Circulating plasma exosomes in obstructive sleep apnoea and reverse dipping blood pressure

Abdelnaby Khalyfa ¹, David Gozal ¹, Wen-Ching Chan², Jorge Andrade² and Bharati Prasad³

Affiliations: ¹Dept of Child Health and the Child Health Research Institute, University of Missouri School of Medicine, Columbia, MO, USA. ²Center for Research Informatics, The University of Chicago, Chicago, IL, USA. ³Division of Pulmonary, Critical Care, Sleep and Allergy, Dept of Medicine, University of Illinois at Chicago and Jesse Brown VA Medical Center, Chicago, IL, USA.

Correspondence: Bharati Prasad, Division of Pulmonary, Critical Care, Sleep, and Allergy, Dept of Medicine, University of Illinois Hospital and Health Sciences System, 840 S. Wood Street, MC 719, 9th Floor, Chicago, IL 60612, USA. E-mail: bprasad@uic.edu

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ABSTRACT

Background: Obstructive sleep apnoea (OSA) increases the risk of an abnormal nondipping 24 h blood pressure profile, an independent risk factor for cardiovascular disease (CVD). We examined differential exosomal microRNA (miRNA) expression in untreated OSA patients with normal dipping blood pressure (NDBP) and reverse dipping blood pressure (RDBP), an extreme form of nondipping, to understand the mechanisms underlying nondipping blood pressure in OSA.

Methods: 46 patients (15 RDBP *versus* 31 NDBP) matched for OSA severity (respiratory event index 32.6 ± 22.5 *versus* 32.2 ± 18.1 events·h⁻¹; $p=0.9$), age (54.8 ± 12.9 *versus* 49 ± 9.9 years; $p=0.09$) and body mass index (36.2 ± 6.6 *versus* 34.4 ± 6.8 kg·m⁻²; $p=0.4$) were included. Plasma exosomes were characterised by flow cytometry and functional *in vitro* reporter assays were conducted on cultured endothelial cells. Exosome miRNA cargo was profiled with microarrays followed by bioinformatics analyses.

Results: Exosomes from RDBP patients increased the permeability of endothelial cell tight junctions and adhesion molecule expression. Principal component analyses of miRNA array data showed strict separation and identification of the two groups. A restricted and validated signature of exosomal miRNAs was identified in the RDBP *versus* NDBP group. Their predicted target genes involved phosphatidylinositol 3-kinase-Akt ($p=0.004$), Ras ($p=3.42E-05$), Wnt ($p=0.003$) and hypoxia inducible factor-1 signalling ($p=0.04$), inflammatory mediator regulation of transient receptor potential channels ($p=0.01$), and several cancer-related pathways.

Conclusions: Patients with RDBP have altered miRNA cargoes in circulating exosomes that invoke *in vitro* endothelial dysfunction. A selected number of circulating exosomal miRNAs play an important role in abnormal circadian regulation of blood pressure and may provide prognostic biomarkers of CVD risk in OSA.

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Introduction

Obstructive sleep apnoea (OSA) is characterised by intermittent hypoxia and sleep fragmentation [1], and promotes the abolition of the normal nocturnal blood pressure decline that occurs during sleep [2–4]. The emergence of a nondipping blood pressure phenomenon is an independent predictor of organ damage and cardiovascular disease (CVD) [5, 6]. Abnormal circadian patterns of blood pressure in OSA range from nondipping (<10% nocturnal decline) to reverse dipping, where a rise, rather than a decline, in average nocturnal blood pressure occurs compared with average diurnal values.

The mechanisms underlying hypertension and nondipping in OSA include increased sympathetic nervous system activity and endothelial dysfunction [7, 8]. In particular, endothelial dysfunction in OSA is an early event preceding the clinical manifestations of CVD [9, 10]. Therefore, examining the molecular basis of nondipping in clinical populations with OSA and hypertension and the potential effects on endothelial function can improve our understanding of the mechanistic underpinnings of OSA-related hypertension.

Exosomes are 30–100 nm extracellular vesicles secreted by cells into the circulation [11–13]. Exosomes carry a biologically active cargo derived from parent cells, which depends on the status of the parent cells at the time of exosome biogenesis [14–16]. Circulating exosomes are captured by acceptor cells, where the exosomal microRNAs (miRNAs), small noncoding RNAs, can regulate gene expression post-transcriptionally, thus acting as vehicles for intercellular communication [17]. The diverse exosomal cargo, which consists of miRNAs, proteins, lipids, DNA and RNA, provides unique opportunities for biomarker discovery and development of noninvasive diagnostic approaches using urine or blood [12, 14, 18]. We have previously shown that exosomal miRNAs play a critical role in multiple cardiometabolic diseases [19–21]. Furthermore, exosomes shed by endothelial cells are involved in CVD [22].

