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## Circulating microparticles from obstructive sleep apnea syndrome patients induce endothelin-mediated angiogenesis



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### ABSTRACT

Microparticles are deemed true biomarkers and vectors of biological information between cells. Depending on their origin, the composition of microparticles varies and the subsequent message transported by them, such as proteins, mRNA, or miRNA, can differ. In obstructive sleep apnea syndrome (OSAS), circulating microparticles are associated with endothelial dysfunction by reducing endothelial-derived nitric oxide production. Here, we have analyzed the potential role of circulating microparticles from OSAS patients on the regulation of angiogenesis and the involved pathway. VEGF content carried by circulating microparticles from OSAS patients was increased when compared with microparticles from non-OSAS patients. Circulating microparticles from OSAS patients induced an increase of angiogenesis that was abolished in the presence of the antagonist of endothelin-1 receptor type B. In addition, endothelin-1 secretion was increased in human endothelial cells treated by OSAS microparticles. We highlight that circulating microparticles from OSAS patients can modify the secretome of endothelial cells leading to angiogenesis.

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### 1. Introduction

Obstructive sleep apnea syndrome (OSAS) is sleep-disordered breathing associated with increased cardiovascular morbidity that could result from intermittent hypoxia-related inflammation, oxidative stress and endothelial dysfunction. Recently, circulating microparticles (MPs), small vesicles of plasma membrane released during cell activation and apoptosis [1], have been proposed to contribute to the pathogenesis of vascular dysfunction in OSAS [2]. Thus, MPs from OSAS patients decrease nitric oxide production without affecting reactive oxygen species (ROS) generation in human endothelial cells [2]. Furthermore, injection of OSAS MPs into mice results in a reduced endothelium-dependent relaxation to acetylcholine [2] and an enhanced vascular contraction to serotonin in the aorta [3]. Interestingly, MPs expressing CD62L are positively correlated with the severity of OSAS according to the apnea–hypopnea index [2,3].

Among the possible mechanisms responsible for the cardiovascular changes described in OSAS patients, activation of the endothelin-1 system has been proposed (for review see [4]). A very recent study shows single nucleotide polymorphisms in endothelin-1 gene, in which allelic frequencies are significantly altered in children with OSAS [5]. In OSAS

patients, plasma endothelin-1 levels are increased [6,7] and positively correlated with the severity of nocturnal hypoxia, and decreased by positive airway pressure treatment [8]. However, another study shows that whereas positive airway pressure treatment improves circulating levels of inflammatory adhesion molecules such as ICAM-1 and plasminogen activator inhibitor-1, enhanced levels of plasmatic endothelin-1 are not corrected [9] suggesting that further treatments against OSAS need to be developed [10]. Furthermore, in an animal model of OSAS, rats exposed to chronic intermittent hypoxia display elevated levels of endothelin-1 as well as decreased endothelium-dependent vasodilation and increased vascular contraction to endothelin-1 which account for the increase of arterial pressure [11,12]. In addition, pharmacological treatment with bosentan abolishes deleterious consequences induced by chronic intermittent hypoxia [13].

Very recent data suggest that intermittent hypoxia in OSAS patients might be involved in the development of cancer [14]. Although the exact mechanism implicated remains to be determined, it is possible that the reoxygenation periods during intermittent hypoxia generate changes in gene expression which may regulate the activity of some transcription factors and signaling pathways involved in tumor growth-inducing angiogenesis [14]. Among the plausible mechanisms involved, an enhanced angiogenesis has been proposed in OSAS patients (for review see [15]). Indeed, an increased coronary collateralization has been described in OSAS patients [16]. In addition, upregulation of proangiogenic vascular endothelial growth factor (VEGF) [17] has

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been demonstrated in OSAS. One of the key features of MPs on cardiovascular system is their ability to modulate angiogenic program [1]. Therefore, the purpose of the present study was to determine whether MPs from OSAS patients can modulate angiogenesis in human aortic endothelial cells. To our knowledge, the study reported here is the first to provide experimental evidence that circulating MPs from OSAS patients are able to induce angiogenesis through the production of endothelin-1 by human endothelial cells via a mechanism sensitive to ETB receptor antagonist.

