Endothelial Cell Apoptosis in Obstructive Sleep Apnea
A Link to Endothelial Dysfunction

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***Rationale*: Patients with obstructive sleep apnea (OSA) are at increased risk for cardiovascular diseases. Injury of endothelial cells has been advanced as an initial trigger to atherosclerosis.**

***Objectives*: To study the association between circulating apoptotic endothelial cells and vasomotor dysfunction as a function of sleep apnea.**

***Methods*: Brachial artery flow-mediated dilation was determined in 14 subjects with documented OSA and 10 healthy control subjects at baseline and 8 weeks after continuous positive airway pressure (CPAP) therapy. Quantification of circulating apoptotic endothelial cells ($CD146^+$ Annexin V^+) was performed by flow cytometry.**

***Measurements and Main Results*: Compared with healthy subjects, patients with OSA had higher numbers of circulating $CD146^+$ Annexin V^+ cells (39.2 $\pm$ 13.6 cells/mL and17.8 $\pm$ 9.4, respectively; $p < 0.001$). Increased apoptotic endothelial cells correlated moderately with abnormal vascular function ($r = -0.61$; $p = 0.001$). A significant correlation was observed between CD146 Annexin V^+ cells and the apnea-hypopnea index ($r = 0.56$; $p = 0.004$). After 8 weeks of treatment with CPAP, the numbers of circulating apoptotic endothelial cells were reduced significantly from 39.2 $\pm$ 13.6 to 22.3 $\pm$ 12.9 apoptotic cells per milliliter ($p < 0.001$) and correlated with improvement in endothelium-dependent vasodilation ($r = 0.49$; $p = 0.07$).**

***Conclusions*: In patients with OSA, impairment of endothelial-dependent vasodilation correlated with the degree of endothelial cell apoptosis. CPAP therapy led to significant decline in circulating apoptotic endothelial cells. These findings provide an additional mechanism for the predisposition of patients with OSA to premature vascular disease.**

Keywords: sleep apnea; endothelium; apoptosis; vasodilation

In the past decade, numerous studies have provided evidence linking OSA to the development of cardiovascular and cerebrovascular diseases (1–3). The associated risk attributable to OSA has been found to be independent of traditional risk factors such as age, sex, and obesity. Increased sympathetic activity and recurrent hypoxia associated with apneic episodes have been advanced as possible mechanisms behind the enhanced *in vitro* release of free oxygen radicals from neutrophils and monocytes (4, 5), reduced levels of nitric oxide derivatives (6, 7), and increased lipid peroxidation (8) of patients with OSA. The cumulative effect of the perturbed vascular milieu is thought to lead to endothelial dysfunction (9, 10), which is recognized as an important early event in the pathogenesis of atherosclerosis.

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Obstructive sleep apnea has been linked to cardiovascular and cerebrovascular morbidity and mortality. Endothelial dysfunction, a precursor of cardiovascular disease, has been associated with sleep apnea.

What This Study Adds to the Field

Patients with obstructive sleep apnea have higher numbers of circulating apoptic endothelial cells, which correlate with abnormal vascular function. Treatment with CPAP reduces the number of circulating apoptic endothelial cells, which correlated with improvement in endothelium-derived vasodilation.

Evidence accumulated in the last decade shows that many of the risk factors linked to endothelial dysfunction can be caused by endothelial apoptosis (11, 12). Apoptotic microparticles have been described in conditions of endothelial cell damage (e.g., in patients with thrombotic thrombocytopenic purpura, preeclampsia, and paroxysmal nocturnal hemoglobinuria) (13, 14). Similarly, increased detection of apoptotic cells, including macrophages, vascular smooth muscle cells, and vascular endothelial cells, has been reported *in vitro* and *ex vivo* in patients with ischemic heart disease (15, 16). Whether endothelial cells are also an apoptotic target in OSA has not been systematically investigated. To answer this question, we hypothesized that enhanced circulating endothelial cell apoptosis in patients with OSA correlates with the severity of sleep disturbance and reduced endothelial function.

METHODS

Study Population

Fourteen consecutive male subjects with polysomnographically verified diagnosis of OSA (apnea-hypopnea index [AHI] > 5/h) were recruited from the Sleep Clinic at the Erie County Medical Center, a University at Buffalo–affiliated hospital. Inclusion criteria included subjects who were nonsmokers, free from other diseases, and taking no medications. Ten healthy nonsmoking volunteers without sleep-disordered breathing were recruited from a wellness clinic at the hospital. Initial evaluation included collection of demographic and anthropometric measurements. A screening fasting venous blood and blood pressure were measured on the morning after polysomnography. The Institutional Review Board of the University at Buffalo approved the protocol, and informed consent was obtained from all participants.