Here, we examined exosomal miRNA cargo in a cohort of untreated OSA patients with hypertension. First, differences in the expression of exosomal miRNAs were identified between two groups of patients within this cohort: those with an abnormal circadian profile on 24 h ambulatory blood pressure (ABP) recordings (reverse dipping blood pressure (RDBP)) compared with a matched group with a normal dipping blood pressure (NDBP) profile. Next, we examined the functional effects of differentially expressed exosomal miRNA on endothelial function by a series of *in vitro* reporter assays, as well as potential differences in exosomal miRNA content between RDBP and NDBP. We hypothesised that differences in exosomal miRNA expression and their functional implications would identify genomic pathways underlying endothelial dysfunction, an early and essential mechanism of OSA-related hypertension.

Material and methods

Participants

The study sample was a subset of participants drawn from a prospective study (n=160) to examine race and sleepiness as predictors of hypertension in OSA. 15 participants in this study were identified to have RDBP (*i.e.* a rise in blood pressure during sleep compared with waking values) on 24 h ABP monitoring. They were matched to 31 participants with normal dipping ABP (NDBP) from the same study based on age, OSA severity and body mass index (BMI). Participants were between 18 and 70 years of age, with hypertension and untreated moderate-to-severe OSA. Hypertension was defined as a physician diagnosis, use of antihypertensive medications or repeated office blood pressure $\geq 140/90$ mmHg within 3 months of enrolment. Home sleep testing (Alice NightOne type 3; Philips North America, Andover, MA, USA) was used to diagnose OSA. A home sleep test was deemed acceptable if the recording was at least 3 h in duration with functional oximetry, respiratory effort and airflow channels. OSA was defined by a respiratory event index (REI) ≥ 15 events·h⁻¹. Home sleep tests were manually scored by a board-certified sleep physician. Apnoeas were scored for flow reduction >90% for ≥ 10 s. Hypopnoeas were scored by $\geq 30\%$ reduction in flow associated with $\geq 3\%$ oxygen desaturation. Participants with $\geq 25\%$ central or mixed apnoeas were excluded. Participants completed the Epworth Sleepiness Scale, 3–7 days of wrist actigraphy (Actiwatch 2; Philips Respironics), ABP monitoring and 24 h urine collection for catecholamines. ABP measurements were taken every 20 min during the day and every 30 min at night, using a Spacelabs 90217 (Spacelabs Medical, Snoqualmie, WA, USA) ABP monitor. Urine catecholamines were measured with quantitative high-performance liquid chromatography-tandem mass spectrometry (ARUP Laboratories, Salt Lake City, UT, USA). Participants on medications that may affect urine catecholamine assays were excluded. Participants on diuretics had normal serum sodium levels. ABP recordings were deemed acceptable if >70% of measurements were completed. Dipping was calculated by the Spacelabs software using: average wake ABP–average sleep ABP/average wake ABP. Dipping status was determined by mean ABP (mean blood pressure=systolic blood pressure+2×diastolic blood pressure/3). Exclusion criteria included previous medical or surgical treatment of OSA, uncontrolled medical or psychiatric disease, hospitalisation within 3 months of enrolment, shift work, occupational driving and

pregnancy. Phlebotomy to collect plasma samples was conducted in the morning. The experimental procedures are shown in figure 1.

This study was conducted per the amended Declaration of Helsinki, and University of Illinois at Chicago (20131086-81081) and Jesse Brown VA (756434-2) Institutional Review Board approvals. Written informed consent was obtained from all patients.

