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Canonical Pathways Associated with Blood Pressure Response to Sleep Apnea Treatment: A Post Hoc Analysis

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Keywords
MicroRNAs · Obstructive sleep apnea · Resistant hypertension · Cardiovascular diseases · Continuous positive airway pressure

Abstract
Background: Several studies have reported an association between microRNAs (miRNAs) and hypertension or cardiovascular disease (CVD). In a previous study performed on a group of 38 patients, we observed a cluster of 3 miRNAs (miR-378a-3p, miR-100-5p, and miR-486-5p) that were functionally associated with the cardiovascular system that predicted a favorable blood pressure (BP) response to continuous positive airway pressure (CPAP) treatment in patients with resistant hypertension (RH) and obstructive sleep apnea (OSA) (HIPARCO score). However, little is known regarding the molecular mechanisms underlying this phenomenon. ***Objectives:*** The aim of the study was to perform a post hoc analysis to investigate the genes, functions, and pathways related to the previously found HIPARCO score miRNAs. ***Methods:*** We performed an enrichment analysis using Ingenuity pathway analysis. The genes potentially associated with the miRNAs were filtered based on their confidence level. Particularly for CVD, only the genes regulated by at least 2 of the miRNAs were studied. ***Results:*** We observed that the miRNAs studied regulate 200–249 molecules associated with several functions and diseases, including extracranial solid tumors and abdominal neoplasms, among others. The cardiac hypertrophy and NF-kB signaling pathways were identified as the cardiovascular pathways most influenced by these 3 miRNAs. ***Conclusions:*** The mechanisms by which CPAP treatment decreases the BP in OSA patients with RH could be related to the cardiac hypertrophy and NF-kB signaling pathways. Further investigations will be necessary to confirm these findings, contributing to the elucidation of new therapeutic targets in patients who do not respond to CPAP treatment.

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Introduction

Obstructive sleep apnea (OSA) is the most common sleep-disordered breathing, with a prevalence of 20% in the middle-aged population [1, 2]. This disorder is caused by the collapse of the upper airway during sleep, which promotes intermittent hypoxemia and hypercapnia. These events are usually accompanied by cortical arousals during the night, leading to daytime somnolence and poor quality of life. The repetitive cycles of upper airway collapse during sleep induce the activation of various pathways related to endothelial dysfunction, oxidative stress, sympathetic activation, inflammation, hypercoagulability, and metabolic dysregulation [3, 4]. This explains, at least in part, the relationship between OSA and atherosclerosis. Basic research and epidemiological and clinical data support the concept that OSA has a role in the initiation or progression of several cardiovascular diseases (CVDs) [5, 6].

Continuous positive airway pressure (CPAP) is the first-choice treatment for sleep apnea. Although CPAP treatment does not demonstrate effectiveness for secondary cardiovascular prevention [7], observational studies show a lower incidence of CVD in patients treated with CPAP [8–10]. Additionally, treatment with CPAP showed effectiveness in controlling blood pressure (BP) in patients with resistant hypertension (RH), which is of extreme importance considering that increased BP is the main factor for CVD development [11, 12]. However, despite good CPAP compliance, substantial variability exists in its effects. This increases the interest and necessity to develop tools to identify patients who could benefit from this treatment in terms of BP reduction.

MicroRNAs (miRNAs) are small RNAs that inhibit protein-coding messenger RNAs (mRNAs) through translational repression and degradation. These molecules regulate a wide range of biological processes, including development, differentiation, proliferation, metabolism, and apoptosis [13]. Accordingly, miRNAs are linked with the pathogenesis of human diseases, such as cancer, viral infections, metabolic disorders, and CVD. Considering this, miRNAs are interesting diagnostic and prognostic biomarkers [14, 15].

Our research group described the existence of a singular cluster of circulating miRNAs that predicts BP responses to CPAP treatment in patients with RH and OSA [2]. This specific expression profile gives a quantitative

