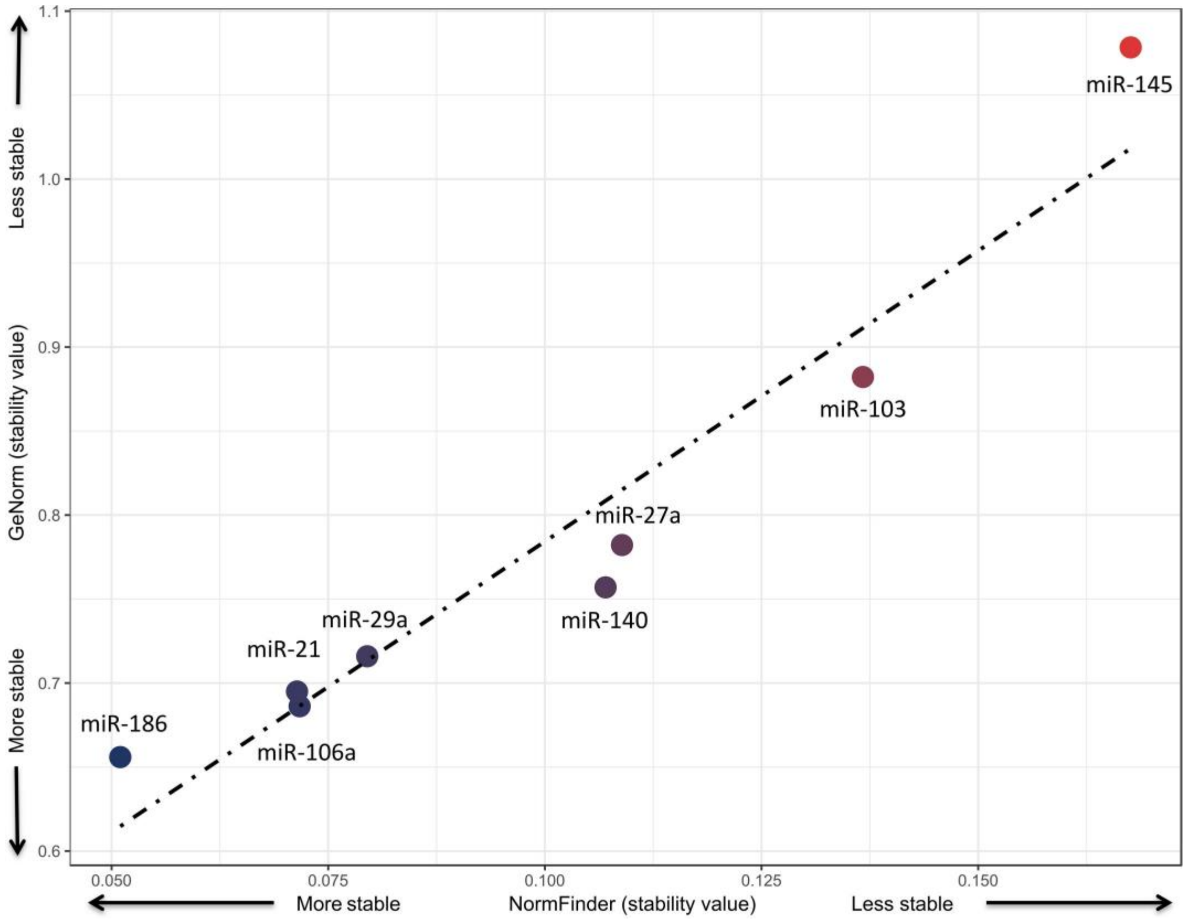


**Fig 4. Correlation plot of stability values from NormFinder and GeNorm.**

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in the search for biomarkers of clinical utility in OSA. These biomarkers could offer insight into the physiopathology of the disease and could have diagnostic and prognostic utility in OSA. The results of this study identified a set of miRNAs that could be used as ECs for standard normalization of miRNA profiling in OSA.

## Supporting information

**S1 Dataset. Baseline characteristics of TLDA patients.**
(TXT)

**S2 Dataset. Baseline characteristics of qPCR patients.**
(TXT)

**S3 Dataset. Raw miRNA Ct data from TLDA cohort.**
(TXT)

**S4 Dataset. Raw miRNA Ct data from qPCR cohort.**
(TXT)

**S5 Dataset. Normalized miRNA Ct from TLDA cohort.**
(TXT)

**S1 Table. Normalized miRNA TLDA data (ΔCt).** CI: Confidence Interval; IQR: Interquartile Range.
(DOC)

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**S1 Table:** normalized miRNA TLDA data (ΔCt).

| | Non-OSA | | OSA | |
|---|---|---|---|---|
| | *Mean (95%CI)* | *Median [IQR]* | *Mean (95%CI)* | *Median [IQR]* |
| **miRNA (ΔCt)** | | | | |
| miR-103 | 0.47 (0.26-0.67) | 0.46 [0.36;0.48] | 0.42 (0.19-0.66) | 0.38 [0.08;0.61] |
| miR-140 | -0.01 (-0.27-0.24) | -0.09 [-0.30;0.26] | 0.07 (-0.34-0.48) | -0.26 [-0.50;0.43] |
| miR-145 | 0.63 (0.4-0.86) | 1.24 [0.68;1.47] | 1.08 (0.6-1.56) | 0.49 [0.35;0.99] |
| miR-186 | 0.3 (0.13-0.47) | 0.52 [0.24;1.03] | 0.6 (0.25-0.94) | 0.26 [-0.02;0.55] |
| miR-21 | -1.38 (-1.5--1.26) | -1.29 [-1.60;-1.15] | -1.38 (-1.64-(-1.11)) | -1.43 [-1.62;-1.20] |
| miR-27a | -0.42 (-0.67-(-0.16)) | -0.29 [-0.55;0.04] | -0.17 (-0.74-0.41) | -0.47 [-0.86;-0.09] |
| miR-29a | 1.37 (1.2-1.54) | 1.45 [1.26;1.50] | 1.37 (1.22-1.53) | 1.36 [1.04;1.56] |
| miR-106a | -4.4 (-4.52-(-4.27)) | -4.43 [-4.60;-4.35] | -4.42 (-4.6-(-4.24)) | -4.44 [-4.60;-4.19] |

CI: Confidence Interval; IQR: Interquartile Range

## ARTÍCULO 2

"*Circulating microRNA profile as a potential biomarker for obstructive sleep apnea diagnosis*". **Fernando Santamaria-Martos**, Iván Benítez, Francisco Ortega, Andrea Zapater, Cristina Girón, Lucía Pinilla, Lydia Pascual, Anunciación Cortijo, Mireia Dalmases, Jose M. Fernandez-Real, Ferran Barbé and Manuel Sánchez-de-la-Torre. *Scientific Reports* 9, Article number: 13456 (2019).

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# Circulating microRNA profile as a potential biomarker for obstructive sleep apnea diagnosis

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**Evaluation of microRNAs (miRNAs) could allow characterization of the obstructive sleep apnea (OSA) and help diagnose it more accurately. We aimed to examine circulating miRNA profiles to establish the differences between non-OSA and OSA patients. Additionally, we aimed to analyse the effect of continuous positive airway pressure (CPAP) treatment on the miRNA profile. This observational, longitudinal study included 230 subjects referred to the Sleep Unit due to suspected OSA. Expression profiling of 188 miRNAs in plasma was performed in 27 subjects by TaqMan-Low-Density-Array. OSA-related miRNAs were selected for validation by RT-qPCR in 203 patients. Prediction models were built to discriminate between non-OSA and OSA: 1) NoSAS-score, 2) differentially expressed miRNAs, and 3) combination of NoSAS-score plus miRNAs. The differentially expressed miRNAs were measured after 6 months of follow-up. From the 14 miRNAs selected for validation, 6 were confirmed to be differentially expressed. The areas under the curve were 0.73 for the NoSAS-score, 0.81 for the miRNAs and 0.86 for the combination. After 6 months of CPAP treatment, miRNA levels in the OSA group seem to approximate to non-OSA levels. A cluster of miRNAs was identified to differentiate between non-OSA and OSA patients. CPAP treatment was associated with changes in the circulating miRNA profile.**

Obstructive sleep apnea (OSA) is a prevalent disease that affects approximately 10–17% of the adult population[1,2]. OSA is characterized by repetitive episodes of upper airway collapse during sleep leading to arousal, nocturnal hypoxemia and changes in intrathoracic pressure. Altogether, these events link OSA to the risk of cardiovascular diseases, metabolic disturbances, and cancer and to higher overall mortality mechanisms[3–6]. This association is of special relevance in the young population, in which OSA could have a higher damaging effect, as the observed cancer incidence is increased in young patients[7] and the impact of OSA on cardiovascular diseases may not be reversible when treatment is delayed[8]. Thus, new methods of early diagnosis of the disease are needed.

Up to 80% of individuals with moderate-to-severe OSA remain undiagnosed[9]. Diagnosis requires overnight recordings, including time- and resource-consuming procedures[9]. High-throughput diagnostic systems may improve the underdiagnoses of the disease and could reduce unnecessary procedures, decreasing the use of healthcare resources.

Currently, the NoSAS score is one of the best-validated tools for screening OSA populations (area under the curve (AUC) 0.74). The NoSAS score performed significantly better than did the STOP-Bang and Berlin scores[10]. NoSAS is based on a questionnaire and does not include any molecular variables that could help with diagnosis. The search for biomarkers that can help physicians not only diagnose OSA but also understand the physiopathology of the disease is a research priority in the field[9,11]. In this context, microRNAs (miRNAs) have emerged as an opportunity in the era of precision medicine for the screening, diagnosis and management of several diseases[12–16]. miRNAs are a class of small non-coding RNAs that negatively regulate gene expression post-transcriptionally by binding to target messenger RNA (mRNA), leading to either degradation or translational repression and protein synthesis[17]. They play a pivotal role in several biological processes, such as stress response, apoptosis, proliferation

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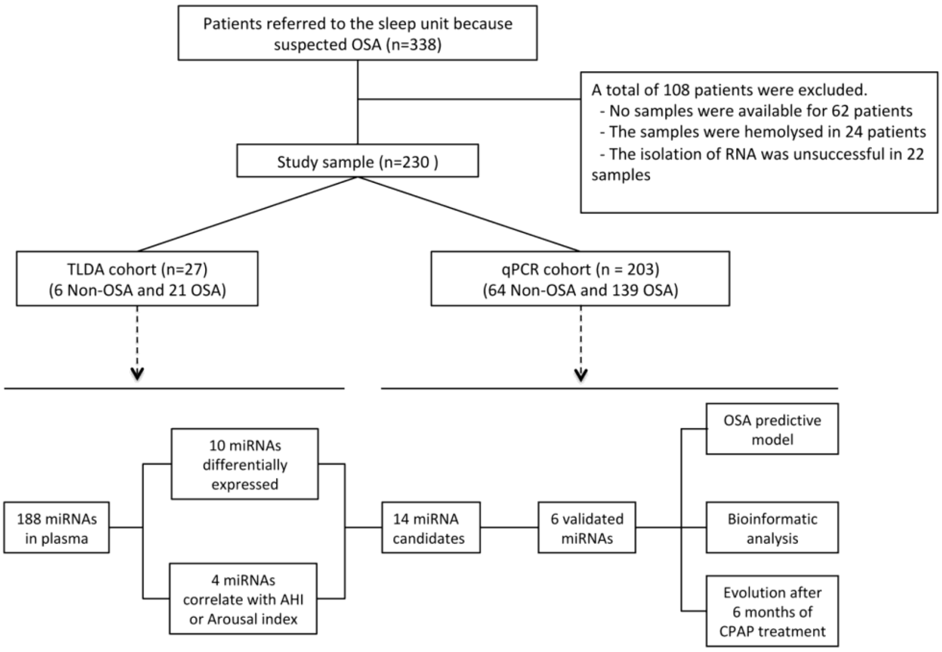


**Figure 1.** Flowchart of the study. Patients who were referred because of suspected OSA were divided into the TLDA cohort and the qPCR cohort and further divided for study on the basis of non-OSA and OSA. Six non-OSA and 21 OSA patients were used to perform a general screening of 188 miRNAs. Differentially expressed miRNAs were validated in an independent cohort. The analyses in the validation cohort were stratified by sex.

and differentiation, and many studies suggest that they are deregulated in several diseases, including cardiovascular diseases, metabolic disturbances and cancer[18]. miRNAs are present in body fluids, resistant to degradation and easily and rapidly (8–10 hours) measurable, fulfilling the criteria of an ideal biomarker in an era of evolving precision medicine[19].

We aimed to examine circulating miRNA profiles to establish the differences between non-OSA and OSA patients and to explore their clinical significance and contribution to disease diagnosis. Moreover, we aimed to evaluate changes in miRNA patterns after 6 months of continuous positive airway pressure (CPAP) treatment.

## Methods

**Study cohort and sample collection.** A total of 230 consecutive subjects who were aged between 18 and 60 years, were referred because of suspected OSA and had undergone full polysomnography were enrolled at the Sleep Unit of the Arnau de Vilanova-Santa Maria University Hospital of Lleida (NCT03513926). Subjects were grouped according to their Apnea-Hypopnea Index (AHI) gain into the following: 1) non-OSA group (subjects without moderate-to-severe OSA: AHI <15 events/h), 2) OSA group (moderate to severe OSA: AHI ≥15 events/h). Patients in the TaqMan low-density array (TLDA) cohort were carefully selected. Only patients taking medications related to the most habitual OSA associated pathologies (e.g., hypertension, dyslipidaemia, and cardiovascular events) were not excluded. No patients were excluded from the validation cohort, except those with previous CPAP use or any condition that, in the opinion of the responsible physician investigator, made the person unsuitable for the study (e.g., pregnancy, drug or alcohol consumption, or less than one year of life expectancy). On the other hand, patients without samples or with a haemolysed sample were excluded from the analyses. Additionally, samples with a recovery of RNA less than 1% were excluded (Fig. 1). All recruited patients signed an informed consent form, and the ethics committee of the centre (Clinical Research Ethics Committee of the Arnau de Vilanova-Santa Maria Hospital University Hospital) approved the study. All methods were performed in accordance with current clinical practice guidelines and regulations.

A venous fasting blood sample was obtained from each patient at baseline in the morning immediately after the sleep study between 08:00 and 09:00 a.m. An additional fasting blood sample was obtained at the 6-month follow-up between 08:00 and 09:00 a.m. The blood samples were centrifuged to separate plasma, and all specimens were immediately aliquoted, frozen, and stored in a dedicated −80 °C freezer. No freeze-thaw cycles were performed during the experiment.

**Clinical measurements.** All patients underwent full polysomnography at baseline. Apnea was defined as an interruption or reduction in oronasal airflow ≥90% that lasted at least 10 seconds. Hypopnoea was defined as a 30% to 90% reduction in oronasal airflow for at least 10 seconds associated with oxygen desaturation of at least 3% or an arousal on the electroencephalogram. The AHI was defined as the number of apnea and hypopnoea events per hour of sleep. The NoSAS score for screening sleep-disordered breathing was evaluated. The NoSAS score ranges from 0–17 based on neck circumference, body mass index (BMI), age, and gender[10]. All patients were evaluated at baseline and after 6 months of follow-up. Following the National Clinical Guidelines[20], patients with OSA were treated with CPAP.

**Circulating RNA extraction and purification.** RNA was extracted from 300 μL of plasma using a mir-Vana PARIS isolation kit (Applied Biosystems, Vilnius, Lithuania) according to the manufacturer's instructions. Non-human cel-miR-39 was spiked into the plasma immediately before extraction. RNA isolation efficiency was examined by RT-qPCR quantification of cel-miR-39 to guarantee homogeneous retrotranscription and cDNA synthesis.

**Circulating miRNA profiling with taqman low density array.** To identify plasma miRNA profiles that could help to diagnose OSA, we performed a general screening of 188 circulating miRNAs that have been reported as the most consistent and reliable miRNAs in human plasma samples[21,22]. This circulating profile was evaluated in a cohort of 27 patients (TLDA cohort) that included 6 non-OSA and 21 OSA patients matched by nearest neighbor matching (a procedure to match each OSA patient to a non-OSA subject with the most similar value of the estimated propensity score)[23] for age and body mass index (Fig. 1). At this point, due to sample size, only male subjects were evaluated. Multiple real-time (RT)-PCRs were performed using a customized TaqMan Low Density Array (TLDA, Life Technologies, Foster City, CA, USA). Briefly, a fixed volume of 3 μL of RNA solution from the 40 μL of RNA isolation eluate was used as the input for the retrotranscription, using a TaqMan MicroRNA Reverse Transcription Kit and TaqMan MicroRNA Multiplex RT Assays, which are customized to run TLDAs. Preamplification was performed using TaqMan PreAmp Master Mix and Megaplex PreAmp Primers for our selected miRNAs. RT-PCR was carried out using an Applied Biosystems QuantStudio™ 7 Flex Real-Time PCR System. Data were processed with the Relative Quantification tool (powered by Thermo Fisher cloud), with a minimal threshold above the baseline ($\Delta Rn = 0.012$) and less than 35 thermal cycles (Ct). The results were normalized using a mean-centre normalization method, the gold-standard method when screening a large number of miRNAs[24].

**Analysis of individual miRNAs.** The most reliable and consistent candidates for endogenous control (i.e., hsa-miR-106a and hsa-miR-186) were identified in TLDA analyses and were used together with spike-in (cel-miR-39) for normalization[25]. Then, individual TaqMan hydrolysis probes (Applied Biosystems) were applied to analyse the expression of the set of differentially expressed miRNAs in the qPCR cohort (Fig. 1). Additionally, to assess the evolution of miRNAs after CPAP treatment, we evaluated the changes in miRNA expression of the validated candidates after 6 months of CPAP treatment, comparing non-OSA subjects with OSA patients treated with CPAP.

**Enrichment analysis.** The web-based computational tools miRWalk, DIANA-mirPath, TargetScan, DAVID and miRanda were used to predict the target genes and altered pathways of the differentially expressed miRNAs.

