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ORIGINAL RESEARCH

Patients with Obstructive Sleep Apnea Have Altered Levels of Four Cytokines Associated with Cardiovascular and Kidney Disease, but Near Normal Levels with Airways Therapy

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Introduction: Obstructive sleep apnea (OSA) results in chronic intermittent hypoxia leading to systemic inflammation, increases in pro-inflammatory cytokines TNF-Alpha and IL-6, and increased risk for a number of life threatening medical disorders such as cardiovascular and kidney disease.

Methods: A BioPlex Array was used to examined the serum levels of four cytokines also expressed in endothelial cells and/or macrophages and associated with cardiovascular and kidney disease risk.

Results: Relative to untreated OSA patients, airways treated OSA patients had a 5.4-fold higher median level of MMP2 ($p = 9.1x10^{-11}$), a 1.4-fold higher level of TWEAK ($p = 1.8x10^{-7}$), a 1.7-fold higher level of CD163 ($p = 1.4x10^{-6}$), but a 2.0-fold lower level of MMP3 ($p = 7.9x10^{-7}$). Airway treatment resulted in levels more similar to or indistinguishable from control subjects. Both t-SNE or UMAP analysis of the global structure of these multi-dimensional data revealed two data clusters, one populated primarily with data for controls and most airways treated OSA patients and a second populated primarily with data for OSA patients.

Discussion: We discuss a concept in which the aberrant levels of these cytokines in untreated OSA patients may represent a chronic response after years of experiencing intermittent nightly hypoxia, which attenuated the acute response to hypoxia. A balanced therapeutic correction of the aberrant levels of these cytokines may limit the progression of CVD and kidney disease in OSA patients.

Keywords: CPAP, OSA, atherosclerosis, cytokines, renal disease, apnea

Plain Language Summary

Obstructive sleep apnea (OSA) results in chronic intermittent hypoxia leading to systemic inflammation, increases in pro-inflammatory cytokines, and increased risk of cardiovascular and kidney disease. Herein, we show that the serum levels of four cytokines associated with cardiovascular and kidney disease risk are altered in OSA patients relative to controls. Airway treatment of OSA patients resulted in levels more similar to or indistinguishable from control subjects. Analysis of the global structure of these multi-dimensional data revealed two data clusters, one populated primarily with data for controls and most airways treated OSA patients and a second populated primarily with data for OSA patients. We discuss a concept in which the aberrant levels of these cytokines in untreated OSA patients may represent a chronic response which attenuated the acute response to hypoxia. A

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balanced therapeutic correction of the aberrant levels of these cytokines may limit the progression of CVD and kidney disease in OSA patients.

Introduction

Obstructive sleep apnea (OSA) is a sleep-related breathing disorder. OSA patients experience fragmented sleep patterns, abnormally long pauses in breathing and/or abnormally low levels of breathing, which result in poor oxygenation of the blood and chronic intermittent tissue hypoxia. Hypoxia leads to tissue inflammation, which may be one major reason that OSA patients have an increased risk of mortality. Their numerous health problems develop over months and years and include cardiovascular disease (CVD)[1] and kidney disease.[2,3] Although there are treatable co-morbidities associated with OSA, such as obesity, continuous positive airways pressure therapy (CPAP), is the mainstay of OSA treatment. For most OSA patients, airway therapy improves oxygenation, reduces inflammation, and in general, reverses the risks associated OSA, including CVD and kidney disease.

Higher serum concentrations of inflammatory cytokines have been linked to cardiovascular and kidney disease risk.[4,5] Cytokine induced inflammatory damage to the endothelium is likely to play a significant part in the development of CVD and kidney disease.[6–8] Most but not all studies on OSA patients show higher levels of the pro-inflammatory TNF-Alpha,[9] IL-6,[10] IL-12,[11] IL-17,[12] and IL-23.[12,13] Airways therapy of OSA patients is not universally effective at restoring altered levels of these cytokines to the levels approaching those in control subjects. Herein, we compare the serum levels of four other inflammatory cytokines, MMP2, MMP3, TWEAK, and CD163, previously linked to CVD and kidney disease between untreated OSA and airways treated OSA patients.