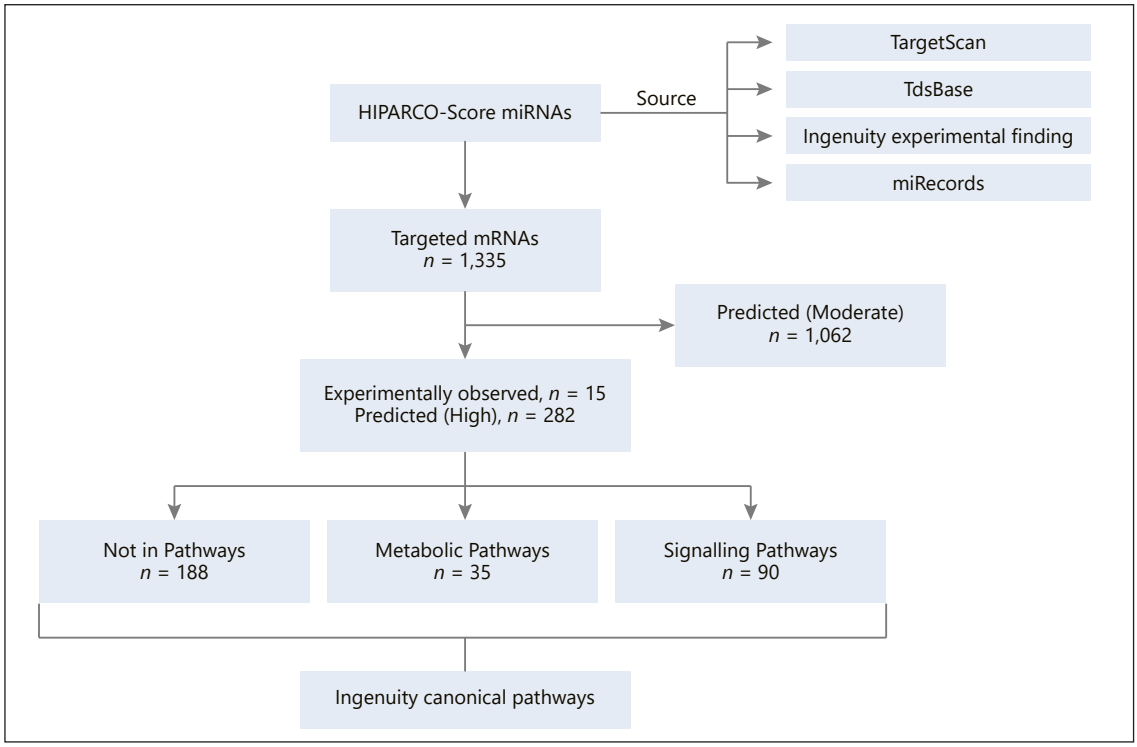

Fig. 1. Flowchart of the study. miRNA, microRNA; mRNA, messenger RNA.

Table 1. Diseases/functions and canonical pathways regulated by the studied miRNAs

Disease or function	*p* value	Molecules
Digestive organ tumor	<0.001	234
Extracranial solid tumor	<0.001	248
Abdominal neoplasm	<0.001	238
Nonhematologic malignant neoplasm	<0.001	244
Solid tumor	<0.001	249
Carcinoma	<0.001	227
Tumorigenesis of tissue	<0.001	229
Cancer	<0.001	245
Adenocarcinoma	<0.001	209
Abdominal cancer	<0.001	221
Pathways	**Genes***	
Axonal guidance signaling	11	
Glucocorticoid receptor signaling	10	
Molecular mechanisms of cancer	10	
Role of macrophages and fibroblasts	10	
Glioblastoma multiforme signaling	9	
Human embryonic stem cell pluripotency	9	
Xenobiotic metabolism signaling	9	
AMPK signaling	9	
Role of osteoblast and osteoclasts	9	
Glioma signaling	8	

miRNA, microRNA; mTOR, mammalian target of rapamycin; IGF1R, insulin-like growth factor 1; GRB2, growth factor receptor-bound protein 2. * Axonal guidance signaling: BMP2; FGFR3; FZD8; GNA13; MRAS; NTF4; NTRK3; PDGFC; PIK3CG; PIK3R; PLCB1. Glucocorticoid receptor signaling: CD247; FCGR1A; FGFR3; FKBP5; KRT24; MRAS; PIK3CG; PPFIA3; PPP3CA; PTEN. Molecular mechanisms of cancer: BMP2; CASP9; CDKN2B; FGFR3; FOXO1; FZD8; GNA13; GRB2; MRAS; mTOR. Role of macrophages and fibroblasts: FCGR1A; FGFR3; FZD8; IL33; IL6ST; MRAS; PDGFC; PIK3CG; PPP3CA; PTEN. Glioblastoma multiforme signaling: FGFR3; FOXO1; FZD8; IGF1R; MRAS; mTOR; PDGFC; PIK3CG; PPFIA3. Human embryonic stem cell pluripotency: BMP2; FGFR3; FOXO1; FZD8; MRAS; NTF4; NTRK3; PDGFC; PIK3CG. Xenobiotic metabolism signaling: CHST2; FGFR3; HS3ST2; HS3ST3B1; MRAS; NR1I3; PIK3CG; PPFIA3; PPP3CA. AMPK signaling: AK7; CHRNA10; FGFR3; FOXO1; MRAS; mTOR; PIK3CG; PPFIA3; PPP3CA. Role of osteoblast and osteoclasts: BMP2; CASP9; FGFR3; FOXO1; FZD8; IL33; PIK3CG; PPP3CA; PTEN. Glioma signaling: CDKN2B; FGFR3; IGF1R; MRAS; mTOR; PDGFC; PIK3CG; PPP3CA.

