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Circulating cell-derived microparticles in patients with minimally-symptomatic obstructive sleep apnoea

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Abstract

Moderate-severe obstructive sleep apnoea (OSA) has been associated with several pro-atherogenic mechanisms and increased cardiovascular risk but it is not known if minimally-symptomatic OSA has similar effects. Circulating cell-derived microparticles have been shown to have pro-inflammatory, pro-coagulant and endothelial function impairing effects, as well as to predict subclinical atherosclerosis and cardiovascular risk.

In 57 patients with minimally-symptomatic OSA, and 15 closely-matched control subjects without OSA, AnnexinV+, platelet-, leukocyte- and endothelial-cell derived microparticles were measured by flow-cytometry.

In patients with OSA, levels of AnnexinV+ microparticles were significantly elevated, 2586 (1566-3964) μl^{-1} compared to control subjects, 1206 (474-2501) μl^{-1} (medians and interquartile range, p=0.007). Levels of platelet-derived and leukocyte-derived microparticles were also significantly higher in patients with OSA 2267 (1102-3592) μl^{-1} and 20 (14-31) μl^{-1} respectively, compared to control subjects, 925 (328-2068) μl^{-1} and 15 (5-23) μl^{-1} respectively (p=0.009 and p=0.02 respectively). Endothelial-cell-derived microparticle levels were similar in patients with OSA, 13 (8-25) μl^{-1}, compared to control subjects, 11 (6-17) μl^{-1} (p=0.21).

In patients with minimally-symptomatic OSA, levels of AnnexinV+, platelet- and leukocyte-derived microparticles are elevated when compared to closely-matched control subjects without OSA. These findings suggest that these patients may be at increased cardiovascular risk, despite being minimally-symptomatic.

Key words: Obstructive sleep apnoea, microparticles, atherosclerosis, cardiovascular risk

Introduction

Obstructive sleep apnoea (OSA) is characterized by repetitive apnoeas/hypopnoeas during sleep, associated with oxygen desaturations and arousals from sleep. It has been estimated that between 2-4% of the adult population in Western countries suffer from symptomatic OSA, and it is becoming more prevalent as the average population body weight rises. [1] The prevalence of minimally symptomatic OSA among middle-aged adults has been shown to be as high as 26% [1], making OSA one of the most frequent disorders and thus of major epidemiologic interest.

Cross-sectional and prospective studies have implicated OSA as an important potential causal factor in the development of cardiovascular disease. [2] The mechanisms underlying the association between OSA and cardiovascular disease are currently not fully understood. Multiple causal factors leading to vessel wall damage and atherosclerotic plaques have been proposed, including reflex sympathetic activation, consequent increases in blood pressure (BP), endothelial dysfunction, activation of platelets, systemic inflammation, reactive oxygen species and hypoxia induced apoptosis of endothelial cells. [3-6]

Microparticles (MP) circulating in peripheral blood originate from the plasma membrane of diverse activated or apoptotic cells of platelet, leukocyte and endothelial origin, and are involved in the pathogenesis of vascular inflammation, thrombosis, endothelial dysfunction and atherosclerosis. [7,8] Plasma levels of MP have been shown to be elevated in patients with cardiovascular risk factors [9,10], to predict subclinical atherosclerosis burden in asymptomatic subjects [11], and appear to be a predictor of the recurrence of myocardial infarction or death in patients with acute coronary syndromes. [12] As a result, there is growing interest in early detection of these novel measures of cardiovascular risk with the anticipation that an intervention could reduce their levels and thus benefit patients who are at increased risk for future cardiovascular events.

However, there are no studies measuring circulating MP in patients with highly prevalent minimally-symptomatic OSA, nor studies investigating diverse subgroups of MP originating from platelets, leukocytes and endothelial cells in patients with OSA.

To explore this novel hypothesis as to the mechanism of vascular damage in OSA, we performed a cross-sectional study measuring circulating cell-derived MP in patients with minimally-symptomatic OSA, compared to a closely-matched control group.