Exosome isolation, characterisation and functional effects on endothelial cells

Exosomes samples were stored at -20°C for short-term use and stored long term at -80°C in several aliquots in top-cap tubes. Exosome isolation, size determination and quantification were performed by methods previously reported and detailed in the supplementary material [19, 23, 24]. *In vitro* materials and methods used to examine differences in exosomes properties and miRNA cargo between RDBP and NDBP groups included: 1) endothelial cell culture (human dermal microvascular endothelial cell (hMVEC-d) monolayer) [20, 25], 2) exosome characterisation by flow cytometry, 3) exosome uptake by endothelial cells using fluorescent linker PKH26-Red [26], and 4) endothelial monolayer barrier function by i) electric cell-substrate impedance sensing (ECIS) [25], ii) trans-endothelial electric resistance (TEER) [27], and iii) examination of tight junctions and adherens junction protein and glycoproteins, namely zonula occludens (ZO)-1, VE-cadherin and intercellular adhesion molecule (ICAM)-1, by immunofluorescence, confocal microscopy and stimulated emission depletion (STED) super-resolution microscopy, respectively. All of the materials and methods are detailed in the supplementary material.

Exosomal miRNA isolation and microarrays

Total RNA, including miRNA, was extracted from isolated exosomes using a miRNeasy Mini Kit column-based system (Qiagen, Valencia, CA, USA) following the manufacturer's instructions as previously described [20]. Microarray results were extracted using Agilent Feature Extraction version 12.0 [19, 28] to quantify signal intensities. The quality control for each miRNAs was evaluated based on their Agilent Feature Extraction quality control report (supplementary material). Functional annotation of differentially expressed miRNA, miRNA target gene predictions, regulatory network construction and quantitative real-time (qRT)-PCR validation of microarray results are further described in the supplementary material.

FIGURE 1 Schematic diagram illustrating the experimental design and data analyses. OSA: obstructive sleep apnoea; NDBP: normal dipping blood pressure; RDBP: reverse dipping blood pressure; miRNA: microRNA. Plasma exosomes were isolated from each of the matched subjects with OSA and either NDBP or RDBP patterns during sleep.

Data analysis

This proof-of-concept study was performed on a subset of matched patients derived from a cohort study. No *a priori* power calculation was performed. All data are expressed as mean with standard deviation, unless otherwise stated. All numerical data were subjected to statistical analysis using paired t-tests or ANOVA followed by *post hoc* tests (Tukey) as appropriate. Chi-squared analysis was performed on categorical data concerning demographic characteristics of the various groups. All analyses were conducted using SPSS version 21.0 (IBM, Armonk, NY, USA). Comparisons between exosome-treated and untreated cells were performed using t-tests or one-way ANOVA with Bonferroni's multiple comparison tests. $p < 0.05$ was considered statistically significant.

Results

Patient characteristics

46 patients were classified into two groups (RDBP $n=15$ versus NDBP $n=31$) matched by OSA severity (REI 32.6 ± 22.5 versus 32.2 ± 18.1 events·h⁻¹; $p=0.9$), age (54.8 ± 12.9 versus 49 ± 9.9 years; $p=0.09$) and BMI (36.2 ± 6.6 versus 34.4 ± 6.8 kg·m⁻²; $p=0.4$) as described in table 1.

Exosome characterisation and quantifications

Plasma exosome isolation, characterisation and quantification derived from RDBP are shown in supplementary figure S1. Confirming published results [19], negative stain electron microscopy showed vesicles in typically shaped morphology (supplementary figure S1a). Supplementary figure S1b shows no difference in the quantification of exosomes derived from NDBP (4.44×10^8 mL⁻¹) versus RDBP (4.50×10^8 mL⁻¹). To determine unique markers specific to exosomes of purified samples, different exosome markers were used, including tetraspanins (CD63; exosome formation and secretion), targeting/adhesion (CD31; exosome maturation and target cell binding) and membrane transport (Rab5b; exosome biogenesis, secretion and cell fusion), as shown in supplementary figure S1c. Flow cytometry of isolated exosomes from NDBP or RDBP groups revealed the presence of tetraspanins, targeting/adhesion and antigen presentation markers as anticipated from highly purified (>96%) exosome fractions (supplementary figure S1c).