## 2. Materials and methods

### 2.1. Patients

Consecutive male patients (19 to 70 years old) investigated by polysomnography in the Sleep Unit of the Department of Respiratory Medicine of Angers University, for suspected OSAS were screened for the study. Exclusion criteria were previous treatment for OSAS, body mass index (calculated as weight in kilograms divided by height in meters squared)  $\geq 35$  kg/m<sup>2</sup>, history of coronary artery disease, heart failure, stroke, hypertension, diabetes mellitus, dyslipidemia, and treatment with any drug known to affect endothelial function. Patients with an apnea–hypoapnea index of  $\geq 5$  events per hour were included in the OSAS group. Patients with an apnea–hypoapnea index  $< 5$  were included in the non-OSAS (N-OSAS) group. All the patients underwent evaluation of clinical profile and daytime sleepiness using the Epworth Sleepiness Scale [18]. Standard in-laboratory overnight polysomnography was performed as previously described [19] using a computerized recording system (CID 102; Cidelec, Angers, France) with the following channels: electroencephalogram, electrooculogram, chin electromyogram, arterial oxygen saturation (finger oximetry), nasal–oral airflow (pressure cannula), tracheal sound (suprasternal microphone), electrocardiogram, chest and abdominal wall motion (piezo electrodes), bilateral tibialis electromyogram, and body position. Respiratory events were scored manually using recommended criteria [20]. Hypopneas had to be associated with  $\geq 4\%$  oxygen desaturation. The University of Angers ethics committee approved the study, and patients gave their informed consent.

### 2.2. MP isolation and characterization

MP characterization was performed in the morning after sleep recording, at approximately 7 or 8 am, before breakfast. Routine laboratory tests, including glucose, glycated hemoglobin, triglycerides, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and blood cell count, were also performed using a morning blood sample. For MP isolation, blood samples were collected in EDTA tubes (Vacutainer; Becton Dickinson, Le Pont de Claix, France) from a peripheral vein using a 21-gauge needle to minimize platelet activation and were processed for assay within 2 h. Samples were centrifuged for 20 min at 270 g, and plasma was then harvested and centrifuged for 20 min at 1500 g to obtain platelet-free plasma (PFP). Two hundred microliters of PFP was frozen and stored at  $-80$  °C until use. As previously described [2,3], the remaining PFP was subjected to two series of centrifugation at 21,000 g for 45 min to eliminate plasma and to pellet MPs for studies, and supernatant was replaced by 0.9% NaCl saline solution. Finally, MP pellets were suspended in 150  $\mu$ L of 0.9% saline salt solution and were stored at 4 °C until subsequent use.

MP subpopulations were discriminated into PFP according to the expression of membrane-specific antigens by flow cytometry. MPs derived from platelets, lymphocytes, and endothelial cells were identified using anti-CD41, anti-CD45, and anti-CD146 antibodies, respectively. Anti-CD62L antibody was used to identify MPs derived from activated L-selectin<sup>+</sup> leukocytes. Irrelevant human IgG was used as an isotype-matched negative control for each sample. Five microliters of PFP was incubated with 5  $\mu$ L of specific antibody (Beckman Coulter, Villepinte, France), and after 45 min of incubation, samples were diluted in

300  $\mu$ L of 0.9% NaCl. Annexin V-FITC (BioVision Research Products, Mountain View, CA) binding was used to count phosphatidylserine-expressing MPs. To determine the MP concentration, equal volumes of sample and FlowCount beads were then added to calculate the MP concentration, and samples were analyzed using a 500 MPL system flow cytometer (Beckman Coulter). Regions corresponding to MPs were identified in forward and side-angle light scatter intensity dot plot representation set at logarithmic gain, depending on their diameter (0.1 to 1.0  $\mu$ m). Sample analysis was stopped after counting 10,000 events.

### 2.3. Cell culture

Human aortic endothelial cells (HAoECs) (Promocell, Heidelberg, Germany) were cultured (37°C, 5% CO<sub>2</sub>) in Endothelial Cell Growth Medium MV2 (Promocell) complemented with the supplements according to the manufacturer's instructions. All ECs were used at  $< 10$  passages. Cells were treated for 24 h in the absence or presence of N-OSAS or OSAS MPs at the circulating levels of MPs detected in the plasma of each patient (OSAS group; range, 3885 to 69,480 MPs per microliter of plasma; N-OSAS group; range, 2,887 to 8,5725 MPs per microliter of plasma), as previously described for other pathologies [21,22].