Methods

Patients

Patients with possible obstructive sleep apnoea were referred to the Oxford Sleep Unit, Oxford Centre for Respiratory Medicine, by general practitioners, ear, nose, and throat surgeons or other hospital consultants because of severe snoring or witnessed apnoeas. Patients were eligible for the study if they were aged between 45 and 75 years, had proven obstructive sleep apnoea with a severity defined as more than 7.5 oxygen desaturations of >4% per hour (ODI>7.5/h), and no history of excessive daytime sleepiness or any other daytime symptom of OSA (such as tiredness or fatigue) which would have justified a trial of CPAP therapy. The level of symptoms was assessed at clinical interview, with no specific upper threshold for the ESS value to exclude entry. All eligible patients were offered participation in the study.

Control subjects were mainly identified and recruited from a general practitioner's database and were eligible for the study if they were between 45 and 75 years with no history of OSA, had less than 5 oxygen desaturations of >4% per hour (ODI<5/h) and an apnoea/hypopnoea index <5/h (AHI<5/h). Control subjects were matched to patients with OSA for gender, age, body mass index (BMI), waist to hip ratio, and cardiovascular co-morbidities. The study was approved by the Oxford

research ethics committee (REC No: 05/Q1604/159), and written informed consent was obtained from all participants.

Sleep study and assessment of sleepiness

In patients**,** OSA was diagnosed from a one-night in-hospital respiratory polygraphic sleep study. Patients' body movements, heart rate and pulse transit time (PTT) changes were recorded as measures of arousal from sleep. Pulse oximetry, snoring and increases in the respiratory swing in PTT were used as markers of breathing pattern and respiratory effort (Win-Visi monitoring system, Stowood Scientific Instruments, Oxford, UK) as previously described and validated. [13,14]

The results of the sleep study were scored automatically, with manual review to ensure accuracy of the data. OSA was diagnosed from review of all data, and the severity was quantified as the number of oxygen desaturations >4% per hour of study (ODI). In control subjects, OSA was excluded by home sleep studies using the ApneaLink™ device (ResMed, MAP medicine technology, Martinsried, Germany). This device records the patient's nasal respiratory pressure signal and finger oximetry during sleep; it has been validated as an accurate instrument to detect snoring, apnoea/hypopnoea and oxygen desaturations. [15] The results of the sleep study were scored automatically with dedicated software (ResMed, MAP medicine technology, Martinsried, Germany), with manual review to ensure accuracy of the data. Apnoeas were defined as a cessation of airflow lasting > 10 seconds, and hypopnoeas as a reduction in airflow of at least 50% lasting > 10 seconds, associated with a drop in oxygen saturation of >4%.

Comparisons of measurements of the ODI in 7 control subjects on two different nights by both sleep study methods (Win-Visi monitoring system and ApneaLink™) revealed a small and non significant bias (mean difference in ODI was -0.29 desaturations/h, p=0.72); all 7 subjects had an ODI of less than 5 in both sleep studies.

Subjective sleepiness was assessed using the Epworth sleepiness score, which assesses the tendency to fall asleep during 8 typical daytime situations. [16] Objective sleepiness was measured with one sleep resistance challenge (Osler test), which tests the ability to stay awake in a darkened and sound isolated room. [17]

Cardiovascular risk score

A cardiovascular risk score ("Framingham index") was used to objectively assess an individuals 5 year risk of death due to cardiovascular events. [18] The risk score is based on 11 factors including: age, sex, systolic blood pressure, serum total cholesterol concentration, height, serum creatinine concentration, cigarette smoking, diabetes, left ventricular hypertrophy (evaluated by resting ECG; Sokolow-Lyon index; $S_{V1}+R_{V5/V6}$ >3.5 mV), history of stroke and myocardial infarction. The risk score is an integer, with points added for each factor according to its association with risk. The sum score, and the corresponding risk of a fatal cardiovascular event within 5 years, were derived from individual patient data according to Pocock et al. [18]

Office blood pressure was measured in the sitting position after a period of rest for 5 minutes with a standard digital automatic monitor (Omron Healthcare Company, Kyoto, Japan). The mean value of three readings was used for analysis.

