UC Irvine
UC Irvine Previously Published Works

Title
Pathways of Microcirculatory Endothelial Dysfunction in Obstructive Sleep Apnea: A Comprehensive Ex Vivo Evaluation in Human Tissue.

Permalink
https://escholarship.org/uc/item/905982q2

Journal
American Journal of Hypertension, 35(4)

ISSN
0895-7061

Authors
Gavrilin, Mikhail A
Porter, Kyle
Samouilov, Alexandre
et al.

Publication Date
2022-04-02

DOI
10.1093/ajh/hpab169

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

ORIGINAL ARTICLE

Pathways of Microcirculatory Endothelial Dysfunction in Obstructive Sleep Apnea: A Comprehensive *Ex Vivo* Evaluation in Human Tissue

Mikhail A. Gavrilin,[1] Kyle Porter,[2] Alexandre Samouilov,[3] and Rami N. Khayat[1,4]

BACKGROUND
The mechanism and markers of cardiovascular disease (CVD) in obstructive sleep apnea (OSA) remain unknown. The microcirculation is the site of early changes in OSA patients who are free of CVD risk.

METHODS
Patients with newly diagnosed moderate to severe OSA (n = 7) were studied before and 12 weeks after intensive treatment with continuous positive airway pressure (CPAP), along with weight and age matched controls (n = 7). Microcirculatory vessels were isolated from gluteal biopsies and changes in critical functional genes were measured.

RESULTS
The following genes changed after 12 weeks of intensive CPAP therapy in the microcirculatory vessels: angiotensin receptor type 1 (*AGTR-1*) (11.6 (3.4) to 6 (0.8); $P = 0.019$); NADPH oxidase (*NOX4*) (0.85 (0.02) to 0.79 (0.11); $P = 0.016$); and dimethylarginine dimethylaminohydrolase (*DDAH 1*) (1 (0.31) to 0.55 (0.1); $P = 0.028$). Despite decreased nitric oxide (NO) availability as measured indirectly through brachial artery flow-mediated dilation, endothelial NO synthase (*NOS3*) did not change with CPAP. Other disease markers of OSA that changed with treatment in the microcirculation were endothelin, hypoxia inducible factor 1a, nuclear factor kappa B, interleukin-8, and interleukin-6.

CONCLUSIONS
In this *ex vivo* evaluation of the microcirculation of patients with OSA and no CVD risk, several pathways of CVD were activated supporting that OSA independently induces microcirculatory endothelial dysfunction and serving as disease-specific markers for future pharmacological targeting of OSA-related CVD risk. The findings support the role of renin–angiotensin activation and endothelial oxidative stress in the decreased microcirculatory NO availability in OSA.

Keywords: blood pressure; endothelial dysfunction; hypertension; nitric oxide; obstructive sleep apnea.

https://doi.org/10.1093/ajh/hpab169

Obstructive sleep apnea (OSA) is present in up to 25% of all middle-aged individuals in the United States.[1] OSA has a causal relationship with hypertension[2–4] and is independently associated with the progression or poor outcome of other cardiovascular disorders including arrhythmia, heart failure, stroke, and coronary artery disease.[5–10] Previously, several studies have supported that the standard therapy of OSA such as continuous positive airway pressure (CPAP) can reverse the immediate cardiovascular effects of OSA such as hypertension, and insulin resistance.[11–13] Recently, however, long-awaited randomized controlled trials evaluating the effect of treating OSA with CPAP on cardiovascular disease (CVD) demonstrated no significant effect on important CVD outcomes including mortality,[14–16] calling into question the role of OSA as a CVD risk factor[17] and/or the role of CPAP in reversing OSA-related CVD.[18] Important questions that remain largely unaddressed include the pathways of OSA-induced CVD; the subpopulations of OSA patients at risk for CVD complications; and the effective therapeutic approaches to reversing CVD risk in OSA.

Microcirculatory vascular endothelial dysfunction (VED) is critical for the pathogenesis of hypertension.[19,20] Therefore, the microcirculation would be an important site for studying the early vascular consequences of OSA. We previously employed an experimental approach in which we study microcirculatory endothelial tissue from younger adults with OSA who are free of CVD risk before and after a short period (12 weeks) of enhanced CPAP therapy (exceeding 70% of sleep time). Using this approach, we found evidence of microcirculatory VED in normotensive patients with OSA).[21–23] We specifically found that decreased nitric

Correspondence: Rami N. Khayat (khayatr@hs.uci.edu).

Initially submitted September 8, 2021; date of first revision October 18, 2021; accepted for publication October 20, 2021; online publication October 25, 2021.

[1]The Sleep Heart Program, The Ohio State University, Columbus, Ohio, USA; [2]The Center for Biostatistics, The Ohio State University, Columbus, Ohio, USA; [3]Department of Medicine, The Division of Cardiovascular Medicine, The Ohio State University, Columbus, Ohio, USA; [4]The Division of Pulmonary and Critical Medicine and the UCI Sleep Disorders Center, Departments of Medicine and Psychiatry, the University of California-Irvine, Irvine, California, USA.

© The Author(s) 2021. Published by Oxford University Press on behalf of American Journal of Hypertension, Ltd. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

oxide (NO) availability in OSA is due to oxidative inhibition in endothelial nitric oxide synthase (eNOS).[21] However, the pathways involved in increased oxidant overproduction in the microcirculatory endothelium in OSA remained unknown. Furthermore, there are minimal to no established OSA disease-specific markers of CVD risk that can serve as surrogates for mechanistic or therapeutic studies in humans. Therefore, we sought to identify markers of early microcirculatory dysfunction that are specific to OSA and can assist in profiling OSA patients, serve as surrogate targets in clinical trials, and provide molecular signature for *in vitro* models.