score parameter, the HIPARCO score, which identifies those patients with RH and OSA who are more likely to exhibit a favorable BP response to CPAP. This approach constitutes the first validated precision medicine tool for RH management in OSA patients. However, the biological mechanisms that underlie this outcome are still unknown. The identification of genes, functions, and pathways related to this molecular signature will improve the understanding of the role of OSA in the pathogenesis of CVD, ultimately benefiting OSA patients with RH who do not present a favorable BP response after CPAP treatment. Considering this, the aim of the present study was to perform a post hoc analysis to investigate the genes, functions, and pathways related to the previously found HIPARCO score miRNAs.

Materials and Methods

Study Population and miRNA Analysis

The selection of miRNAs was based on a study by Sánchez-de-la-Torre et al. [2]. In brief, the study was conducted with patients diagnosed with RH and OSA from the HIPARCO study (NCT00616265), which was carried out in 24 hospitals in Spain. Patients with RH and OSA who used CPAP for >4 h per night were selected to determine the responder patients in relation to BP levels and therefore establish whether there was an miRNA profile associated with a positive response to CPAP treatment.

To identify this profile, patients were divided into training and validation sets with 2 different groups regarding their response to CPAP treatment. Patients with reductions in mean BP greater than the observed median of 4.5 mm Hg were classified as responders to CPAP treatment. Otherwise, patients with a mean BP lower than that observed with a median of 4.5 mm Hg were classified as nonresponders to CPAP treatment. Human cardiovascular system-specific miRNA arrays (84 miRNAs; Qiagen) were used with the blood plasma of the patients to identify differentially expressed miRNAs. Seven differentially expressed miRNAs were found, of which 3 miRNAs (miR-378-3p, miR-438-5p, and miR-100-5p) had the highest statistical associations, were linked to an average favorable BP response to CPAP treatment, and were chosen for in silico analysis to investigate the genes, functions, and pathways related to the HIPARCO score miRNAs. This study was conducted with permission to use the patients' records, and the confidentiality was maintained (CEIC number 48/2008. Consorci Hospital Universitari General de València).

Data Analysis

The overall design and data analysis workflow are shown in Figure 1. We performed enrichment analysis using Ingenuity pathway analysis (IPA) (Ingenuity Systems, Redwood City, CA, USA) to link the miRNAs with their mRNA target genes. The IPA miRNA Target Filter tool was used to identify the genes regulated by each miRNA. This software identifies a list of potentially regulated genes based on 4 databases (TargetScan, miRecords, Ingenuity Knowledge Base, and TarBase). This platform provides a literature-curated analysis of networks, biological processes, canonical pathways, diseases, and functions associated with miRNAs and target genes. The genes potentially associated with the miRNAs were filtered based on their confidence level, and only experimentally observed and highly predicted target genes were used in the analysis (Fig. 1).

The associations among the predicted miRNA target genes and networks, canonical pathways, diseases, and functions were ana-

lyzed. We first established the mRNAs regulated by these miRNAs. Then, from the list of mRNAs/genes, the canonical pathways were examined. Different diseases and associated pathways were also explored, especially cardiovascular-related ones due to our previous experience exploring the role of these miRNAs in RH. (For this, only the genes regulated by at least 2 of the miRNAs were studied.)

Results

Canonical Pathway Analysis

The HIPARCO score molecular signature comprises 3 miRNAs (miR-378a-3p, miR-100-5p, and miR-486-5p). The diseases and functions most significantly associated with these miRNAs are summarized in Table 1. The studied miRNAs regulated 200–249 molecules in each disease or function, including digestive organ tumors, extracranial solid tumors, abdominal neoplasms, nonhematologic cancers, solid tumors, and carcinomas. Regarding the canonical pathways, we observed that these miRNAs target 8–11 genes in each of the processes in which they are involved (Table 1).

Cardiovascular-Targeted Canonical Pathway Analysis

We explored the cardiovascular-related functions and canonical pathways significantly altered by at least 2 of the miRNAs studied (Table 2). The major cardiovascular functions associated with these miRNAs are related to the development of blood vessels and cardiovascular tissues (Table 2). We observed 30, 26, and 18 molecules altered in angiogenesis, vasculogenesis, and cardiogenesis, respectively (p value <0.05).