### 2.4. Plasmatic VEGF

Measurement of plasmatic VEGF was performed on PFP using a commercially available ELISA assay (Pierce Biotechnology, Rockford, IL).

### 2.5. VEGF expression by Western Blotting

MPs (30  $\mu$ g of proteins) were separated on a 4–12% NuPAGE gels (Invitrogen, Carlsbad, CA). Blots were probed with anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA). Tubulin (Santa Cruz Biotechnology) was used to visualize protein gel loading. The membranes were then washed at least three times in Tris buffer solution containing 0.05% Tween and were incubated for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ). The protein–antibody complexes were detected by enhanced chemiluminescence plus reagent (Amersham Biosciences) according to the manufacturer's instructions.

### 2.6. Apoptosis measurement by flow cytometry

HAoECs were exposed to MPs or actinomycinD (1  $\mu$ M as positive control; Sigma-Aldrich, St. Louis, MO) for 24 h and then fixed in 70% ethanol at 4 °C for at least 4 h. After a centrifugation at 15,000 g for 5 min, cells were re-suspended in PBS containing 0.05 mg/mL RNase (Sigma-Aldrich) and 10  $\mu$ g/mL propidium iodide (Sigma-Aldrich). Cellular DNA content was analyzed on a Cytomics FC 500 MPL flow cytometer (Beckman Coulter). In all cases at least 10,000 events were collected for analysis.

### 2.7. Cell adhesion assay

Evaluation of adherent cells was performed using crystal violet staining. Then,  $5 \times 10^4$  cells per well were seeded into 96-well plates and were treated for 24 h. After incubation, the plate was shaken for 15 s. The supernatant with non-adherent cells was removed by three washes with washing buffer (0.1% BSA in medium without serum). Attached cells were fixed with 4% of paraformaldehyde for 15 min at room temperature. Cells were rinsed two times with washing buffer, stained with crystal violet (Sigma-Aldrich) (1 mg/mL in 2% of ethanol) for 10 min at room temperature and extensively washed with distilled water. Then, sodium dodecyl sulfate 2% was added and incubated for 30 min at room temperature. Absorbance was then evaluated using a microplate reader at 550 nm (Synergy HT, Biotek).

## 2.8. Cell proliferation assay

Effects of MPs on HAoECs proliferation were analyzed by using CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). Briefly,  $5 \times 10^4$  cells per well were seeded into 96-well plates and allowed to attach overnight and then cells were treated with MPs for 24 h. After growth medium removal, dye-binding solution was added into each microplate well and cells were incubated at 37 °C for 30 min. The fluorescence levels were read on a fluorescent microplate reader (Synergy HT, Biotek) with filters for 485 nm excitation and 530 nm emission.

## 2.9. In vitro capillary network formation on Matrigel®

HAoECs, preincubated for 45 min or not with endothelin-1 receptor antagonist BQ-788 (5  $\mu$ M, Sigma-Aldrich), were treated for 24 h with either VEGF (20 ng/mL) or in the absence or in the presence of N-OSAS or OSAS MPs at the circulating levels of MPs detected in the plasma of each patient [2,3]. Then, cells were detached with trypsin-EDTA and seeded with a density of  $150 \times 10^3$  cells per well precoated with Matrigel® (Sigma-Aldrich). Briefly 150  $\mu$ L of ECM gel® substrate diluted with FBS-free medium (1:1 dilution) was added into a four-well plate and allowed to solidify for 1 h at 37 °C. Then 75  $\mu$ L of Matrigel® substrate was added and allowed to solidify for another 1 h at 37 °C. Then, cells were incubated with medium and allowed to adhere for 1 h after which the different stimuli were added. Tube formation was examined by phase-contrast microscopy (MOTIC AE21; 100 $\times$  magnification) after 24 h and was quantified using ImageJ software. The capillary length was counted in five randomly selected microscopic fields for each experiment.

## 2.10. Measurement of endothelin-1 concentration

After incubation with MPs, endothelin-1 concentration was measured in cells using Human Endothelin-1 Immunossay (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

## 2.11. Statistical analysis

Data were analyzed using GraphPad Prism Software (San Diego, CA). Data are expressed as mean  $\pm$  SEM, and  $n$  = number of patients or experiments performed. Statistical analyses were performed with non-parametric Mann-Whitney U or  $\chi^2$  tests as appropriate.  $P < 0.05$  was considered to be statistically significant.