METHODS

Our experimental approach includes the evaluation of relatively younger adults with newly diagnosed OSA who are free of CVD risk factors. We provide intensive CPAP adherence program to achieve ≥5 hours of CPAP use during the 12-week period treatment. Our research design assumes that the changes in microcirculation with short term (12 weeks) of intensive CPAP therapy (>5 hours/night) are an indication of resolution of the OSA effects on the microcirculation. We still compare the change to weight and age matched control for added precision. This rationale and selected duration of follow-up is supported by several previous studies.[22,24,25]

Participants

Patients with OSA Newly diagnosed patients were recruited from an academic Sleep center after their diagnostic polysomnography and prior to the initiation of CPAP therapy. Only patients with an apnea–hypopnea index >15 events/hour of sleep were included. Very low cardiovascular risk status (≤2%) was required in all participants using the published Framingham Heart Study 10-year cardiovascular event calculators.[26,27] Therefore, the exclusion criteria included any diagnosis or ongoing treatment of hypertension, hypercholesterolemia (total cholesterol >200 mg/dl regardless of age), a diagnosis of diabetes, or any current or history of smoking. None of the participants were on any prescribed medications; and any supplements were discontinued at least a week prior to participation. The purpose of the restrictive inclusion criteria is to ensure that the microcirculatory dysfunction in participants is due to OSA only and not to other subclinical CVD risk factors.

Non-OSA controls We enrolled healthy participants with no OSA to serve as controls. The same inclusion and exclusion criteria were used in selecting controls. Specifically, the same Framingham risk score was required. Attempts were made to frequency-match OSA patients with similar sex, weight, and age controls. All non-OSA participants underwent either a polysomnography or home sleep test to rule out OSA.

Procedures

OSA patients were asked to provide a baseline visit within 2 weeks of initial OSA diagnosis, prior to starting CPAP; and a second conclusion visit after 12 weeks of CPAP therapy. Participants with OSA received an adherence intervention with regular reminder calls and their usage of CPAP was verified with device download during the conclusion visit. Patients who used CPAP more than 5 hours/night proceeded to complete the second visit with the biopsy and endothelial reactivity studies. Non-OSA volunteers underwent both procedures once.

Gluteal subcutaneous biopsy and vessel isolation Incisional skin biopsy techniques were used to obtain 1–2 cm^3 of subcutaneous tissue from the lateral superior gluteal region, as described in a previous publication.[28] The tissue from the subcutaneous biopsy was immediately dissected on ice using a dissecting Leica microscope and microsurgical tools to isolate microcirculatory vessels (50–300 μm), which were placed in an Eppendorf tube, immediately frozen in liquid nitrogen, and then kept at −80 °C prior to RNA isolation.

Endothelial reactivity studies Doppler ultrasound was used to measure flow-mediated dilation (FMD) of the brachial artery. Image acquisition was done with a linear array transducer (7 MHz frequency) and color spectral Doppler (GE Vivid 7). FMD measurements were performed according to published guidelines[29] and our previously reported studies.[21,22] Briefly, participants underwent the measurement between 7 and 9 AM prior to the biopsy procedure in a climate-controlled room. All participants were instructed to abstain from caffeine for at least 12 hours prior to the measurements. None of the participants used tobacco, and all discontinued any supplements at least 1 week prior to participation.

Peripheral blood mononuclear cells isolation Blood was collected in 5 ml heparinized tubes (green top) and spun down at 1,200 g for 10 minutes. Plasma was collected, aliquoted, and stored at −80 °C for cytokine analysis. After plasma collection, equal volume of Ca/Mg free phosphate-buffered saline was added to the blood cells, and peripheral blood mononuclear cells (PBMCs) were isolated by Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation at 600 × *g* for 20 minutes at room temperature. The mononuclear layer was removed and washed twice in RPMI 1640 (BioWhittaker, Walkersville, MD). The cells were either immediately lysed for RNA and protein isolation or placed in a 12-well plate (3×10^6) in RPMI 1640 supplemented with 10% fetal bovine serum (Atlas Biological). The cells were resolved in Trizol reagent (Invitrogen, Live Technologies, Carlsbad, CA) immediately after isolation, and RNA extraction was done according to the manufacturer's protocol.

RNA isolation and gene studies in the microcirculatory vessels 0.5 ml of Trizol reagent (Invitrogen, Live Technologies, Carlsbad, CA) was added to the tube with the isolated microvessels. Later, the frozen vessels were homogenized in TissueLyser LT (Qiagen) with a prechilled adaptor and mix of beads for 1 minute. The tissue lysate was then collected and 0.5 ml of fresh Trizol was added, and the homogenization step was repeated. Lysates from 2 homogenization steps were combined, and RNA extraction was continued according to the manufacturer's protocol. After isolation, 1–2 μg of total

RNA was reverse transcribed to cDNA by ThermoScript RNase H-Reverse Transcriptase (Invitrogen Life Technologies) and then diluted to 60 µl by RNAse/DNAse free water (ThermoFisher Scientific). The converted cDNA (20–60 ng) was used for quantitative reverse transcriptase polymerase chain reaction (rtPCR) with SYBR Green PCR Master Mix in the StepOne Plus system (Applied Biosystems, Foster City, CA).

Ethics statement

The protocol was approved by the OSU Institutional Review Board (protocol number 2009H0212). The study was registered in the National Clinical Trials database (NCT01027078).

Analysis

Patient characteristics are reported for OSA patients and non-OSA controls as mean and SD for continuous variables and count and percentage for sex. These characteristics were compared between groups using Wilcoxon rank-sum tests for continuous variables. FMD was compared pre-CPAP vs. post-CPAP within patient using paired *t*-tests.

FMD was measured according to published guidelines and our previously published studies.[21,22,28] Vessel images were obtained at baseline and at 60 seconds postrelease of occlusion. The images were immediately deidentified with dates removed to be measured later by a blinded vascular imaging technologist.

We used relative quantification to evaluate the expression of selected gene markers linked to endothelial cells function. All primers were designed with the following similar criteria: to give an 80- to 200-nt amplicon, GC range of 30%–70%, melting temperature of 59–60 °C, free of secondary structure, and a free-energy change (ΔG) of less than or equal to −5 kcal/mol. Once selected, primer pairs were validated by PCR, melting curve and high-resolution gel electrophoresis to have a single band of the desired size that were free of primer dimers. For the selected genes, PCR products were sequenced. After optimization, only primer pairs that satisfied our criteria were used. Amplification of genomic DNA was used to verify that the PCR conditions did not amplify any contaminating genomic DNA. Gene expression was calculated in relative copy numbers (RCNs) with the following equation:

$$\mathrm{RCN} = E^{-\Delta \mathrm{Ct}} \times 100,$$

where E is the efficiency of PCR, and ΔCt (cycle threshold) is the difference (Ct target – Ct reference). Ct reference is the average of 2 housekeeping genes, GAPDH and CAP-1. PCR efficiency was calculated by the equation $E = 10^{\left(-\frac{1}{\mathrm{slope}}\right)}$ as previously reported.[30,31]

RESULTS

Participant characteristics

Table 1 includes details of patients and control participant characteristics. No significant differences were present at baseline. All participants had a Framingham risk score less than 2%. One patient with OSA provided partial data on the second visit with only FMD but did not wish to proceed with the repeat biopsy. Data from this participant are reported in Table 1 since the participant was part of the BMI and age matching process with the controls. The changes with treatment in the OSA group are presented in Table 2. Notably, significant change in FMD occurred with CPAP in the OSA group.