Table 2. CVDs and functions regulated by the studied miRNAs

Disease and function	p value	Molecules
Angiogenesis	7.57E−04	30
Vasculogenesis	5.79E−04	26
Cardiogenesis	2.27E−04	18
Hypertrophy of heart	3.51E−02	10
Heart failure	2.44E−02	10
Development of artery	1.92E−04	8
Apoptosis of heart	3.45E−02	7
Formation of coronary vessel	1.76E−05	7
Hypertrophy of cardiac muscle	2.24E−02	6
Formation of heart tissue	3.05E−04	6
Proliferation of heart cells	2.63E−02	4
Blood vessel collateralization of coronary artery	7.77E−04	4
Development of atrioventricular valve	5.53E−04	3
Hypoplasia of myocardium	7.20E−03	2
Injury of heart tissue	1.71E−02	1
Injury of left ventricle	8.61E−03	1
Hypoplasia of ventricular compact zone	8.61E−03	1
Oxidative stress response of cardiomyocytes	4.23E−02	1
Atresia of tricuspid valve	3.40E−02	1
Experimentally induced heart failure	1.71E−02	1
Thrombosis of glomerular capillary	4.23E−02	1

miRNA, microRNA; CVD, cardiovascular disease.

Discussion/Conclusion

In the present study, we performed an enrichment analysis using IPA to observe the pathways regulated by the HIPARCO score miRNAs (miR-378a-3p, miR-100-5p, and miR-486-5p). Our main objective was to investigate the main biological functions regulated by these 3 miRNAs that are involved in the favorable response to CPAP treatment in patients with OSA and RH.

We observed the contribution of miR-378a-3p, miR-100-5p, and miR-486-5p in the regulation of a variety of genes and pathways, especially in those related to cardiovascular function and cancer. Specifically, we observed a role for these miRNAs in heart hypertrophy, heart failure, apoptosis, and heart tissue injury. This finding is corroborated by a variety of studies in the literature demonstrating an association between miRNAs, CVD, and hypertension [16, 17]. Similarly, studies have shown a strong relation between OSA and these events [18–20]. Therefore, it has been reported that OSA is associated with hypertension and with hypertension-associated end-stage organ diseases such as stroke, coronary heart disease, and arrhythmia [21]. Moreover, previous studies have suggested that OSA has a role in the initiation and progression of several CVDs [18]. Considering this, the effect of CPAP treatment in RH patients could be mediated by these miRNAs through their actions on cardiovascular-associated genes and functions.

In the cardiac hypertrophy signaling canonical pathway, miR-100 regulates the expression of mammalian target of rapamycin (mTOR), and together with miR-378a, it regulates insulin-like growth factor 1. miR-378a-3p also affects the expression of the growth factor receptor-bound protein 2 and interleukin 6 receptor. Moreover, miR-486 interferes with the regulation of the expression of eukaryotic translation initiation factor 4E (eIF4E), p70S6 kinase and the complex transforming growth factor-β-activated kinase 1 (TAK1)/TAB1 (Fig. 2). The 3 miRNAs explored also participate in the regulation of the nuclear factor of activated T-cells