Methods

Patient Recruitment

Following polysomnography a total of 46 study subjects were enrolled in the study after obtaining informed consent. This including nineteen subjects with untreated OSA (ie, not on airways therapy), and nineteen airways treated OSA patients (Table 1, Supplemental Data File SD1). Eighteen of the nineteen treated OSA patients acknowledged the recent use of primary CPAP and one a dental airways device for six or more months. Dental airways devices have been shown to produce a similar treatment effect for patients who cannot tolerate CPAP,[14,15] although their relative effectiveness is still debated. The eight control individuals had undergone polysomnography and did not have sleep-disordered breathing. Sleep studies were conducted in American Academy of Sleep Medicine accredited diagnostic sleep centers in Georgia and followed AASM's recommended procedures.[16] Subject characteristics (age, gender), anthropometrics (BMI), history of CVD, medication history, Apnea-Hypopnea Index (AHI), average low oxygen saturation levels during each breath cycle (SaO_2 low % saturation), and daytime sleepiness (Epworth Sleepiness Score/Scale, ESS), fasting cholesterol, glucose, and hs-CRP were evaluated at the time of recruitment, which for the airways treated group followed at least six months of airways therapy. Treated and untreated OSA patients are well matched for nearly all parameters (p values Table 1). The control group was considerably younger and leaner and were recruited for their potential to display more nearly optimal cytokine levels. All samples were collected prior to the COVID-19 Pandemic. None of the patients had a fever at the time of the blood draw.

Measurement of Serum Cytokine Levels

The picogram per milliliter levels of inflammatory cytokines were examined using multiplex kits that quantify biomarkers of human inflammation (BioRad #171AL001M). Multiple 96 well plates were assayed using Bio-Rad Bio-Plex instrument at UGA's Cytometry Shared Resource Laboratory. The Bio-Plex system has the advantage that hundreds of individual fluorescent reporter beads each estimate each cytokine level in each well, which improves the statistical accuracy, increases the dynamic range and has greater sensitivity of each individual well estimate for each cytokine relative to colorimetric immunoassays. Serum samples were taken at the time of recruitment which for airway treated subjects was after airways therapy. Multiple serum samples for each patient were snap frozen on dry ice and stored at −80°C so that no serum sample would be thawed twice. Serum samples and standards were prepared as per the manufacturer's instructions (Bulletin 10044281), except that samples were run in triplicate and standards in quadruplicate instead of in duplicate. Each serum sample was diluted 4-fold and 50 microliters assayed in each single well assay. The picogram per milliliter output data for each cytokine was normalized to the concentration of standards. The standard error of the lowest concentration standards used to estimate the lowest cytokine concentration among

Table 1 Patient Biometric, Laboratory and Sleep Data

	Control Subjects (n=8)	Control vs Airways Treated p value	Airways Treated Patients (n=19)	Airways Treated vs Apneic p value	Apneic Patients (n=19)	Control vs Apneic p value
Female/Male	6/2		7/12		7/12	
Age	37.7±12.1	p=0.000270	60.6±10.5	p=0.559	58.2±12.4	0.00105
Hypertension or heart disease Y/N	2Y/6N		11Y/8N		11Y/8N	
Race C/H/B(A/M)/A	4C/0H/3B/1A		17C/0H/2B/0A		11C/1H/6B/1A	
BMI	25.7±5.21	p=0.0344	33.1±9.07	p=0.414	35.0±9.22	p=0.0286
ESS	6±3.30	p=0.711	7.16±5.80	p=0.228	7.94±4.02	p=0.146
AHI at time of diagnosis	1.58±1.64	p=0.042	35.7±24.8	p=0.190	26.8±25.7	p=0.0437
SaO2 low %	92.4%±1.92%	p=0.00120	80.7%±5.44%	p=0.273	76.4%±10.3%	0.00168
Glucose	94.4±9.21	p=0.121	106±18.7	p=0.849	104±12.1	p=0.0433
Cholesterol	170±23.8	p=0.161	181±25.1	p=0.365	179±43.1	p=0.640
HDL	53.8±14.7	p=1	51.8±18.1	p=0.307	45.7±17.3	p=0.302
LDL	99.2±22.4	p=0.363	104±27.6	p=0.6826	106±35.8	p=0.6579
hs-CRP	1.07±0.736	p=0.0491	4.84±7.65	p=0.448	3.48±5.60	p=0.201
Chronic Meds Y/N	2Y/6N		16Y/3N		16Y/3N	

patient samples was generally less than 15% and much less than that for higher concentrations.