**Statistical analysis.** To prevent biases due to differential miRNA pattern expression, all analyses were stratified by gender. Comparability between non-OSA and OSA patients was assessed using the Mann-Whitney U test for quantitative characteristics and Fisher's exact test for qualitative variables. The differences in miRNA expression between groups were evaluated by relative quantification using linear models for arrays (LIMMA)[26]. Spearman's rank correlation coefficient was used to evaluate the association between miRNA levels and polysomnography parameters. False discovery rate-adjusted p-values were calculated to adjust for the performance of multiple paired comparisons.

Three logistic models were fitted for OSA risk modelling: 1) miRNAs showing differential expression in the validation phase, which were dichotomized by selecting the cut-off point as the point on the receiver operating characteristic (ROC) curve closest to the upper left corner of the unit square and were included in the model; 2) NoSAS score as a continuous variable; and 3) The combination of NoSAS and differentially expressed miRNAs. The Hosmer-Lemeshow test was used to test model calibration. A nonparametric test comparing the AUC of ROC curves[27] was evaluated. The AUC of the combined model (NoSAS and miRNA) was evaluated in a 10-fold cross-validation. The effect of CPAP on the changes in miRNA expression at the six-month follow-up was estimated by the difference in the mean change between treated OSA patients and non-OSA patients. R software (R Project for Statistical Computing, Vienna, Austria) was used for statistical analysis.

## Results

**Patient characteristics.** Of the 338 patients evaluated, 230 were ultimately included (Fig. 1). Patients were mainly middle aged, overweight-obese and male, especially in the OSA group (Table 1). Significant differences were identified in baseline characteristics between non-OSA and OSA groups in age (median 47 vs. 51 years old, p-value = 0.002), BMI (median 27.5 vs. 31.7 kg/m$^2$, p-value = 0.015) and sex (68.6% vs. 83.8% male, p-value < 0.001).

**Identification of plasma miRNAs relevant to OSA.** The miRNA profiles were used to identify miRNAs related to OSA in the TLDA cohort (a cohort of men paired by BMI and age). After the analysis, a subset of 14 miRNAs was selected based on an individual p-value < 0.05 when comparing non-OSA and OSA groups and/or a high correlation (r > 0.4) with the AHI or arousal index (Table 2). miR-451, miR-486-3p and miR-133a were selected based on their p-values. On the other hand, miR-181a, hsa-let-7d, miR-199a and miR-199b were selected because of their high correlations with the sleep parameters. Finally, miR-181a-2, miR-495, miR-486, miR-660,

| | All | Non-OSA (AHI < 15) | OSA (AHI ≥ 15) | |
|---|---|---|---|---|
| | N = 230 | N = 70 | N = 160 | p-value |
| Demographic and clinical variables | | | | |
| Age (years) -median [IQR]- | 49.0 [44.0;55.0] | 47.0 [40.0;52.0] | 51.0 [45.0;55.0] | 0.002 |
| Sex (men), n (%) | 182 (79.1%) | 48 (68.6%) | 134 (83.8%) | 0.015 |
| BMI (kg/m$^2$) -median [IQR]- | 30.9 [26.5;34.7] | 27.5 [25.4;31.9] | 31.7 [28.1;35.2] | <0.001 |
| Systolic blood pressure (mmHg) -median [IQR]- | 134 [123;146] | 127 [116;137] | 136 [127;148] | <0.001 |
| Diastolic blood pressure (mmHg) -median [IQR]- | 86.0 [79.5;94.5] | 81.8 [75.2;87.8] | 88.5 [82.5;95.0] | <0.001 |
| Smoking status: -n(%)- | | | | 0.564 |
| Non-smoker | 81 (35.5%) | 28 (40.0%) | 53 (33.5%) | |
| Former smoker | 79 (34.6%) | 24 (34.3%) | 55 (34.8%) | |
| Smoker | 68 (29.8%) | 18 (25.7%) | 50 (31.6%) | |
| Respiratory parameters | | | | |
| AHI (events/h) -median [IQR]- | 29.5 [12.1;51.0] | 8.44 [5.00;11.4] | 43.8 [27.4;63.9] | <0.001 |
| TSat90 (%) -median [IQR]- | 2.40 [0.21;12.7] | 0.05 [0.00;0.58] | 5.15 [2.00;20.6] | <0.001 |
| Arousal index (events/h) -median [IQR]- | 33.8 [21.1;53.6] | 18.9 [13.7;25.9] | 43.3 [32.1;60.7] | <0.001 |
| Minimum SaO2 (%) -median [IQR]- | 82.0 [73.0;87.0] | 89.0 [86.0;91.0] | 79.0 [71.0;83.0] | <0.001 |
| Mean SaO2 (%) -median [IQR]- | 93.0 [91.0;94.0] | 94.0 [93.0;95.0] | 93.0 [91.0;94.0] | <0.001 |
| ESS (0–24) -median [IQR]- | 10.0 [7.00;13.0] | 10.0 [7.00;14.0] | 10.0 [7.00;13.0] | 0.469 |

**Table 1.** Baseline characteristics of the patients. Abbreviations: BMI = Body Mass Index; AHI = Apnea-Hypoapnea Index; TSat90 = Night Time with Oxygen Saturation Less Than 90%; ESS = Epworth Sleepiness Scale.

| miRNA | Fold change | p-value | AHI correlation | Arousal index correlation |
|---|---|---|---|---|
| hsa-miR-181a-2 | 3.72 | 0.001 | −0.51 | −0.42 |
| hsa-miR-495 | 2.22 | 0.004 | −0.58 | −0.79 |
| hsa-miR-451 | 0.52 | 0.006 | 0.18 | −0.06 |
| hsa-miR-486 | 0.52 | 0.007 | 0.54 | 0.28 |
| hsa-miR-660 | 0.65 | 0.015 | 0.42 | 0.22 |
| hsa-miR-345 | 0.69 | 0.021 | 0.47 | 0.41 |
| hsa-miR-340 | 1.51 | 0.026 | −0.61 | −0.49 |
| hsa-miR-107 | 1.62 | 0.029 | −0.63 | −0.45 |
| hsa-miR-486-3p | 0.55 | 0.03 | 0.38 | 0.31 |
| hsa-miR-133a | 0.37 | 0.049 | −0.13 | −0.06 |
| hsa-miR-181a | 0.73 | 0.066 | 0.47 | 0.5 |
| hsa-let-7d | 1.35 | 0.1 | −0.52 | −0.67 |
| hsa-miR-199a | 1.3 | 0.157 | −0.28 | −0.41 |
| hsa-miR-199b | 0.81 | 0.332 | 0.42 | 0.36 |

**Table 2.** miRNA candidates that were differentially expressed between non-OSA and OSA male subjects. Fourteen miRNAs were selected based on their fold change and their correlation with the AHI and arousal index.

miR-345, miR-340 and miR-107 were selected because they met both criteria. From the 14 miRNAs selected, 8 seemed to have less expression in the OSA patients than in the non-OSA patients.

**Validation of plasma miRNAs relevant to OSA.** All potential OSA-related miRNAs selected from TLDA were validated in the qPCR cohort. Among men, the analysis confirmed that the circulating concentrations of hsa-miR-181a, hsa-miR-199b, hsa-miR-345, hsa-miR-133a, hsa-miR-340 and hsa-miR-486-3p were decreased in the OSA group after adjustment for BMI and age (Table 3 and Fig. 2). Of these, miR-181a seemed to be correlated with the AHI (rho = 0.29) and with the arousal index (rho = 0.25) and miR-345 with the AHI (rho = 0.21). To further explore the results, mild OSA patients were removed in order to confidently report the results between non-OSA (AHI <5 events/h) and OSA (AHI ≥15 events/h) subjects. This analysis reported increased differences between non-OSA and OSA subjects (except for miR-199b and miR-486-3p) (see e-Table 3).

Among women, only hsa-miR-451 (fold change = 1.6 and p-value = 0.057) seemed to be differentially expressed after adjustment for BMI and age, but this significance is lost after FDR correction (corrected p-value = 0.54) (see e-Table 4).

| miRNA | Fold change | p-value | FDR correction |
|---|---|---|---|
| hsa-miR-181a | 0.59 | 0.001 | 0.013 |
| hsa-miR-199b | 0.42 | 0.008 | 0.056 |
| hsa-miR-345 | 0.71 | 0.014 | 0.056 |
| hsa-miR-133a | 0.47 | 0.016 | 0.056 |
| hsa-miR-340 | 0.67 | 0.029 | 0.072 |
| hsa-miR-486-3p | 0.64 | 0.031 | 0.072 |
| hsa-miR-181a2 | 0.51 | 0.066 | 0.133 |
| hsa-miR-199a | 0.54 | 0.184 | 0.318 |
| hsa-miR-660 | 0.79 | 0.214 | 0.318 |
| hsa-miR-451 | 0.82 | 0.227 | 0.318 |
| hsa-let-7d | 0.93 | 0.591 | 0.752 |
| hsa-miR-486 | 1.06 | 0.665 | 0.776 |
| hsa-miR-107 | 1.07 | 0.817 | 0.832 |
| hsa-miR-495 | 0.93 | 0.832 | 0.832 |

**Table 3.** Validation of miRNA candidates. Six miRNAs were found to be differentially expressed between non-OSA and OSA patients. P-values adjusted for age and BMI. FDR <0.1 was considered statistically significant.

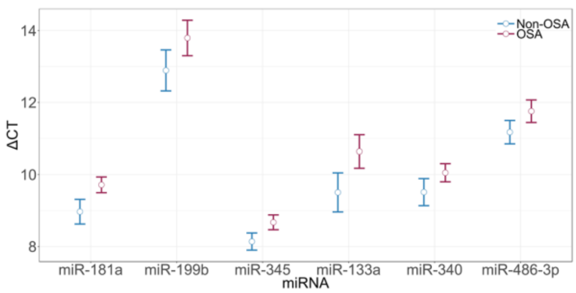


**Figure 2.** Delta Ct plot means of validated miRNAs (FDR < 0.1).

**Prediction model.** Logistic regression models were generated for male patients as a tool to differentiate between non-OSA and OSA patients. Three multivariate models were fitted to classify the patients: A) the NoSAS score; B) the validated miRNAs and C) a combination of NoSAS score and validated miRNAs (Fig. 3). All the models exhibited good calibration and discrimination. The AUCs were 0.73 for the NoSAS score, 0.81 for the miRNAs and 0.86 for the combination. A comparison of the models based on AUC showed that the combination of NoSAS score and miRNAs was the best model for discriminating OSA patients (Table 4). The AUC of the combined model showed similar results in cross-validation (AUC = 0.83).

**Changes in validated plasma miRNAs after CPAP treatment.** We evaluated the miRNAs that were differentially expressed and validated, at baseline and after 6 months of follow-up. We found that in OSA CPAP-treated patients (median adherence 5.24 h/night), miRNA levels seem to increase, except for miR-486-3p, compared to those in non-OSA patients. Despite this observation, only changes in miR-345 reached statistical significance (Table 5).

**Bioinformatic analysis.** The enrichment analysis, including the 6 miRNAs confirmed as differentially expressed between non-OSA and OSA patients at baseline, revealed 69 dysregulated pathways. A total of 1,743 genes were identified to be deregulated; 352 genes were targeted by miR-133a, 188 genes by miR-181, 167 genes by miR-199b, 47 genes by miR-340, 185 genes by miR-345, and 804 genes by miR-486-3p. Of the 1,743 predicted targeted genes, only 626 were unique genes, and IGF1R, KRAS, RUNX1T1 and SLC9A8 were regulated by 3 miRNAs.The most frequently enriched pathways were categorized under biological processes connected with cancer (renal cell carcinoma, melanoma, etc.), cardiovascular diseases (dilated cardiomyopathy, hypertrophic cardiomyopathy, etc.), and signalling (sphingolipid signalling, adrenergic signalling of cardiomyocytes, etc.) (see e-Table 6 and e-Fig. 6).

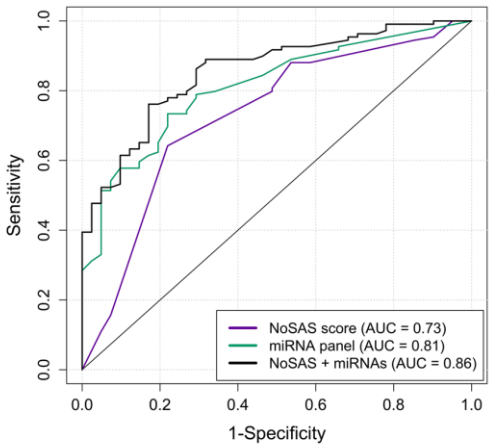


**Figure 3.** Discriminatory ROC plot of the models for screening OSA using (**A**) NoSAS; (**B**) 6 miRNAs; and (**C**) a combination of NoSAS and miRNAs.

| Comparison | AUC difference | p-value | Correlation |
|---|---|---|---|
| A vs. B | −0.08 | 0.16 | 0.01 |
| A vs. C | −0.13 | 0.002 | 0.54 |
| B vs. C | −0.04 | 0.065 | 0.76 |

**Table 4.** Comparison of ROC models. A) NoSAS scores, B) miRNA model, C) combination.

| miRNA | Estimate difference | Lower bound | Upper bound | p-value |
|---|---|---|---|---|
| hsa-miR-181a | −0.514 | −1.524 | 0.495 | 0.314 |
| hsa-miR-199b | −0.372 | −1.714 | 0.971 | 0.581 |
| hsa-miR-345 | −1.419 | −2.416 | −0.423 | 0.006 |
| hsa-miR-133a | −0.216 | −1.443 | 1.012 | 0.728 |
| hsa-miR-340 | −0.418 | −1.477 | 0.642 | 0.435 |
| hsa-miR-486-3p | 0.550 | −0.567 | 1.668 | 0.330 |

**Table 5.** Post-pre mean differences in differentially expressed miRNAs between non-OSA and OSA patients after 6 months of CPAP treatment.

## Discussion

A singular male-specific cluster of miRNAs functionally associated with sleep, cardiovascular diseases, metabolic disorders and cancer significantly contributes to the discrimination between non-OSA and OSA patients in the sleep unit. The present study also showed that CPAP use is associated with changes in the miRNA profile that could influence the overall risk of suffering OSA-related diseases.

Circulating concentrations of 6 miRNAs were significantly reduced in OSA subjects compared with those in non-OSA subjects. We also investigated the discriminatory ability of the miRNAs. We compared the NoSAS score, miRNAs and a combination of both. The NoSAS score AUC was consistent with previous results, with an AUC of 0.73, but lower than that obtained with miRNAs (AUC = 0.81)[10,28]. The correlation between both AUCs is very low (0.01), which could be argued because NoSAS and miRNAs could explain different components of OSA, resulting in a higher AUC of 0.86 for the combination of both, which could represent a very powerful tool that combines anthropometric and molecular variables to explain OSA variability. Finally, the longitudinal study showed that after CPAP treatment, miRNAs seem to increase their circulating concentration, at least partially recovering the non-OSA phenotype. Decreased blood pressure could have contributed to the miRNA changes detected after CPAP treatment. In fact, as previously reported by our group, CPAP treatment changes the miRNA profile, and these changes correlate with blood pressure changes that occur following CPAP treatment. Additionally, these changes are associated with the response to treatment of the patient[14].

This study identifies the utility of miRNAs as biomarkers that could contribute to the personalized management of OSA in the sleep unit. Recent studies have demonstrated the importance of miRNAs in the control of many processes in health and disease[14,19,21]. To the best of our knowledge, this is one of the first studies to use miRNAs to identify a signature and characterize OSA in patients who are referred to a sleep unit. A similar study was conducted[29], in which the researchers identified that in a preliminary phase, 104 miRNAs were differentially expressed in serum between OSA and non-OSA subjects. Of these miRNAs, only 4 were validated by qPCR. We also attempted to validate miR-107 and miR-199 in our study. In both studies, miR-199 was downregulated when comparing OSA patients vs. non-OSA subjects; however, in our study, miR-107 was not confirmed to be differentially expressed. These possible discrepancies could be due to the different sample types utilized (plasma vs. serum) and our different normalization methods (endogenous control + spike-in vs. spike-in). Additionally, the data reported in this previous study did not seem to be adjusted by confounding variables.

A total of 6 miRNAs have been identified to be differentially expressed between non-OSA and OSA patients. This set of miRNAs seems to have the ability to differentiate between non-OSA and OSA patients, which is effective and practical for clinical use and enables the identification of patients with OSA. Recently, a seminal study developed by our group identified miRNAs as a useful tool in the management of patients with OSA and resistant hypertension[14]. In this study, we demonstrated the utility of miRNAs by identifying a singular cluster of miRNAs (HIPARCO-Score) that identifies those patients who will have a favourable blood pressure response to CPAP treatment. The present study corroborates the clinical usefulness of miRNAs for the diagnosis of OSA at sleep unit level.

The set of 6 miRNAs that are differentially expressed between non-OSA and OSA patients is related to relevant canonical pathways. First, we identified that miRNAs are misregulating pathways related to cardiovascular diseases, specifically several cardiomyopathies, in concordance with the hypothesis of a difference in cardiovascular risk between non-OSA and OSA patients. Second, identifying the molecular pathways related to both cancer and OSA is one of the hallmarks of OSA research. Additionally, an early diagnosis of OSA could be especially important because the association of OSA with cancer incidence is limited to young people[7]. Several studies have shown the association of OSA with several types of cancer, especially in young patients[30]. In the present study, we identified several cancer-related pathways that could be altered in patients with OSA, and some of the altered pathways are related to melanoma. These results are in accordance with previous literature in which the impact of OSA on melanoma and some of the intermediate pathways have been addressed[31,32]. Similar results were found in a previous work[25], where bioinformatic analyses showed that OSA could alter cardiovascular and cancer-related pathways.