Measurement of Microparticles

Microparticles were measured by flow cytometry as previously described by Biro et al. [19] and blind of the patient group.

Briefly, blood was drawn from fasting participants in the morning between 09.00 and 10.00 am using a 19 G needle. Within 1 min of the blood being taken, the tubes were centrifuged at 1550g for 20 min at 20°C, to produce platelet-free-plasma. 250 μl of plasma was aliquoted, frozen immediately and stored at -80°C.

The 250 μl samples were thawed on melting ice for one hour and then spun at 18,000g for 30 min at 20°C. 225 μl of supernatant was removed, and 225 μl of phosphate-buffered saline (PBS) -citrate 0.32%, was added. The samples were spun again at 18,000g for 30 min at 20°C. 225 μl of supernatant was again removed and replaced with 75 μl of PBS-citrate 0.32%. The diluted monoclonal antibodies (MAbs) were spun again at 18,000g for 5 min, to remove MAb-MAb complexes, and the supernatant transferred to a fresh tube.

Annexin-V fluoresceinisothiocyanate (AnnV-FITC, Becton Dickinson, BD, Oxford, UK) was used to stain all phosphatidylserine-positive MPs of endothelial, platelet and lymphocyte origin. AnnV-FITC in the absence of calcium was used as a negative control.

CD31-phycoerythrin (CD31-PE), CD41-phycoerythrin-Cy5 (CD41-PE-Cy5) (BD, Oxford UK) were used to differentiate between platelet-derived MPs (PMPs, CD31+CD41+) and endothelial cell-derived MPs (EMPs, CD31+CD41-). CD45-allophycocyanin (CD45-APC, BD, Oxford, UK) was used as a marker for lymphocyte-derived MPs (LMPs, CD45+). Appropriate PE, PE-Cy5 and APC isotypes were used as negative controls.

Each sample was stained in two tubes, the first containing AnnV-FITC, CD31-PE and CD41-PE-Cy5 to detect PMPs and EMPs, the second containing AnnV-FITC and CD45-APC, to detect LMPs.

5 μl of diluted AnnV-FITC or MAbs were added to PBS-Ca 2.5 mmol/L to make up a total volume of 50 μl. 10 μl of sample was incubated with the appropriate MAbs for 30 min at room temperature, protected from light. After the incubation, 900 μl of PBS-Ca was added.

Acquisition of Samples

Samples were acquired using a BD FACSCalibur®. Compensations were checked by acquiring single-colour stained tubes. The microparticle gate was checked at each run by acquiring a tube containing 10 μl of microparticles and 5 μl of 1:1000 dilution of 1 μM beads (Sigma L-2778) in 950 μl of PBS-Ca.

Data analysis

Data are expressed as mean (SD) if data were normally distributed, and as medians (inter-quartile range, IQR) if data were not normally distributed. All statistical analyses were performed with Statistica V6.0 (StatSoft, Tulsa, OK, USA). Differences between the OSA and the control group in patients' characteristics and MPs were compared by independent *t* tests if data were normally distributed, or by the Mann-Whitney U test if data were not normally distributed. For comparison of frequencies, χ^2 test of independence was used. Spearman's rank test was used for correlation analysis. A p value < 0.05 was considered to be statistically significant.

Results

Subject characteristics

Fifty-seven patients with minimally-symptomatic OSA and 15 control subjects without OSA were recruited. Control subjects were well matched for age, gender, BMI, waist to hip ratio, and cardiovascular risk profile. There was no statistically significant difference in prescribed anti-hypertensive, cholesterol- and glucose-lowering medications between the two groups. As would be expected, patients with OSA had a higher ODI, a greater neck circumference, and tended to have a higher ESS although objective sleepiness assessed by the Osler-test was similar in the two groups (table 1).