TABLE 1 Demographic characteristics of subjects with normal dipping blood pressure (NDBP) and reverse dipping blood pressure (RDBP) recruited and included in the study

	NDBP	RDBP	p-value
Subjects n	31	15	
Age years	49 ± 9.9	54.8 ± 12.9	0.09
Race n			
African-American	18	10	0.6
Caucasian	8	3	
Other	5	2	
Male	27	14	0.5
BMI kg·m⁻²	34.4 ± 6.8	36.2 ± 6.6	0.4
REI events·h⁻¹	32.6 ± 22.5	32.2 ± 18.1	0.9
ODI3 events·h⁻¹	28.7 ± 18.9	28.5 ± 23.0	0.9
Nadir S_{po₂} %	77.2 ± 1.6	78.4 ± 1.6	0.6
T<90 min	81.3 ± 95.9	46.6 ± 50.1	0.2
ESS	11.2 ± 5.0	12.5 ± 4.9	0.4
TST h	6.0 ± 1.7	5.2 ± 1.6	0.1
Systolic ABP mmHg	127.7 ± 10.53	130.3 ± 12.6	0.4
Systolic ABP dipping mmHg	0.14 ± 0.03	-0.07 ± 0.05	<0.05 [#]
Diastolic ABP mmHg	78.2 ± 8.8	77.9 ± 7.2	0.9
Diastolic ABP dipping mmHg	0.17 ± 0.03	-0.10 ± 0.07	<0.05 [#]
Mean ABP mmHg	93.8 ± 7.85	94.8 ± 7.11	0.7
Mean ABP dipping mmHg	0.15 ± 0.03	-0.08 ± 0.05	<0.05 [#]
Urine epinephrine µg·24 h ⁻¹	5.5 ± 2.7	6.0 ± 3.9	0.6
Urine norepinephrine µg·24 h ⁻¹	56.7 ± 26.7	71.5 ± 45.8	0.1

Data are presented as mean±SD, unless otherwise stated. BMI: body mass index; REI: respiratory event index; ODI3: 3% oxygen desaturation index; S_{po₂}: arterial oxygen saturation measured by pulse oximetry; T<90: time <90% S_{po₂}; ESS: Epworth Sleepiness Scale; TST: total sleep time; ABP: ambulatory mean blood pressure. [#]: statistically significant.

Exosomes uptake using PKH26

To test whether exosomes isolated from the RDBP and NDBP groups were incorporated into human naive endothelial cells, the culture media of the cells was supplemented individually with PKH26-labelled exosomes (figure 2). Endothelial uptake of exosomes from NDBP and RDBP was similar for the six patients tested from each group. The PKH26 signal was observed in the lipid cell membrane of cells grown in medium supplemented with PKH26-labelled exosomes, whereas no signal was observed in cells grown in medium supplemented without exosomes to which PKH26 was also added. After validation of the quality of purified exosome samples using known exosomal markers, we determined the bioactivity of exosomes by examining cell uptake after the isolation procedures (figure 2). In this setting, cells exhibiting exosome uptake showed that both RNA and protein colocalised with 4',6-diamidino-2-phenylindole in the nucleus, while lipid localisation was restricted to the cell membrane. Exosome uptake was dose dependent, as there were increased spot numbers, total fluorescence and median pixel intensities of the dyes with increasing exosome concentrations (data not shown).

ECIS experiments

Exosomes from RDBP patients induced greater disruption of the endothelial monolayer barrier integrity compared with NDBP patients (figure 3a and b). The normalised resistance change was higher in the RDBP group ($-78 \pm -7.2\%$) compared with the NDBP group ($-57 \pm -5.3\%$) ($p=0.001$) (figure 3b).

TEER is a sensitive method to confirm the integrity and permeability of the cell monolayer. Increased TEER is directly correlated with decreased endothelial cell paracellular permeability [29, 30]. We found that the TEER values in transwells treated with exosomes derived from RDBP were $3.61 \pm 0.13 \Omega \cdot \text{cm}^{-2}$ compared with $6.61 \pm 0.57 \Omega \cdot \text{cm}^{-2}$ for NDBP ($p=0.002$) (figure 3c).

Effects of exosomes on tight junctions

Immunohistochemistry assessments confirmed that exosomes incubated with hMVEC-d monolayers induce disruption of cell membrane integrity, as illustrated by the altered topographic distribution of the tight junction protein ZO-1 (figure 4). We found that exosomes from NDBP and RDBP groups disrupted

FIGURE 2 Confocal microscope images illustrating exosome uptake by naive human endothelial cells. DAPI: 4',6-diamidino-2-phenylindole; NDBP: normal dipping blood pressure; RDBP: reverse dipping blood pressure. Exosomes were isolated from plasma of NDBP and RDBP subjects and labelled with PKH26. Human endothelial cells were grown on coverslips for 24 h and the labelled exosomes derived from NDBP or RDBP with PKH26 were added to the cells at 37°C for 24 h. Cells were washed and nuclei (blue) stained with DAPI. As controls, no exosomes were used, but PKH26 was added. Exosome uptake was measured using confocal laser scanning microscopy (Leica SP5; Leica Microsystems, Buffalo Grove, IL, USA) at 490 nm excitation and 502 nm emission. $n=6$. Scale bar: 10 μm .