Specifically, these miRNAs play an important role in some pathways related to common diseases associated with OSA. OSA is well known to be related to deregulated nutrient sensing, especially glucose. Patients with OSA are more likely to develop insulin resistance due to oxidative stress and a lower concentration of oxygen in pancreatic ß-cells[33]. Recently, miR-486 has been associated with the HOMA index and fasting levels of glucose and insulin[34]. Moreover, miR-340 seems to deregulate IGF1R gene expression, as shown in bioinformatic analyses. Previous studies observed associations between OSA and melanoma. Additionally, miR-340 is known to regulate MITF gene expression, which is a gene that plays a key role in melanocyte development. Recently, one study found that miR-340 regulates RAS–RAF–mitogen activated protein kinase (MAPK) signalling by modulating the expression of multiple components of this pathway[35], and this association could explain, at least in part, the relationship between OSA and melanoma. Several genes regulated by these miRNAs are involved in cardiovascular diseases. miR-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart[36] and is dysregulated in human myocardial infarction[37]. Finally, miR-199b is involved in the response to the hypoxia-repressing HIF1A gene[38]. HIF1A is a key gene in the response to hypoxic stress, and its expression is deregulated in OSA patients[39]. Moreover, IGF1R, KRAS, RUNX1T1 and SLC9A8 are regulated by 3 miRNAs. All these genes are related to melanoma pathways and play an important role in the transformation of melanocytes. Recently, one study assessed the molecular relationship between OSA and melanoma, concluding that OSA increased circulating VCAM-1 levels in melanoma patients[31]. This relationship could be an indirect consequence of IGF1R, KRAS, RUNX1T1 and SLC9A8 regulation.

Notably, all miRNAs validated in the present study were downregulated in patients with OSA. As previously reported, chronic hypoxia impairs Dicer (DICER1) expression and activity, resulting in global downregulation of miRNA expression[40].

Diagnostic and personalized therapeutic decision-making tools are needed to manage OSA, especially in the young population[9,10,41]. Together, these results suggest that the use of miRNAs in OSA management could be a powerful tool for the diagnosis of OSA and to establish the benefits of the treatment for this complex chronic disease.

The present study has several limitations that deserve comment. First, only patients between 18 and 60 years old were studied, and larger studies should be performed to determine the validity of this tool in patients of other ages. The validation showed poor results in women, reflecting the different physiopathology of OSA between women and men. Recent studies have shown that miRNAs have sex-dimorphic expression patterns[42]. Because of the ages of the subjects, oestrogens could play a very important role in these expression patterns. As previously reported, sex-related differences may cause abnormal miRNA expression, and for this reason, stratified analyses are recommended. Further studies are needed to evaluate the whole profile of miRNAs in women with OSA. Despite, RNA isolation efficiency was measured with spike-in cel-miR-39 to guarantee homogeneous retrotranscription and cDNA synthesis; no data about RNA integrity or quantity are available. Although we performed a quantification of a large number of miRNAs (a total of 188 miRNAs) that were previously identified as the most stable miRNAs in plasma and were quantified in at least 70% of the subjects[21,22], we did not perform whole miRNA quantification. This approximation permits us to use gold standard technology and enables an accurate quantification in a short period of time, with the highest dynamic range[43]. By last, this tool is not able to distinguish mild OSA patients from non-OSA subjects; further studies including a higher number of patients with AHI $<5$ events/h is required. The strengths of this study reside in a large number of patients who were analysed in two different sets for the TLDA and qPCR cohorts, which permitted us to detect high-magnitude associations.

Additionally, some of the basal associations were modified after CPAP use, confirming the importance of these miRNAs in OSA. Finally, all patients were recruited from the sleep unit, performing gold-standard methods for OSA diagnosis (polysomnography) and for miRNA measurement (RT-qPCR).

A singular male-specific cluster of miRNAs was identified that specifically differentiates between non-OSA and OSA patients. This miRNA model, which improves the discriminatory ability, is based in anthropometric and clinical variables. Finally, the use of CPAP treatment was associated with changes in the circulating miRNA profile that partly recovered the non-OSA phenotype.

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## Author Contributions

M.S.T. is the guarantor of the study. F.S.M., I.B., J.M.F.R., F.B., F.O. and M.S.T. contributed to study design F.S.M., A.Z., C.G., L.P., L.P., A.C., M.D., F.O., M.S.T. contributed to data acquisition F.S.M., I.B., F.O., M.S.T. contributed to data analysis and interpretation F.S.M., I.B., A.Z., C.G., L.P., L.P., A.C., M.D., J.M.F.R., F.B., F.O. and M.S.T. contributed to drafting of the manuscript F.S.M., I.B., A.Z., C.G., L.P., L.P., A.C., M.D., J.M.F.R., F.B., F.O. and M.S.T. contributed to revision of the manuscript for intellectual content and approval of the final version.

## Additional Information


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**Competing Interests:** The authors declare no competing interests.

## SUPPLEMENTARY MATERIAL

### 1. TaqMan Low Density Array determinations

1.1. Baseline characteristics of the cohort.

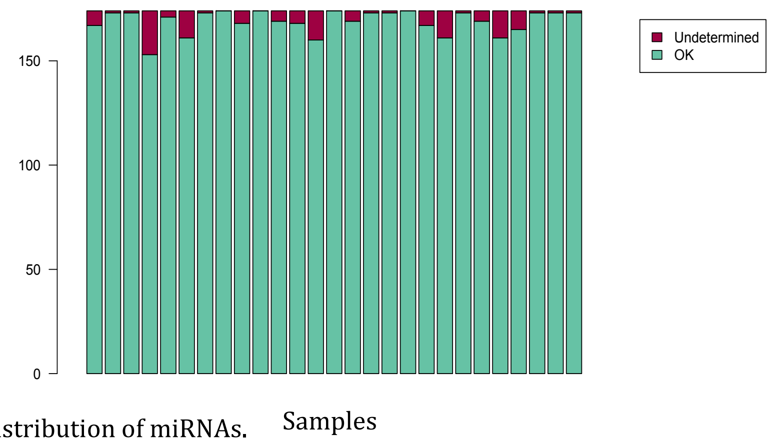
e-Table 1: Baseline characteristics of the cohort.

| | All N=27 | Non-OSA (AHI<15) N=6 | OSA (AHI ≥15) N=21 | p value |
|---|---|---|---|---|
| Demografic and Clinical variables | | | | |
| Age (years) -median [IQR]- | 49.0 [41.0;55.0] | 43.5 [39.2;47.8] | 51.0 [44.0;56.0] | 0.115 |
| Sex (men), n (%) | 27 (100%) | 6 (100%) | 21 (100%) | |
| BMI (kg/m2) -median [IQR]- | 27.2 [25.4;30.4] | 25.1 [23.1;29.3] | 27.2 [26.2;30.5] | 0.180 |
| Smoking status: -n(%)- | | | | 0.723 |
| Non smoker | 8 (29.6%) | 1 (16.7%) | 7 (33.3%) | |
| Former smoker | 12 (44.4%) | 3 (50.0%) | 9 (42.9%) | |
| Smoker | 7 (25.9%) | 2 (33.3%) | 5 (23.8%) | |
| Respiratory parameters | | | | |
| AHI (events/h) -median [IQR]- | 29.7 [18.3;42.7] | 7.36 [6.18;10.4] | 32.4 [28.1;49.2] | <0.001 |
| TSat90 (%) -median [IQR]- | 0.60 [0.22;6.70] | 0.03 [0.00;0.09] | 2.28 [0.33;8.37] | 0.002 |
| Arousal index (events/h) -median [IQR]- | 36.8 (17.0) | 15.9 (10.1) | 42.8 (13.6) | <0.001 |
| ESS (0-24) -median [IQR]- | 9.96 (5.59) | 12.3 (7.09) | 9.21 (5.03) | 0.351 |

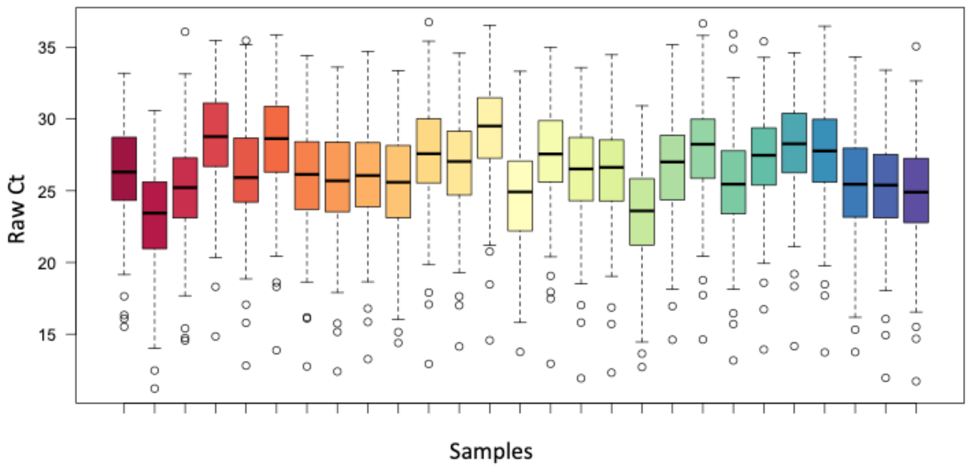
Abbreviations: BMI = Body Mass Index; AHI = Apnoea-Hypoapnoea Index; TSat90 = nighttime with oxygen saturation less than 90%; ESS= Epworth Sleepiness Scale.

1.2. Quality control

e-Figure 1: Number of determinations/missings.

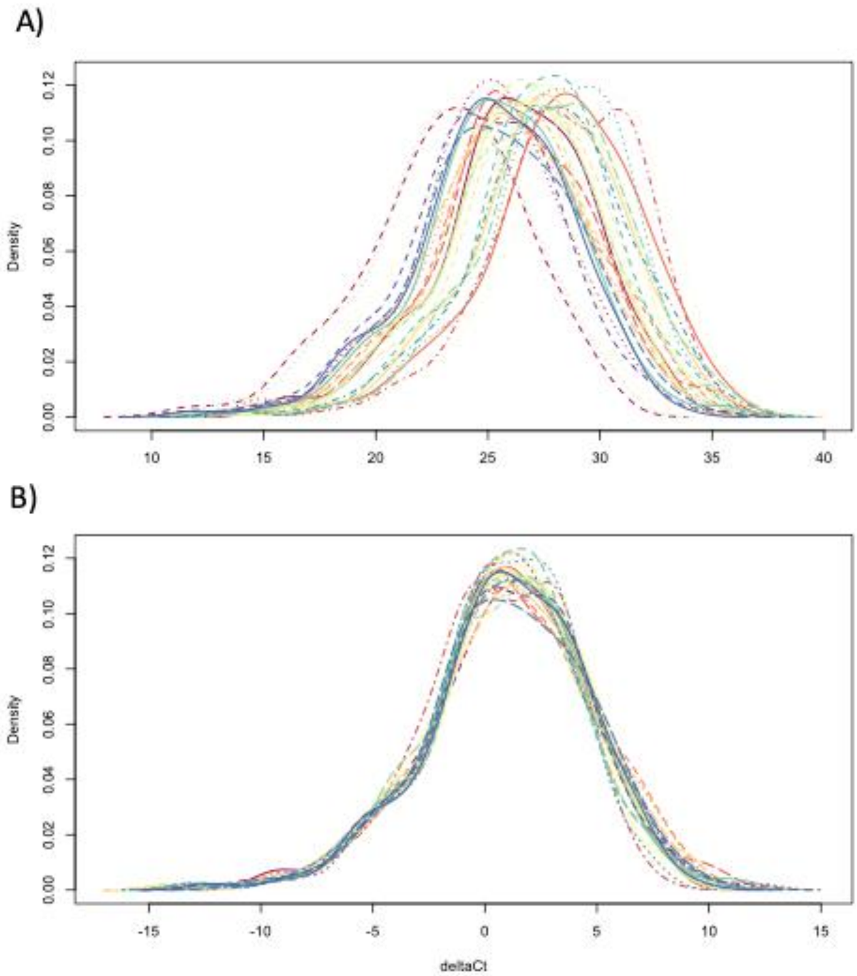



e-Figure 2: Raw Ct distribution of miRNAs.




1.3. miRNA normalization.

e-Figure 3: Density plot of miRNA normalization. A) density of raw Cts of each subject. B) Mean-center normalized density plot.




**2. qPCR determinations**

## 2.1. Baseline characteristics of the cohort

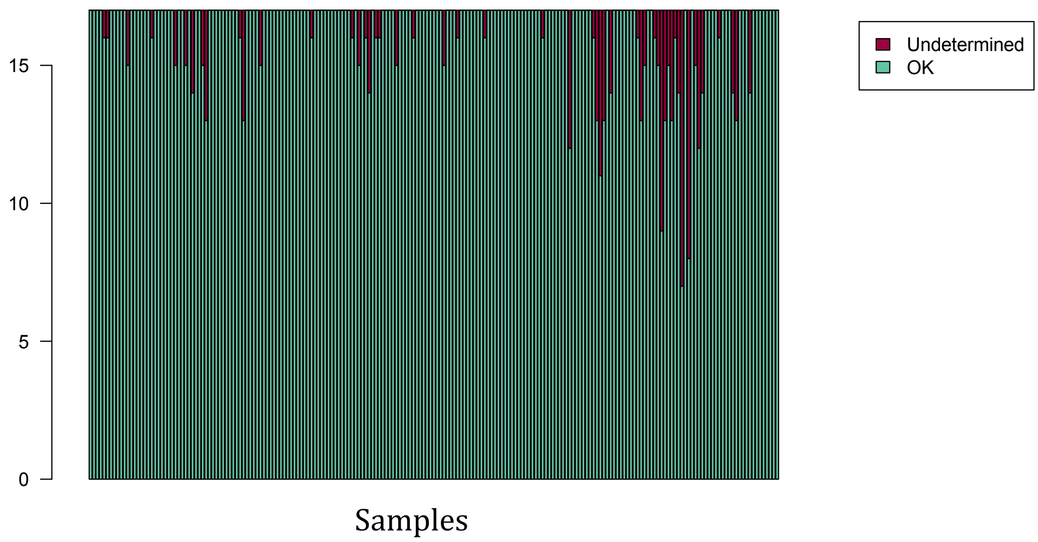
e-Table 2: Baseline characteristics of the cohort

| | Men | | | Women | | |
|---|---|---|---|---|---|---|
| | Non-OSA (AHI<15) N=42 | OSA (AHI ≥15) N=113 | p value | Non-OSA (AHI<15) N=22 | OSA (AHI ≥15) N=26 | p value |
| **Demografic and Clinical variables** | | | | | | |
| Age (years) -median [IQR]- | 47.0 [41.2;52.0] | 49.0 [44.0;55.0] | 0.142 | 45.5 [38.5;53.8] | 53.5 [49.2;57.0] | 0.001 |
| BMI (kg/m2) -median [IQR]- | 27.1 [25.6;29.6] | 32.5 [29.1;35.7] | <0.001 | 29.1 [24.1;34.7] | 33.3 [28.5;35.8] | 0.153 |
| Smoking status: -n(%)- | | | 0.281 | | | 0.793 |
| Non smoker | 16 (38.1%) | 31 (27.7%) | | 11 (50.0%) | 15 (60.0%) | |
| Former smoker | 15 (35.7%) | 41 (36.6%) | | 5 (22.7%) | 5 (20.0%) | |
| Smoker | 11 (26.2%) | 40 (35.7%) | | 6 (27.3%) | 5 (20.0%) | |
| **Respiratory parameters** | | | | | | |
| AHI (events/h) -median [IQR]- | 9.29 [5.65;12.0] | 45.6 [29.4;64.6] | <0.001 | 5.09 [2.88;9.17] | 39.3 [23.3;66.5] | <0.001 |
| TSat90 (%) -median [IQR]- | 0.12 [0.00;0.60] | 5.40 [2.30;21.3] | <0.001 | 0.02 [0.00;0.29] | 6.43 [2.08;20.7] | <0.001 |
| Arousal index (events/h) -median [IQR]- | 19.0 [13.6;25.7] | 45.3 [33.1;62.4] | <0.001 | 15.4 [14.2;26.8] | 36.2 [23.7;58.4] | <0.001 |
| ESS (0-24) -median [IQR]- | 9.00 [7.00;12.0] | 10.0 [6.50;13.0] | 0.553 | 11.5 [9.0;15.0] | 11.0 [7.0;15.0] | 0.453 |

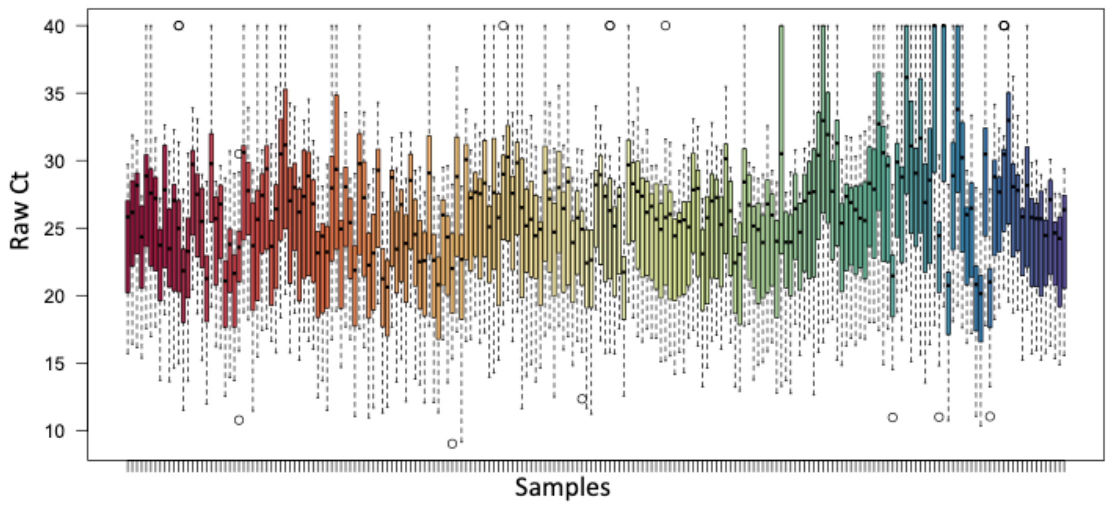
Abbreviations: BMI = Body Mass Index; AHI = Apnoea-Hypoapnoea Index; TSat90 = nighttime with oxygen saturation less than 90%; ESS= Epworth Sleepiness Scale.