AnnexinV+ microparticles

The median level of AnnV+ microparticles was significantly higher in patients with minimally-symptomatic OSA, 2586 (IQR 1566-3964) μl^{-1}, than in matched control subjects, 1206 (IQR 474-2501) μl^{-1} (p=0.007), as illustrated in figure 1.

There was no correlation between the level of AnnV+ microparticles and oxygen desaturation index (r=0.12, p=0.31).

CD31+CD41+ platelet-derived microparticles (PMP)

Patients with OSA had significantly higher median levels of PMP, 2267 (IQR 1102-3592) μl^{-1}, than matched control subjects without OSA, 925 (IQR 3289-2068) μl^{-1} (p=0.009) (figure 2). There was no correlation between the level of PMP and oxygen desaturation index (r=0.09, p=0.43).

CD31+CD41- endothelial-cell-derived microparticles (EMP)

Levels of peripheral blood EMP were slightly higher in patients with OSA, 13 (IQR 8-25) μl^{-1} compared to control subjects, 11 (IQR 6-17) μl^{-1}, although this did not reach statistical significance (p=0.21), as shown in figure 3. There was no correlation between the level of EMP and oxygen desaturation index (r=0.12, p=0.32).

CD45+ leukocyte-derived microparticles (LMP)

The median level of LMP was significantly higher in patients with minimally-symptomatic OSA, 20 (IQR 14-31) μl^{-1}, than in matched control subjects, 15 (IQR 5-23) μl^{-1} (p=0.02), as illustrated in figure 4. There was a statistically significant correlation between the level of LMP and oxygen deasturation index (r=0.30, p=0.01).

Discussion

To our best knowledge, this is the first study looking at circulating microparticles originating from different cell-types in patients with OSA. We have found that patients with minimally-symptomatic OSA have higher blood levels of AnnexinV+, platelet- and leukocyte-derived microparticles when compared to a well-matched control group without OSA. Although levels of endothelial cell-derived microparticles were slightly higher in patients with OSA compared to control subjects, this did not reach statistical significance.

There has been growing interest in circulating cell-derived microparticles (MP) in recent years, as plasma levels of MP have been shown to be elevated in patients with cardiovascular risk factors [9,10], to predict subclinical atherosclerosis burden in asymptomatic subjects [11], and appear to be a predictor of the recurrence of myocardial infarction or death in patients with acute coronary syndromes. [12] Therefore, quantification of MP seems to be a valuable tool to explore cardiovascular risk in asymptomatic patients.

Our finding that patients with minimally-symptomatic OSA had twice the plasma level of AnnV+ MP compared to closely-matched control subjects without OSA (figure 1) suggests that OSA could play an independent role in the formation of MP. There are several possible links between OSA and the creation of MP: platelets have been shown to release MP after activation and in response to high vascular shear stress (e.g. during acute rises in blood pressure) [20], endothelial cells and leukocytes release MP after activation by inflammatory cytokines (e.g. TNF-α), aggregated low-density lipoproteins or reactive oxygen species. [21,22] All of these mechanisms of MP formation have previously been shown to be associated with OSA. [3,23]

We found that levels of platelet-derived CD31+CD41+ microparticles (PMP) were more than twice as high in OSA patients than in control subjects (figure 2). This is

in contrast to the report of Geiser et al. [24] who found no difference in PMP levels between 12 patients with moderate-severe OSAS and 6 healthy controls. Possible explanations for the negative finding may be the small sample size and the different technique used to detect PMP in the study by Geiser et al. [24]

PMP have been shown to have prothrombogenic/proatherogenic effects in experimental vascular models by enhancing monocyte arrest on activated endothelium through P-selectin, GPIIb/IIIa, RANTES and ICAM-1. [25,26] In addition, PMP express CD40L, which has been shown to stabilize arterial thrombi [27]. As elevated levels of P-selectin, CD40L and ICAM-1 have been associated with OSA in previous studies [28,29], the elevated PMP levels we found may be a novel important link between these proatherogenic factors and OSA.