## 3. Results

### 3.1. Characterization of patients

Thirty five male patients (19 to 70 years old) were included in this study. According to the apnea-hypopnea index, 20 patients were included in the OSAS group and 15 in the N-OSAS group. As shown in Table 1, there was no statistically significant difference between OSAS and N-OSAS patients for age, BMI, Epworth Sleepiness Scale, lipid metabolism, glucose metabolism and percentage of current smokers (Table 1). For all experiments OSAS and N-OSAS groups were matched for age, BMI and biological data.

### 3.2. Circulating MPs harbor the pro-angiogenic factor, VEGF

As previously described [2], total number of circulating MPs was not significantly different between the two groups. In contrast, CD62L<sup>+</sup> MP levels were enhanced in OSAS patients when compared to N-OSAS group (Table 1). Other subtypes of MPs were not significantly different between the two groups (not shown). These results confirm that OSA

**Table 1**

Characterization of population included in the study.

	N-OSAS	OSAS	p value
n	15	20	
Age, years	40 $\pm$ 3.3	46.5 $\pm$ 2	NS
Body mass index, kg/m <sup>2</sup>	25.3 $\pm$ 0.8	27.8 $\pm$ 0.8	NS
Weight (kg)	80 $\pm$ 3.6	84.6 $\pm$ 3.1	NS
Current smokers, n (%)	2 (13)	6 (30)	NS
Plasma glucose, mmol/L	5.2 $\pm$ 0.3	5.3 $\pm$ 0.1	NS
Total cholesterol, mmol/dL	5.8 $\pm$ 0.3	5.6 $\pm$ 0.2	NS
Triglycerides, g/L	1.3 $\pm$ 0.2	1.4 $\pm$ 0.1	NS
HDL cholesterol, g/dL	1.3 $\pm$ 0.1	1.3 $\pm$ 0.1	NS
LDL cholesterol, g/dL	3.9 $\pm$ 0.2	3.6 $\pm$ 0.2	NS
Epworth sleepiness scale	8.8 $\pm$ 1.3	10 $\pm$ 1	NS
Apnea-hypopnea index, events/h	2.2 $\pm$ 0.5	22.8 $\pm$ 3.7	<0.0001
Mean SaO <sub>2</sub> , %	94.2 $\pm$ 0.4	93.2 $\pm$ 0.3	0.038
4% oxygen desaturation index, events/h	0.8 $\pm$ 0.3	19.7 $\pm$ 3.4	<0.0001
Total circulating MPs/ $\mu$ L	17,533 $\pm$ 6,031	21,591 $\pm$ 3,780	NS
CD62L <sup>+</sup> MPs/ $\mu$ L	146 $\pm$ 35	255 $\pm$ 31	<0.01

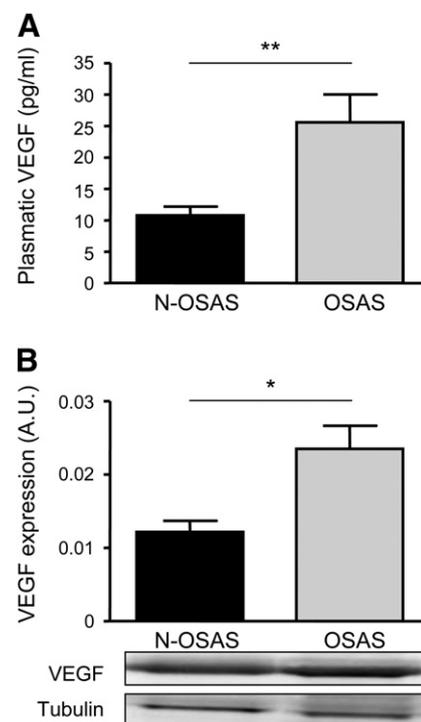
N-OSAS: non-obstructive sleep apnea patients; OSAS: obstructive sleep apnea patients; HDL: high density lipoprotein; LDL: low density lipoprotein; SaO<sub>2</sub>: oxygen saturation; MPs: microparticles. Values expressed as mean  $\pm$  SEM.

patients included in the present study display the same characteristics concerning MP levels than those previously described.

VEGF was 2.5-fold increased in platelet-free plasma from OSAS patients (Fig. 1A). Both N-OSAS MPs and OSAS MPs carried VEGF, interestingly the MPs from OSAS patients displayed a greater VEGF expression (Fig. 1B).