Determination of Vascular Function

The method for measuring endothelium-dependent brachial artery dilation has been described previously (17). Data were reported as baseline diameter and percentage change in diameter (absolute change/baseline

× 100), known also as percentage flow-mediated dilatation (FMD). The end point of measurement was the percentage of change in diameter in response to reactive hyperemia (FMD). All ultrasound studies were performed by the same operator, who was blinded to the severity of sleep-disordered breathing or treatment of the study subjects. The variability in baseline diameter measurements expressed as intrasession variability was determined previously at 0.6%. Additional detail is provided in the online supplement.

Detection of Apoptotic Circulating Endothelial Cells

These studies were performed at the time of the vascular measurements between 8:30 AM and 9:30 AM. Twenty milliliters of blood were obtained by venipuncture, with the first 2 ml discarded to avoid contamination from the punctured vessel wall. Peripheral blood mononuclear cells were isolated with Ficoll-Hypaque (Amersham, Uppsala, Sweden) and after suspension of 10^6 cells/ml in phosphate-buffered saline, ethylenediaminetetraacetic acid, and 2% horse serum incubated with mouse anti-human CD146 (Chemicon, Temecula, CA), conjugated to phycoerythrin (PE) or isotype control. With the exception of certain tumor lines, CD146 (also known as MCAM, S-Endo-1, and Mel-CAM) is exclusively expressed on mature endothelial cells (18). To further confirm the specificity of CD146 expression as a marker of endothelial cell lineage, we performed two-color flow cytometry using anti-CD3, anti-CD21, and anti-CD14 monoclonal antibodies (BD Biosciences, Rockville, MD) as markers of T-cell, B-cell, and monocyte lineage, respectively. CD146 cells did not coexpress the latter markers. Apoptosis was evaluated by resuspending these cells in 100 μl Annexin V Binding Buffer and incubating them with 5 μl Annexin V–phycoerythrin (phycoerythrin; BD Biosciences) and 5 μl of 7-amino actinomycin D (7-AAD) for 15 minutes. The latter differentiates dead (7-AAD–positive) from apoptotic (7-AAD–negative) cells. Annexin V detects the externalization of phosphatidylserine during apoptosis and represents an early and sensitive marker for this type of cell death (19). Cells were analyzed by using a fluorescence-activated cell sorter scan flow cytometer (BD Biosciences) to assess the cells that were positive for CD146 and Annexin V ($CD146^{AnnV+}$) and negative for 7-AAD. A total of 10,000 events were measured per sample. On the basis of the peripheral blood mononuclear cell counts, we calculated the absolute number of circulating $CD146^{AnnV+}$/ml.

CPAP Therapy

After continuous positive airway pressure (CPAP) titration, all patients with OSA were treated with nasal CPAP using the S6 CPAP device (ResMed, Hampton, VA). Nasal CPAP compliance was directly measured using compliance software provided by the CPAP manufacturer. At 8 weeks after nasal CPAP was started, samples of venous blood were obtained, and vascular reactivity was repeated as described previously.

Statistical Analysis

Data are expressed as mean ± SD or median and range. Continuous variables were tested for normal distribution using the Kolmogorov-Smirnov test. Group comparisons were performed by Student's *t* test or the Wilcoxon rank-sum test. Univariate and nonparametric bivariate correlations were performed using the Spearman correlation coefficient. Cook's distance was used to assess for potential outliers. Statistical significance was assumed when a null hypothesis could be rejected at $p < 0.05$. Statistical analysis was done using SPSS 12.0 for Windows (Chicago, IL).

RESULTS

Fourteen consecutive patients with documented OSA and 10 control subjects were eligible for participation in the study. Table 1 depicts the baseline demographic, anthropomorphic, polysomnographic, and vascular reactivity of the brachial artery data of the study population. The patients with OSA and the control group were comparable with respect to age, sex, blood pressure, and metabolic profile. Only the body mass index was higher in subjects with OSA compared with control subjects, although the difference was not statistically significant.

After OSA diagnosis, all subjects completed 8 weeks of treatment with nasal CPAP. The average pressure setting for the CPAP device was 9.7 ± 1.9 mm Hg (range, 7–14 mm Hg). The use of CPAP ranged from 4 to 7 hours per night, with an average of 5.2 ± 1.1 hours per night. At the end of the study, there were no significant differences in anthropomorphic and metabolic profile compared with baseline measurements. However, there was a marked improvement in brachial artery vascular reactivity (Table 2).