The two-fold elevation in PMP levels we found in patients with OSA compared with the control group is comparable to the difference in PMP levels reported between patients with severe arterial hypertension and normotensive control subjects. [9] Therefore, it appears that the difference we found may be of clinical importance.

OSA is considered to represent a pro-inflammatory state as evidenced by increased levels of pro-inflammatory proteins and cytokines, and thereby leading to endothelial dysfunction and atherosclerosis. [23] Our finding that leukocyte-derived microparticles (LMP) levels were higher in patients with minimally-symptomatic OSA compared to control subjects (figure 4) suggests that even minimally-symptomatic OSA may be associated with systemic inflammation, as it has been shown that LMP formation is enhanced by inflammatory stimuli [30], and in turn LMP induce IL-6, IL-8 and MCP-1 production. [31] The clinical significance of our finding is supported by a recently published study which reported that LMP levels predict subclinical atherosclerosis, as measured by plaque burden of carotid, abdominal aorta, and femoral arteries in 216 asymptomatic patients without overt cardiovascular disease. [11]

The level of endothelial cell-derived microparticles (EMP) has been shown to be an index of endothelial injury in patients with coronary artery disease and is possibly useful for identifying asymptomatic patients with diabetes mellitus at increased risk for coronary artery disease. [32,33] Although levels of CD31+CD41- EMP were slightly higher in patients with OSA compared to control subjects in our study, this did not reach statistical significance (figure 3). This in contrast to the report of El Solh and co-workers [6] who found increased levels of apoptotic endothelial cells in 14 patients with moderate-severe OSA when compared to healthy control subjects. It must be mentioned that in the latter study the authors aimed to measure apoptotic circulating endothelial cells rather than EMP (apoptotic endothelial cells are considerably larger than microparticles), and the detection of endothelial cells in their study was based on a relatively nonspecific marker (CD146) which is also recognized on other cell lines such as tumor, smooth muscle, dendritic, and stroma cells. [34]

However, a possible explanation for our negative finding could be that patients with minimally-symptomatic OSA may not yet have structural damage of endothelial cells and therefore do not produce high levels of CD31+CD41- EMP which have been shown to reflect more structural damage of endothelial cells rather than endothelial cell activation. [35] Therefore in future studies looking at EMP in patients with OSA, markers that are more specific for endothelial cell activation, such as CD62E+ EMP [35], should be analysed as well. Furthermore, the relatively small number of EMP in plasma might have reduced the power to detect a significant difference between patients with OSA and the control group.

At this point it must be mentioned that although the OSA and control group were matched as closely as possible, it still remains possible that there are other unmeasured and important differences between the two groups that might account for the differences in MP found. Possible evidence of this is that OSA patients had a larger

neck circumference, despite identical body mass index and waist-to-hip ratio. This may indicate a difference in fat distribution that in turn may reflect metabolic differences. Hence this study can only generate the hypothesis that minimally-symptomatic OSA could raise the cardiovascular risk and that this is reflected in elevated MP levels. Controlled interventional trials are needed to see if such changes are reversible before cause and effect can be implied.

Furthermore, the question of whether circulating levels of MP represent a cause or simply a marker of vascular disease in patients with OSA remains unsolved. It is possible that intermittent hypoxia may directly induce cell apoptosis and thereby leads to elevated levels of MP.

In conclusion, we found elevated levels of total, platelet-derived and leukocyte-derived microparticles in patients with minimally-symptomatic OSA when compared to well-matched control subjects without OSA, although this was not associated with a significantly increased level of endothelial-cell-derived microparticles. The elevated levels of microparticles we found may provide an important link between OSA and proatherogenic mechanisms such as thrombosis, inflammation and endothelial dysfunction. Our findings suggest that patients with minimally-symptomatic OSA might have increased cardiovascular risk, but any cause and effect relationship has to be proven in randomized-controlled interventional studies.