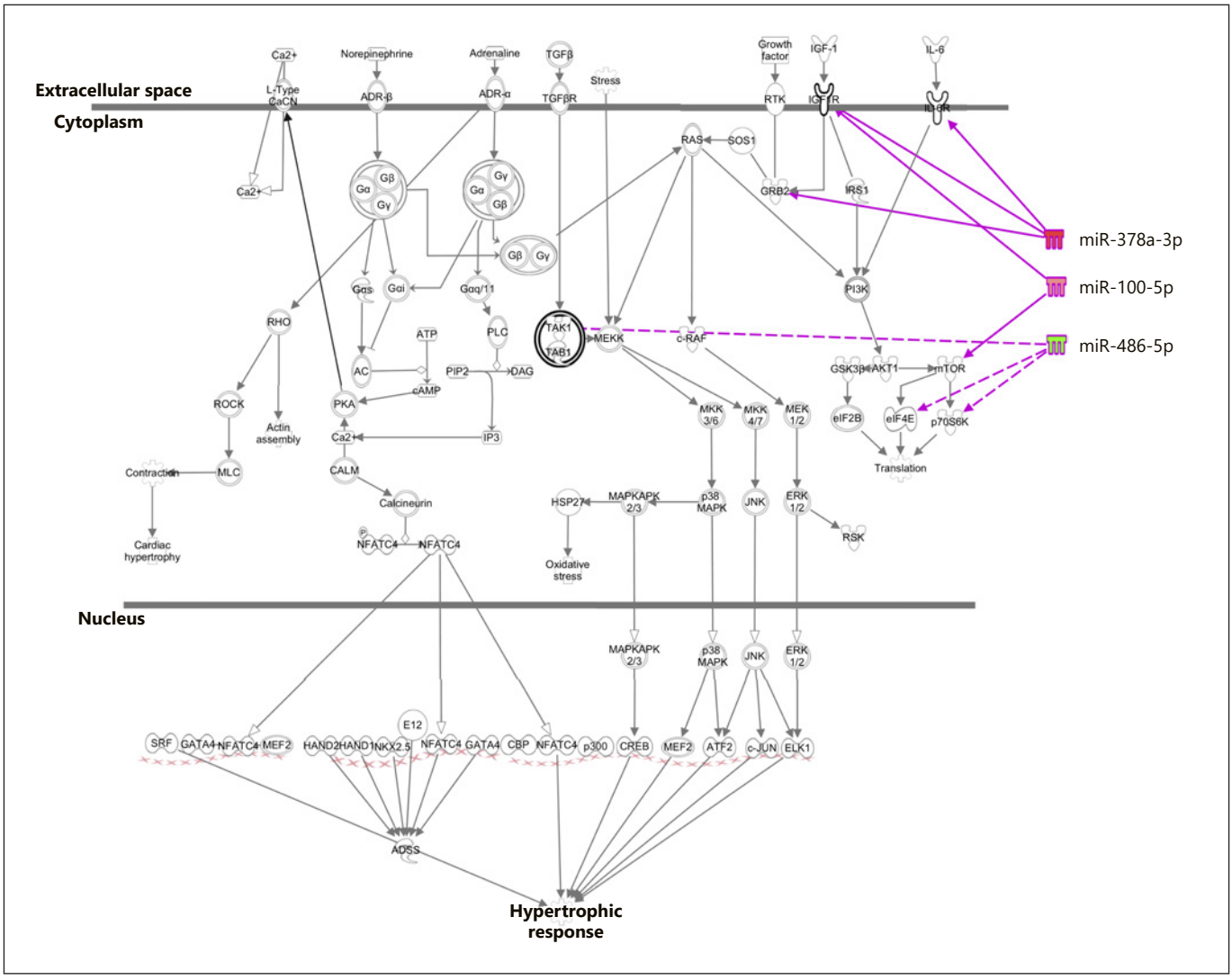

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Fig. 2. miRNA target genes in the cardiac hypertrophy signaling canonical pathway: miR-100-5p directly regulates mTOR. Additionally, miR-100-5p and miR-378a-3p directly modulate the IGF1 membrane receptor. miR-378a-3p also regulates the membrane receptor of IL-6 and GRB2 at the cytoplasmic level. miR-486-5p has an indirect effect on eIF4E, p70S6K and on the TAK1/TAB1 complex. miRNA, microRNA; mTOR, mammalian target of rapamycin; GRB2, growth factor receptor-bound protein 2; p70S6K, p70S6 kinase; TAK1, transforming growth factor-β-activated kinase .

(NFAT). miR-100-5p and miR-378a-3p jointly regulate the expression of leukemia inhibitory factor and insulin-like growth factor 1, while miR-486 regulates TAK1. In addition, miR-378a-3p alters the expression of the glycoprotein 130 dimer and SHC-growth factor receptor-bound protein 2-SOS complex (Fig. 3). Diverse studies reported an association between cardiac hypertrophy and the severity of OSA [22, 23]. In addition, it is important to address that RH may lead to cardiac hypertrophy [24].

We observed that the studied miRNAs were also associated with the NF-kB signaling pathway, which is known to upregulate the transcription of genes involved in endothelin signaling. miR-486-5p and miR-378a-3p regulate the expression of TAK1 and tumor necrosis factor receptor-associated factor 6, respectively, leading to an alteration in the activation of the NF-kB pathway (Fig. 4). This ultimately leads to an alteration in the mechanisms of cell survival and cell proliferation. Thus, the mechanism by which CPAP treatment decreases BP may be associated