2.2. Quality control

e-Figure 4: Number of determinations/missings.




e-Figure 5: Raw Ct distribution of miRNAs.




2.3. miRNA validation and models

2.3.1. Validation of miRNA candidates excluding mild-OSA.

e-Table 3: Validation of miRNA candidates excluding mild-OSA. There were 6 non-OSA patients reducing the power of this sub-analysis.

| miRNA | Fold change | p-value |
|---|---|---|
| hsa-mir-181a | 0.315 | 0.002 |
| hsa-miR-133a | 0.324 | 0.054 |
| hsa-miR-345 | 0.556 | 0.081 |
| hsa-miR-495 | 0.441 | 0.086 |
| hsa-miR-199b | 0.522 | 0.139 |
| hsa-miR-340 | 0.620 | 0.198 |
| hsa-let-7d | 0.732 | 0.328 |
| hsa-miR-660 | 0.791 | 0.406 |
| hsa-miR-486-3p | 0.745 | 0.512 |
| hsa-miR-451 | 0.809 | 0.551 |
| hsa-miR-181a2 | 0.768 | 0.614 |
| hsa-miR-486 | 1.109 | 0.743 |
| hsa-miR-199a | 0.968 | 0.948 |
| hsa-miR-107 | 1.015 | 0.960 |

2.3.2. Validation of differentially expressed miRNAs in Women.

e-Table 4: Validation of miRNA candidates in women

| miRNA | Fold change | p-value | FDR correction |
|---|---|---|---|
| hsa-miR-451 | 1.641617132 | 0.057011678 | 0.53960381 |
| hsa-miR-199a | 3.968594886 | 0.127989504 | 0.53960381 |
| hsa-miR-340 | 2.114076774 | 0.185507751 | 0.53960381 |
| hsa-miR-107 | 2.596445604 | 0.205911237 | 0.53960381 |
| hsa-miR-133a | 2.079198266 | 0.212445111 | 0.53960381 |
| hsa-mir-181a | 1.78621407 | 0.290038965 | 0.53960381 |
| hsa-miR-181a2 | 2.380875818 | 0.290888298 | 0.53960381 |
| hsa-miR-486 | 1.286395732 | 0.308345035 | 0.53960381 |
| hsa-miR-199b | 1.847589946 | 0.380614767 | 0.592067416 |
| hsa-miR-486-3p | 1.454902206 | 0.433785555 | 0.596972855 |
| hsa-miR-495 | 1.827937146 | 0.4690501 | 0.596972855 |
| hsa-let-7d | 1.128385545 | 0.64411447 | 0.751466882 |
| hsa-miR-345 | 1.018070318 | 0.946577378 | 0.995775644 |
| hsa-miR-660 | 0.99700101 | 0.995775644 | 0.995775644 |

2.3.3. Univariate logistic regression analysis.

e-Table 5: Univariate logistic regression analysis of the miRNAs.

| | Estimate | SE | z-value | p-value | OR | LB (95% CI) | UB (95% CI) |
|---|---|---|---|---|---|---|---|
| hsa-mir-181a | 1.37 | 0.39 | 3.53 | <0.001 | 3.92 | 1.87 | 8.59 |
| hsa-miR-199b | 1.96 | 0.63 | 3.11 | <0.001 | 7.12 | 2.38 | 30.75 |
| hsa-miR-345 | 1.37 | 0.39 | 3.53 | <0.001 | 3.92 | 1.87 | 8.59 |
| hsa-miR-133a | 1.3 | 0.4 | 3.27 | <0.001 | 3.68 | 1.73 | 8.34 |
| hsa-miR-340 | 1.34 | 0.46 | 2.95 | <0.001 | 3.83 | 1.65 | 10.06 |
| hsa-miR-486-3p | 1.32 | 0.52 | 2.56 | 0.01 | 3.75 | 1.47 | 11.58 |

2.4.4. Bioinformatic analysis of differentially expressed miRNAs.

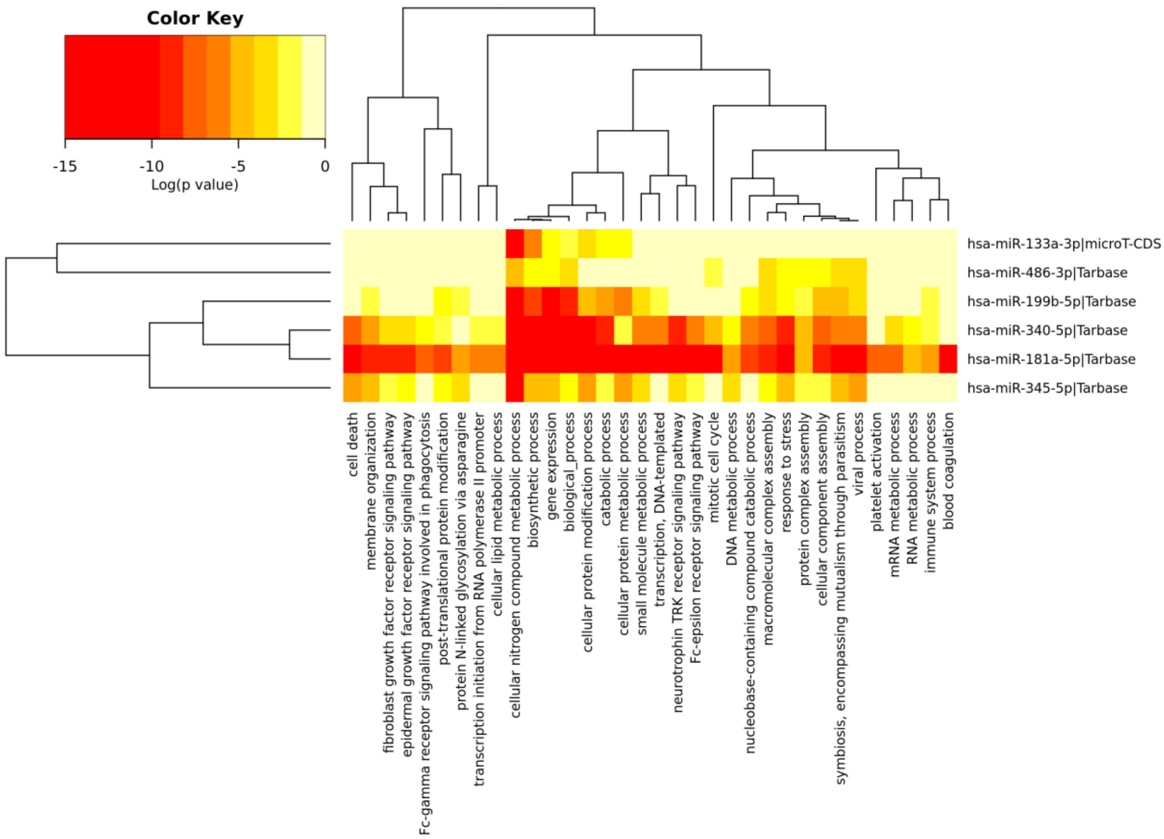
e-Table 6: Pathways enrichment analysis.

| Kegg Pathway | Number of genes | Benjamini |
|---|---|---|
| MAPK signaling pathway | 128 | 6,8E-5 |
| Sphingolipid signaling pathway | 67 | 4,3E-4 |
| Dilated cardiomyopathy | 50 | 6,1E-4 |
| Endocytosis | 124 | 4,6E-4 |
| Hypertrophic cardiomyopathy (HCM) | 47 | 4,7E-4 |
| Adrenergic signaling in cardiomyocytes | 76 | 8,7E-4 |
| Oxytocin signaling pathway | 81 | 7,9E-4 |
| Ras signaling pathway | 109 | 7,4E-4 |
| Renal cell carcinoma | 40 | 7,3E-4 |
| Pathways in cancer | 175 | 7,1E-4 |
| Insulin resistance | 58 | 1,8E-3 |
| Neurotrophin signaling pathway | 63 | 1,8E-3 |

| | | |
|---|---|---|
| cAMP signaling pathway | 95 | 2,1E-3 |
| Retrograde endocannabinoid signaling | 54 | 3,0E-3 |
| Axon guidance | 65 | 2,9E-3 |
| Rap1 signaling pathway | 99 | 2,8E-3 |
| Calcium signaling pathway | 86 | 3,4E-3 |
| Insulin signaling pathway | 69 | 3,6E-3 |
| Fc epsilon RI signaling pathway | 39 | 3,6E-3 |
| Morphine addiction | 49 | 3,7E-3 |
| T cell receptor signaling pathway | 54 | 3,9E-3 |
| Cell adhesion molecules (CAMs) | 70 | 4,5E-3 |
| Cholinergic synapse | 57 | 4,7E-3 |
| Dopaminergic synapse | 64 | 4,8E-3 |
| Oocyte meiosis | 56 | 4,8E-3 |
| Glioma | 37 | 4,9E-3 |
| Regulation of actin cytoskeleton | 97 | 5,8E-3 |
| ErbB signaling pathway | 46 | 7,1E-3 |
| Osteoclast differentiation | 64 | 8,6E-3 |
| mTOR signaling pathway | 33 | 9,5E-3 |
| FoxO signaling pathway | 65 | 9,2E-3 |
| GnRH signaling pathway | 47 | 1,0E-2 |
| Signaling pathways regulating pluripotency of stem cells | 67 | 1,1E-2 |
| VEGF signaling pathway | 34 | 1,1E-2 |
| Leukocyte transendothelial migration | 58 | 1,1E-2 |
| Progesterone-mediated oocyte maturation | 45 | 1,1E-2 |
| Long-term potentiation | 36 | 1,2E-2 |
| Chemokine signaling pathway | 85 | 1,2E-2 |
| AMPK signaling pathway | 59 | 1,4E-2 |
| Hepatitis B | 68 | 1,6E-2 |
| Pancreatic cancer | 35 | 1,7E-2 |
| Non-small cell lung cancer | 31 | 1,8E-2 |

| | | |
|---|---|---|
| Neuroactive ligand-receptor interaction | 119 | 1,8E-2 |
| Arrhythmogenic right ventricular cardiomyopathy (ARVC) | 37 | 2,3E-2 |
| Melanoma | 37 | 2,3E-2 |
| Inositol phosphate metabolism | 37 | 2,3E-2 |
| Prolactin signaling pathway | 37 | 2,3E-2 |
| HIF-1 signaling pathway | 48 | 2,5E-2 |
| Long-term depression | 32 | 2,9E-2 |
| Proteoglycans in cancer | 88 | 2,9E-2 |
| Glucagon signaling pathway | 48 | 3,0E-2 |
| Ubiquitin mediated proteolysis | 63 | 3,3E-2 |
| Cocaine addiction | 27 | 3,3E-2 |
| cGMP-PKG signaling pathway | 74 | 3,8E-2 |
| Wnt signaling pathway | 63 | 3,8E-2 |
| Gap junction | 43 | 3,8E-2 |
| Phosphatidylinositol signaling system | 47 | 3,8E-2 |
| Aldosterone synthesis and secretion | 40 | 4,0E-2 |
| Choline metabolism in cancer | 48 | 4,1E-2 |
| TNF signaling pathway | 50 | 4,1E-2 |
| Focal adhesion | 89 | 4,0E-2 |
| SNARE interactions in vesicular transport | 20 | 4,2E-2 |
| Thyroid hormone signaling pathway | 53 | 4,3E-2 |
| Estrogen signaling pathway | 47 | 4,2E-2 |
| Chronic myeloid leukemia | 36 | 4,3E-2 |
| NF-kappa B signaling pathway | 42 | 4,5E-2 |
| Adipocytokine signaling pathway | 35 | 4,7E-2 |
| Small cell lung cancer | 41 | 4,9E-2 |
| GABAergic synapse | 41 | 4,9E-2 |

e-Figure 6: Main biological processes regulated by the differentially expressed miRNAs.



## ARTÍCULO 3

"*MicroRNA profile of cardiovascular risk in patients with obstructive sleep apnea*". **Fernando Santamaria-Martos**, Iván Benítez, Lucía Pinilla, Francisco Ortega, Andrea Zapater, Cristina Girón, Olga Mínguez, Silvia Gómez, Rafaela Vaca, Jose M. Fernandez-Real, Ferran Barbé and Manuel Sánchez-de-la-Torre. (Submitted).

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# MicroRNA profile of cardiovascular risk in patients with obstructive sleep apnea

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**Keywords:** Obstructive sleep apnea, microRNA, profiling, management, cardiovascular, personalized medicine.

# ABSTRACT

Obstructive sleep apnea (OSA) is a common disease caused by repeated episodes of collapse of the upper airway during sleep and is associated with the development of cardiovascular disease (CVD). However, there is high heterogeneity in the impact of OSA on patients. Until now, the profile of OSA patients at risk of developing CVD has not been defined, including the measurable variables that could be used to predict the CVD risk of a patient with OSA.

To identify the microRNA (miRNA) profile associated with CVD in patients with OSA.

This is an observational, cross-sectional study that included 132 male patients. Three groups were defined: OSA patients, OSA patients with hypertension and OSA patients who developed a major cardiovascular event. Polysomnography and ambulatory blood pressure measurements were performed. The expression profiling of 188 miRNAs in plasma was performed in 21 subjects (matched by body mass index and age) by the TaqMan-Low-Density-Array (TLDA). miRNAs differentially expressed in the different subgroups of patients were selected for validation by RT-qPCR in the 111 remaining patients.

From the TLDA analysis, 5 miRNAs were selected for validation. Differential expression was not confirmed in any of the miRNAs. miR-143 was associated with nocturnal systolic blood pressure. miR-107 correlated with 24-h blood pressure parameters and with nocturnal hypertension.

The circulating profile of miRNAs does not seem to be different in any of the subgroups of patients with OSA and different cardiovascular risk factors. Nevertheless, miR-107 and miR-143 are associated with specific blood pressure parameters in patients with OSA.

## INTRODUCTION

Obstructive sleep apnea (OSA) is a prevalent disease that affects approximately 10-17% of the adult population [1, 2]. OSA is characterized by repetitive episodes of collapse of the upper airway during sleep leading to arousals, nocturnal hypoxemia and changes in intrathoracic pressure [3]. These consequences, through intermediate mechanisms, link OSA with cardiovascular diseases (CVDs) (i.e., high blood pressure, stroke, myocardial infarction, etc.) and a higher overall mortality risk [3–5]. In fact, OSA is one of the most frequent causes of hypertension, and hypertension is the principal risk factor for cardiovascular events (CVEs) [6]. Although numerous studies have identified a significant number of pathophysiological processes that relate OSA to CVD [3], the set of biomarkers that establish this relationship and that would allow a better characterization of OSA and CVD have not yet been fully defined [7, 8]. The identification of biomarkers that can help physicians establish different phenotypes of cardiovascular risk is a research priority in the field.

microRNAs (miRNAs) are a class of small non-coding RNAs that negatively regulate gene expression post-transcriptionally by binding to target mRNAs, thereby leading to either mRNA degradation or translational repression [9]. miRNAs play a pivotal role in several biological processes, such as stress response, apoptosis, proliferation and differentiation, and evidence suggests that they are deregulated in several diseases, including CVD [9–11]. miRNAs are present in body fluids, highly resistant to degradation and easily measurable, fulfilling the criteria of an ideal biomarker in an era of evolving precision medicine [12]. Our group previously identified the potential of miRNAs in patients with resistant hypertension and OSA [10].

Nevertheless, the potential miRNAs for the characterization of the different phenotypes of OSA and CVD have not been defined.

In the present study, we aimed to examine circulating miRNAs to establish a potential specific miRNA profile for the clinically relevant phenotypes of cardiovascular risk in patients with OSA. To accomplish this, we explored the miRNA profiles of OSA patients without CVD, OSA patients with hypertension, and OSA patients who have suffered a major cardiovascular event. These three populations represent the natural history of CVD in patients with OSA.

## METHODS

### *Study cohort*

Recruitment took place in the Sleep Unit of the University Hospital Arnau de Vilanova-Santamaria of Lleida (Spain) between 2014 and 2017 (ClinicalTrials.gov Registration number: NCT03513926). Patients were referred to the sleep unit for suspected OSA. All recruited patients signed an informed consent form, and the ethics committee of the centre (Clinical Research Ethics Committee of the Arnau de Vilanova-Santa Maria Hospital University Hospital) approved the study. All methods were performed in accordance with current clinical practice guidelines and regulations. Male subjects were eligible if they were between 18 and 60 years old. The OSA diagnosis was made using a conventional polysomnographic sleep study. A 24-h blood pressure evaluation was performed by ambulatory blood pressure monitoring (ABPM). Subjects with an apnea-hypopnea index (AHI) of 15 or more events/h were grouped according to their clinical cardiovascular risk profile into the following groups: 1) OSA group: OSA patients without CVD (AHI ≥15 events/h, normal ABPM and no previous history of CVEs); 2) OSA hypertensive group: OSA patients with hypertension (AHI ≥15 events/h, abnormal ABPM or previous diagnosis of hypertension, without previous CVEs); or 3) OSA cardiovascular group: OSA patients with a previous major CVE (AHI ≥15 events/h and previous stroke or myocardial infarction). The initial exclusion criteria included patients with previous use of continuous positive airway pressure (CPAP) and any condition that, in the opinion of the responsible physician investigator, made a subject unsuitable for the study (e.g., pregnancy, drug use or alcohol consumption).