### 3.3. Circulating MPs from OSAS patients favor angiogenesis through the endothelin-1 release

Neither N-OSAS MPs nor OSAS MPs modified the apoptosis in HAoECs indicating that MPs did not affect cell viability (Fig. 2A).



**Fig. 1.** Analysis of vascular endothelial growth factor (VEGF) expression. (A) Quantification by ELISA assay of VEGF levels in plasma from non-OSAS (N-OSAS) and OSAS patients ( $n = 7-9$ ). (B) Thirty  $\mu$ g of proteins of microparticles from N-OSAS and OSAS patients were analyzed by Western blot using antibodies against VEGF and tubulin. Data are representative of four separate blots, and the densitometry values are expressed in arbitrary units (A.U.) as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ .

Actinomycin D was used as positive control of apoptosis induction. Also, both types of MPs did not modify HAoEC adhesion (Fig. 2B). However, OSAS MPs, but not N-OSAS MPs, were able to increase cell proliferation (Fig. 2C).

*In vitro* treatment during 24 h of HAoECs with OSAS-MPs induced increased capillary formation in a similar manner than VEGF, reflecting angiogenesis (Fig. 3A and B). N-OSAS MPs had no effect on capillary length. Endothelin-1 concentration in HAoECs (under basal conditions  $32.2 \pm 6.4 \text{ pg ml}^{-1}$ ) was increased by 27% in cells treated by N-OSAS MPs and 46% in cells treated by OSAS MPs (Fig. 3A). Interestingly, the enhanced angiogenesis induced by OSAS MPs was abolished in the presence of the antagonist of endothelin-1 receptor type B (ETB), BQ-788 (Fig. 3B and C), suggesting that OSAS MPs might stimulate the angiogenesis process in OSAS patients resulting from endothelin-1 release sensitive to blockade of endothelin-1 receptor type B.

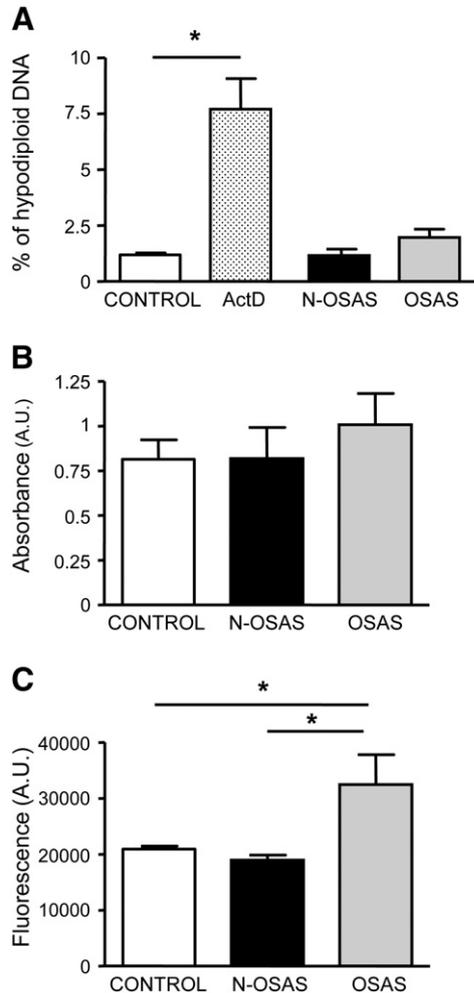
#### 4. Discussion

MPs have been described to act directly through the interaction ligand/receptor or indirectly on angiogenesis (i) by modulating soluble factor production involved in endothelial cell differentiation, proliferation, migration, and adhesion, (ii) by reprogramming endothelial