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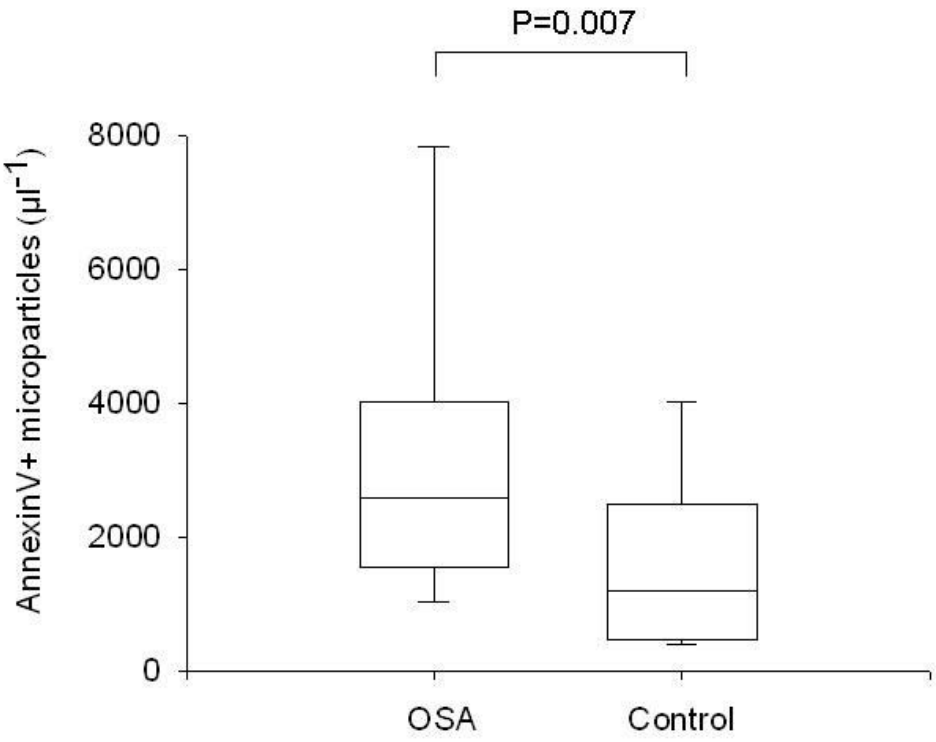
Figure legends

Figure 1:

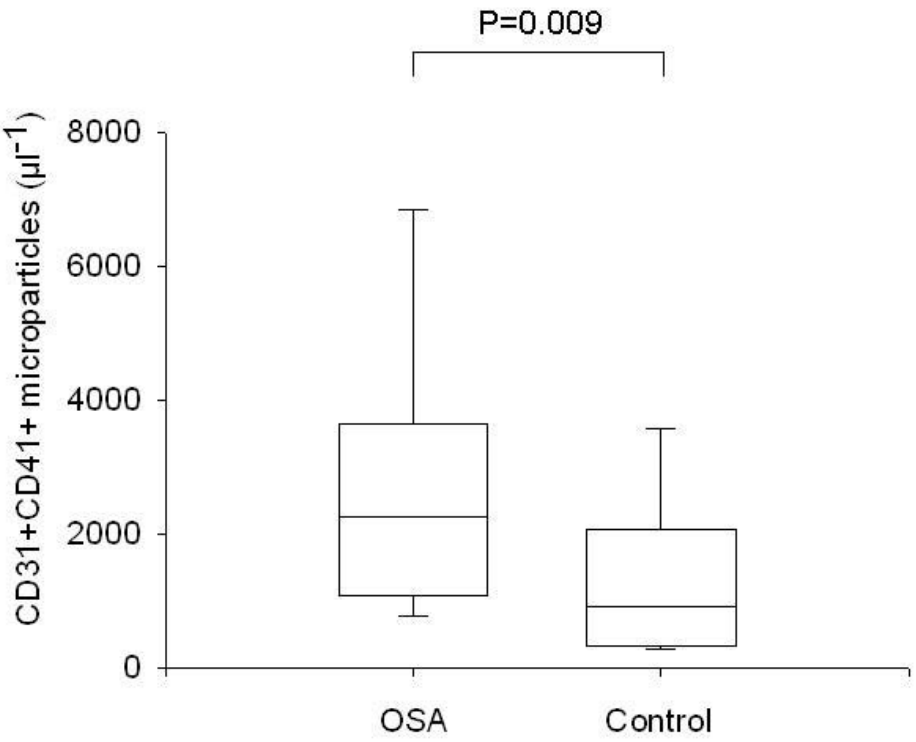
Annexin-V+ microparticle levels, as assessed by flow cytometry, were statistically significantly higher in patients with minimally-symptomatic OSA than in control subjects without OSA. Box extremities, 25^{th} and 75^{th} percentiles; error bars, 10^{th} and 90^{th} percentiles; solid line in box median.