*Sample collection*

A venous fasting blood sample was obtained from each patient at baseline in the morning immediately after the sleep study between 08:00 and 09:00 a.m. The blood samples were centrifuged to separate plasma, and all specimens were immediately aliquoted, frozen, and stored in a dedicated -80°C freezer. No freeze-thaw cycles were performed during the experiment.

*Clinical measurements*

All patients underwent polysomnography. Apnea was defined as an interruption or reduction in oronasal airflow of ≥ 90% that lasted at least 10 seconds. Hypopnoea was defined as a 30% to 90% reduction in oronasal airflow for at least 10 seconds associated with an oxygen desaturation of at least 3% or an arousal on the electroencephalogram. The AHI was defined as the number of apnea and hypopnea events per h of sleep. All participants were subjected to 24-h ABPM (Mortara Ambulo 2400, Milwaukee, EE.UU.). Hypertension was defined by the use of antihypertensive drugs or was based on the ABPM measurements according to the guidelines [13]. Hypertension was defined as a 24 h systolic BP ≥ 130 mmHg or a 24 h diastolic BP ≥ 80 mmHg.

*Circulating RNA isolation and purification*

RNA extraction was performed from 300 μL of plasma using a mirVana PARIS isolation kit (Applied Biosystems, Vilnius, Lithuania) according to the manufacturer's instructions. For quality control, non-human cel-miR-39 was spiked into the plasma immediately before extraction. RNA concentration and integrity were examined by RT-qPCR quantification of cel-miR-39.

*Circulating miRNA profiling with TLDA*

To identify the plasma miRNA profiles of cardiovascular risk in OSA patients, we screened 188 circulating miRNAs that have been reported as the most consistent and reliable miRNAs in human plasma samples [14, 15]. This circulating profile was evaluated in a cohort of 21 patients (TaqMan Low Density Array (TLDA) cohort), including 8 OSA patients, 7 OSA hypertensive patients and 6 OSA with CVE patients matched for age and body mass index (BMI) (Figure 1). Multiple real-time RT-PCRs were performed using a customized TLDA (Life Technologies, Foster City, CA, USA). Briefly, a fixed volume of 3 μL of RNA solution from the 40 μL of RNA isolation eluate was used as the input for the retrotranscription, using the TaqMan MicroRNA Reverse Transcription Kit and TaqMan MicroRNA Multiplex RT Assays, which were customized to run TLDAs. Preamplification was performed using TaqMan PreAmp Master Mix and Megaplex PreAmp Primers for our selected miRNAs. RT-PCR was carried out using an Applied Biosystems QuantStudio™ 7 Flex Real-Time PCR System. Data were processed with the Relative Quantification tool (powered by Thermo Fisher cloud), with a minimal threshold above the baseline ($\triangle$Rn=0.012) and less than 35 thermal cycles (Ct). The results were normalized using the mean-center normalization method, which is the gold-standard method when screening a large number of miRNAs [16].

*Analysis of individual miRNAs*

The most reliable and consistent candidates for endogenous control (i.e., hsa-miR-106a and hsa-miR-186) were identified in the TLDA analyses and were used together with the spike-in control (cel-miR-39) for normalization [17]. Then, individual TaqMan hydrolysis probes (Applied Biosystems) were applied to analyse the expression of the differentially expressed miRNAs in the qPCR cohort (Figure 1).

*Statistical analysis*

Comparisons between the subgroups of OSA patients were assessed using the Kruskal-Wallis test for quantitative variables and Fisher's exact test for qualitative variables. The differences in miRNA expression between groups were evaluated using linear models for microarray data (LIMMA) [18]. Spearman's rank correlation coefficient was used to evaluate the association between miRNA levels and clinical parameters. False discovery rate-adjusted p-values were calculated to adjust for the performance of multiple paired comparisons. R software (R Project for Statistical Computing, Vienna, Austria) was used for statistical analysis.

**RESULTS**

*Characteristics of patients in the TLDA cohort*

The patients were middle-aged, overweight-obese and male (Table 1). The OSA group was slightly younger and had a lower BMI than the other groups. A similar AHI and arousal index was found between groups, but the time with oxygen saturation under 90% was higher in the OSA with hypertension group and the OSA with CVE group than in the OSA without CVD group.

*Identification of plasma miRNAs differentially expressed in OSA populations with cardiovascular risk*

The miRNA profiles were used to identify miRNAs related to cardiovascular risk in the OSA patients in the TLDA cohort. After miRNA qPCR TLDA-based expression analysis, a subset of 4 differentially expressed miRNAs (based on an individual p-value lower than 0.05) was selected for subsequent analysis. miR-148b and miR-301 were significant when comparing the OSA group vs. the OSA hypertensive group. miR-143b and miR-140-3p were significant when comparing the OSA hypertensive group vs. the OSA with CVE group. Although miR-107 did not reach statistical significance, it was selected because previous literature related it to arterial hypertension in OSA patients (Table 2) [19].

*Characteristics of patients in the validation cohort*

An independent set of patients was selected for the validation process. They were middle-aged, and the patients in the OSA group with CVE were slightly older than those in the other groups. The OSA with CVD group had a higher BMI than the OSA group. Respiratory parameters were worse in the OSA with hypertension group, especially TSat 90 (See Table 3).

*Validation of differentially expressed miRNAs*

All potential cardiovascular risk-related miRNAs selected from TLDA were validated in the qPCR cohort. The analyses did not confirm that the circulating concentrations of miR-148b, miR-301, miR-143, miR-140-3p and miR-107 were altered after the adjustment for BMI and age (Table 4). Nevertheless, miR-143 and miR-107 are noteworthy. miR-143 is associated with the arousal index (rho= -0.27, p-value= 0.012) and nocturnal systolic blood pressure (rho= 0.23, p-value= 0.035). miR-107 seems to correlate with 24 h blood pressure parameters (rho= 0.19, p-value= 0.091 for 24-h systolic blood pressure and rho= 0.18, p-value= 0.108 for 24-h diastolic blood pressure) and especially with nocturnal hypertension (rho= 0.23, p-value= 0.057 for nocturnal systolic blood pressure and rho= 0.21, p-value= 0.058 for nocturnal diastolic blood pressure).

**DISCUSSION**

In the present study, we evaluated the circulating epigenetic profile of cardiovascular risk in OSA patients. This observational study showed that miRNAs do not seem to be associated with the clinical categories of cardiovascular risk in OSA patients. Despite this finding, in OSA patients, miR-143 and miR-107 were associated with ambulatory blood pressure parameters.

Recent studies have demonstrated the importance of miRNAs in the control of many processes in health and disease [12, 14]. Our previous study related the response in blood pressure reduction after CPAP treatment in patients with OSA and resistant hypertension with a miRNA signature [10]. To the best of our knowledge, this is one of the first studies to explore miRNAs to characterize different OSA populations in patients who are referred to the sleep unit, focusing on cardiovascular risk. The circulating concentrations of miRNAs were not significantly deregulated when comparing healthy OSA patients (without cardiovascular comorbidities) with OSA patients with hypertension or when comparing OSA patients with hypertension with OSA patients with a previous major CVE. The clinical differences measured in cardiovascular history and hypertension did not show a specific circulating epigenetic fingerprint. Similar results were found in a previous work [19], where researchers identified differences between non-OSA and healthy OSA patients and between non-OSA and OSA with arterial hypertension patients, but not between healthy OSA and OSA with hypertension patients. This could be because chronic hypoxia impairs Dicer (DICER1) expression and activity, resulting in a global downregulation of miRNA expression [20]. A general downregulation of miRNA expression could mask the differences between groups, as the hypoxia produced by OSA is a saturating element in the expression of miRNAs, limiting the use of miRNAs as potential biomarkers of cardiovascular risk in OSA.

Nevertheless, miR-143 and miR-107 correlate especially with nocturnal ABPM measurements. miR-107 has been identified to contribute to vascular remodelling in some hypoxic conditions by targeting HIF-1β [21]. The intermittent hypoxia caused by OSA could contribute, through the downregulation of miR-107, to worse vascular remodelling. This could be one of the possible mechanisms that associate OSA with high blood pressure. Additionally, miR-143 is associated with a loss of vascular myogenic tone [22], altering smooth muscle cell maintenance and vascular homeostasis [23], being one of the most useful biomarkers in CVD [24]. Additional studies are needed to elucidate the implications of miR-107 and miR-143 in hypertension in patients with OSA.

The present study has several limitations that deserve comment. First, only patients between 18 and 60 years old were studied, and larger studies should be performed to determine the validity of miRNAs in detecting cardiovascular risk profiles in patients of different ages. Due to the different pathophysiologies of OSA in women and the previously demonstrated sex bias in miRNA expression [25], further studies are needed to evaluate the profile of miRNAs in women with OSA. The strengths of this study reside in a large number of patients who were analysed in two different sets (the TLDA and validation cohorts), which allowed us to detect high-magnitude associations. Finally, all patients were recruited from the sleep unit, with the use of gold-standard methods for OSA diagnosis (polysomnography), blood pressure measurements (ABPM) and miRNA measurement (RT-qPCR).

The circulating profile of miRNAs does not seem to be altered in different OSA cardiovascular risk populations, making it unsuitable as a biomarker to assess cardiovascular risk in patients with OSA. Nevertheless, miR-107 and miR-143 could explain the mechanisms of hypertension in patients with OSA.

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## TABLES

Table 1: Baseline characteristics of the TaqMan Low Density Array (TLDA) cohort, matched by age and BMI.

| | OSA N=8 | OSA+HBP N=7 | OSA+CVE N=6 | p-value |
|---|---|---|---|---|
| Demographic and clinical variables | | | | |
| Age (years) median [IQR] | 43.0 [26.5;47.5] | 54.0 [50.0;55.5] | 55.0 [51.0;56.8] | 0.078 |
| BMI (kg/m2) median [IQR] | 25.6 [24.3;28.2] | 27.2 [26.4;28.3] | 30.5 [28.5;31.1] | 0.09 |
| Smoking status: n (%) | | | | 0.012 |
| Non-smoker | 4 (50.0%) | 2 (28.6%) | 1 (16.7%) | |
| Smoker | 4 (50.0%) | 0 (0.00%) | 1 (16.7%) | |
| Former smoker | 0 (0.00%) | 5 (71.4%) | 4 (66.7%) | |
| Respiratory parameters | | | | |
| AHI (events/h) median [IQR] | 34.9 [26.8;43.5] | 31.6 [23.9;38.6] | 40.7 [30.3;77.5] | 0.414 |
| TSat 90 (%) median [IQR] | 0.46 [0.27;3.57] | 4.10 [1.07;12.4] | 4.29 [1.81;7.85] | 0.397 |
| Arousal index (events/h) median [IQR] | 41.4 (13.5) | 39.9 (13.7) | 48.0 (14.4) | 0.553 |
| ESS (0-24) median [IQR] | 9.00 (5.40) | 12.3 (4.50) | 5.80 (2.86) | 0.093 |
| ABPM parameters | | | | |
| 24 h systolic blood pressure (mmHg) median [IQR] | 114 [111;121] | 146 [140;151] | 125 [112;142] | 0.009 |
| 24 h diastolic blood pressure (mmHg) median [IQR] | 73.7 (2.50) | 89.6 (4.23) | 79.1 (10.00) | <0.001 |
| Daytime systolic blood pressure (mmHg) median [IQR] | 119 (6.37) | 148 (11.6) | 129 (19.7) | 0.002 |
| Daytime diastolic blood pressure (mmHg) median [IQR] | 75.1 (2.55) | 91.1 (5.23) | 81.5 (12.8) | 0.003 |
| Nighttime systolic blood pressure (mmHg) median [IQR] | 110 [108;114] | 139 [136;144] | 119 [101;138] | 0.003 |
| Nighttime diastolic blood pressure (mmHg) median [IQR] | 71.0 [67.7;73.2] | 84.6 [83.2;88.8] | 74.2 [66.8;81.4] | <0.001 |

HBP= High blood pressure; CVE; Cardiovascular event; BMI = Body mass index; AHI = Apnea-Hypopnea Index; TSat 90 = nighttime oxygen saturation less than 90%; ESS= Epworth Sleepiness Scale.

Table 2: miRNA candidates for cardiovascular risk in OSA patients. Five miRNAs were selected based on their fold change value and previous literature.

| miRNA | Comparison | Fold change | p-value |
|---|---|---|---|
| hsa-miR-148b | HBP vs. OSA | 0.63 | 0.02 |
| hsa-miR-301 | HBP vs. OSA | 0.68 | 0.02 |
| hsa-miR-143 | CVE vs. HBP | 0.44 | 0.02 |
| hsa-miR-140-3p | CVE vs. HBP | 1.43 | 0.05 |
| hsa-miR-107 | CVE vs. HBP | 1.52 | 0.09 |

HBP= High blood pressure; CVE; Cardiovascular event.

Table 3: Baseline characteristics of the validation cohort.

| | OSA N=27 | OSA+HBP N=64 | OSA+CVE N=20 | p-value |
|---|---|---|---|---|
| Demographic and clinical variables | | | | |
| Age (years) median [IQR] | 44.0 [39.5;53.5] | 48.0 [45.0;54.0] | 53.0 [51.0;56.0] | 0.006 |
| BMI (kg/m2) median [IQR] | 29.9 (6.46) | 33.8 (5.30) | 32.4 (5.89) | 0.015 |
| Smoking status: n (%) | | | | 0.098 |
| Non-smoker | 11 (40.7%) | 17 (27.0%) | 3 (15.0%) | |
| Smoker | 6 (22.2%) | 27 (42.9%) | 6 (30.0%) | |
| Former smoker | 10 (37.0%) | 19 (30.2%) | 11 (55.0%) | |
| Respiratory parameters | | | | |
| AHI (events/h) median [IQR] | 38.3 [25.7;52.4] | 49.3 [35.1;80.0] | 45.6 [29.1;62.1] | 0.044 |
| TSat 90 (%) median [IQR] | 2.70 [1.00;4.80] | 12.6 [3.93;30.2] | 3.38 [0.79;12.6] | 0.001 |
| Arousal index (events/h) median [IQR] | 39.5 [29.2;57.9] | 45.5 [35.7;68.6] | 52.8 [39.9;60.7] | 0.34 |
| ESS (0-24) median [IQR] | 10.5 (4.88) | 9.66 (4.69) | 10.6 (4.47) | 0.616 |
| ABPM parameters | | | | |
| 24 h systolic blood pressure (mmHg) median [IQR] | 118 [115;124] | 136 [128;147] | 141 [121;144] | <0.001 |
| 24 h diastolic blood pressure (mmHg) median [IQR] | 74.7 (3.83) | 85.2 (6.28) | 79.4 (7.09) | <0.001 |
| Daytime systolic blood pressure (mmHg) median [IQR] | 122 [118;128] | 138 [130;149] | 140 [124;146] | <0.001 |
| Daytime diastolic blood pressure (mmHg) median [IQR] | 76.9 [74.8;79.8] | 86.2 [83.0;91.1] | 82.0 [77.8;85.3] | <0.001 |
| Nighttime systolic blood pressure (mmHg) median [IQR] | 108 [100;113] | 128 [117;145] | 129 [113;140] | <0.001 |
| Nighttime diastolic blood pressure (mmHg) median [IQR] | 69.3 [66.5;71.5] | 79.4 [74.7;85.0] | 75.2 [69.9;79.0] | <0.001 |

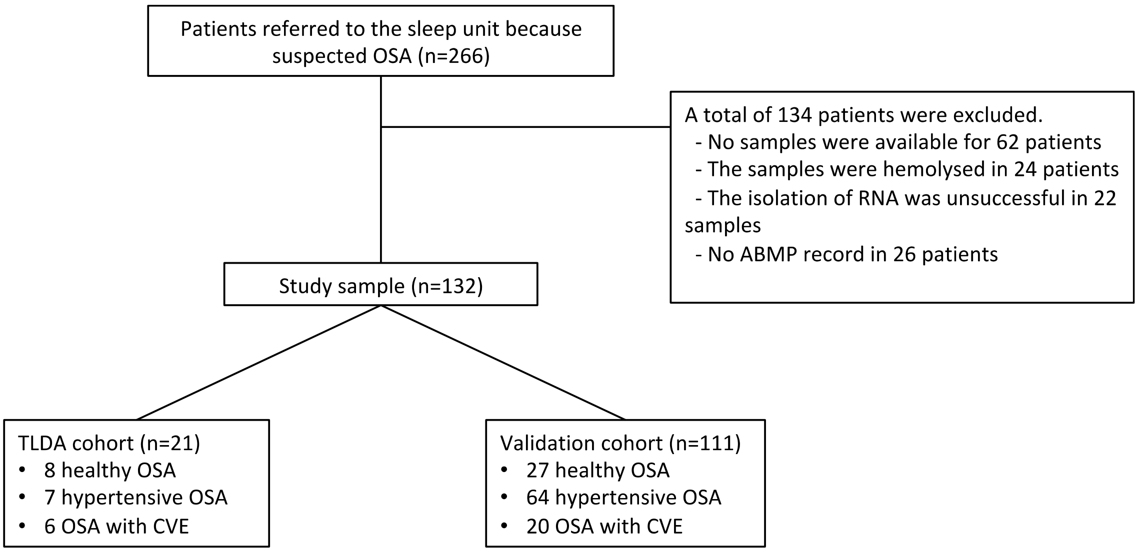
HBP= High blood pressure; BMI = Body mass index; AHI = Apnea-Hypopnea Index; TSat 90 = nighttime oxygen saturation less than 90%; ESS= Epworth Sleepiness Scale.

Table 4: Validation of miRNA candidates. P-values are adjusted for age and body mass index.

| miRNA | Comparison | Fold change | p-value | FDR correction |
|---|---|---|---|---|
| hsa-miR-148b | HBP vs. OSA | 0.87 | 0.55 | 0.67 |
| hsa-miR-301 | HBP vs. OSA | 0.83 | 0.45 | 0.67 |
| hsa-miR-143 | CVE vs. HBP | 1.41 | 0.33 | 0.86 |
| hsa-miR-140-3p | CVE vs. HBP | 1.08 | 0.79 | 0.86 |
| hsa-miR-107 | CVE vs. HBP | 1.04 | 0.86 | 0.86 |

## FIGURES

Figure 1: Flowchart of the study.