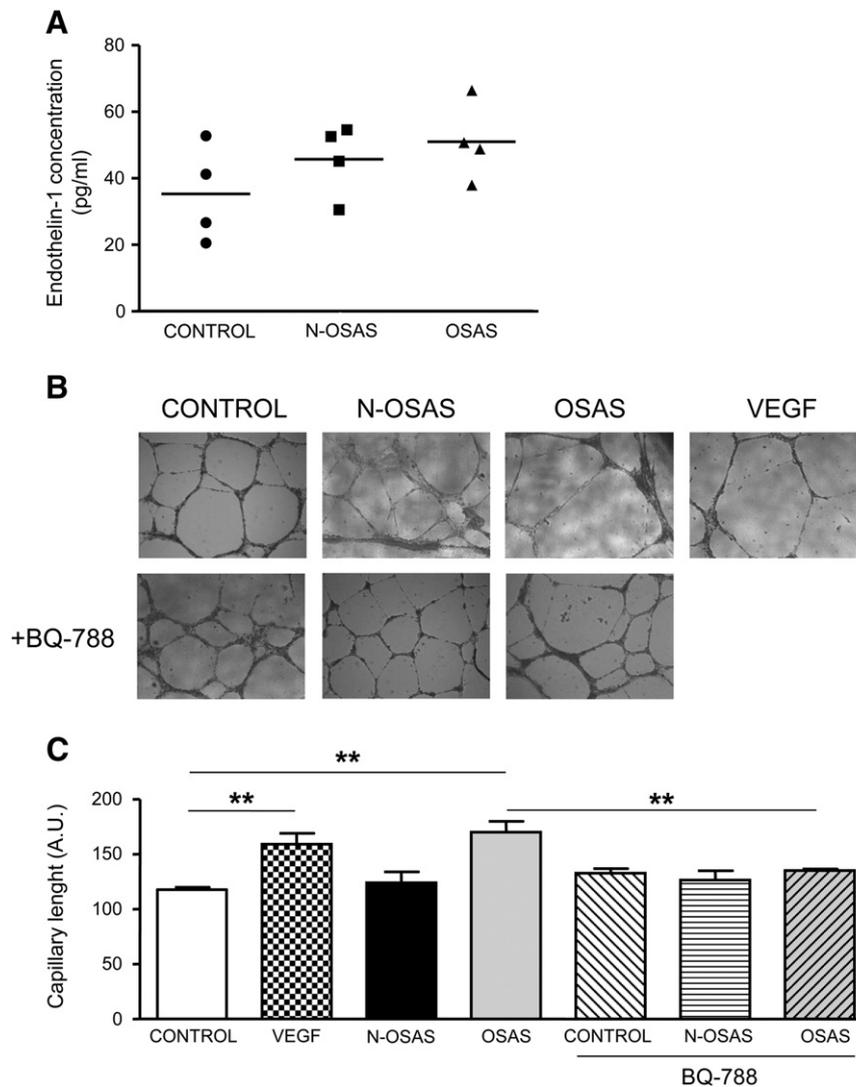
mature cells, and (iii) by inducing changes in levels, phenotype, and function of endothelial progenitor cells (for review see [1]). In the present study, we show that MPs from OSAS, but not those obtained from non-OSAS patients, induce both endothelial cell proliferation and angiogenesis to the similar extent than VEGF. Other studies have shown that MPs from patients with different pathologies are able to regulate endothelial angiogenesis by different mechanisms. Indeed, circulating MPs from atherosclerotic patients have been shown to enhance the pro-angiogenic ability of circulating angiogenic cells via RANTES pathway activation [23]. Also, MPs isolated from vitreous from patients with proliferative diabetic retinopathy increase endothelial cell proliferation and new vessel formation and [24]. In contrast, circulating MPs from diabetic retinopathy and diabetic foot ulcer patients induce unstable capillary-like tube networks that collapsed over time [25]. Taken together, these results suggest that in pathologies associated with an exacerbated angiogenesis, MPs can participate in favoring new vessel formation; in contrast, in those in which failed angiogenesis is described, MPs may be involved in the process of destabilization of new capillary formation. In the present study, intermittent hypoxia, a powerful regulator of angiogenesis, observed in OSAS may be responsible of the generation of MPs harboring VEGF. In this way, Gaustad et al. [26] have shown that acute cyclic hypoxia induced angiogenesis in melanoma tumors resulting in increased density of small-diameter vessels. Also, mice exposed to acute cyclic hypoxia showed increased incidence of pulmonary metastases, and the primary tumors of these mice showed increased blood perfusion, microvascular density and vascular endothelial growth factor-A (VEGF-A) expression [27].

