Respiration

Clinical Investigations

Respiration 2020;99:1122–1128
DOI: 10.1159/000511093

Received: April 27, 2020
Accepted: August 20, 2020
Published online: November 18, 2020

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
Background: 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. ***Objective:*** The aim of this study was to identify the microRNA (miRNA) profile associated with CVD in patients with OSA. ***Method:*** This is an observational, cross-sectional study that included 132 male patients. Three groups were defined as 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 BMI and age) by the TaqMan low density array (TLDA). miRNAs differentially expressed in the different subgroups of patients and miRNAs that correlated with the cardiovascular risk SCORE were selected for validation by RT-qPCR in the 111 remaining patients. ***Results:*** From the TLDA analysis, 7 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. miR-486 was associated with the cardiovascular risk SCORE. ***Conclusions:*** 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 and miR-486 is associated with the cardiovascular risk SCORE.

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

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mechanisms, link OSA with cardiovascular diseases (CVDs) (i.e., high blood pressure, stroke, and myocardial infarction) 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 3 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-Santa Maria 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 center (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 aged between 18 and 60 years. 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 s. Hypopnoea was defined as a 30–90% reduction in oronasal airflow for at least 10 s 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 hour of sleep. All participants were subjected to 24-h ABPM (Mortara Ambulo 2400, Milwaukee, WI, USA). 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 mm Hg or a 24-h diastolic BP ≥ 80 mm Hg. The cardiovascular risk SCORE [14] was calculated for all the patients.

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 TaqMan Low Density Array

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 [15, 16]. 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 BMI (Fig. 1). Multiple real-time RT-PCRs were performed using a customized TLDA (Life Technologies, Foster City, CA, USA).

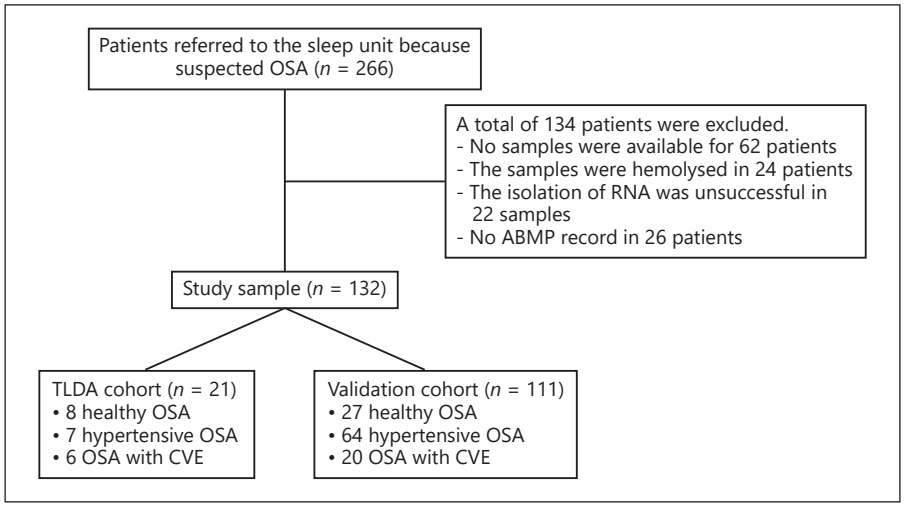

Fig. 1. Flowchart of the study. TLDA, TaqMan low density array; OSA, obstructive sleep apnea; CVE, cardiovascular event.

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 (ΔRn = 0.012) and >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 [17].