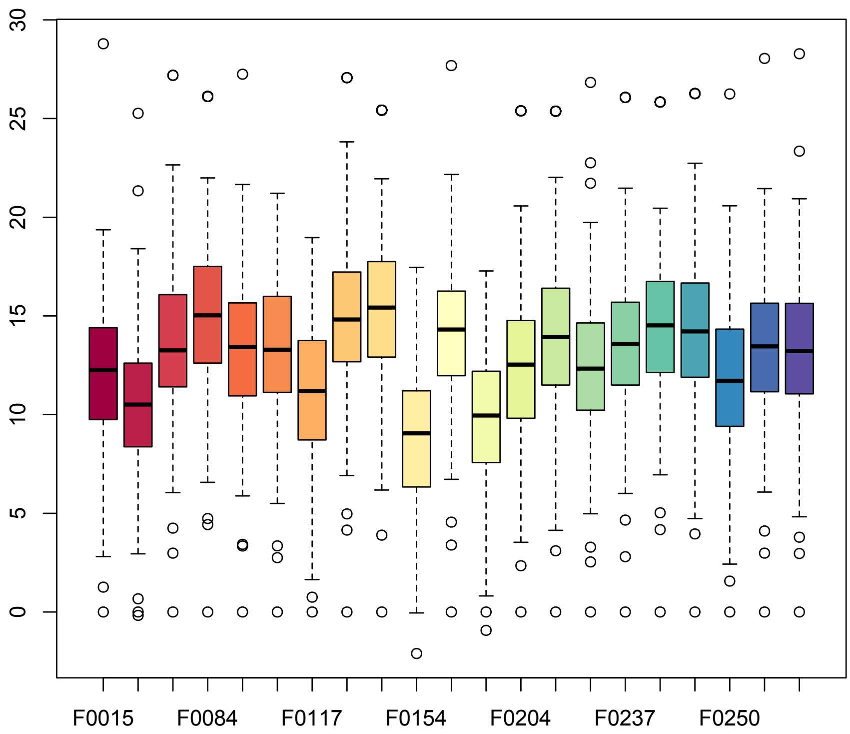



## SUPPLEMENTARY MATERIAL

### 1. TaqMan Low Density Array determinations

1.1. Quality control

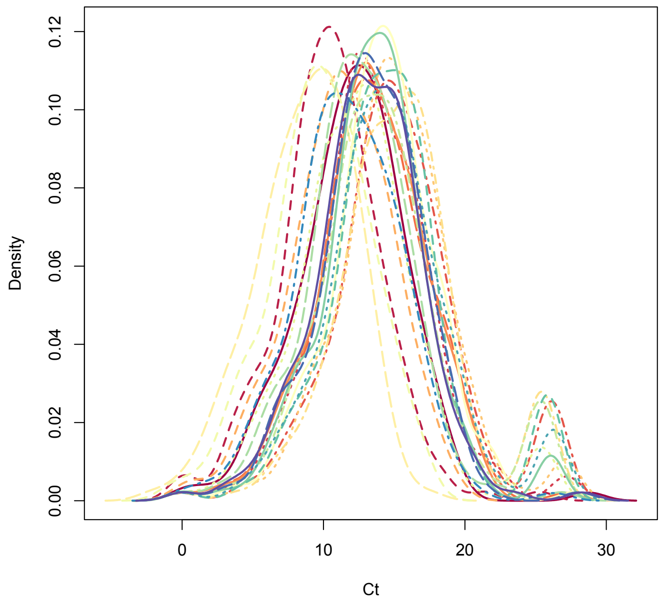
e-Figure 1: Raw Ct distribution of miRNAs.



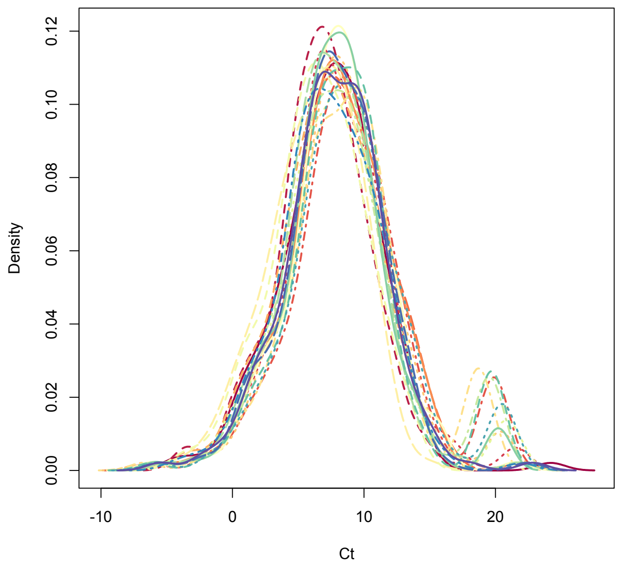
1.2. miRNA normalization.

e-Figure 2: Density plot of miRNA normalization. A) density of raw Cts of each subject. B) Mean-center normalized density plot.

A)



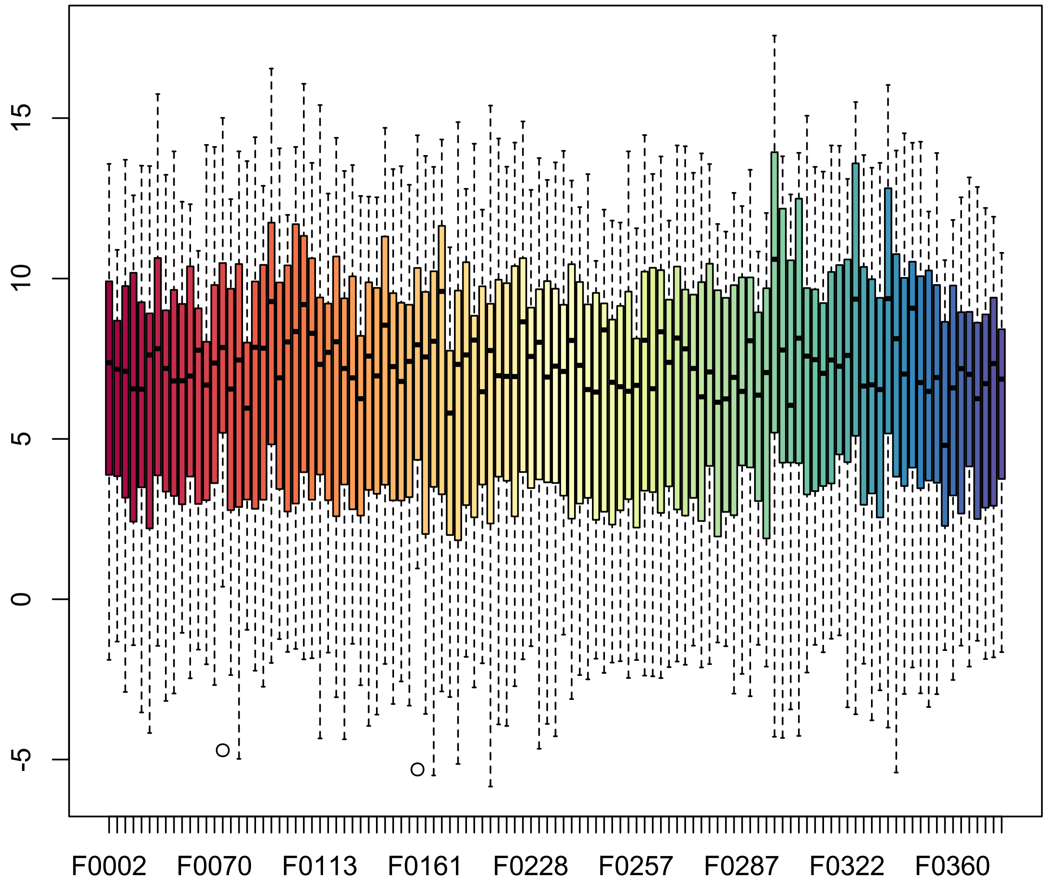

B)



## 2. qPCR determinations

2.1. Quality control

e-Figure 3: Raw Ct distribution of miRNAs.



## ARTÍCULO 4

"*Biomarkers of carcinogenesis and tumour growth in patients with cutaneous melanoma and obstructive sleep apnoea".* **Fernando Santamaria-Martos**, Ivan Benítez, Cristina Girón, Ferran Barbé, Miguel-Angel Martínez-García, Luis Hernández, Josep M. Montserrat, Eduardo Nagore, Antonio Martorell, Francisco Campos-Rodriguez, Jaime Corral, Valentin Cabriada, Jorge Abad, Olga Mediano, Maria F. Troncoso, Irene Cano-Pumarega, Ana Maria Fortuna Gutierrez, Trinidad Diaz-Cambriles, Maria Somoza-Gonzalez, Isaac Almendros, Ramon Farre, David Gozal and Manuel Sánchez-de-la-Torre, on behalf of The Spanish Sleep Network. ". European Respiratory Journal. 2018 Mar 15;51(3). pii: 1701885.

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# Biomarkers of carcinogenesis and tumour growth in patients with cutaneous melanoma and obstructive sleep apnea

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**Keywords:** Obstructive sleep apnea, cancer, melanoma, biomarkers, VCAM-1

**Take-Home Message:** In patients with cutaneous melanoma, OSA is associated with an increased expression of a biomarker of tumorigenesis.

**Abstract**

The goal of this study was to assess the relationship between the severity of OSA and the levels of carcinogenesis- and tumour growth-related biomarkers, in patients with cutaneous melanoma (CM).

This multicentre observational study included patients who were newly diagnosed with melanoma. The patients were classified as non-OSA (apnoea–hypopnoea index (AHI); 0-5 events/h), mild-OSA (AHI; 5-15 events/h), and moderate to severe OSA (AHI>15 events/h). Enzyme-linked immunosorbent assays (ELISAs) were performed to analyze the serum levels of hypoxia- and tumour adhesion-related biomarkers (VEGF, IL8, ICAM-1, VCAM-1), and markers of tumour aggressiveness (S100B, MIA). A logistic model adjusted for age, sex and body mass index (BMI) was fitted to each biomarker, and the AHI served as the dependent variable.

A total of 360 patients were included (52.2% male, median [IQR]; 55.5 [43.8-68.0] years, AHI of 8.55 [2.8-19.5] events/h). The levels of VEGF, IL-8, ICAM-1, S100B and MIA were not related to the severity of OSA. The levels of VCAM-1 were higher in patients with OSA than those without OSA (mild OSA: OR=2.07,p=0.021; moderate-severe OSA: OR=2.35,p=0.013).

In patients with cutaneous melanoma, OSA was associated with elevated circulating levels of VCAM-1 that could indicate the contribution of OSA in tumorigenesis via integrin-based adhesion.

## Introduction

Obstructive sleep apnea (OSA) is a prevalent disease that affects at least 10% of the adult population [1–3]. OSA is characterized by a repetitive obstruction of the upper airway that leads to intermittent hypoxia and sleep disruption. OSA is associated with an increased risk of cardiovascular and neurocognitive consequences [4].

Cutaneous melanoma (CM) is a tumour with a melanocytic origin. The incidence of melanoma has increased worldwide, and 10-30 new melanoma cases per 100,000 inhabitants are estimated to occur in Europe and the USA. Melanomas account for 90% of deaths associated with cutaneous tumours [5]. No effective treatments are available once this malignant tumour has spread [6–8]. The oncogenic transformation of melanocytes to melanoma is the result of complex changes in multiple physiopathological pathways that control cell cycle progression and apoptosis [9]. The identification of treatable diseases that accelerate the progression of melanoma and the pathways involved in this progression can be crucial for reducing the mortality from melanoma.

Recently, OSA has been shown to be associated with a higher cancer prevalence, incidence and mortality [10–12]. Specifically, two recent studies have demonstrated the existence of a relationship between OSA and CM aggressiveness [6, 13]. Systemic inflammation due to hypoxia-reoxygenation cycles in OSA may activate diverse physiopathological pathways that enhanced tumour progression [14, 15]. Both diseases are associated with elevated circulating levels of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) [16–18] and vascular cell adhesion molecule-1 (VCAM-1) [17, 19, 20], inflammation cytokines, such as vascular endothelial growth factor (VEGF) [21, 22] and interleukin-8 (IL-8) [7, 16, 23], and biomarkers of the prognosis and severity of melanoma, such as S100 calcium binding protein B (S100B) [7, 24] and melanoma inhibitory activity (MIA) [7].

Patients with OSA could be at an increased risk of suffering from neoplastic processes, but the specific roles played by OSA have not been clearly established to date [25]. The objective of the present study was to evaluate the relationship between the biomarkers of carcinogenesis and tumour growth and OSA. Therefore, we measured six biomarkers that represent different physiopathological pathways that may link OSA and melanoma.

## Methods

### Study design

This ancillary, observational, prospective, cross-sectional and multicentre clinical study was conducted in 29 teaching hospitals in Spain and included patients with CM. Briefly, the relationship between the presence and severity of sleep disordered breathing and melanoma aggressiveness was analysed. This study was approved by the ethics committees of each of the participating hospitals. All participants provided signed informed consent to participate in the study.

### Patient selection

The patients were consecutively recruited at the dermatology or oncology units of each participating centre. The patients were initially eligible for participation in the study if they had a diagnosis of invasive CM, were older than 18 years and signed the informed consent to participate in the study. The initial exclusion criteria included primary unknown melanoma, extracutaneous melanoma, *in situ* melanoma, pregnancy, daytime respiratory or heart failure, and current or previous use of continuous positive airway pressure therapy (CPAP). Because the metastatic status of the patients may strongly influence the levels of the circulating biomarkers of cancer, those patients with metastatic status were excluded (Figure 1).

### Sleep studies

The patients included in the study underwent polygraphy for a maximum of 6 months after the CM diagnosis at the sleep unit of each centre. The respiratory polygraphy included a continuous recording of the oronasal flow and pressure, heart rate, thoracic and abdominal respiratory movements, and oxygen saturation ($SaO_2$). Trained personnel manually scored the polygraphy data. Apnea was defined as an interruption of the oronasal airflow for >10 seconds. Hypopnea was defined as a 30-90% reduction in the oronasal airflow for >10 seconds, which was associated with an oxygen desaturation ≥3%. The apnea-hypopnea index (AHI) was defined as the number of apnea events plus hypopnea events per hour during the recording, while the $Tsat_{90}$ was defined as the percentage of the recording time with an $SaO_2$<90%. The baseline saturation, mean saturation, minimum saturation, and oxygen desaturation index (ODI) at 3% and 4% were also measured.

The patients were divided into the following three groups: the non-OSA group (AHI: 0-5 events·$h^{-1}$), the mild sleep apnea group (AHI: 5-15 events·$h^{-1}$) and the moderate-severe sleep apnea group (AHI: >15 events·$h^{-1}$).

Evaluation of the growth rate of CM

CM growth rate was calculated as the Breslow index (in millimetres) divided by the difference between the date of the excision of the tumour and the date on which the patient noticed changes suggestive of malignant transformation in a stable, pre-existing lesion or the time he/she noticed the appearance of a new and changing lesion [6]. CM growth rate was categorized as follows: slow-intermediate growth rate (<0.49 mm·$month^{-1}$) and fast growth rate (≥0.5 mm·$month^{-1}$) [26].

Measurements of circulating molecules

Fasting venous blood samples were drawn between 08.00 and 09.00 a.m. The blood samples were centrifuged to separate the serum, and all specimens were immediately aliquoted, frozen, and stored at -80°C.

Two different methods, i.e., multiplex analyses and traditional enzyme-linked immunosorbent assays (ELISAs), were used to quantify the circulating molecules. Briefly, the Q-Plex TM ELISA technology allows for the simultaneous measurement and detection of multiple proteins at the microscale level. This technology involves an array of quantitative sandwich ELISAs that use standard curves for data interpolation. Using the sandwich ELISAs, capture antibodies are spotted into a 96-well microtiter plate and bind to target proteins in the sample, and secondary or detection antibodies bind to the different epitopes on the target proteins. Streptavidin-HRP is pre-conjugated to secondary antibodies and used to generate a signal. The measured signal is proportional to the amount of the target protein in the sample.

The two multiplexed proteins (including VEGF and IL-8) are analysed using the Q-View Imager. Individual ELISAs were performed to detect VCAM-1, ICAM-1, MIA and S100B following the manufacturer's recommendations. For each assay, standard curves were generated to determine the concentrations of the proteins. The sensitivity and ELISA trademark for each protein are presented in e-Table 1.

Measurements of molecules in postexcisional melanoma tumour tissues

The expression levels of VEGF and the levels of hypoxia inducible factor 1 alpha (HIF-1α) were measured and previously reported in this cohort of patients [27] to determine whether assessments of the abundance of these biomarkers in post-excisional melanoma tumour tissues may lead to a more accurate determination of tumour aggressiveness. For the present study, we evaluated the association of the expression levels of these biomarkers with OSA severity. The levels of VEGF and HIF-1α were assessed by immunohistochemistry on 4-μm-thick sections of formalin-fixed, paraffin-embedded melanoma samples. Immunostaining was performed on a Leica Bond-III stainer (Leica Biosystems, Newcastle, UK) using the Leica Bond Polymer Refine Kit (Leica Biosystems). Melanoma samples were stained with a 1:2 dilution of anti-VEGF prediluted antibody (ab27620; Abcam, Cambridge, UK) and with a 1:100 dilution of anti-HIF-1α antibody (ab51608; Abcam). The staining results were analysed independently by two expert pathologists who were blinded to the staging and clinical features of the patients. VEGF-specific staining was scored considering both the percentage of the positive (<25, 25–50, 51–75, and >75%) and the expression intensity (negative-low or high). HIF-1α-specific nuclear staining was scored considering the presence or absence of positive cells.