Ayers et al. Fig.1

Figure 2:

CD31+CD41+ PMP levels were statistically significantly higher in patients with minimally-symptomatic OSA than in the control group without OSA. Box extremities, 25th and 75th percentiles; error bars, 10th and 90th percentiles; solid line in box median.

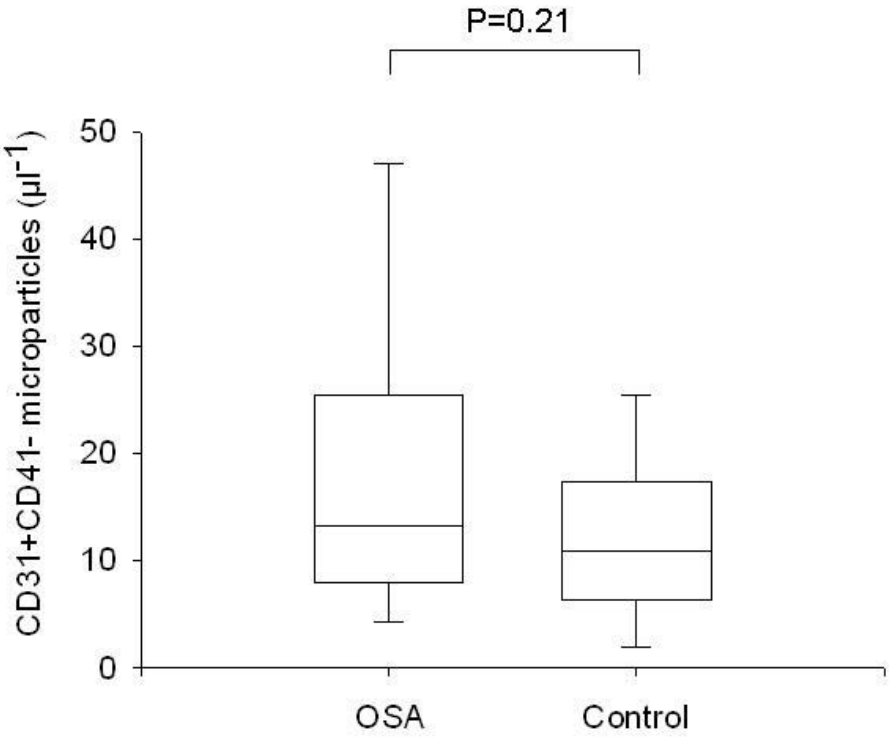


Ayers et al. Fig.2

Figure 3:

There was no statistical significant difference between CD31+CD41- EMP levels in patients with minimally-symptomatic OSA compared with the matched control group