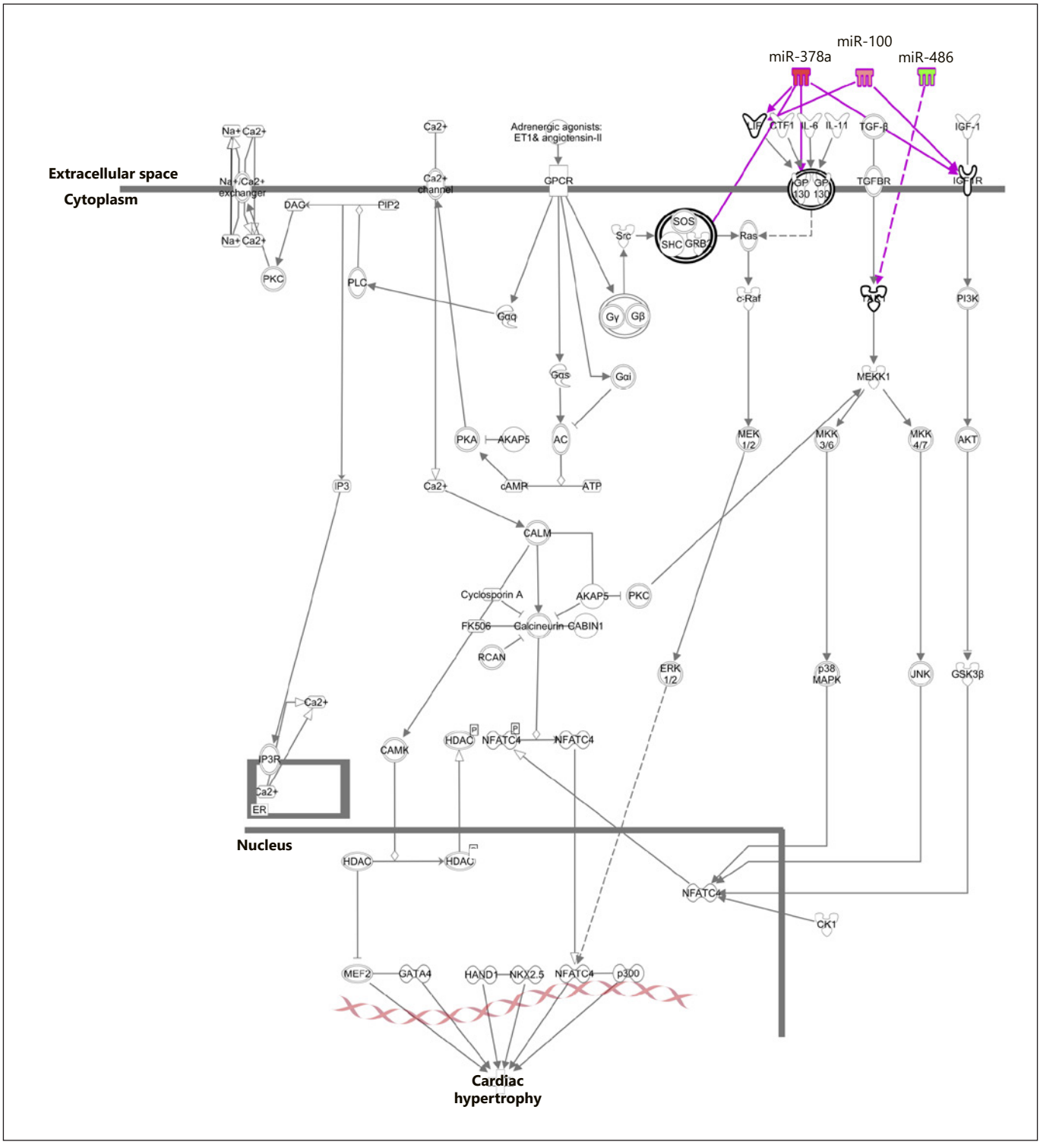

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Fig. 3. miRNA target genes in the NFAT pathway: miR-100-5p and miR-378a-3p directly regulate the IGF1 membrane receptor and LIF. miR-378a-3p acts on the formation of the SCH-GRB2-SOS complex at the cytoplasmic level. In addition, this miRNA is directly involved in the activity of the GP130 dimer. miR-486-5p is indirectly implicated in the regulation of TAK1. miRNA, microRNA; GRB2, growth factor receptor-bound protein 2; NFAT, nuclear factor of activated T-cells; LIF, leukemia inhibitory factor; TAK1, transforming growth factor-β-activated kinase 1; GP130, glycoprotein 130.