Impact of OSA on the microcirculation

The genes measured in this study were selected to assay established or highly suspected critical pathways of microcirculatory endothelial function based on previous studies done in OSA patients. Comparisons between pre- and post-CPAP as well as between OSA and non-OSA participants are provided. The genes are grouped by function in Table 3 and discussed further below.

Vasomotion pathways

In the microcirculation of OSA patients, key endothelial genes including: endothelin 1 (*EDN1*), endothelin receptor type-b (*EDNRB*), and angiotensin II receptor type1 (*AGTR1*) genes were upregulated compared with non-OSA participants and decreased after 12 weeks of CPAP in the OSA patients. There were no significant differences in *TGFB1* (tumor growth factor type-B) and the surface adhesion molecule cadherin (*CDH5*). All gene changes measurements are reported in Table 2 and significant changes in related genes

Table 1. Characteristics of participants

Characteristics, mean (SD)	Non-OSA (n = 7)	OSA (n = 7)	P value[a]
Age (y)	37 (9.6)	40 (10.7)	0.22
Sex (male)	3	4	
Race (Black)	2	1	
BMI (kg/m^2)	37.2 (10.5)	39.2 (7.4)	0.48
SBP (mm Hg)	121 (8.9)	127 (7.6)	0.27
DBP (mm Hg)	78 (3.2)	86 (6.4)	0.03
ESS	7.7 (3.3)	10 (3.1)	0.79
AHI (events/h)	2.2 (1.7)	37 (20.6)	0.003
Desaturation index (4%) (events/h)	2.7 (1.5)	32 (17.4)	0.006
TST below 90% (%)	0.5 (0.75)	16.6 (7.7)	0.01
FMD (%)	8.8 (9.1)	4 (3.9)	0.094

Abbreviations: AHI, apnea–hypopnea index; BMI, body mass index; DBP, diastolic blood pressure; desaturation index (4%) is the number of decreases by 4% or greater in the oxygen saturation per hour of sleep; ESS, Epworth sleepiness scale; FMD: flow-mediated dilation in the brachial artery; OSA, obstructive sleep apnea; SBP, systolic blood pressure; TST below 90%: is the percent of total sleep time spent with oxygen saturation below 90%. All participants had a Framingham risk score <5%.

[a]Wilcoxon rank-sum tests except for sex and race.

as well as platelet and adhesion molecule (*PECAM1*) are shown in Figure 1.

Table 2. Cardiovascular and respiratory changes in participants with OSA

Mean (SD)	Pre (*n* = 6)	Post (*n* = 6)	*P* value
Treatment AHI (events/h)	37 (10.5)	4.4 (3.4)	0.003
CPAP average nightly usage (h/night)	5.85 (3)		
SBP (mm Hg)	124 (10.9)	121.6 (11.4)	0.337
DBP (mm Hg)	80 (5.3)	74 (7.8)	0.067
HR (beats/min)	83 (16)	76 (7.1)	0.14
FMD (%)	4 (3.9)	9.0 (1.8)	0.013

Abbreviations: AHI, apnea–hypopnea index; CPAP, continuous positive airway pressure; DBP, diastolic blood pressure; ESS, Epworth sleepiness scale; FMD, flow-mediated dilation in the brachial artery; HR, heart rate; SBP, systolic blood pressure.

Hypoxia sensing, oxidative function, and NO synthesis

These groups of genes were selected to assay the pathways of NO production and availability based on previous findings from human studies.[21,28,32] Endothelial nitric oxide (*NOS3*) expression was not different between OSA and non-OSA participants and there was no significant change with CPAP. Notably, significant changes were present in dimethylarginine dimethylaminohydrolase (*DDAH1*, *DDAH2*) and arginase (*ARG1*). These genes are critical for NO availability. Figure 2 depicts the changes in genes related to these interconnected pathways. Notably, the profile of changes in NADPH oxidase (*NOX4*) and superoxide overproduction are other contributors to NO availability in the OSA endothelium.

Inflammatory genes

Selected genes involved in the endothelial inflammatory response to hypoxia and CVD were measured and are depicted in Table 3 and Figure 3. Expression of NFkB subunits p50 and p65 (*NFKB1* and *RELA*, respectively), along with several interleukins decreased in the microcirculation after 12 weeks of CPAP.

Table 3. Changes in microcirculatory endothelial genes

Gene (relative copy number)	Non-OSA (*n* = 7)	OSA pre-CPAP (*n* = 6)	OSA post-CPAP (*n* = 6)	*P* value OSA pre-CPAP vs. OSA post-CPAP	*P* value non-OSA vs. OSA pre-CPAP
EDN1 (endothelin 1)	4.24 (0.68)	10.07 (1.80)	5.38 (0.811)	**0.047**	**0.021**
EDNRB	3.12 (2.12)	6.30 (1.05)	2.68 (0.22)	**0.024**	0.06
CHD5	5.05 (1.16)	15.90 (4.65)	4.80 (1.6)	0.085	0.08
PECAM1	25.56 (5.6)	34.48 (7.22)	11.84 (1.17)	**0.032**	0.25
AGTR1	11.61 (3.42)	18.86 (3.90)	6.07 (0.78)	**0.019**	0.13
TGFB1	6.23 (1.61)	8.94 (1.29)	6.40 (2.74)	0.206	0.17
NOS3 (eNOS)	7.23 (1.25)	10.81 (1.97)	7.66 (2.58)	0.254	0.146
DDAH1	1.0 (0.31)	1.44 (0.33)	0.55 (0.1)	**0.028**	0.199
DDAH2	83.03 (3.69)	64.63 (3.58)	72.26 (5.91)	0.222	**0.014**
ARG1	0.06 (0.02)	0.49 (0.18)	0.39 (0.16)	0.356	**0.044**
HIF1A	4.91 (0.81)	7.30 (1.05)	3.22 (0.484)	**0.016**	0.124
SOD1	215.93 (25)	234.1 (17.4)	197.4 (11.2)	0.094	0.316
NOX4	0.85 (0.19)	1.81 (0.30)	0.79 (0.11)	**0.016**	**0.024**
NFKB1 (NFκB p50)	4.43 (0.88)	4.50 (0.90)	1.20 (0.20)	**0.013**	0.482
RELA (NFκB p65)	2.89 (0.59)	5.24 (0.94)	2.27 (0.345)	**0.049**	0.093
CCL2 (MCP-1)	19.84 (5.99)	5.69 (1.11)	14.75 (1.77)	**0.006**	0.060
IL8	0.57 (0.18)	1.09 (0.27)	0.13 (0.001)	**0.0033**	0.153
IL6	0.33 (0.11)	0.36 (0.08)	0.07 (0.01)	**0.0027**	0.439
IL1B	0.31 (0.02)	1.19 (0.21)	0.24 (0.04)	**0.0021**	**0.0027**