Effect Sizes and Powering the Study

The BioPlex system provides a wider dynamic range, greater sensitivity, and more statistical significance for each assay than conventional immunoassays. Hence, in sample size planning the potential for medium to large effect sizes for the differences in the levels of the four cytokines examined and study costs was considered,[17,18] as recommended by the FDA study guidelines for estimating minimum appropriate patient sample size and cost.[19] Within the context of testing the hypothesis that there would be significant differences in the levels of each cytokine between OSA patient and airways treated patients and controls, we performed effect size estimates using three methods, (1) the absolute value of r, (2) Cliff's delta, and (3) Vargha and Delaney's A. Table A of Supplementary Data File SD2 summarizes the standard values for small, medium, large effect sizes. An empirical power calculation was applied to the Wilcoxon rank sum test of the cytokine data to determine if there was sufficient power with the number of test subjects for all comparisons of all four cytokine levels among patient groups (Table C of Supplementary Data File SD2).

Data Curation

The data output from separate Bio-Plex plates and patient biometric data were combined to make a single excel data file, with one sheet for each of the four cytokines assayed (Supplemental Data File SD1). The data were then moved into R v3.5.1 for further statistical analysis. The combined data for patient groups were examined using Boxplot. After applying the Kolmogorov–Smirnov test in R, it was clear that the airways treated patient data for cytokine levels were not normally distributed ($p < 0.05$) and often fell into two groups of values. Hence, the nonparametric Wilcoxon rank-sum test was used to estimate p values for the significance of pairwise differences in cytokine levels among OSA patients, airways treated OSA patients, and controls and for the biometric data. Without any assumption of distribution, the test determines if it is likely that an observation in one group is greater than an observation in the other, with significance level (alpha) = 0.05 =5%[20] shown as p-values in Table 2. To visualize the similarity and clustering among high-dimensional data for the levels

Table 2 Cytokine Levels Among Apneic Patients and Controls

Cytokine	Gene Name	Control Individuals Median pg/mL Cytokine	Airways Treated Median pg/mL Cytokine	OSA Patients Median pg/mL Cytokine	Fold Change OSA to Airways Treated	Control vs OSA p value	Control vs Airways Treated p value	Airways Treated vs OSA p value
MMP-2	*MMP2*	4426	4257	796	5.4	$2.8x10^{-12}$	0.26	$9.14x10^{-11}$
MMP-3	*MMP3*	487	6465	1312	−2	$1.1x10^{-08}$	0.33	$7.88x10^{-07}$
TWEAK	*TNFSF12*	58	36.5	26.3	1.4	$2.4x10^{-11}$	$4.7x10^{-06}$	$1.80x10^{-07}$
CD163	*CD163*	28,509	25,848	14,939	1.7	$1.1x10^{-6}$	0.27	$1.4x10^{-06}$

of all cytokines among all patients in a two-dimensional map, we applied the t-distributed Stochastic Neighbor Embedding method (t-SNE)[21] using the Rtsne statistical package available on line.[22] T-SNE reduces dimensionality by first examining a Gaussian distance to analyze the similarity among data points in high-dimensional space and then projecting these data into two dimensional space. The robustness of the t-SNE analysis was tested by employing an alternative method to obtaining a visual projection of high dimensional data into two dimensions, Uniform Manifold Approximation and Projection (UMAP).[23,24] UMAP analysis proceeds quite differently from t-SNE in that it first estimates a topology for the high-dimensional data and then uses the topology information to project data into two dimensional space.[23] It has been argued that UMAP may be superior to and/or equivalent to t-SNE at recovering the global structure among high dimensional data.