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 [18]. Then, individual TaqMan hydrolysis probes (Applied Biosystems) were applied to analyze the expression of the differentially expressed miRNAs in the qPCR cohort (Fig. 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 [19]. 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 using the Benjamini-Hochberg procedure [20] to adjust for the performance of multiple paired comparisons. The hypotheses tested included 188 in the discovery phase and 5 in the validation phase. A type 1 error of 0.05 and a coefficient of variation of 0.5 were assumed. Regarding the TLDA phase, the comparison between groups had a power of 75% to detect fold changes greater than 2 (or 0.5 in the case of downregulation). Concerning the validation phase, the tests obtained a minimum power of 80% to detect fold changes of 1.5 (or 0.667 in the case of downregulation). 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 were 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 versus the OSA hypertensive group. miR-143b and miR-140-3p were significant when comparing the OSA hypertensive group versus 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) [21]. Moreover, the correlation between each individual miRNA and cardiovascular risk SCORE was evaluated. Five miRNAs were

Table 1. Baseline characteristics of the 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, median [IQR], years	43.0 [26.5; 47.5]	54.0 [50.0; 55.5]	55.0 [51.0; 56.8]	0.078
BMI, median [IQR], kg/m^2	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, median [IQR], events/h	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, median [IQR], events/h	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, median [IQR], mm Hg	114 [111; 121]	146 [140; 151]	125 [112; 142]	0.009
24-h diastolic blood pressure, median [IQR], mm Hg	73.7 (2.50)	89.6 (4.23)	79.1 (10.00)	<0.001
Daytime systolic blood pressure, median [IQR], mm Hg	119 (6.37)	148 (11.6)	129 (19.7)	0.002
Daytime diastolic blood pressure, median [IQR], mm Hg	75.1 (2.55)	91.1 (5.23)	81.5 (12.8)	0.003
Nighttime systolic blood pressure, median [IQR], mm Hg	110 [108; 114]	139 [136; 144]	119 [101; 138]	0.003
Nighttime diastolic blood pressure, median [IQR], mm Hg	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; AHI, apnea-hypopnea index; TSat, 90, nighttime oxygen saturation less than 90%; TLDA, TaqMan low density array; ESS, Epworth Sleepiness Scale.

correlated with the SCORE in the TLDA cohort: miR-206, miR-328, miR-486, miR-579, and miR-92a (based on an individual p value lower than 0.05). Two of these miRNAs that did not display missing values were selected for subsequent analysis.

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) (see Table 5). miR-486, but

Table 2. miRNA candidates for cardiovascular risk in OSA patients

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

Five miRNAs were selected based on their fold change value and previous literature. 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, median [IQR], years	44.0 [39.5; 53.5]	48.0 [45.0; 54.0]	53.0 [51.0; 56.0]	0.006
BMI, median [IQR], kg/m^2	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, median [IQR], events/h	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, median [IQR], events/h	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, median [IQR], mm Hg	118 [115; 124]	136 [128; 147]	141 [121; 144]	<0.001
24-h diastolic blood pressure, median [IQR], mm Hg	74.7 (3.83)	85.2 (6.28)	79.4 (7.09)	<0.001
Daytime systolic blood pressure, median [IQR], mm Hg	122 [118; 128]	138 [130; 149]	140 [124; 146]	<0.001
Daytime diastolic blood pressure, median [IQR], mm Hg	76.9 [74.8; 79.8]	86.2 [83.0; 91.1]	82.0 [77.8; 85.3]	<0.001
Nighttime systolic blood pressure, median [IQR], mm Hg	108 [100; 113]	128 [117; 145]	129 [113; 140]	<0.001
Nighttime diastolic blood pressure, median [IQR], mm Hg	69.3 [66.5; 71.5]	79.4 [74.7; 85.0]	75.2 [69.9; 79.0]	<0.001

HBP, high blood pressure; AHI, apnea-hypopnea index; TSat 90, nighttime oxygen saturation less than 90%; ESS, Epworth Sleepiness Scale.

not miR-92a, tended to be correlated with the cardiovascular risk SCORE (r = −0.18, p = 0.061).

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, 15]. 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 [21] where researchers identified differences between non-OSA and healthy OSA patients and between non-OSA and OSA with arterial hypertension

Table 4. Validation of miRNA candidates

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

p values are adjusted for age and BMI. CVE, cardiovascular event; OSA, obstructive sleep apnea; HBP, high blood pressure.