Abbreviations: AGTR1, angiotensin receptor type 1; ARG1, arginase; CH5, cadherin; CPAP, continuous positive airway pressure; DDAH, dimethylarginine dimethylaminohydrolase (DDAH 1 and 2); EDNRB, endothelin receptor type-B; eNOS, endothelial nitric oxide synthase; HIF1A, hypoxia inducible factor 1A; IL-1B, Interleukin 1B; IL-6, interleukin-6; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; NFκB p50, nuclear factor kappa-B subunit 50; NFκB p65, nuclear factor kappa-B subunit p65; NOX4, NADPH oxidase; OSA, obstructive sleep apnea; PECAM1, platelet and endothelial adhesion molecule 1; SOD1, superoxide dismutase-1; TGFB, tumor growth factor type-B.

P values for significant comparisons are highlighted in bold.

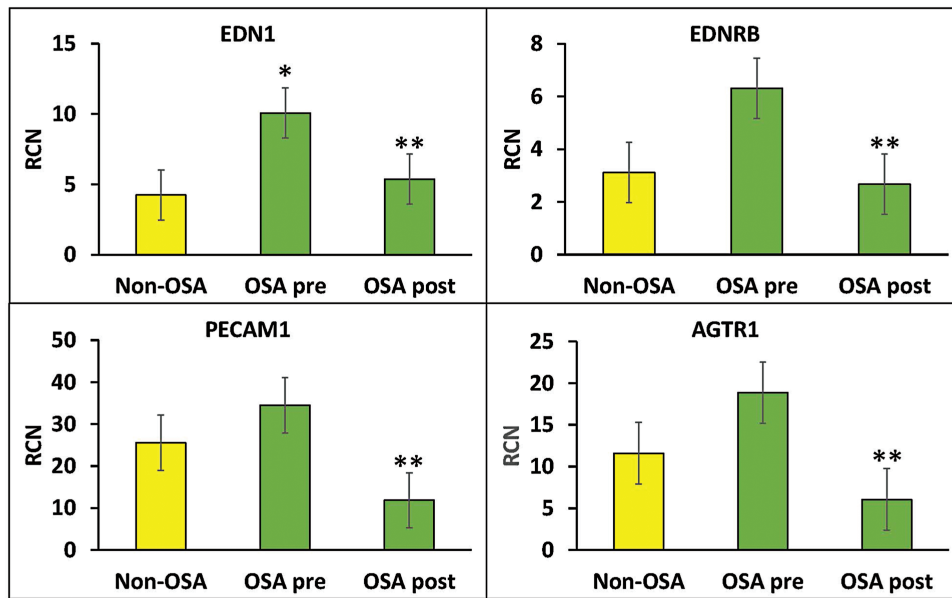

Figure 1. Changes in microcirculatory endothelial vasomotion and injury markers with OSA treatment. Abbreviations: AGTR1, angiotensin II receptor type 1; END1, endothelin; EDNRB, endothelin receptor type-B; OSA, obstructive sleep apnea; PECAM1, platelet and endothelial cell adhesion molecule 1. OSA pre: patients with OSA before CPAP; OSA post: OSA patients post-CPAP. **P* < 0.05 for OSA pre vs. non-OSA; ***P* < 0.05 for OSA pre-CPAP vs. OSA post-CPAP.

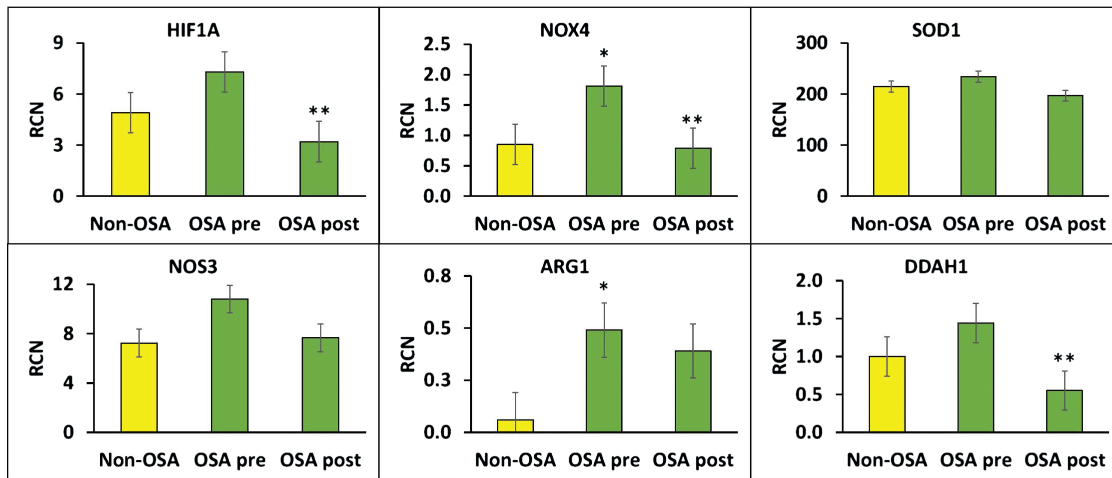

Figure 2. Changes in selected endothelial genes related to hypoxia, oxidative stress, and nitric oxide production. Abbreviations: ARG1, arginase; DDAH1, dimethylarginine dimethylaminohydrolase; HIF1A, hypoxia inducible factor 1a; NOS3, endothelial nitric oxide synthase; NOX4, NADPH oxidase subunit 4; SOD1, superoxide dismutase. OSA-pre: patients with OSA before CPAP; OSA post: OSA patients post-CPAP. **P* < 0.05 for OSA pre vs. non-OSA; ***P* < 0.05 for OSA pre-CPAP vs. OSA post-CPAP.