When compared with healthy control subjects, patients with OSA had higher numbers of circulating $CD146^{AnnV+}$ cells (17.8 ± 9.4 and 39.2 ± 13.6 cells/mL, respectively; $p < 0.001$) (Figure 1A). Figure 1B shows box-plots of $CD146^{AnnV+}$ cells from a healthy control subject and a patient with OSA. Overall, there was a significant correlation between the presence of circulating apoptotic endothelial cells and AHI ($r = 0.56$; $p = 0.004$) (Figure 2) and time with oxygen saturation less than 90% ($r = 0.44$; $p = 0.01$) but not with arousal index ($r = 0.23$; $p = 0.15$).

Univariate analysis identified a significant correlation between $CD146^{AnnV+}$ cells and an impaired endothelial dependent vasodilation ($r = -0.61$; $p = 0.001$). Figure 3 shows that patients with increased circulating number of apoptotic endothelial cells displayed a strong impairment of endothelial-dependent vasorelaxation. After 8 weeks of CPAP therapy, circulating apoptotic endothelial cells was reduced in almost all patients with OSA from a mean of 39.2 ± 13.6 to 22.3 ± 12.9 apoptotic cells per milliliter ($p < 0.001$) (Figure 4). Only two patients with OSA, with AHI of 22 and 41, respectively, had a rise in the circulating apoptotic cells after therapy. The decrease in circulating apoptotic

TABLE 1. BASELINE DEMOGRAPHIC AND CLINICAL DATA

	OSA (*n* = *14*)	Control (*n* = *10*)	p Value
Age, yr	45.9 ± 5.0	42.8 ± 6.7	0.25
BMI, kg/m^2	30.6 ± 4.7	27.8 ± 3.6	0.11
Systolic blood pressure, mm Hg	124 ± 9	119 ± 8	0.34
Diastolic blood pressure, mm Hg	75 ± 6	71 ± 6	0.20
Total cholesterol, mg/dl	198 ± 28	186 ± 14	0.12
Glucose, mg/dl	95.0 ± 16.0	91.2 ± 16	0.57
Hemoglobin A1c, %	5.2 ± 0.6	4.9 ± 0.8	0.30
AHI, per hour	27.3 ± 12.5	3.9 ± 1.1	<0.001
Time spent with $Sa_{O_2} < 90\%$, min	53.1 ± 5.6	0.8 ± 0.4	<0.001
Lowest Sa_{O_2}, %	71.4 ± 4.8	89.1 ± 3.6	<0.001
Arousal index, per hour	18.2 ± 6.4	2.1 ± 1.4	<0.001
Brachial artery baseline diameter, mm	4.1 ± 0.7	4.0 ± 0.6	0.7
FMD, %	5.8 ± 1.8	7.7 ± 1.5	0.01

Definition of abbreviations: AHI = apnea hypopnea index; BMI = body mass index; FMD = flow-mediated dilation.

TABLE 2. ANTHROPOMORPHIC AND METABOLIC PROFILE FOR PATIENTS WITH OBSTRUCTIVE SLEEP APNEA AT BASELINE AND AFTER CONTINUOUS POSITIVE AIRWAY PRESSURE THERAPY

	Baseline (*n* = 14)	After CPAP (*n* = 14)	p Value
BMI, kg/m^2	30.6 ± 4.7	30.3 ± 4.0	0.5
Body weight, kg	85.0 ± 18.7	83.6 ± 14.8	0.43
Systolic blood pressure, mm Hg	124 ± 9	122.1 ± 8	0.24
Diastolic blood pressure, mm Hg	75 ± 6	74 ± 4	0.44
Total cholesterol, mg/dl	198 ± 28	195 ± 23	0.32
Glucose, mg/dl	95.0 ± 16.0	94.5 ± 14.5	0.87
FMD, %	5.8 ± 1.8	9.4 ± 2.1	<0.001

Definition of abbreviations: BMI = body mass index; CPAP = continuous positive airway pressure; FMD = flow-mediated dilation.

endothelial cells compared with baseline showed a parallel trend with the improvement in vascular reactivity, although the degree of correlation did not achieve statistical significance (r = 0.49; p = 0.07).