It has been largely described that MPs from different cell origins can affect endothelial function by mainly decreasing NO production, increasing generation of ROS and cyclooxygenase derivatives [2,3,22,28]. In particular, we have previously shown that MPs from OSAS patients induce endothelial dysfunction mediated by reduced NO bioavailability and vascular hyperactivity to vasoconstrictors as results to cyclooxygenase metabolite production [2,3]. Another factor such as endothelin-1 released by endothelial cells might participate in the vascular alterations observed in OSAS patients. By acting through ETA receptors localized in smooth muscle cells, endothelin-1 induces vasoconstriction via phospholipase C activation, 1,4,5-inositol triphosphate formation and the consequent release of calcium from intracellular stores [29]. In contrast, ETB receptors are predominantly expressed on endothelial cells and mediate vasodilation by increasing NO and prostacyclin production [29]. Here, we show that treatment of human endothelial cells with MPs from OSAS patients induces endothelin-1 release and most importantly, OSAS MP-induced capillary tube formation is completely prevented when ETB receptors are blocked. These results are in agreement with those of Salani [30] showing that endothelin-1 is able to promote *in vitro* human endothelial cell proliferation, migration and invasion and *in vivo* mice neovascularization, and BQ 788, an ETB receptor antagonist, block the angiogenic effects induced by endothelin-1. Most interestingly, hypoxic stress evokes a significant increase of endothelin-1 release by brain endothelial cells that is associated with a decrease of endothelial NO synthase expression [31]. Several works show that intermittent hypoxia increases plasmatic levels of endothelin-1 in OSAS patients [5,9] and in animal models [13] suggesting that endothelin-1 antagonists may be used as therapeutic tools in OSAS [13]. Further studies are needed to determine whether MP-induced angiogenesis represents an adaptive mechanism to local hypoxia and may impact on the development of cardiovascular abnormalities in OSAS patients.

One potential limitation of the present study is that we only included male patients. The male predominance of OSAS is well known with a 2–3:1 ratio of male to female OSA cases in the general population, and a 8:1 or greater ratio in clinical populations. Gender-related interactions were also observed between OSAS, metabolic and cardiovascular abnormalities [32,33]. Therefore, the findings of the present study may not be extrapolated to women.



**Fig. 2.** Effects of microparticles on apoptosis, adhesion and proliferation of human aortic endothelial cells. (A) Quantification of propidium iodide staining by flow cytometry under different conditions (Control, actinomycin D (ActD), N-OSAS microparticles and OSAS microparticles). (B) Adherent cells in the absence (Control) or in the presence of microparticles from non-OSAS (N-OSAS) or OSAS patients were evaluated by crystal violet staining. (C) Proliferative effects of microparticles from OSAS patients, but not from non-OSAS (N-OSAS) patients, compared with vehicle (Control). Results are means  $\pm$  SEMs from five independent experiments. \* $P < 0.05$ .



**Fig. 3.** Implication of endothelin-1 on the pro-angiogenic effects of microparticles. (A) Quantification by ELISA assay of endothelin-1 levels in human aortic endothelial cells in the absence (Control) or in the presence of microparticles from non-OSAS (N-OSAS) or OSAS patients ( $n = 4$ ). (B) Phase-contrast micrographs showing the effects of microparticles from N-OSAS and OSAS patients on capillary-like structure formation in human aortic endothelial cells. Cells were incubated in the absence (Control) or in the presence of the antagonist of endothelin-1 receptor type B, BQ-788 (5  $\mu$ M). VEGF treatment was used as positive control. (C) Capillary length was used to quantify angiogenesis and expressed in arbitrary units (A.U.) ( $n = 6$ ). \* $P < 0.05$ , \*\* $P < 0.01$ .

In summary, we underscore a novel role of MPs in the vascular pathogenesis in OSAS patients in which increased circulating endothelin-1 is highlighted. Furthermore, OSAS MPs display increased VEGF expression and enhanced release of endothelin-1 from endothelial cells to promote the tube formation. Thus, MPs can also act as vector of information to induce release endothelin-1 responsible for increased of both angiogenesis at the endothelial side and arterial pressure resulting from vascular contraction at the level of smooth muscle.

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#### Authors' contribution

S. Tual-Chalot performed research and analyzed data. W. Trzepizur, P. Priou and Gagnadoux analyzed clinical data. R. Andriantsitohaina and M.C. Martinez designed research. F. Gagnadoux and M.C. Martinez

wrote the paper. All authors read and approved the final version of the manuscript.

#### Disclosure statement

There is no conflict of interest for this study.

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