FIGURE 3 Effects of exosomes derived from reverse dipping blood pressure (RDBP) and normal dipping blood pressure (NDBP) subjects on endothelial cell monolayer resistance *in vitro* using two different approaches. TEER: trans-endothelial electrical resistance; ECIS: electric cell-substrate impedance sensing. First, the ECIS device was used to monitor barrier integrity continuously. **a)** Ensemble-averaged curves of ECIS-measured endothelial cell barrier resistance changes over time after administration of exosomes from adult patients with NDBP and RDBP compared with endothelial cells incubated with plasma-free media and empty exosomes. **b)** Evaluation of ECIS-measured endothelial cell barrier resistance changes of all 15 individual patients per group after exosome administration. The quantification averages of the disruption of endothelial cell monolayer resistance for all 15 subjects per group were $-78 \pm 7.2\%$ (RDBP) compared with $-57 \pm 5.3\%$ (NDBP) ($p=0.001$). Secondly, endothelial cells were grown on transwell membranes to measure the integrity and permeability of the monolayer cells. **c)** Continuous measurement of cell monolayer barrier function (TEER) using membrane inserts in multiple wells to measure the resistance across the transwell membrane over time. TEER values for the average of each group: no exosomes, NDBP and RDBP. $n=15$.

endothelial cell tight junctions compared with untreated cells. Furthermore, exosomes derived from the RDBP group showed greater disruption of endothelial cell tight junctions compared with the NDBP group (figure 4a). STED super-resolution microscopy showed a very high magnification of ZO-1 immunoreactivity (figure 4b), allowing for visual identification of intact endothelial cell tight junctions *versus* disrupted tight junctions and the adhesion junctions between two cells. ZO-1 was linearly distributed in untreated cells, suggesting that exosomes mediate the disruption of tight junctions between cells. ZO-1 is a central regulator of VE-cadherin-dependent endothelial junctions that organise the cytoskeleton, tune cell-cell tension and control barrier formation [31]. Thus, we studied *in vitro* VE-cadherin protein expression on coverslips using naive human endothelial cells. We found that plasma exosomes from NDBP and RDBP samples incubated on naive endothelial cells diminished VE-cadherin adhesion junctions and tight junctions compared with untreated cells. The endothelial cell monolayer breakdown, as shown by a decrease in VE-cadherin, was observed only when exosomes derived from RDBP were used (figure 5a).

Since ICAM-1 is critical for the firm arrest and transmigration of leukocytes from blood vessels, we examined the effect of exosomes by ICAM-1 expression on barrier function (figure 5b). We found that immunostaining for ICAM-1 was weaker in untreated endothelial cells compared with endothelial cells treated with exosomes derived from the NDBP or RDBP groups.

Exosomal miRNA profiling and validation

Using human miRNA arrays, we identified 87 unique exosomal miRNAs that were detectable above the background level (figure 6a and b, and supplementary figure S2). Principal component analysis (PCA) and

FIGURE 4 Effect of plasma exosomes on naive endothelial cell zonula occludens (ZO)-1 tight junctions. DAPI: 4',6-diamidino-2-phenylindole; NDBP: normal dipping blood pressure; RDBP: reverse dipping blood pressure. Human endothelial cell monolayers were treated with exosomes derived from NDBP and RDBP subjects for 24 h. Labelling was carried out as described in the Materials and methods. a) Representative images of at least eight separate experiments illustrating exosome-induced changes in expression of ZO-1. n=8. Scale bar: 10 μ m. b) Stimulated emission depletion microscopy-derived image for ZO-1. As opposed to the intact tight junction of the cells not treated with exosomes, the tight junction integrity is markedly disrupted between two neighbouring cells after exosomes from RDBP are added to the media. n=8. Scale bar: 500 μ m.