DISCUSSION

The major findings of this investigation are: (*1*) Patients with OSA have increased density of circulating apoptotic endothelial cells compared with non-OSA subjects; (*2*) levels of apoptotic endothelial cells are correlated with abnormal endothelial vasorelaxation, a precursor of atherosclerosis related events; and (*3*) treatment with nasal CPAP therapy reduced the level of apoptotic endothelial cells in the circulation.

To the best of our knowledge, this is the first study to demonstrate evidence of circulating apoptotic endothelial cells in the peripheral circulation of patients with OSA. If specifically searched for, apoptotic endothelial cells are rarely found in vessels that are free of vascular pathology (20), but, when afflicted with diseases, circulating apoptotic endothelial cells have been reported to be an *ex vivo* indicator of vascular injury. In these cases, the level of circulating apoptotic cells varies according to the extent of the endothelial lesion. Previous investigations have demonstrated high CD31$^+$/Annexin V$^+$ microparticles associated with widespread vascular damage in multiple sclerosis, lupus anticoagulant, and cardiovascular disease (21–23). The current findings extend these observations to the syndrome of OSA, where a positive correlation between the severity of sleep apnea and the degree of circulating apoptotic endothelial cells has been depicted. This correlation was also reproducible for the time spent with oxygen saturation less than 90%, suggesting a role of hypoxia in the induction of endothelial apoptosis. Previous investigators have shown that when human aortic endothelial cells were exposed to hypoxia for 24 hours, there was a significant decrease in cell number owing to necrosis and an increased number of apoptotic cells exhibiting DNA fragmentation compared with cells grown under normoxic conditions (24). However,

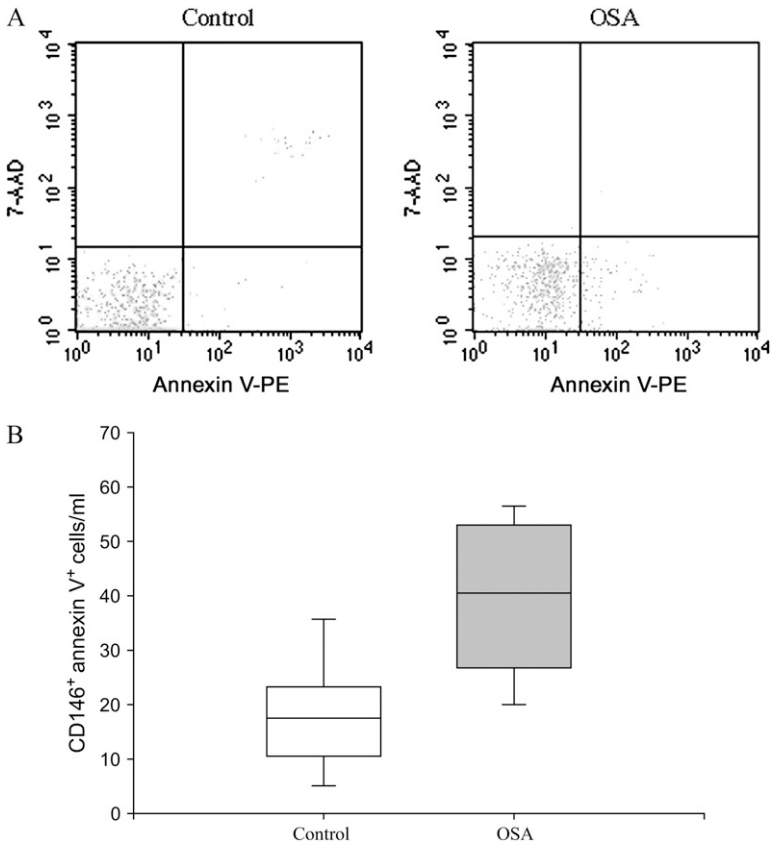

Figure 1. Patients with obstructive sleep apnea (OSA) have increased numbers of circulating apoptotic endothelial cells. (*A*) Fluorescence-activated cell sorter analysis displaying circulating apoptotic endothelial cells in a healthy control subject and in a patient with OSA. On the basis of the peripheral blood mononuclear cell counts, the absolute number of circulating CD146$^+$ expressing Annexin V$^+$ per volume of blood (milliliter) is derived from the density represented in the bottom right quadrant. 7-AAD = 7-amino actinomycin D. (*B*) Box-whisker plots of circulating CD146$^+$ expressing Annexin V$^+$ per volume of blood (milliliter) in healthy control subjects and patients with OSA. PE = phycoerythrin.