Table 5. Correlations between miRNA candidates and ABPM parameters

miRNAs	ABPM parameters							
	24-h systolic BP		24-h diastolic BP		nocturnal systolic BP		nocturnal diastolic BP	
	rho	*p* value	rho	*p* value	rho	*p* value	rho	*p* value
hsa.miR.148b	−0.03	0.809	−0.06	0.589	0.11	0.314	−0.05	0.66
hsa.miR.301	0.05	0.662	0.07	0.531	0.1	0.348	−0.05	0.633
hsa.miR.143	0.16	0.156	0.08	0.479	0.23	0.035	0.04	0.691
hsa.miR.140.3p	−0.1	0.369	−0.08	0.47	−0.04	0.732	−0.11	0.325
hsa.miR.133a	0.18	0.096	0.15	0.155	0.17	0.117	0.03	0.804
hsa.miR.107	0.19	0.091	0.18	0.108	0.21	0.057	0.21	0.058

p values are adjusted for age and BMI. ABPM, ambulatory blood pressure monitoring.

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 [22]. 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 remodeling in some hypoxic conditions by targeting HIF-1β [23]. The intermittent hypoxia caused by OSA could contribute, through the downregulation of miR-107, to worse vascular remodeling. 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 [24], altering smooth muscle cell maintenance and vascular homeostasis [25], being one of the most useful biomarkers in CVD [26]. Additional studies are needed to elucidate the implications of miR-107 and miR-143 in hypertension in patients with OSA. Moreover, miR-486 tended to be correlated with the cardiovascular risk SCORE. The cardiovascular risk SCORE is one of the best tools for identifying the individual cardiovascular risk of a population [14]. This finding is interesting because miR-486 has previously been described as being correlated with the response to CPAP treatment in patients with resistant hypertension and OSA (3) and is also related to the discrimination between OSA and non-OSA patients (4). miR-486 has been suggested to play a role in muscle atrophy in cardiomyocytes (5) and cardiovascular diseases [27].

The present study has several limitations that deserve comment. First, only patients aged between 18 and 60 years 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 [28], further studies are needed to evaluate the profile of miRNAs in women with OSA. Additionally, an AHI ≥ 15 was selected as cutoff point; for instance, mild OSA patients were not included in the study.

The strengths of this study reside in a large number of patients who were analyzed in 2 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 male OSA cardiovascular risk populations aged under 60 years, 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. Moreover, miR-486 seems to play a role in the relationship between OSA and cardiovascular risk.

Statement of Ethics

All recruited patients in the study (ClinicalTrials.gov, registration number: NCT03513926) signed an informed consent form, and the Ethics Committee of the center (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.

Conflict of Interest Statement

All authors who have contributed to the submitted manuscript declare that they have no competing interests and have submitted a signed copy of the Submission Statement.

Funding Sources

The funding sources have no role in the writing, data collection, analysis, or interpretation of the study. The project is supported by PI 14/01266 and PI 18/00449 from the Instituto de Salud Carlos III (ISCIII), Fondo Europeo de Desarrollo Regional (FEDER) "Una manera de hacer Europa," Sociedad Española de Neumología y Cirugía Torácica (SEPAR), and Societat Catalana de Pneumologia (SOCAP). The work is supported by IRBLleida Biobank (B.0000682) and PLATAFORMA BIOBANCOS PT17/0015/0027." F.S.-M. is the recipient of a predoctoral fellowship from "AGAUR-University of Lleida" and Convocatòria d'Ajuts 2018 de Promoció a la Recerca en Salut.

Author Contributions

F.S.M., L.P., A.Z., F.B., and M.S.d.T. conceived and designed the study; F.S.M., L.P., C.G., O.M., S.G., and R.V. acquired the data; F.S.M., I.B., L.P., F.O., A.Z., C.G., J.M.F.R., F.B., and M.S.d.T. analyzed and interpreted the data; and F.S.M., I.B., L.P., F.O., A.Z., C.G., O.M., S.G., R.V., J.M.F.R., F.B., and M.S.d.T. drafted the manuscript, critically revised the manuscript for important intellectual content, and approved the final version. M.S.d.T. is the guarantor of the paper.

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