Impact of OSA and CPAP on inflammatory response in PBMCs

Selected inflammatory gene transcription was measured in the PBMCs isolated from patients before and after 12 weeks of CPAP, and in non-OSA controls. The profile of change is depicted in Table 4 and Figure 4.

DISCUSSION

In this study, we examined several established and highly suspected pathways of microcirculatory dysfunction in patients with OSA. The study was done in its entirety in freshly procured microcirculatory tissue from OSA patients, before and after adequate elimination of OSA with CPAP, and from weight and age matched controls. The findings of the study have several implications. The changes in the endothelial gene profiles provide a comprehensive view of the preclinical changes OSA produces in the human microcirculation. The profile of changes in the NO pathway along with upregulation of NOX gene provides an explanation for eNOS uncoupling, decreased NO and increased superoxide in the microcirculatory endothelium of OSA patients.[21,22] Furthermore, the

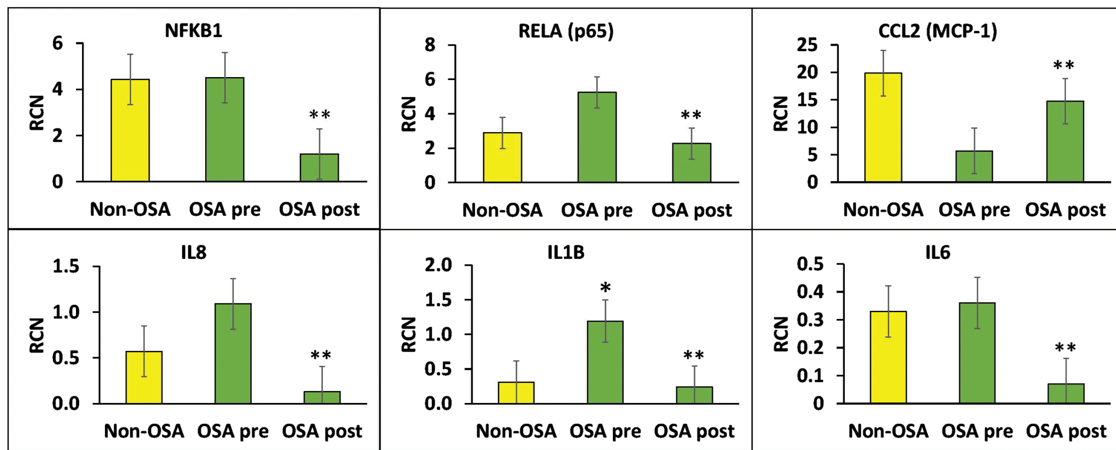

Figure 3. Changes with OSA treatment in selected inflammatory genes in the microcirculatory endothelium. Abbreviations: CCL2 (MCP-1), monocyte chemoattractant protein-1; IL1B, interleukin-1B; IL6, interleukin-6; IL8, interleukin-8; NFKB1, nuclear factor kappa-B subunit 50; OSA, obstructive sleep apnea; RELA, nuclear factor kappa-B subunit p65. OSA-pre: patients with OSA before CPAP; OSA post: OSA patients post-CPAP. **P* < 0.05 for OSA pre vs. non-OSA; ***P* < 0.05 for OSA pre-CPAP vs. OSA post-CPAP.

Table 4. Inflammatory gene changes in PBMC

PBMC gene	Non-OSA (*n* = 7)	OSA pre-CPAP (*n* = 6)	OSA post-CPAP (*n* = 6)	*P* value OSA pre-CPAP vs. OSA post-CPAP	*P* value non-OSA vs. OSA pre-CPAP
PECAM1	9.49 (2.15)	11.72 (3.7)	8.83 (0.94)	0.48	0.60
CCL2 (MCP-1)	0.34 (1.10)	1.77 (1.11)	0.19 (0.06)	0.5	0.409
IL6	0.23 (0.08)	0.40 (0.04)	0.10 (0.04)	**0.022**	0.38
IL1B	1.14 (0.13)	3.82 (0.53)	2.05 (0.23)	**0.046**	**0.015**
CAMP (LL37)	3.35 (0.65)	6.38 (3.07)	1.40 (0.25)	0.44	0.53

Abbreviations: CPAP, continuous positive airway pressure; IL-1B, interleukin-1B; IL-6, interleukin-6; IL-8, interleukin-8; LL-37, human cathelicidin antimicrobial peptide LL-37; MCP-1, monocyte chemoattractant protein-1; OSA, obstructive sleep apnea; PBMC, peripheral blood mononuclear cell; PECAM1, platelet and endothelial cell adhesion molecule 1.

P values for significant comparisons are highlighted in bold.

significant activation of several inflammatory genes in the microcirculation provides a novel insight into the effect of OSA on vascular inflammation. The ability to simultaneously measure several genes that are highly relevant to CVD risk in patients with OSA before and after treatment, in addition to its important mechanistic insights, provides a new suite of disease-specific biomarkers that can be used in both mechanistic and interventional studies. Despite the relatively, albeit minimally, invasive nature of the subcutaneous biopsies, these techniques can be highly relevant to clinical trials evaluating surrogates of CVD risk in this population. In this regard, the changes reported in the PBMC can even offer a truly noninvasive set of disease-specific biomarkers to stratify disease severity, determine endotypes, or measure treatment effects.