Results

The serum pg/mL levels of MMP2, MMP3, TWEAK, and CD163 in airways treated OSA patients were compared to untreated OSA patients and to control subjects and visualized using Box Plots (Figure 1). The median pg/mL serum level of MMP2 was 5.5-fold higher in airways treated OSA patients relative to untreated OSA patients ($p = 9.1x10^{-11}$) (Figure 1A, Table 2). The median level in airways treated patients was not distinguishable from the median level in controls ($p = 0.26$). Contrary to what we found for MMP2, the median level of MMP3 (pg/mL) was 2.0-fold lower for the airways treated OSA patients relative untreated OSA patients ($p = 7.9x10^{-7}$) (Figure 1B, Table 2), and again indistinguishable from that in controls ($p = 0.33$). The median level of soluble TWEAK (pg/mL) was 1.4-fold higher in airways treated OSA patients relative to untreated OSA patients ($p = 1.8x10^{-9}$, Figure 1C, Table 2), but still 1.6-fold lower than in control subjects ($p = 4.7x10^{-6}$). The median pg/mL level of soluble CD163 was 1.7 fold higher ($p = 1.4x10^{-6}$) in airways treated OSA patients than untreated OSA patients (Figure 1D, Table 2) and statistically indistinguishable from the median level in control subjects ($p = 0.27$). There were five airways treated patients (#12, #26, #34, #47, and #70), whose levels of all four cytokines were more similar to untreated OSA patients (encircled data points in Figure 1). The patient using the dental airways device was not among these outliers.

Considering the significant differences in pg/mL cytokine levels between some patient groups we made estimates of effect sizes and empirical power. Table A of Supplementary Data File SD2 summarizes the standard values for small, medium, large effect sizes estimated using the absolute value of r, Cliff's delta, and Vargha and Delaney's A. By at least two of the three metrics, comparisons of each of the four cytokine levels between airways treated OSA patients or controls and untreated OSA patients produced large effect sizes (Table B of Supplementary Data File SD2). An empirical power calculation applied to the Wilcoxon rank sum test of the cytokine data demonstrated that there was sufficient power with the number of test subjects for all comparisons of all four cytokine levels between OSA patients and either airways treated OSA patients or control that would avoid both false positive and false negative errors (Table C of Supplementary Data File SD2).

We used two methods to visualize in two dimensions (Figure 2) the potential for global structure among these high-dimensional datasets of cytokine levels (Figure 1, Table 2). T-SNE analysis revealed two groups of data for the 3 patient populations (Figure 2A). Group 1 contains the data for all, but one, control subject (red data points) and fourteen of the nineteen airways treated OSA subjects (green data points) and data two of the nineteen OSA