FIGURE 5 Effects of exosomes derived from reverse dipping blood pressure (RDBP) and normal dipping blood pressure (NDBP) subjects on tight junctions and adhesion molecule expression in naive primary human endothelial cells. DAPI: 4',6-diamidino-2-phenylindole; ICAM: intracellular adhesion molecule. a) Representative images of exosome-induced changes in expression of VE-cadherin adherens junctions in primary human endothelial cells after treatment with exosomes from RDBP and NDBP subjects. VE-cadherin protein is shown in red and nuclei are stained with DAPI (blue). Disruption of adherens junctions and tight junctions is more apparent in cells treated with exosomes from RDBP compared with NDBP subjects. b) Representative images of ICAM-1 immunohistochemistry in naive human primary endothelial cells following treatment with exosomes from RDBP and NDBP subjects. Exosomes from RDBP subjects induced increased ICAM-1 expression compared with NDBP subjects. ICAM-1 is shown in red and nuclei are stained with DAPI (blue). Images are representative of n=8 per group. Scale bar: 10 μ m.

FIGURE 6 Plasma-derived exosome microRNA (miRNA) profiling. PCA: principal component analysis; NDBP: normal dipping blood pressure; RDBP: reverse dipping blood pressure; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes. **a)** PCA plot of exosomal miRNAs from RDBP and NDBP subjects. PCA clustering of miRNA expression by subject group is denoted by different colours, and reveals complete and accurate separation of miRNA expression across the subjects from the two groups. **b)** Heatmap illustrating miRNA expression patterns in exosomes (dark red: increased miRNA expression; light blue: reduced miRNA expression). The dendrograms show hierarchical clustering representing the similarities and dissimilarities in expression profiles among individuals and miRNAs. *In silico* target gene predictions were used for GO, KEGG pathways and gene network interactions.

heatmap clustering revealed consistent group separation and categorical assignments concordant with the experimental groups, and showed substantial differences in miRNA expression profiles (figure 6a and b). We found that 27 out of the 87 miRNAs were significantly upregulated in RDBP-derived exosomes (figure 6b). We validated four of these differentially expressed miRNAs using qRT-PCR. Furthermore, we validated four miRNAs overexpressed in RDBP compared with NDBP patients (figure 6b): hsa-miR-1234-3p (1.86 ± 0.14 fold difference; $p=0.001$), hsa-miR-1304-3p (1.91 ± 0.11 fold difference; $p=0.003$), hsa-miR-4313 (2.01 ± 0.18 fold difference; $p=0.001$) and hsa-miR-940 (2.19 ± 0.17 fold difference; $p=0.001$). These validated miRNAs showed the same trends as the expression differences identified in the miRNA arrays.

Gene targets prediction

We used several computational databases for the target predictions of 27 miRNAs and identified 5288 individual genes. To evaluate the biological role of the differentially expressed miRNA target genes, we performed a Gene Ontology (GO) classification enrichment analysis. Genes that showed a significance level $p < 0.01$ were selected and tested against the background set of all genes with GO annotation using established computational algorithms. GO revealed concordant pathways associated with vascular dysfunction (figure 7a and supplementary table S1).

We also performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis for differentially expressed miRNA target genes. The significant KEGG pathways and their genes are shown in table 2. Here, we highlight some of the pathways with potential biological significance in OSA, such as the AMP-activated protein kinase (AMPK) signalling pathway ($p=1.65E-05$), Ras signalling pathway ($p\text{-value}=2.05E-05$), cAMP signalling pathway ($p=5.53E-05$), phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway ($p=5.63E-04$), insulin secretion ($p=0.002$), insulin resistance ($p=0.023$) and hypoxia inducible factor (HIF)-1 signalling pathway ($p=0.024$).

FIGURE 7 Gene Ontology (GO) for the differentially expressed microRNAs (miRNAs) in exosomes derived from reverse dipping blood pressure (RDBP) and normal dipping blood pressure (NDBP) subjects. GO analysis was used to assess a) cellular components, b) biological processes and c) molecular functions. The classification shows the top ranked significant GO terms. d) Gene target prediction genes derived from differentially expressed miRNAs in RDBP compared with NDBP subjects and their potential involvement in human disease. 1: metabolic; 2: cardiovascular; 3: neurological; 4: psychiatric; 5: haematological; 6: developmental; 7: renal; 8: ageing. The highest significance values were for metabolic and cardiovascular genes.

Gene networks

We constructed gene networks for predicted target genes involved in the pathways shown in table 2. Based on potential biological significance, we constructed two pathways: 1) for insulin resistance (figure 8a) [32–34] and 2) for HIF-1 signalling pathway (figure 8b) [35].