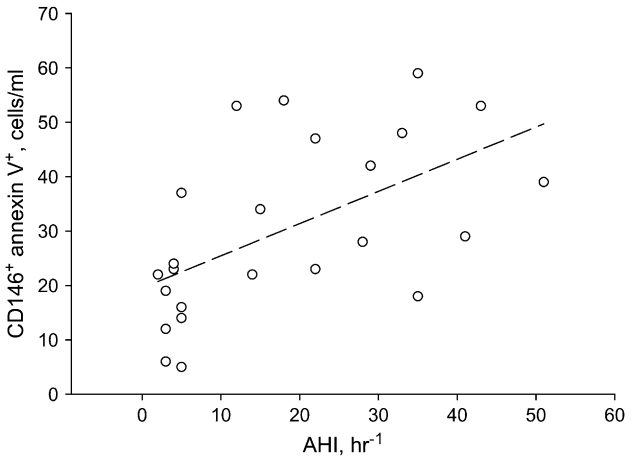

Figure 2. Increased circulating apoptotic endothelial cells correlate positively with severity of sleep apnea ($r = 0.56$; $p = 0.004$). AHI = apnea-hypopnea index.

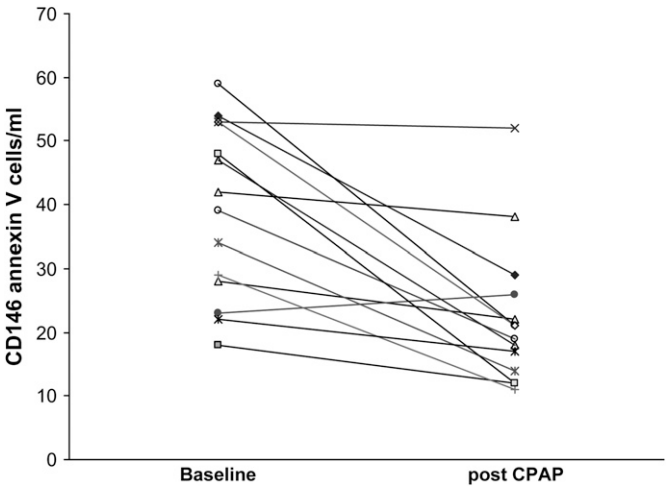

Figure 4. Circulating apoptotic endothelial cells of 14 patients with obstructive sleep apnea at baseline and after 8 weeks of continuous positive airway pressure (CPAP) therapy ($p < 0.001$).

later studies performed in vascular endothelial cells noted that exposure to hypoxia *per se* for 24 hours did not induce cell death. Only when exposed to nitric oxide under hypoxic conditions, at levels shown to be nontoxic under normoxic conditions, was apoptotic cell death markedly increased (25). These observations suggest that the hypoxic conditions encountered during apneic episodes in patients with OSA may serve to sensitize cells to apoptosis-inducing stimuli.

The increased propensity for vascular disease in OSA, despite adjustment for risk factors, is at least 4.6-fold higher for coronary artery disease (26) and eightfold higher for stroke (27). Individuals with OSA with no prior vascular event have evidence of incipient vascular dysfunction as evidenced by subtle alterations in vascular function and asymptomatic perfusion abnormalities, suggestive of altered coronary flow reserve (28, 29). A number of OSA-specific mechanisms have been hypothesized to play a role in the pathogenesis of this vasculopathy. These mechanisms include the phenomenon of hypoxia reoxygenation (30), oxidative stress (31), and release of proinflammatory markers (32), all of which have been described to cause endothelial cell apoptosis. In this line of evidence, our data underline the importance of pronounced endothelial cell apoptosis *in vivo* in the development of impaired endothelial function in patients with OSA. We have shown that circulating $CD146^{AnnV+}$ apoptotic cells highly predicted the degree of endothelial dysfunction in humans with OSA. Similar findings were observed in patients with acute coronary syndromes (33). In addition, plasma levels of apoptotic endothelial microparticles correlated with indexes of arterial stiffness in hemodialyzed patients, suggesting that high plasma apoptotic microparticles also reflect alterations in arterial properties (34). These findings indicate that the more circulating apoptotic endothelial residues are detected, the patient's arteries are less likely to normally vasodilate in response to increases in flow rate or to acetylcholine. Although the question of whether circulating levels of apoptotic endothelial cells cause or result from endothelial dysfunction is debated, *in vitro* evidence indicates that the augmented plasma levels of apoptotic endothelial cells could result from endothelial injury or apoptosis (35). Cytokines, activated platelets, or oxidized low-density lipoprotein could play a role as well. Aside from its ability to promote inflammation in atheromas, oxidized low-density lipoprotein has been found to induce endothelial cell apoptosis (36, 37). The endothelial cell loss through apoptosis results in further decreased NO production and hence accelerates the attenuation of endothelium-dependent vasorelaxation.