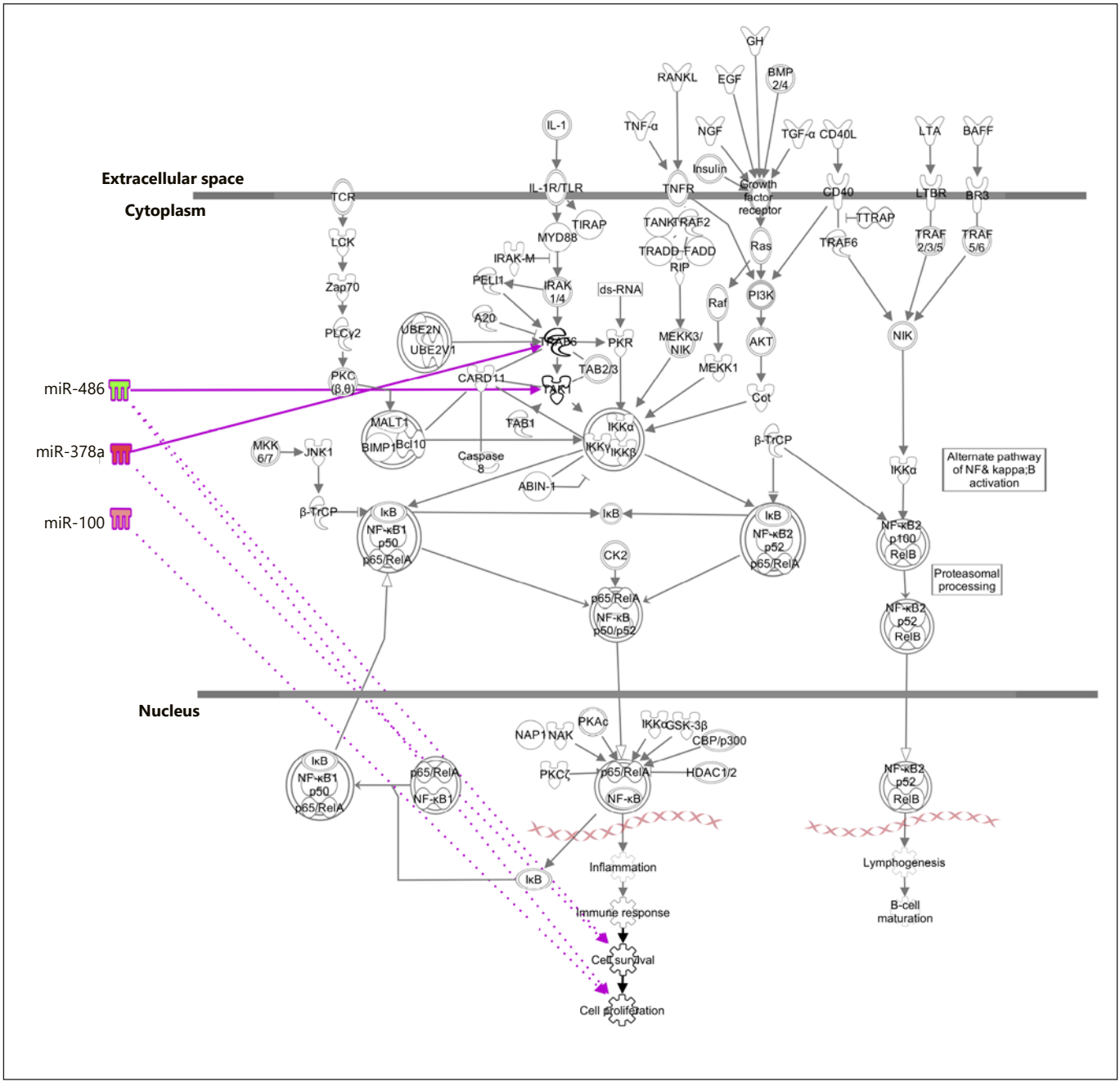

Fig. 4. miRNA target genes in the NF-kB signaling canonical pathway: miR-486-5p directly regulates TAK1, and miR-378a-3p directly modulates TRAF6 at the cytoplasmic level. miR-486-5p, miR-378a-3p and miR-100-5p indirectly alter the functions of cellular survival and proliferation. miRNA, microRNA; TAK1, transforming growth factor-β-activated kinase 1; TRAF6, tumor necrosis factor receptor-associated factor 6.

with the involvement of these miRNAs with the NF-kB signaling pathway, ultimately affecting the endothelin signaling. Previous studies have reported that hypoxia, a potential major factor contributing to the pathogenesis of OSA-related comorbidities, induces endothelin gene expression and secretion in the cultured human endothelium [25]. Moreover, it has been reported that OSA increases BP and plasma levels of endothelin-1. Interestingly, both are reduced after CPAP treatment [26]. These authors postulate that vasoconstrictor and mitogenic ef-

fects of endothelin-1 may be implicated in increased cardiovascular risk in patients with OSA [27].