The Regulatory Network of Target Genes was constructed using STRING (<http://string-db.org>). In this network, several genes are ranked as highly significant in insulin resistance, e.g. *SLC2A4*, *AKT3*, *CPT1B*, *CREB1* and *CREB3L1*, while in HIF-1 signalling the genes with high statistical significance were *AKT3*, *ANGPT2*, *BCL2*, *CAMK2A*, *CAMK2G*, *CDKN1B* and *CREBBP*.

Discussion

In this study, we found that RDBP in OSA alters functional properties of plasma exosomes, which are more disruptive of the permeability of the endothelial barrier, reduce ZO-1 and VE-cadherin, and increase ICAM-1 expression. Furthermore, the exosomes carry differentially expressed miRNAs in RDBP patients that regulate potentially important genomic pathways such as HIF-1 signalling and insulin resistance. Conceivably, these changes promote vascular damage *in vivo*, and underlie endothelial dysfunction and abnormal circadian blood pressure regulation in OSA.

TABLE 2 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways identified in target prediction genes that were found in differentially expressed microRNAs in reverse dipping blood pressure *versus* normal dipping blood pressure

Terms	KEGG pathway	p-value
hsa04152	AMPK signalling pathway [#]	1.65E-05
hsa04014	Ras signalling pathway [#]	2.05E-05
hsa04024	cAMP signalling pathway [#]	5.53E-05
hsa05231	Choline metabolism in cancer	1.04E-04
hsa04520	Adherens junction	1.13E-04
hsa04068	FoxO signalling pathway	1.46E-04
hsa04012	ErbB signalling pathway	1.53E-04
hsa04713	Circadian entrainment	2.51E-04
hsa04010	MAPK signalling pathway	4.54E-04
hsa04151	PI3K-Akt signalling pathway [#]	5.63E-04
hsa04911	Insulin secretion [#]	0.00225
hsa04310	Wnt signalling pathway	0.00397
hsa04350	TGF- β signalling pathway	0.00724
hsa04931	Insulin resistance [#]	0.023119
hsa04066	HIF-1 signalling pathway [#]	0.02485

AMPK: AMP-activated protein kinase; MAPK: mitogen-activated protein kinase; PI3K: phosphatidylinositol 3-kinase; TGF: transforming growth factor; HIF: hypoxia inducible factor. #: pathways with potential biological significance in obstructive sleep apnoea.

Exosomes are released from various tissues and carry a specifically sorted cargo of signalling molecules, including miRNAs for intercellular communication and regulation of organ function [12, 14, 36, 37]. Dysregulation of the miRNA content in the exosomes can provide valuable insights into disease mechanisms and candidate biomarkers [38, 39]. We have shown that exosomes induce *in vitro* endothelial

FIGURE 8 Gene network representation of interactions between gene target predictions of exosomal miRNAs derived from reverse dipping blood pressure compared with normal dipping blood pressure that involve a) insulin resistance and b) hypoxia inducible factor-1 signalling pathways as shown in table 2.

dysfunction in healthy humans exposed to acute intermittent hypoxia [20] and reported similar findings in severe OSA patients with obesity hypoventilation [21].

The integration, transmission and regulation of mechanical forces at sites of adhesion in the endothelium drive the progression of hypertension [40]. Interendothelial cell junctions include adherens junctions and tight junctions, which play pivotal roles in tissue integrity, barrier function and cell–cell communication [41]. The disruption of tight junctions leads to increased paracellular permeability and endothelial dysfunction [42]. Previously, we have shown that exosomes from children [19] and adults [21] with untreated OSA disrupt ZO-1 tight junctions compared with either healthy controls or effectively treated patients. In this study, we focused on the effects of exosomes, derived from a particularly high-risk phenotype (*i.e.* RDBP), on endothelial barrier function [19, 43, 44]. RDBP exosomes reduced ZO-1 and VE-cadherin expression and increased ICAM-1 expression when delivered to naive endothelial cells compared with NDBP exosomes. Thus, exosomes from RDBP patients exhibit pathophysiological properties that promote disruption of tight junctions and adherens junctions. VE-cadherin in endothelial cell adherens junctions maintains the integrity of tight junction activity [41]. ZO-1 is a junctional adaptor protein that interacts with other junctional components to stabilise the tight junction intercellular interface [45]. Finally, ICAM-1 transmembrane protein is constitutively expressed in low concentrations in leukocytes and endothelial cells, but with increased expression, it facilitates the slowing, rolling and activation of macrophages to cross the endothelial junctions and foster matrix changes associated with vascular stiffness. Thus, the increases in ICAM-1 elicited by RDBP exosomes reflect an enhanced propensity for the initiation of vascular stiffness [12, 20]. Furthermore, RDBP exosomes induced changes in endothelial permeability compared with NDBP exosomes through the loosening of adherens junctions (ECIS measurements). Thus, the exosomal cargo and its structural and functional effects on the endothelium differ between RDBP and NDBP patients, providing inferential assumptions about mechanisms underlying the onset and progression of nocturnal hypertension in OSA.