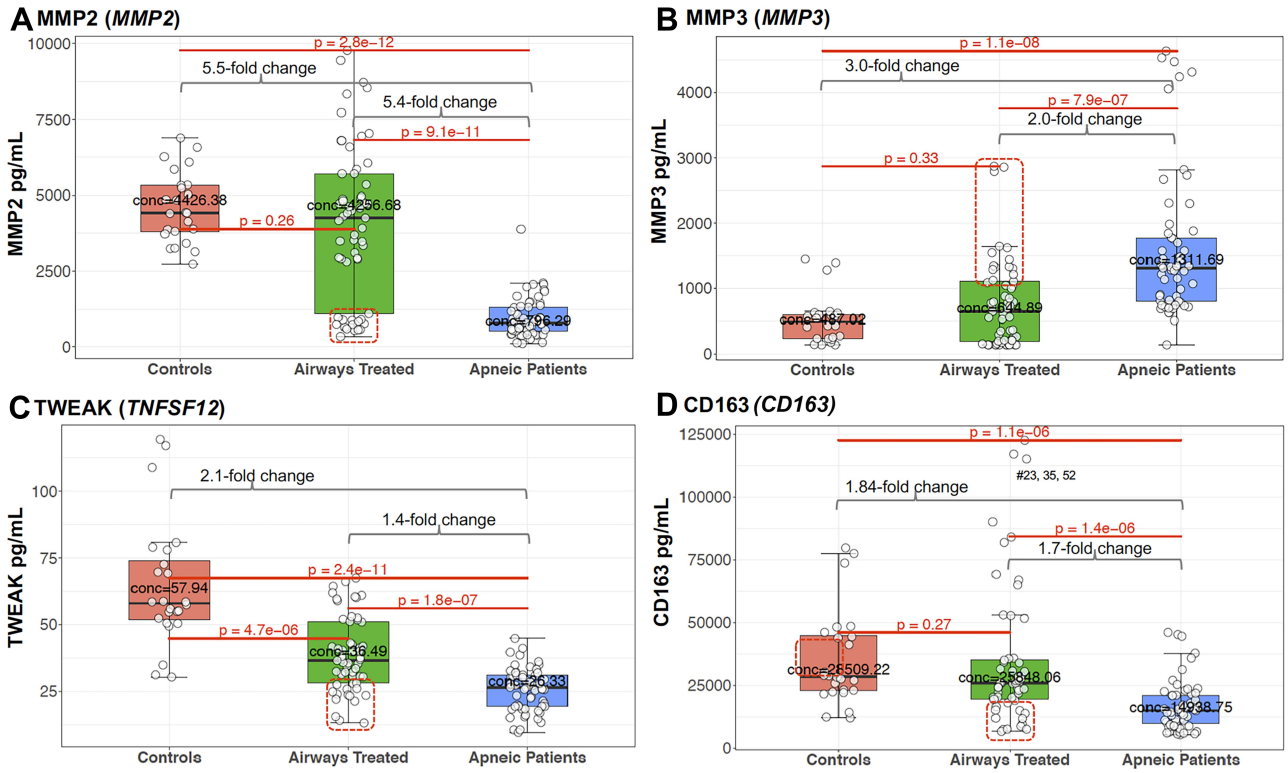

Figure 1 The levels of four cytokines involved in the CVD and renal disease risk are significantly altered in airways treated OSA patients and more similar to the levels in control subjects than untreated OSA patients. The serum picogram per milliliter (pg/mL) levels of (**A**) MMP2, (**B**) MMP3, (**C**) TWEAK, and (**D**) CD163 for the nineteen OSA patients not receiving airways therapy, nineteen airways treated OSA patients and eight control individuals are summarized in box blots. Median levels are indicated by a black line. The top box encloses the third quartile and is bounded by median pg/mL value, the box below the median level encloses the first quartile. The whiskers indicate the greatest/least values excluding statistical outliers. Each of the three independent Bio-Plex estimates of a cytokine level for each patient is represented by separate data point. The data for the five airways treated OSA subjects that showed cytokine levels more like those in untreated patients are outlined in a red dotted line.

patients (blue data points patients). Group 2 contains the data for seventeen of the untreated OSA patients, five of the airways treated patients (#12, #26, #34, #47, and #70), and one control subject (#4). UMAP also produced two groups of data (Figure 2B), and group membership was the same as for t-SNE. In short, both methods of analysis suggest that airways therapy produced cytokine levels near the levels found in control subjects for most, but not all, of the airways treated subjects.

We considered the source of variability in the data for patient groups and potential outliers revealed in Figures 1 and 2. We did not find any obvious biostatistical, laboratory, or measures of sleep to explain observed variances in cytokine levels in some subjects to explain various outlying data except that control subject #4 had a relatively low BMI of 18.7. To look at underlying trends in these data, we performed a linear regression analysis, plotting the levels of MMP2, MMP3, TWEAK, and CD163, against age, BMI, heart rate, ESS, AHI, and SaO_2 low %, glucose, total cholesterol, HDL, LDL, and hs-CRP. None of these individual variables accounted for significant variance in cytokine levels in any one group or across all three groups". All of the airways treated subjects self-reported that they were adherent to OSA treatment including the five outliers.

Discussion

We cannot explain the outlying cytokine data among several airway treated patients. The one patient using a dental airways device was not among them. However, confounding health issues such as depression and discomfort with CPAP devices are known to reduce compliance with airways therapy to as low as 60%.[25–30] Perhaps misreported compliance accounts for the outlying airways treated OSA patient data. As an additional explanation, a significant fraction of patients receiving long-term airway therapy develop treatment emergent central sleep apnea, resulting in fragmented sleep (Nigam et al, 2016; Nigam et al, 2018), which may have led to aberrant cytokine expression in some of our airways treated subjects.