NO availability is a critical determinant of endothelial function. The pathways leading to NO deficiency in OSA remain unknown. In 1 study, expression of microcirculatory eNOS protein did not change, while phosphorylated eNOS increased with CPAP.[21] Certain types of NOS phosphorylation cause dysfunction and $O_2^{-\cdot}$ Overproduction (uncoupling).[33] Furthermore, overexpression of eNOS by gene transfer can increase NO activity in the vessel wall, while constitutive overexpression of eNOS paradoxically accelerated atherosclerosis in association with increased oxidative stress.[34,35] The findings of unchanged eNOS transcription with CPAP in this study further support that eNOS dysfunction rather than decreased expression is responsible for the decrease in NO availability. We have previously found that microcirculatory eNOS in otherwise health OSA patients demonstrated "uncoupling"[36,37] with decreased NO production and overproduction of superoxide.[21] We further confirmed that eNOS uncoupling was dependent, at least partially, on depletion of the critical cofactor tetrahydrobiopterin (BH4) by oxidation.[21] To this effect, the novel finding in this study of NADPH upregulation provides a source of superoxide overproduction in the OSA microcirculation and explains the oxidative inactivation of BH4 and possibly of eNOS protein modification[38] in OSA. Furthermore, this study demonstrated upregulation of *AGTR1* in OSA pretreatment patients. This finding, along with previous findings of increased angiotensin receptors (both types 1 and 2) in the OSA microcirculatory endothelium[28] confirms the role of renin–angiotensin system (RAS) activation in OSA-related VED. The typical effect of angiotensin II includes activation of NOX-dependent

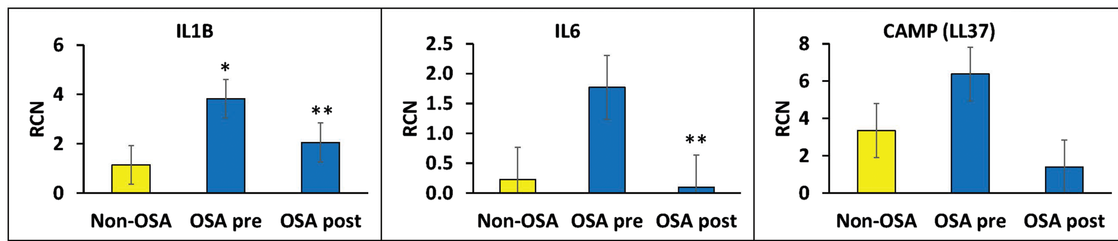

Figure 4. Significant changes in inflammatory PBMC genes from OSA patients and controls. Abbreviations: CAMP (LL-37), human cathelicidin antimicrobial peptide LL-37; IL-1B, interleukin-1beta; IL-6, interleukin-6; OSA, obstructive sleep apnea; PBMC, peripheral blood mononuclear cell. OSA-pre: patients with OSA before CPAP; OSA post: OSA patients post-CPAP. *$P < 0.05$ for OSA pre vs. non-OSA; **$P < 0.05$ for OSA pre-CPAP vs. OSA post-CPAP.

oxidant production.[39,40] In an animal model of intermittent hypoxia, AT-1 overexpression mediated VED.[41] In experimental intermittent hypoxia in healthy adults, the oxidative response was abolished with angiotensin receptor blockade.[42] We also found that angiotensin receptor blockade reversed eNOS uncoupling *ex vivo* in OSA patients.[23] Other studies have examined the RAS pathway in the pathogenesis of vascular disease in OSA patients and yielded mixed results.[43–45] Our findings represent the first simultaneous evaluation of these pathways in humans with OSA and provide evidence of early RAS activation resulting in NOX activation and superoxide overproduction in the microcirculation of OSA patients who are otherwise free of CVD risk factors.

This study should not be viewed as an evaluation of the effect of CPAP on reversing CVD risk. Rather, the use of CPAP in this study was designed to counteract the effects of OSA over a set period that has been shown to be long enough to reverse OSA- and intermittent hypoxia-related CVD changes.

The use of CPAP for a short period of time allows for the elimination for the obstructive apnea conditions while the period of 12 weeks sufficiently long to reverse these changes based on previously published studies, this duration is not long enough to allow for significant changes in lifestyle and healthful behavior. Therefore, CPAP use in this study represents an experimental intervention that was made possible by the study design. Nevertheless, the lack of untreated OSA arm is a limitation in the study. This limitation is somewhat addressed by the enrollment of body mass index and age and sex matched controls. Furthermore, studies of lifestyle and behavioral changes do not produce these robust changes in gene expressions in several highly specific pathways for intermittent hypoxia. Finally, the small sample size represents a limitation of this study. This is somewhat mitigated by the finding of consistent changes in several genes representing each tested pathway.

This study demonstrates activation of inflammatory pathways in both PBMC's and endothelial cells. This large-scale activation of NFkB pathway and upregulation of interleukin-6 supports that OSA is also an inflammatory disorder. The presence and extent of the inflammatory activation in OSA may be a disease severity indicator. In this regard, it is possible that certain individuals with OSA may be exposed to more severe levels of hypoxia in some tissues or organs beyond that is reflected by their apnea–hypopnea index or desaturation index. Also, inflammatory activation may be marker of the impact of OSA on sleep continuity and quality.[46]

In this study, we performed an extensive examination of the microcirculatory tissue of OSA patients who are free of CVD risk and found that several pathways of VED were activated and were reversible with elimination of OSA. These pathways are critical for the development of vascular disease and provide disease-specific biomarkers as well as therapeutic targets for future treatments addressing the vascular risk of OSA. Further, the technical approaches used in this study provide a platform for clinical studies as well as the option to guide bench studies by providing disease-specific molecular signature of OSA.

FUNDING

This research was supported by a grant from NIH-NHLBI (R56HL127079).

DISCLOSURE

The authors declared no conflict of interest.