Statistical analysis

The quantitative variables are expressed as the medians and interquartile ranges (IQR) since these variables were non-normally distributed. Absolute and relative frequencies were used to describe the qualitative variables. We performed a bivariate analysis to analyze the studied variables according to the AHI group. The Kruskal-Wallis test was performed to compare the quantitative variables, and the Pearson's chi-squared test or Fisher exact test was performed to compare the qualitative variables. A logistic regression model (adjusted for age, sex and body mass index (BMI)) was fitted for each of the ELISAs as the explanatory variable(s), and the AHI, which was categorized by severity (non-OSA, mild OSA, and moderate/severe OSA), was the dependent variable. The calibration was assessed using the Hosmer-Lemeshow goodness-of-fit test. All tests were two-tailed, and a statistical significance level of 0.05 was used. The statistical analyses were performed using R version 3.3.1.

## Results

Patient characteristics

Of the 476 patients who were initially recruited, 360 patients with a diagnosis of CM who underwent a sleep study and provided at least one serum sample were included in the final analysis (Figure 1). The clinical characteristics and demographic variables of the patients are shown in Table 1. The study population included middle-aged men and women who were overweight and had mild sleep apnea (an AHI of 8.55 events·$h^{-1}$ [range: 2.80-19.5 events·$h^{-1}$]).

Analysis of serum markers

The results of the measurements of the circulating levels of VEGF, IL-8, ICAM-1, VCAM-1, S100B and MIA are presented in Figure 2. The serum levels of IL-8, ICAM-1, S100B and MIA were similar among the non-OSA, mild OSA and moderate/severe OSA patients ($p = 0.436$, $p = 0.259$, $p = 0.560$, and $p = 0.328$, respectively). These biomarkers, which are related to the inflammation, progression, and prognosis in CM, were not significantly related to the severity of OSA. In contrast, the serum levels of VEGF exhibited an increased tendency ($p = 0.165$) in both the mild OSA (odds ratio (OR) = 1.26 [95% C.I.: 0.70-2.28]) and moderate-severe sleep apnea (OR = 1.61 [95% C.I.: 0.85-3.06]) groups (see Figure 2 and Table 2). Similarly, we observed that the serum levels of VCAM-1 were significantly higher in the sleep apnea patients than those in the non-OSA patients, for mild OSA (OR = 2.07 [95% C.I.: 1.12-3.89]; $p = 0.021$) and for moderate-severe OSA (OR = 2.35 [95% C.I.: 1.20-4.66]; $p = 0.013$) after adjusting for BMI, age, and gender (Table 2 and Figure 2). Similar results were obtained by comparing the groups according to the ODI terciles (data not shown).

Analysis of tumour tissues samples

Assessment of the levels of VEGF and HIF-1α expression in the tissue samples from the resected tumour are presented in Table 3. Although a tendency was observed in the HIF-1α proportion of positive stained tumours in patients with moderate/severe OSA, the adjusted analysis reported similar proportions among non-OSA patients, mild OSA (OR = 1.52 [95% C.I.: 0.64-3.75]; $p = 0.35$) and moderate/severe OSA (OR = 1.82 [95% C.I.: 0.73-4.77]; $p = 0.21$) (Table 3). For VEGF, the proportion of stained cells in tumour tissues samples was higher than 75% for the three groups. OR was obtained for VEGF expression in tissue samples of the resected tumour and no significant differences emerged between groups (Table 3).

Relationship between CM growth rate and VCAM-1

VCAM-1 levels were categorized as follows according to the mean levels: high levels of VCAM-1 (above the mean) and low levels of VCAM-1 (below the mean). It was observed a tendency of high serum levels of VCAM-1 positively associated with a fast CM growth rate (Figure 3) (1.59 [95% C.I.: 0.91-2.76], but this association did not reach statistical significance ($p = 0.112$).

**Discussion**

In the present study we described that in a cohort of patients with cutaneous melanoma, the OSA severity was related to the serum levels of the VCAM-1 tumour adhesion-related biomarker, but not to the serum levels of markers related with tumour aggressiveness, such as VEGF, IL-8, ICAM-1, S100B and MIA, or levels of VEGF and HIF-1α expression in tissue samples of the tumour.

The increase in the circulating levels of ICAM-1 and the correlations between ICAM-1 and AHI have been previously reported [16, 19, 28]. The circulating levels of VEGF are frequently increased in OSA patients and play a role in the regulation of tissue oxygen delivery [22, 28]. The levels of IL-8 have been reported to be higher in OSA patients than those in healthy controls [16, 29], while the S100B levels are increased in OSA patients but do not correlate with the severity of the disease [24, 30]. We only observed increased levels of VCAM-1 and moderate increased levels of VEGF, which may be due to the differences between the populations since our cohort included patients with CM and OSA; the CM effect, that is independently related with the increase of VEGF [31, 32], together with IL-8 [28, 32], ICAM-1 [18, 33], S100B [7, 34, 35] and MIA [7, 35, 36], could mask the OSA effect on these proteins, which may explain why we did not observe the previously reported differences in these biomarkers in the patients with OSA. Although the possibility exists that the presence of CM may exert a potential ceiling effect on the contributions of OSA to the biomarkers of interest, the study was not designed to address this issue, which therefore must remain speculative at present. In the present study, we were not able to evaluate the specific separate effects of OSA and CM. This further buttresses the need to disassociate the relative contributions of OSA and VM to biomarkers such as those explored in the study. However, the study was not designed to address this specific possibility.

Indeed, we would have needed to include two additional matched groups, no CM and no OSA, OSA alone in the absence of CM, and then further evaluate the effect of treating OSA on the biomarkers of interest to segregate the independent and overlapping contributions of CM and OSA to these biomarkers.

Several pathophysiological mechanisms, including key intermediate molecules, link OSA to the aggressiveness of cancer. Patients with OSA have higher serum levels of VCAM-1 than healthy controls [17, 19, 28], which is primarily due to hypoxic stress. OSA could enhance the aggressiveness of CM and increase the levels of VCAM-1.

The secreted form of VCAM-1 is due to proteolytic cleavage upon its release from the cell surface via the activity of neutrophil-derived serine proteases. VCAM-1 is mainly involved in leukocyte transendothelial migration and leukocyte retention into tissues [37]. VCAM-1 plays a central role in the recruitment of inflammatory cells, and its expression is rapidly induced by proinflammatory cytokines, such as VEGF [38]. VCAM-1 plays important roles due to its multiple functionalities in directing the spread of tumours, the formation of metastatic niches and supporting the angiogenic process [20]. Thus, OSA due to hypoxic stress could elevate the serum levels of VEGF and stimulate the expression of VCAM-1, which promotes tumorigenesis. This hypothesis is supported by the relationship between the VCAM-1 circulating levels and the growth rate of melanoma. Even though the result was not statistically significant, a tendency to a positive association could exist, and should be further explored.

For the first time, a biomarker for tumorigenesis has been reported to be elevated in patients with OSA who already suffer from CM. These findings could highlight the importance of VCAM-1 in tumorigenesis in patients with OSA, as a possible pathway that relates OSA with CM. In the present study, we observed a tendency toward increased HIF-1α in post-excisional melanoma tumour tissues as the severity of OSA increased, although the adjusted analysis yielded similar expression levels between mild OSA and moderate/severe OSA. No significant association was observed between VEGF levels in tumour samples and OSA severity. The fact that the biomarkers that were significantly associated with OSA in patients with CM consisted of levels measured in circulating blood samples may be relevant as far as their clinical utility, considering their accessibility. The circulating levels of VEGF and VCAM-1 could be helpful in the identification of patients with melanoma and OSA that are at a higher risk of carcinogenesis and tumour growth. Additionally, measuring the VEGF and VCAM-1 circulating levels is important not only in patients with CM but also in patients with only OSA because these biomarkers play an important role in the initial stages of tumorigenesis, and OSA patients with elevated serum levels of VCAM-1 and VEGF could be more likely to suffer from tumorigenesis. These findings could support the hypothesis that treating patients with CM and OSA, with increased levels of circulating VEGF and VCAM-1 could reduce tumorigenesis and improve tumour outcomes.

Intermittent hypoxia and arousal, the hallmark features of OSA, are recognized as the major pathophysiological consequences of OSA that promote the activation of intermediate mechanisms that associate OSA with its deleterious consequences and may contribute to an increased cancer risk [25]. Consistent with the evidence from laboratory and animal experiments [39], previous observational trials have reported cancer incidence to be augmented with increasing OSA levels [40, 41]. These observational studies suggested an association between nocturnal hypoxemia and cancer mortality in patients with OSA. Notably, in patients who remained untreated, the association between the hypoxemic index and cancer incidence was slightly stronger. The application of treatment that allows the abolition of the apnea may contribute to the reduction in these angiogenic biomarkers associated with cancer. Although there are data clearly suggesting an association between sleep apnea and cancer risk, there is an absolute lack of evidence of the possible positive effect of CPAP treatment on decreasing tumour aggressiveness in patients with OSA. Although the effects of CPAP treatment in patients with OSA and cancer have not been explored, basic research studies have identified the role of CPAP treatment in mechanisms related to tumorigenesis. Indeed, Gharib *et al.* [42] studied the whole-genome expression of peripheral blood leucocytes in 18 patients with OSA before and after one month of CPAP treatment. Gene-set enrichment analysis comparing leucocytes in each patient from pre- and post-CPAP treatment indicated that some gene sets involved in neoplastic processes were down-regulated by OSA treatment. Moreover, Hernández-Jiménez *et al.* [43] demonstrated that OSA induces changes in the functionality of circulating monocytes and NK cells, which could favour tumour immune escape. This finding was not observed in CPAP-treated patients. Finally, Almendros *et al.* [44] evaluated in a murine model whether the chronic intermittent hypoxia that characterizes OSA leads to release of tumor-promoting exosomes in the circulation.

These investigators observed that the exosomes released during IH mimicking OSA increase the malignancy properties of TC1 lung tumour cells in vitro. In this study, the authors also evaluated exosomes from 10 adult human patients with OSA for 6 weeks before and after CPAP treatment and reported that exosomes from patients with OSA increase proliferation and migration when compared with the same patients after 6 weeks of adherent CPAP treatment.

CPAP treatment is the gold standard for treating OSA and should be considered the first line therapeutic option for OSA. Mandibular advancement devices have also been considered for OSA treatment, particularly in mild-to-moderate OSA [45]. Effective OSA treatment in patients with CM and OSA may contribute to the reduction of the levels of markers related to cancer. Because of the lack of evidence of the possible positive effects of CPAP treatment in cancer progression this possibility should be considered speculative at present. Further studies are necessary to evaluate the effect of CPAP treatment in tumorigenic biomarkers and the prognosis of patients with OSA and cancer.

The main strengths of the study are related to its prospective nature and the relatively large cohort size that enables appropriate gauging of the potential effects of OSA severity on biomarkers related with carcinogenesis and tumour growth. Nevertheless, the present study present several limitations that deserve comments. First, the sleep habits and shift-work was not reported, variables that are related to cancer [12]. Second, the results of this study are specific related to cutaneous melanoma. Because of that the relationship observed for VCAM-1 and VEGF could not be present in other types of cancer. Third, the present study evaluates the relationship between the severity of OSA and the levels of cancer-related biomarkers, in patients with cutaneous melanoma; however, no information is available regarding the effect of CPAP treatment on these biomarkers.

In conclusion, our study suggests that in patients with cutaneous melanoma, OSA is independently associated with high levels of circulating VCAM-1 tumour adhesion-related biomarker, but not to circulating levels of markers related with tumour aggressiveness. The activation of pathways related with tumour adhesion could be related with the observed relationship between OSA severity and melanoma. Further studies should be explored to identify the intermediate pathways linking OSA severity and cancer.

**Acknowledgements**

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## Tables and Figures

Table 1. Anthropometric and clinical characteristics of the study groups.

| | All | Non-OSA | Mild OSA | Moderate/severe OSA | p-value |
|---|---|---|---|---|---|
| | N = 360 | N = 132 | N = 109 | N = 119 | |
| Physiological characteristics | | | | | |
| Gender *n (%)* | | | | | |
| Male | 188 (52.2%) | 51 (38.6%) | 54 (49.5%) | 83 (69.7%) | **<0.001** |
| Age (years) *median [IQR]* | 55.0 [43.8-68.0] | 44.0 [37.0-52.2] | 59.0 [46.0-68.0] | 67.0 [56.0-72.5] | **<0.001** |
| BMI (kg/m$^2$) *median [IQR]* | 27.0 [24.2-29.7] | 24.5 [22.9-27.2] | 28.3 [25.1-30.8] | 28.3 [26.2-31.5] | **<0.001** |
| Tobacco habit *n (%)* | | | | | 0.303 |
| Non-smoker | 184 (51.3%) | 62 (47.0%) | 60 (55.0%) | 62 (52.5%) | |
| Active smoker | 69 (19.2%) | 32 (24.2%) | 20 (18.3%) | 17 (14.4%) | |
| Former smoker | 106 (29.5%) | 38 (28.8%) | 29 (26.6%) | 39 (33.1%) | |
| Melanoma site *n (%)* | | | | | 0.457 |
| Head/neck | 52 (14.4%) | 16 (12.1%) | 16 (14.7%) | 20 (16.8%) | |
| Arms | 50 (13.9%) | 17 (12.9%) | 18 (16.5%) | 15 (12.6%) | |
| Legs | 93 (25.8%) | 34 (25.8%) | 33 (30.3%) | 26 (21.8%) | |
| Trunk | 148 (41.1%) | 60 (45.5%) | 35 (32.1%) | 53 (44.5%) | |
| Acral | 16 (4.44%) | 5 (3.79%) | 7 (6.42%) | 4 (3.36%) | |
| Sleep apnea variables | | | | | |
| AHI (events/h) *median [IQR]* | 8.55 [2.80-19.5] | 1.95 [0.70-3.02] | 9.00 [7.00-12.0] | 25.7 [19.6-40.5] | **<0.001** |
| ODI (events/h) *median [IQR]* | 4.90 [1.10-12.8] | 0.80 [0.25-1.60] | 5.05 [3.38-8.38] | 15.4 [10.5-25.8] | **<0.001** |

IQR: interquartile range; BMI: body mass index; AHI: apnea-hypopnea index (number of events·h$^{-1}$); ODI: oxygen desaturation index. The statistically significant p values (p values <0.05) are denoted in bold.

Table 2. Adjusted logistic regression models of the concentrations of the circulating biomarkers in the study groups

| | OR [95% C.I.]* | p-value |
|---|---|---|
| VEGF | | |
| Mild OSA | 1.26 [0.70-2.] | 0.431 |
| Moderate/severe OSA | 1.61 [0.85-3.06] | 0.143 |
| IL-8 | | |
| Mild OSA | 0.75 [0.42-1.35] | 0.345 |
| Moderate/severe OSA | 1.04 [0.55-1.97] | 0.902 |
| ICAM | | |
| Mild OSA | 1.11 [0.62-1.96] | 0.724 |
| Moderate/severe OSA | 0.69 [0.37-1.30] | 0.253 |
| VCAM | | |
| Mild OSA | 2.07 [1.12-3.89] | **0.021** |
| Moderate/severe OSA | 2.35 [1.20-4.66] | **0.013** |
| S100B | | |
| Mild OSA | 1.27 [0.70-2.34] | 0.434 |
| Moderate/severe OSA | 0.94 [0.49-1.76] | 0.883 |
| MIA | | |
| Mild OSA | 1.20 [0.63-2.31] | 0.582 |
| Moderate/severe OSA | 0.70 [0.34-1.40] | 0.312 |

VEGF; Vascular endothelial growth factor. IL-8: Interleukin 8, ICAM: Intracellular adhesion molecule, VCAM: Vascular cell adhesion molecule 1, S100B: S100 calcium-binding protein B, MIA: Melanoma inhibitory activity.
*The odds ratios (OR) were calculated using a logistic regression; the response variables were the individual biomarkers, and the explanatory variables were the categories of OSA. The model was adjusted for gender, age and BMI. The statistically significant p values (p values <0.05) are denoted in bold.

Table 3. Adjusted logistic regression models of the expression of the biomarkers in the resected tumour samples in the groups analysed.

| | OR [95% C.I.]* | p-value |
|---|---|---|
| VEGF | | |
| Mild OSA | 1.69 [0.85-3.39] | 0.14 |
| Moderate/severe OSA | 1.35 [0.64-2.85] | 0.43 |
| HIF-1α | | |
| Mild OSA | 1.52 [0.64-3.75] | 0.35 |
| Moderate/severe OSA | 1.82 [0.73-4.77] | 0.21 |

VEGF; Vascular endothelial growth factor. HIF-1α; Hypoxia inducible factor 1 alpha.
*The odds ratio (OR) was calculated using a logistic regression; the response variables were the individual biomarkers, and the explanatory variables were the categories of OSA. The model was adjusted for gender, age and BMI.