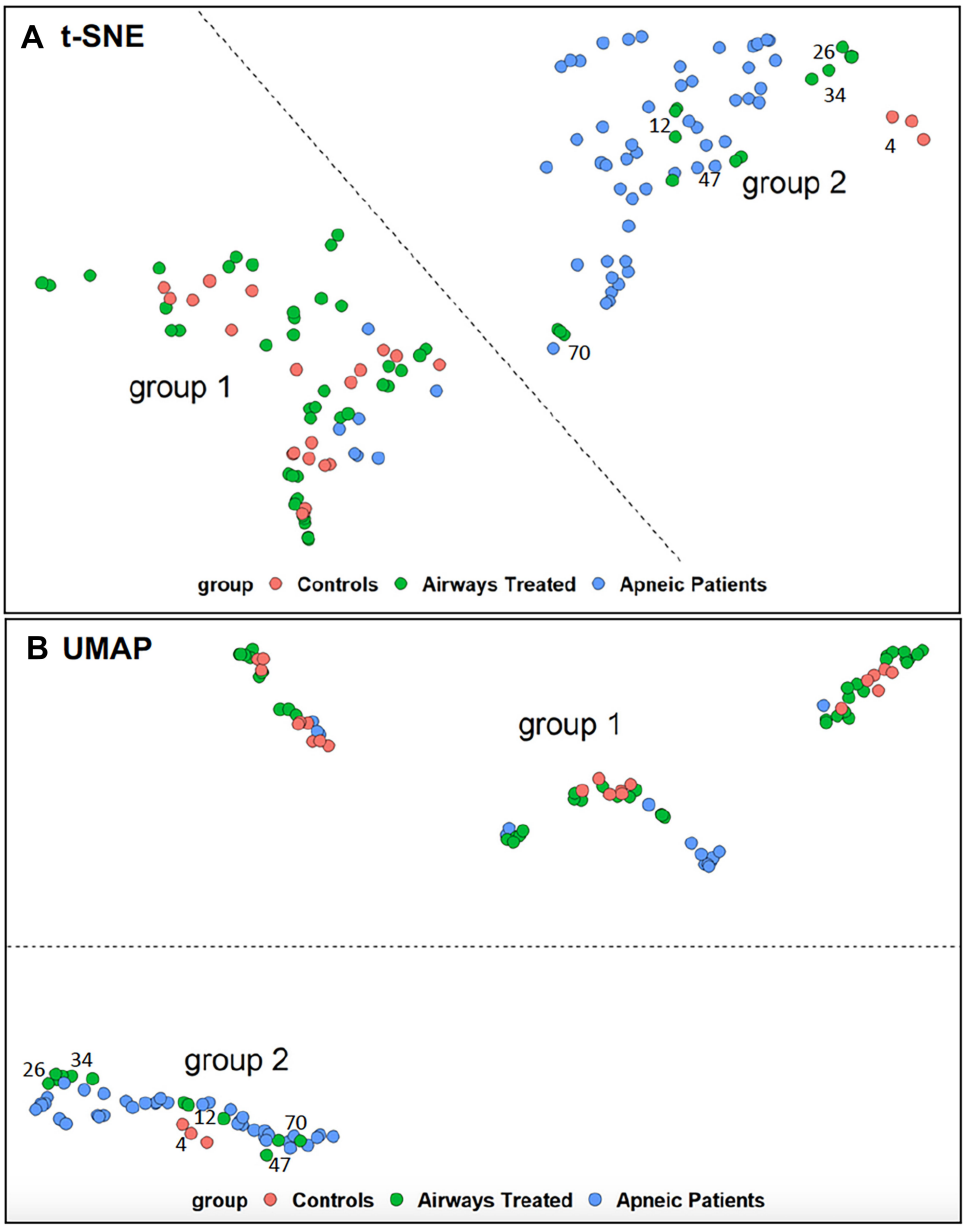

Figure 2 t-SNE and UMAP analysis of patient cytokine data. The cytokine levels of MMP2, MMP3, TWEAK, and CD163 for all patients and controls were examined by (**A**) t-SNE and (**B**) UMAP to visualize local structure among these high dimensional data in two dimensions. Both methods produced two data groups, which are separated by dotted lines. Patient constituency is the same for Group 1 and Group 2 using either t-SNE or UMAP. Group 1 represents the cytokine data for seven of the eight controls, fourteen of the nineteen airways treated OSA patients, and two of the untreated OSA patients. Group 2 represents the cytokine data for seventeen of the nineteen untreated OSA patients, a five of the airways treated OSA patients, and one control subject. Each patient is represented by three separate data points. Data points for outlying patients worthy of discussion are numbed.

We screened a battery of inflammatory cytokines for novel cytokines showing aberrant levels in OSA patients, but more normal levels in airways treated patients (See Materials and Methods). Among those identified, we observed that MMP2, MMP3, TWEAK, and CD163 had been previously linked to CVD and kidney disease, but their aberrant expression had not been associated with OSA. The potential roles of MMP2, MMP3, TWEAK, and CD163 in CVD and kidney disease will be discussed in the light of our data showing OSA subjects had significantly different levels from most airways treated OSA patients and controls.

Genetic polymorphisms at the *MMP2* and *MMP3* loci and their altered expression levels are risk factors for CVD, atherosclerosis, and kidney disease.[31–37] MMP2 is expressed in cells at the leading edge of wound repair and is positively regulated by the inflammatory cytokine TNF-Alpha. MMP2 cleaves collagen from the extracellular

matrix. MMP2 also proteolytically processes and activates cytokines to produce diffusion gradients that attract T-lymphocytes, monocytes, and eosinophils to sites of injury for vascular remodeling and repair and the recruitment of cardiovascular precursor cells to wounded tissues necessary for both angiogenesis and cardiomyogenesis.[38–40] In the balance, MMP2's activities are beneficial to wound healing and repair.