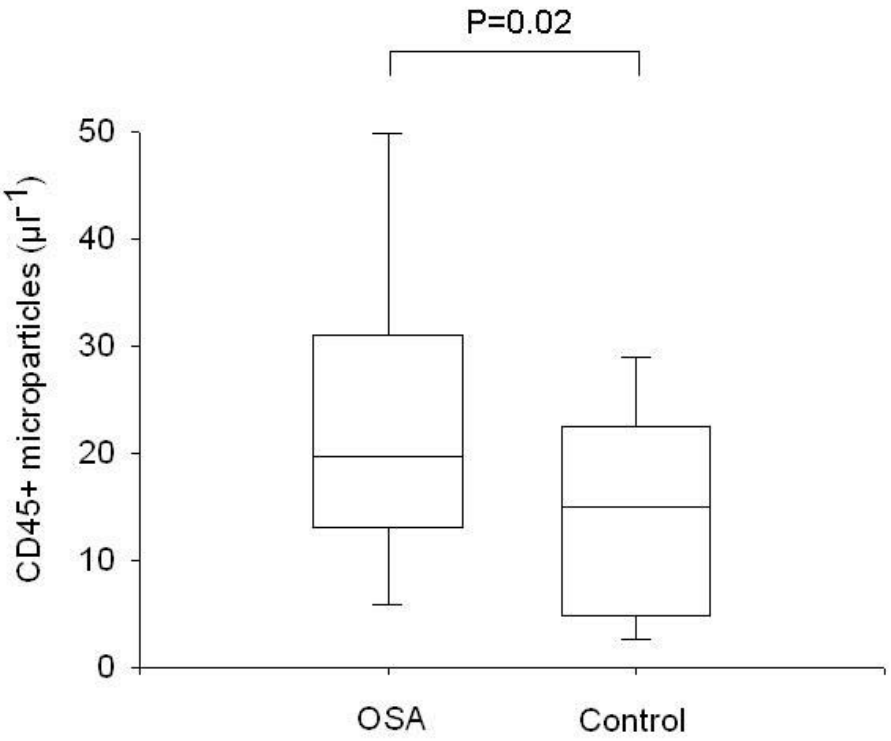
without OSA. Box extremities, 25th and 75th percentiles; error bars, 10th and 90th percentiles; solid line in box median.



Ayers et al. Fig.3

Figure 4:

CD45+ LMP levels were statistically significantly higher in patients with minimally-symptomatic OSA than in the control group without OSA. Box extremities, 25th and 75th percentiles; error bars, 10th and 90th percentiles; solid line in box median.



Ayers et al. Fig.4

Table 1. Patients characteristics

	Patients with OSA n=57	Controls without OSA n=15	P value
Age (years)	56.6 (6.6)	58.1 (6.9)	0.42
BMI (kg/m^2)	32.0 (5.3)	31.7 (2.6)	0.81
Waist/hip circumference ratio	0.97 (0.05)	0.97 (0.06)	0.93
Neck circumference (cm)	43.3 (3.6)	40.8 (3.0)	0.02
Females (%)	10.5	13.3	0.76
Current smokers (%)	15.8	13.3	0.81
Ex-smokers* (%)	35.1	33.3	0.90
Hypertension* (%)	40.4	46.7	0.66
Mean BP (mmHg)	102.3 (11.3)	98.1 (10.2)	0.20
Anti-hypertensive medication (%)	40.4	46.7	0.66
Total cholesterol (mmol/l)	5.5 (1.1)	5.2 (1.2)	0.40
Statin medication (%)	30.9	33.3	0.86
Diabetes* (%)	15.8	6.7	0.36
Fasting glucose (mmol/l)	5.9 (1.3)	6.0 (0.8)	0.82
CAD* (%)	10.5	13.3	0.76
Cardiovascular risk score (%)	2.4 (3.83)	3.0 (4.6)	0.66
Oxygen saturation dips >4% (per hour of sleep)	23.1 (15.0)	2.9 (1.1)	<0.0001

ESS	8.3 (3.8)	6.5 (3.4)	0.09
Osler (min)	32.9 (10.7)	35.2 (10.2)	0.45

Values are means (SD). BMI=body mass index; *defined by medical history; BP=blood pressure; CAD=coronary artery disease; Cardiovascular risk score estimates the risk of death (in percent) in the next 5 years due to a cardiovascular event; ESS=Epworth Sleepiness Score.