Figure 1. Flowchart of the study

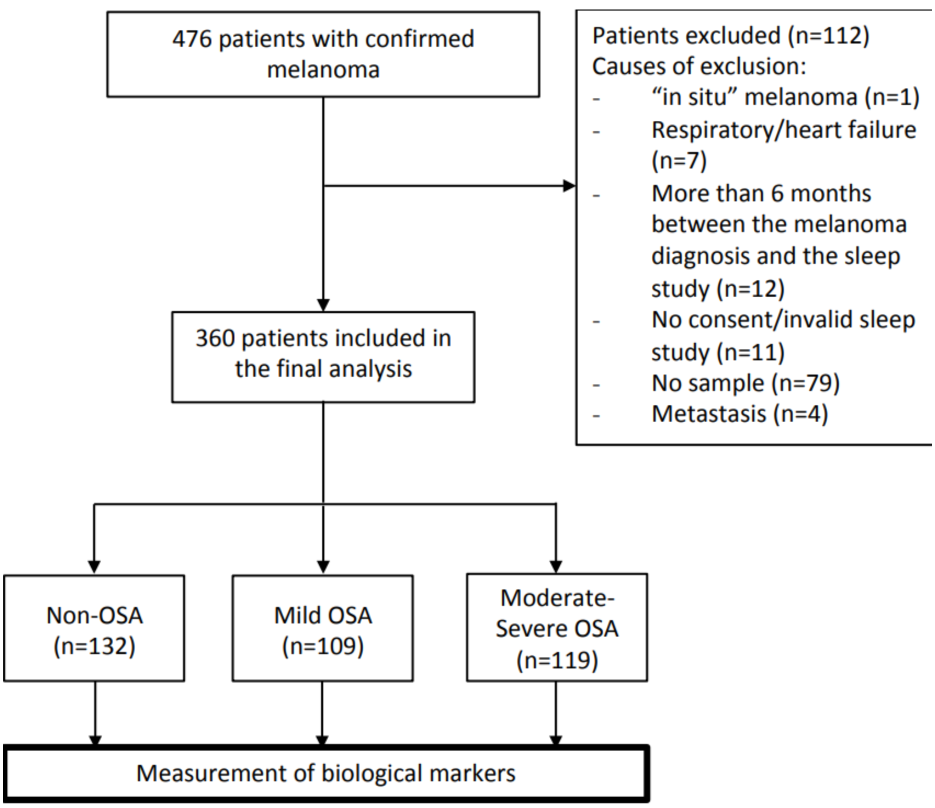


Figure 2. Circulating levels of the biomarkers

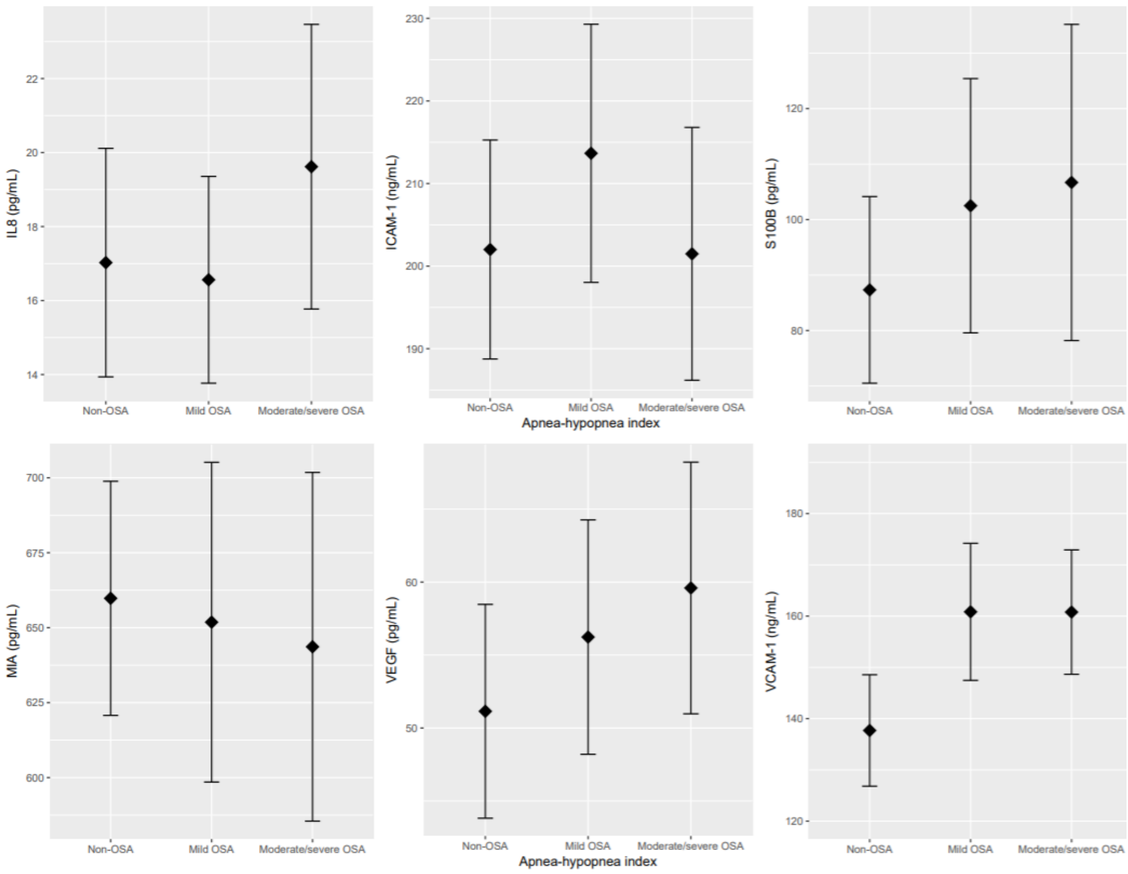
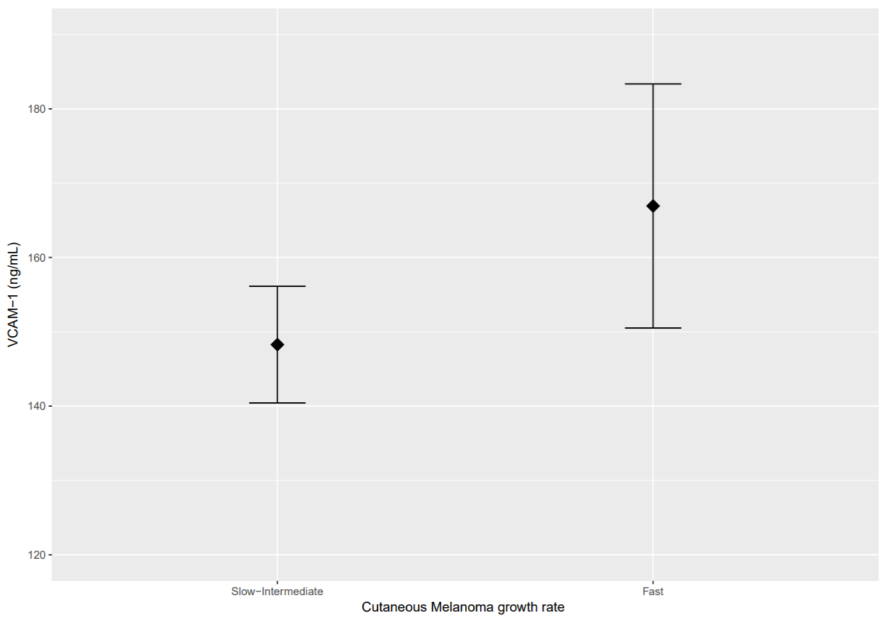



Figure 3. Relationship between the circulating levels of VCAM-1 and the CM growth rate



# 4. DISCUSIÓN

Los resultados de la presente Tesis Doctoral han contribuido en el avance del conocimiento en el campo de la medicina de precisión en pacientes con apnea obstructiva del sueño. Así, los trabajos que se presentan en esta memoria de tesis doctoral han tenido como objetivo evaluar el uso de biomarcadores para el manejo del paciente con SAOS, tanto a nivel de diagnóstico, estratificación del riesgo y evolución con el tratamiento. Concretamente, se ha evaluado el uso de miRNAs para el diagnóstico, evolución con el tratamiento y estratificación del riesgo cardiovascular; y de biomarcadores proteicos para el riesgo de agresividad y crecimiento tumoral.

Los miRNAs han sido reconocidos como biomarcadores fiables y no invasivos que juegan un papel clave en el desarrollo y prognosis de diferentes enfermedades. Su uso va más allá que un simple biomarcador ya que puede ayudar a identificar diferentes vías fisiopatológicas implicadas. Su utilidad ha sido demostrada en el manejo de diversas enfermedades [92], así cómo en el manejo personalizado del SAOS [96]. El uso de miRNAs circulantes representa un avance en el campo de los biomarcadores mínimamente invasivos [120]. Debido a su simple y rápida cuantificación cientos de miRNAs pueden ser evaluados simultáneamente en varios pacientes, haciendo de ellos una herramienta muy potente para la medicina de precisión [121]. El principal problema que existe actualmente con la cuantificación relativa de los miRNAs es su variabilidad técnica. No existe un método universal que permita reducir esta variabilidad. Se han propuesto diferentes técnicas, como el uso de *spike-in*, normalización por genes y normalización por RNAs pequeños nucleares, pero los resultados no han sido replicables [122, 123]. El *gold* estándar se basa en métodos de centrado en la media y es muy útil cuando se utilizan aproximaciones high-troughput, pero no es una opción válida para el análisis de un número bajo de miRNAs. En estos casos, el uso de controles endógenos resulta ser la mejor opción [110, 124]. Los controles endógenos son específicos y deben ser identificados para cada patología y tejido. La identificación y validación de controles endógenos específicos y adecuados es probablemente el paso más crítico en el análisis de miRNAs y permite evitar una interpretación errónea de los resultados.

En el presente trabajo de tesis doctoral se identifica, por primera vez, un conjunto de miRNAs que actúan como controles endógenos para el uso de estos biomarcadores en SAOS. Estos miRNAs permiten estandarizar el proceso de normalización de miRNAs circulantes en pacientes con SAOS, y ha dado paso a los dos siguientes estudios. Por primera vez se describe el uso de miRNAs para el diagnóstico y caracterización de los pacientes con SAOS referidos a la unidad de sueño. Mediante el uso de 6 miRNAs y 5 variables clínico-antropométricas (cuestionario NoSAS [10]) es posible identificar específicamente al paciente con SAOS (AUC=0.86). Además, se muestra como el perfil de miRNAs tiende a recuperar el fenotipo del paciente sin SAOS a los 6 meses de tratamiento con CPAP. Por otro lado, también se ha evaluado si el perfil epigenético circulante se asocia con diferentes fenotipos clínicos de riesgo cardiovascular en pacientes con SAOS. El estudio no consigue demostrar que en las poblaciones de riesgo escogidas (pacientes con solo SAOS, pacientes con SAOS e hipertensión y pacientes SAOS con ECV) exista una diferencia en el perfil de miRNAs. A pesar de ello, se han identificado en los pacientes con SAOS dos miRNAs relacionados con parámetros de tensión arterial, especialmente con los valores de tensión nocturna y este podría ser uno de los mecanismos que relacionaría SAOS con hipertensión.

El último estudio identifica por primera vez, un biomarcador de tumorigénesis que se encuentra elevado en pacientes SAOS que padecen melanoma cutáneo. Diversos mecanismos fisiopatológicos relacionan el SAOS con la agresividad del cáncer. El estudio, que aquí se presenta, sugiere que el SAOS, debido al estrés hipóxico, podría aumentar los niveles circulantes de VEGF que estimularía la expresión de VCAM-1, factor que podría promocionar el desarrollo de tumorigénesis.

Los hallazgos realizados en el presente trabajo de tesis doctoral permiten varios avances significativos en el campo de la medicina de precisión en pacientes con apnea del sueño. Por primera vez, se establece un flujo de trabajo satisfactorio y viable para el análisis integral de miRNAs utilizando qPCR. Hasta la fecha se han realizado varios trabajos en el campo del SAOS y los miRNAs [96, 113, 125, 126]. Estos trabajos utilizan métodos de normalización generales que, aunque pueden ser fiables en ciertos casos, no son los mejores para reducir la variabilidad técnica y podrían llevar a una interpretación errónea de los resultados. En el presente trabajo, se ha conseguido estandarizar el proceso y reducir la variabilidad técnica mediante el uso de 2 miRNAs estables en pacientes con sospecha de SAOS. Este hallazgo permite que en cualquier laboratorio se pueda aplicar el uso de miRNAs circulantes como biomarcadores de SAOS, y que los resultados sean altamente replicables entre ellos, debido a la reducción de la variabilidad técnica conseguida. A su vez, esto ha permitido realizar los dos siguientes estudios en los que se evalúa el papel de los miRNAs en el manejo del paciente con SAOS. De ellos se deriva que los miRNAs pueden jugar un papel importante en el diagnóstico de estos pacientes. La principal utilidad de los resultados es la posibilidad de aplicar esta técnica de manera masiva. Mientras que los métodos diagnósticos actuales requieren de una elevada cantidad de tiempo, personal y recursos económicos, el método de diagnóstico aquí propuesto permite simultáneamente evaluar a un elevado número de pacientes a partir de una sola muestra de sangre. Además, este diagnóstico podría acompañarse de la evaluación de los miRNAs que correlacionan con tensión arterial, y así poder sugerir la prueba por MAPA de estos pacientes, independientemente de sus valores de presión arterial en consulta. De esta manera se puede evaluar el riesgo cardiovascular y valorar el posible beneficio de la CPAP en los mismos. Además, la medición de los miRNAs a los 6 meses de seguimiento contribuiría a evaluar, de una manera objetiva, el posible beneficio del tratamiento a nivel molecular.

Por otro lado, el análisis de biomarcadores proteicos, específicamente VCAM-1 y VEGF, nos permitiría evaluar de una manera mínimamente invasiva los pacientes SAOS que tendrían un elevado riesgo de carcinogénesis y crecimiento tumoral. Dado que ambas moléculas juegan un papel clave en las etapas más tempranas de la tumorigénesis, pacientes SAOS con concentraciones elevadas de VCAM-1 y VEGF podrían tener una mayor susceptibilidad a padecer cáncer. Además, estos hallazgos refuerzan la hipótesis de que el tratamiento con CPAP en pacientes con melanoma y SAOS que presentan niveles elevados de VCAM-1 y VEGF, podría contribuir significativamente en la reducción de los procesos tumorogénicos.

El uso de biomarcadores circulantes en SAOS tiene una serie de limitaciones que merecen ser identificadas y comentadas. En primer lugar, existe una limitación en el uso de los biomarcadores circulantes de manera masiva. Existen una barrera en el acceso a las técnicas utilizadas para la evaluación de estos biomarcadores al tratarse de técnicas que conllevan cierta complejidad y especilización técnica. No obstante, una vez instaurado un flujo de trabajo adecuado, las determinaciones se vuelven sencillas, rutinarias y baratas si se realizan a gran escala por el personal previamente preparado. En segundo lugar, varios de los resultados obtenidos en la presente tesis no han podido ser validados en la población de mujeres. Estudios previos han descrito que el perfil de miRNAs varía entre hombres y mujeres. Recientemente se ha completado el reclutamiento de la población de mujeres necesaria para tener una potencia estadística suficiente para replicar el trabajo realizado en la población de hombres. Dicha evaluación se realizará próximamente. Por otro lado, sería necesario evaluar otros usos de la medicina de precisión en el contexto del SAOS. Especialmente cabría destacar la caracterización de fenotipos de la enfermedad, como pueden ser aquellos con una mayor susceptibilidad a padecer enfermedades cardiovasculares, predición de la respues clínica al tratamiento con CPAP, predicción de la adherencia a la CPAP y predicción de otras comorbilidades asociadas al SAOS. De manera similar, resultaría interesante utilizar los hallazgos encontrados en la presente tesis en modelos celulares y animales, para ver si las posibles vías moleculares descritas a partir de herramientas bioinformáticas se validan.

En resumen, el uso de la medicina de precisión, mediante el análisis de biomarcadores en pacientes SAOS, resulta prometedor y seguirá creciendo a medida que se descubran nuevas maneras de simplificar las herramientas *ómicas* disponibles y caracterizar de una forma simple y barata el perfil molecular individual de cada paciente. Actualmente, el ahorro de recursos y tiempo haría de la medicina de precisión una herramienta útil y fácilmente aplicable en laboratorios especializados en biología molecular, para el diagnóstico y la caracterización de diferentes fenotipos del SAOS.

Por lo tanto, se concluye que, mediante el análisis de biomarcadores circulantes, a partir de una sola muestra sanguínea se podría evaluar la probabilidad de un paciente de manifestar SAOS, identificar pacientes hipertensos nocturnos en los que sería aconsejable una prueba con MAPA y evaluar el riesgo de carcinogénesis y crecimiento tumoral en pacientes SAOS. Una segunda muestra sanguínea, permitiría valorar los cambios a nivel molecular de los pacientes con el tratamiento.

Los resultados del presente trabajo de tesis doctoral suponen una pieza adicional de información que contribuye a señalar la necesidad de explorar la utilidad del uso de biomarcadores en el manejo integral y personalizado del SAOS tanto a nivel de diagnóstico, estratificación del riesgo, y predicción de la evolución clínica.

# 5. CONCLUSIONES

Las conclusiones más relevantes de los estudios presentados son:

1. En pacientes con sospecha de SAOS existe un conjunto de miRNAs que puede ser utilizado como control endógeno para la normalización estándar de los perfiles de miRNAs en SAOS.

2. Un clúster singular de miRNAs diferencia específicamente los pacientes SAOS respecto a los no SAOS. Este modelo basado en miRNAs, mejora la habilidad discriminatoria basada en variables clínicas y antropométricas. Finalmente, el uso de la CPAP se asocia con cambios en el perfil circulante de miRNAs recuperando parcialmente el fenotipo de los sujetos sin SAOS.

3. El perfil circulante de miRNAs no parece estar alterado en diferentes poblaciones de riesgo cardiovascular en pacientes SAOS. A pesar de ello, el miR-107 y el miR-143 podría contribuir a la identificación de los mecanismos fisiopatológicos de la hipertensión en pacientes SAOS.

4. En pacientes con melanoma cutáneo el SAOS está independientemente asociado con niveles circulantes elevados de VCAM-1, un biomarcador de adhesión celular, pero no con los niveles circulantes de marcadores relacionados con la agresividad. La activación de vías relacionadas con la adhesión podría relacionarse con la relación observada entre la gravedad del SAOS y del melanoma.

5. Biomarcadores circulantes como miRNAs y otros biomarcadores protéicos, son una herramienta útil en el manejo personalizado del paciente con SAOS. La evaluación de dichos biomarcadores contribuye, tanto a nivel de diagnóstico, como estratificación del riesgo y pronóstico del paciente.

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