REFERENCES

1. Young T, Palta M, Dempsey J, Skatrud J, Weber S, Badr S. The occurrence of sleep-disordered breathing among middle-aged adults. *N Engl J Med* 1993; 328:1230–1235.
2. Nieto FJ, Young TB, Lind BK, Shahar E, Samet JM, Redline S, D'Agostino RB, Newman AB, Lebowitz MD, Pickering TG. Association of sleep-disordered breathing, sleep apnea, and hypertension in a large community-based study. Sleep Heart Health Study. *JAMA* 2000; 283:1829–1836.
3. Brooks D, Horner RL, Kozar LF, Render-Teixeira CL, Phillipson EA. Obstructive sleep apnea as a cause of systemic hypertension. Evidence from a canine model. *J Clin Invest* 1997; 99:106–109.
4. Martínez-García MA, Capote F, Campos-Rodríguez F, Lloberes P, Díaz de Atauri MJ, Somoza M, Masa JF, González M, Sacristán L, Barbé F, Durán-Cantolla J, Aizpuru F, Mañas E, Barreiro B, Mosteiro M, Cebrián JJ, de la Peña M, García-Río F, Maimó A, Zapater J, Hernández C, Grau SanMarti N, Montserrat JM; Spanish Sleep Network. Effect of CPAP on blood pressure in patients with obstructive sleep apnea and resistant hypertension: the HIPARCO randomized clinical trial. *JAMA* 2013; 310:2407–2415.
5. Drager LF, Bortolotto LA, Figueiredo AC, Krieger EM, Lorenzi GF. Effects of continuous positive airway pressure on early signs of atherosclerosis in obstructive sleep apnea. *Am J Respir Crit Care Med* 2007; 176:706–712.

6. Peker Y, Hedner J, Kraiczi H, Löth S. Respiratory disturbance index: an independent predictor of mortality in coronary artery disease. *Am J Respir Crit Care Med* 2000; 162:81–86.
7. Arzt M, Young T, Finn L, Skatrud JB, Bradley TD. Association of sleep-disordered breathing and the occurrence of stroke. *Am J Respir Crit Care Med* 2005; 172:1447–1451.
8. Bassetti CL, Milanova M, Gugger M. Sleep-disordered breathing and acute ischemic stroke: diagnosis, risk factors, treatment, evolution, and long-term clinical outcome. *Stroke* 2006; 37:967–972.
9. Tishler PV, Redline S, Ferrette V, Hans MG, Altose MD. The association of sudden unexpected infant death with obstructive sleep apnea. *Am J Respir Crit Care Med* 1996; 153:1857–1863.
10. Marin JM, Carrizo SJ, Vicente E, Agusti AG. Long-term cardiovascular outcomes in men with obstructive sleep apnoea-hypopnoea with or without treatment with continuous positive airway pressure: an observational study. *Lancet* 2005; 365:1046–1053.
11. Iftikhar IH, Valentine CW, Bittencourt LR, Cohen DL, Fedson AC, Gíslason T, Penzel T, Phillips CL, Yu-sheng L, Pack AI, Magalang UJ. Effects of continuous positive airway pressure on blood pressure in patients with resistant hypertension and obstructive sleep apnea: a meta-analysis. *J Hypertens* 2014; 32:2341–2350; discussion 2350.
12. Gottlieb DJ, Punjabi NM, Mehra R, Patel SR, Quan SF, Babineau DC, Tracy RP, Rueschman M, Blumenthal RS, Lewis EF, Bhatt DL, Redline S. CPAP versus oxygen in obstructive sleep apnea. *N Engl J Med* 2014; 370:2276–2285.
13. Lam JC, Lam B, Yao TJ, Lai AY, Ooi CG, Tam S, Lam KS, Ip MS. A randomised controlled trial of nasal continuous positive airway pressure on insulin sensitivity in obstructive sleep apnoea. *Eur Respir J* 2010; 35:138–145.
14. McEvoy RD, Antic NA, Heeley E, Luo Y, Ou Q, Zhang X, Mediano O, Chen R, Drager LF, Liu Z, Chen G, Du B, McArdle N, Mukherjee S, Tripathi M, Billot L, Li Q, Lorenzi-Filho G, Barbe F, Redline S, Wang J, Arima H, Neal B, White DP, Grunstein RR, Zhong N, Anderson CS; SAVE Investigators and Coordinators. CPAP for prevention of cardiovascular events in obstructive sleep apnea. *N Engl J Med* 2016; 375:919–931.
15. Peker Y, Glantz H, Eulenburg C, Wegscheider K, Herlitz J, Thunström E. Effect of positive airway pressure on cardiovascular outcomes in coronary artery disease patients with nonsleepy obstructive sleep apnea. The RICCADSA randomized controlled trial. *Am J Respir Crit Care Med* 2016; 194:613–620.
16. Cowie MR, Woehrle H, Wegscheider K, Angermann C, d'Ortho MP, Erdmann E, Levy P, Simonds AK, Somers VK, Zannad F, Teschler H. Adaptive servo-ventilation for central sleep apnea in systolic heart failure. *N Engl J Med* 2015; 373:1095–1105.
17. Bibbins-Domingo K, Grossman DC, Curry SJ, Davidson KW, Epling JW Jr, García FA, Herzstein J, Kemper AR, Krist AH, Kurth AE, Landefeld CS, Mangione CM, Phillips WR, Phipps MG, Pignone MP, Silverstein M, Tseng CW; US Preventive Services Task Force. Screening for obstructive sleep apnea in adults: US Preventive Services Task Force recommendation statement. *JAMA* 2017; 317:407–414.
18. Yu J, Zhou Z, McEvoy RD, Anderson CS, Rodgers A, Perkovic V, Neal B. Association of positive airway pressure with cardiovascular events and death in adults with sleep apnea: a systematic review and meta-analysis. *JAMA* 2017; 318:156–166.
19. Linder L, Kiowski W, Bühler FR, Lüscher TF. Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation in vivo. Blunted response in essential hypertension. *Circulation* 1990; 81:1762–1767.
20. Rossi M, Taddei S, Fabbri A, Tintori G, Credidio L, Virdis A, Ghiadoni L, Salvetti A, Giusti C. Cutaneous vasodilation to acetylcholine in patients with essential hypertension. *J Cardiovasc Pharmacol* 1997; 29:406–411.
21. Varadharaj S, Porter K, Pleister A, Wannemacher J, Sow A, Jarjoura D, Zweier JL, Khayat RN. Endothelial nitric oxide synthase uncoupling: a novel pathway in OSA induced vascular endothelial dysfunction. *Respir Physiol Neurobiol* 2015; 207:40–47.
22. Patt BT, Jarjoura D, Haddad DN, Sen CK, Roy S, Flavahan NA, Khayat RN. Endothelial dysfunction in the microcirculation of patients with obstructive sleep apnea. *Am J Respir Crit Care Med* 2010; 182:1540–1545.
23. Khayat RN, Varadharaj S, Porter K, Sow A, Jarjoura D, Gavrilin MA, Zweier JL. Angiotensin receptor expression and vascular endothelial dysfunction in obstructive sleep apnea. *Am J Hypertens* 2018; 31:355–361.
24. Gao S, Emin M, Thoma T, Pastellas K, Castagna F, Shah R, Jimenez A, Patel N, Wei Y, Jelic S. Complement promotes endothelial von Willebrand factor and angiopoietin-2 release in obstructive sleep apnea. *Sleep* 2021; 44.
25. Jelic S, Padeletti M, Kawut SM, Higgins C, Canfield SM, Onat D, Colombo PC, Basner RC, Factor P, LeJemtel TH. Inflammation, oxidative stress, and repair capacity of the vascular endothelium in obstructive sleep apnea. *Circulation* 2008; 117:2270–2278.
26. Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. *Circulation* 1998; 97:1837–1847.
27. D'Agostino RB Sr, Vasan RS, Pencina MJ, Wolf PA, Cobain M, Massaro JM, Kannel WB. General cardiovascular risk profile for use in primary care: the Framingham Heart Study. *Circulation* 2008; 117:743–753.
28. Khayat RN, Varadharaj S, Porter K, Sow A, Jarjoura D, Gavrilin MA, Zweier JL. Angiotensin receptor expression and vascular endothelial dysfunction in obstructive sleep apnea. *Am J Hypertens* 2018; 31:355–361.
29. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R; International Brachial Artery Reactivity Task Force. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol* 2002; 39:257–265.
30. Gavrilin MA, Bouakl IJ, Knatz NL, Duncan MD, Hall MW, Gunn JS, Wewers MD. Internalization and phagosome escape required for *Francisella* to induce human monocyte IL-1beta processing and release. *Proc Natl Acad Sci USA* 2006; 103:141–146.
31. Fahy RJ, Exline MC, Gavrilin MA, Bhatt NY, Besecker BY, Sarkar A, Hollyfield JL, Duncan MD, Nagaraja HN, Knatz NL, Hall M, Wewers MD. Inflammasome mRNA expression in human monocytes during early septic shock. *Am J Respir Crit Care Med* 2008; 177:983–988.
32. Kaczmarek E, Bakker JP, Clarke DN, Csizmadia E, Kocher O, Veves A, Tecilazich F, O'Donnell CP, Ferran C, Malhotra A. Molecular biomarkers of vascular dysfunction in obstructive sleep apnea. *PLoS One* 2013; 8:e70559.
33. Chen CA, Druhan LJ, Varadharaj S, Chen YR, Zweier JL. Phosphorylation of endothelial nitric-oxide synthase regulates superoxide generation from the enzyme. *J Biol Chem* 2008; 283:27038–27047.
34. Shi W, Wang X, Shih DM, Laubach VE, Navab M, Lusis AJ. Paradoxical reduction of fatty streak formation in mice lacking endothelial nitric oxide synthase. *Circulation* 2002; 105:2078–2082.
35. Ozaki M, Kawashima S, Yamashita T, Hirase T, Namiki M, Inoue N, Hirata K, Yasui H, Sakurai H, Yoshida Y, Masada M, Yokoyama M. Overexpression of endothelial nitric oxide synthase accelerates atherosclerotic lesion formation in apoE-deficient mice. *J Clin Invest* 2002; 110:331–340.
36. List BM, Klösch B, Völker C, Gorren AC, Sessa WC, Werner ER, Kukovetz WR, Schmidt K, Mayer B. Characterization of bovine endothelial nitric oxide synthase as a homodimer with down-regulated uncoupled NADPH oxidase activity: tetrahydrobiopterin binding kinetics and role of haem in dimerization. *Biochem J* 1997; 323(Pt 1):159–165.
37. Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R, Channon KM. Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation* 2002; 105:1656–1662.
38. Chen CA, Wang TY, Varadharaj S, Reyes LA, Hemann C, Talukder MA, Chen YR, Druhan LJ, Zweier JL. S-glutathionylation uncouples eNOS and regulates its cellular and vascular function. *Nature* 2010; 468:1115–1118.
39. Lassègue B, Sorescu D, Szöcs K, Yin Q, Akers M, Zhang Y, Grant SL, Lambeth JD, Griendling KK. Novel gp91(phox) homologues in vascular smooth muscle cells: nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circ Res* 2001; 88:888–894.
40. Rajagopalan S, Kurz S, Münzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* 1996; 97:1916–1923.