Previously, we compared the effects of continuous positive airway pressure (CPAP) treatment of OSA on plasma miRNA expression patterns in OSA patients with resistant hypertension. Two groups were compared: favourable response to CPAP *versus* nonsignificant reduction in blood pressure despite adherent CPAP treatment (24 h mean blood pressure reduction ≥ -4.5 *versus* < -4.5 mmHg) [7]. Here, we identified a cluster of five circulating miRNAs that enabled accurate prediction of significant blood pressure response to CPAP. Furthermore, we have identified several exosomal miRNA candidates that differentiate children with OSA or with obesity and endothelial dysfunction from matched children without endothelial dysfunction, and demonstrated the mechanistic role of one such miRNA in reproducing endothelial dysfunction both *in vitro* and in murine models [19]. In the current study, we found a selected cluster of 27 miRNAs to be upregulated in RDBP compared with matched NDBP patients, suggesting that these miRNAs play a role in nocturnal hypertension in OSA. To further understand the functional significance of the upregulated exosomal miRNAs in RDBP, we examined their gene targets using multiple *in silico* bioinformatic tools. Although miRNAs generally influence the expression of their target genes negatively *via* mRNA degradation and translational repression, there is evidence that they may also enhance the expression of genes [46]. To avoid an excessive number of genes predicted by single bioinformatics approaches, we applied a strict curation of such lists through combinatorial bioinformatic analyses and found that 15% of the target genes were involved in cardiometabolic processes. We showed that 32% of the predicted target genes are associated with metabolism and 27% with CVD. Important pathways significantly altered in RDBP include the AMPK signalling pathway, Ras signalling pathway, cAMP signalling pathway, adherens junction, circadian entrainment, mitogen-activated protein kinase signalling pathway, PI3K-Akt signalling pathway, insulin secretion, Wnt signalling pathway, transforming growth factor- β signalling pathway, insulin resistance and HIF-1 signalling pathway. All of these pathways have been implicated in CVD. Therefore, further studies examining the specific pathways in RDBP patients and their responses to treatment, as well as murine models targeting these pathways through selective alterations of exosomal miRNA cargo, will provide mechanistic insights and potential therapeutic targets.

Some strengths and limitations are worth noting in the present study. First, our patients were carefully phenotyped as RDBP or NDBP and matched for potential confounders. However, this proof-of-concept study does not include a normotensive OSA control group and, due to the cross-sectional design, causal inferences are not possible. Our *in vitro* reporter studies do not demonstrate that nocturnal dipping patterns will be altered *in vivo* by the exosomal miRNAs that are differentially expressed in the RDBP circulating exosomes [14]. The transfer and translation of exosomal mRNA and miRNA in target cells to suppress or enhance gene expression is a breakthrough in exosome biology [18, 19, 37]. The selectivity of miRNA loading into exosomes and their stability prompted our search for potential differences in miRNA exosomal cargo using microarray approaches [19, 20, 47, 48]. However, it is possible that additional

constitutive exosome cargo components such as proteins, RNAs, DNAs or lipids play a functional role, which should be explored in future studies. The restricted quantities of plasma that were available from the participants were constrained by requirements of the bioethical committee and precluded exploration of differences in miRNA exosomal cargo in a cell-source-specific manner. In addition, the lack of quantification of the immunostaining signal and ICAM mRNA expression in our samples are salient limitations. These limitations should be considered in future validation studies.

Conclusions

Exosomes derived from OSA with RDBP enhance disruption of endothelial cell monolayer tight junctions and increase endothelial dysfunction. A selective cluster of miRNAs regulates relevant biological pathways that underlie endothelial dysfunction and RDBP in OSA. We propose that a unique set of circulating exosomal miRNAs plays a mechanistic role in nocturnal hypertension associated with OSA. Thus, exosomal miRNAs may serve as both biomarkers and therapeutic targets in OSA-related hypertension.

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