We observed that miR-378a-3p, miR-100-5p, and miR-486-5p are involved in the regulation of several molecules related to activation pathways that lead to a hypertrophic response at the cellular level. The miR-378a-3p mediated the activation of genes involved in hypertrophy and survival pathways [28]. Consequently, the translation of various signals that targets mTOR also occurs. Interestingly, Wen et al. [29] demonstrated that intermittent hypoxia, which is one of the main features of OSA, led to the inhibition of mTOR phosphorylation and activation of AMPK phosphorylation, inducing cellular apoptosis.

miR-378a-3p, miR-100-5p, and miR-486-5p also seem to regulate the effect of pro-inflammatory cytokines in beta cells via NF-kB [30, 31]. The explored miRNAs modify NF-kB activity, which is pivotal for activating or preventing the overstimulation of the TLR pathway [30]. On the other hand, rapid reoxygenation at the end of apneas/hypopneas leads to the production of free radicals, inducing oxidative stress and the upregulation of NF-kB. In addition, there is evidence indicating that CPAP treatment reduces the levels of inflammatory mediators, such as interleukin-6, tumor necrosis factor-α, and C-reactive protein [32].

A substantial number of studies have reported the oncogenic and tumor-suppressing roles of miRNAs. Additionally, several miRNAs are markers for the early diagnosis of cancer [33, 34]. In fact, the present study suggests a role for the studied miRNAs in a variety of cancer-related pathways. We found that the explored miRNAs targeted 10 genes associated with the molecular mechanisms of cancer.

Our study had several limitations. First, the canonical pathways explored in this study were associated with specific miRNAs explored in males. Due to the different pathophysiology of OSA in females and the previously demonstrated sex bias in miRNA expression [35], further studies are needed to evaluate the profile of miRNAs in females and to explore the canonical pathways associated. Second, we explored a human cardiovascular system-specific miRNA arrays including 84 miRNAs. Additional miRNAs that could also contribute to identify additional canonical pathways associated with BP response to CPAP treatment should be further explored. Third, the design of the study includes an in silico analysis to investigate the genes, functions, and pathways related to the HIPARCO score miRNAs. An evaluation of their functional impacts on putative target genes and pathways will be necessary to confirm these findings. Fourth, it is important to note that there are still considerable limitations in miRNA studies, and more investigations are needed to confirm the role of miR-378a-3p, miR-100-5p, and miR-486-5p in CVD. Considering that these miRNAs were measured in the plasma, no distinction was made between miRNAs derived from exosomes and those derived from other events, such as cell death. Thus, the results discussed in the present exploratory post hoc analysis should be interpreted with caution. Regardless, the observed significant change in the circulating profile of miRNAs from plasma samples is sufficient as a biomarker to be interpreted by itself.

As a conclusion, we observed the contribution of miR-378a-3p, miR-100-5p, and miR-486-5p in the regulation of a variety of genes and pathways, especially in those related to cardiovascular function and cancer. The cardiac hypertrophy and NF-kB signaling pathways were identified as the cardiovascular pathways most influenced by these 3 miRNAs. Considering this, the mechanisms by which CPAP treatment decreases the BP in OSA patients with RH could be related to these pathways. Improved knowledge of the pathways altered by these 3 miRNAs might ultimately lead to a better understanding of the molecular mechanisms underlying lowered BP levels after CPAP treatment. In addition, this will contribute to the elucidation of new therapeutic targets in patients who do not respond to CPAP treatment.

Future perspectives

1. In the present study, we identified specific pathways associated with CVD in which the explored miRNAs could play a relevant role to be explored that could contribute to understanding the cardiovascular physiopathology associated with OSA.
2. It could be hypothesized that the studied miRNAs associated with a favorable response to CPAP treatment could be involved in the regulation of long-term cardiovascular adverse outcomes. This highlights the importance of these miRNAs not only as biomarkers but also as possible therapeutic targets.
3. The present study suggests a role for the studied miRNAs in a variety of cancer-related pathways. This leads to a possibility for which there is still no evidence that the phenotype of good CPAP responders in terms of BP might be associated with other characteristics, such as different prognoses in tumor-related diseases.

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Statement of Ethics

The HIPARCO study was approved by the Ethics Commitee of each participating center (02/05/08_48), and all patients provided written informed consent.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A.Z., F.S., F.B., and M.S. contributed to the study concept and design; A.Z., F.S., A.S., I.B., M.A.M., F.B., and M.S. contributed to the data acquisition; A.Z., F.S., A.T., L.P., A.S., F.B., and M.S. contributed to the data analysis and interpretation; and A.Z., F.S., A.T., L.P., I.B., A.S., M.A.M., F.B., and M.S. contributed to the manuscript draft, critically revised the manuscript for important intellectual content, and approved the final version. M.S. is the guarantor of the paper.

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