41. Marcus NJ, Philippi NR, Bird CE, Li YL, Schultz HD, Morgan BJ. Effect of AT1 receptor blockade on intermittent hypoxia-induced endothelial dysfunction. *Respir Physiol Neurobiol* 2012; 183:67–74.
42. Pialoux V, Foster GE, Ahmed SB, Beaudin AE, Hanly PJ, Poulin MJ. Losartan abolishes oxidative stress induced by intermittent hypoxia in humans. *J Physiol* 2011; 589:5529–5537.
43. Lykouras D, Theodoropoulos K, Sampsonas F, Lagiou O, Lykouras M, Spiropoulou A, Flordellis C, Alexandrides T, Karkoulias K, Spiropoulos K. The impact of obstructive sleep apnea syndrome on renin and aldosterone. *Eur Rev Med Pharmacol Sci* 2015; 19:4164–4170.
44. Zalucky AA, Nicholl DD, Hanly PJ, Poulin MJ, Turin TC, Walji S, Handley GB, Raneri JK, Sola DY, Ahmed SB. Nocturnal hypoxemia severity and renin-angiotensin system activity in obstructive sleep apnea. *Am J Respir Crit Care Med* 2015; 192:873–880.
45. Nicholl DD, Hanly PJ, Poulin MJ, Handley GB, Hemmelgarn BR, Sola DY, Ahmed SB. Evaluation of continuous positive airway pressure therapy on renin-angiotensin system activity in obstructive sleep apnea. *Am J Respir Crit Care Med* 2014; 190:572–580.
46. Shearer WT, Reuben JM, Mullington JM, Price NJ, Lee BN, Smith EO, Szuba MP, Van Dongen HP, Dinges DF. Soluble TNF-alpha receptor 1 and IL-6 plasma levels in humans subjected to the sleep deprivation model of spaceflight. *J Allergy Clin Immunol* 2001; 107:165–170.