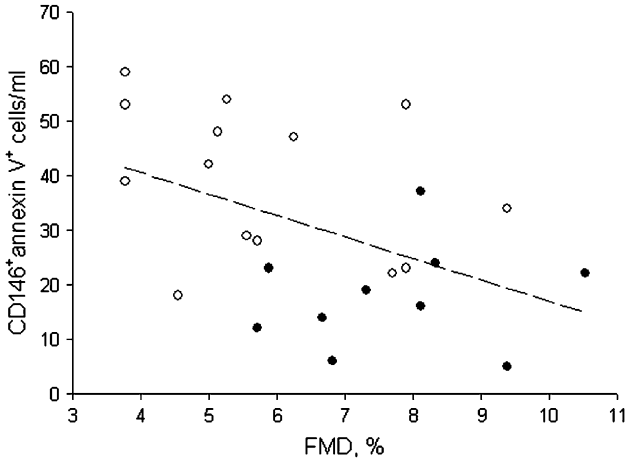

Figure 3. Increased circulating apoptotic endothelial cells correlate negatively with endothelial-dependent vasodilation ($r = -0.61$; $p = 0.001$). OSA group (*open circles*); control group (*closed circles*). FMD = flow-mediated dilatation.

The profound decrease in circulating apoptotic endothelial cells in patients after CPAP therapy underlines the role of OSA in the initiation of apoptotic machinery. The salutary effect of CPAP could be explained by a reduction in proapoptotic stimuli in the serum of OSA patients or by the blockade of intracellular apoptosis signaling pathway, which could lead to reduced sensitivity of endothelial cells to proapoptotic stimuli. There is no clear evidence to support either hypothesis in patients with OSA. We have not repeated the polysomnogram after CPAP therapy to ensure the stability of sleep indices. However, previous studies that repeated polysomnography after CPAP titration found sustained improvement in sleep indices weeks after initiation of CPAP therapy (38, 39). The rigorous inclusion criteria requiring normalization of sleep disturbances and the elimination of recurrent oxygen desaturations at the CPAP titration would lessen further the likelihood of residual sleep abnormalities.

A limitation of our study is that we did not perform a thorough assessment for excluding coronary artery disease in our study

population. Short of conducting a coronary angiography on all participants, the presence of coronary artery disease would represent a confounding variable in the interpretation of our results. However, we have designed our project to assess circulating apoptotic cells before and after treatment with nasal CPAP, which would reflect the contribution of OSA to the pathogenesis of endothelial apoptosis. It could be argued that the improvements in apoptosis in the treated OSA group may have been the effect of time. Although this scenario is plausible, the optimal design to assess the impact of time on endothelial apoptosis would have been the recruitment of a parallel group of patients with OSA matched for age, sex, and severity of disease who would be denied CPAP treatment. Given what we know about the increased morbidity and mortality of untreated OSA, such a design would be problematic. The healthy subjects and the OSA patients were not matched precisely with regard to age and body weight. We believe that this lack of homogeneity does not modify our conclusion. Both groups had comparable blood pressure and lipid profile at study entry. Furthermore, FMD in OSA has been found to improve after several weeks of CPAP without any changes in body mass index during that period. The exclusion of women from our study would raise potential criticism of whether these results would apply to the female sex, but it is well known that women, particularly those of reproductive age, have far better FMD responses (40). Further investigation is needed to determine whether sex exhibits differential endothelial apoptosis. Finally, the inclusion of subjects with moderate sleep apnea might have accounted for the relatively modest correlation between endothelial cell apoptosis and endothelial dysfunction. It is possible that a stronger association might have been established had we included patients with severe OSA, but the coexistence of comorbid diseases in these patients would have introduced confounding variables that would be difficult to control for.

In conclusion, our findings suggest that endothelial apoptosis is associated with endothelial dysfunction in patients with OSA. This enables novel insights into the cellular and molecular determinants of atherogenesis. Furthermore, circulating $CD146^{AnnV+}$ cells may serve as a novel marker of vascular dysfunction, allowing risk stratification and monitoring of athero-modifying regimens.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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