MMP2 expression appears positively linked to acute hypoxia in vitro,[41] hence we anticipated increased levels of MMP2 in OSA patients. Contrary to this expectation, we observed that the median level of MMP2 was several-fold lower in the serum of OSA patients than in control individuals or airways treated patients. Perhaps the chronic intermittent hypoxia experienced over a period of years by apneic patients attenuates the normal increases in MMP2 associated with acute hypoxia and subsequent inflammation. The dramatically reduced expression we observed for MMP2 among apneic patients may be harmful to cardiovascular repair and may contribute to the increased risk of other inflammatory diseases.[42] Finally, when exogenous MMP2 is added to an in vitro wound assay of human epithelial cells, MMP2 significantly accelerated wound healing,[38] suggesting therapeutic supplementation of MMP2 to OSA patients with cardiovascular or kidney disease might be beneficial to vascular tissue development. Hyaluronic acid levels are altered by hypoxia in OSA patients,[43] effect MMP2 expression,[44] and are involved in inflammatory cascades leading to endothelial dysfunction and kidney disease.[43,45] Hence, it might be useful to include assays of the serum levels of hyaluronic acid in future expanded studies of inflammatory cytokine levels in airways treated OSA patients.

MMP3 is expressed in cells adjacent to the leading edge of epithelial wound repair and behind those expressing MMP2.[39] MMP3 proteolytically processes cadherin, laminin, collagen, and TGF-Beta-1, which all have roles in tissue repair. *MMP3-/-* null mice show reduced influx of neutrophils and macrophages into inflamed tissues, supporting the idea that MMP3's processed protein targets stimulate cellular inflammatory responses. Transient overexpression of hypoxia-inducible factor HIF-1-Alpha in bone marrow-derived mesenchymal stem cells and synoviocytes increase MMP3 transcript expression.[46,47] Rats subjected to ischemic hypoxia have increased levels of MMP3 in their cerebral cortex.[48] Therefore, it seemed likely that low oxygenation of the blood should increase MMP3 expression. Consistent with this view, we observed that MMP3 levels were significantly elevated in the serum of OSA patients relative to control individuals and at control levels in airways treated patients. While it is possible that the increase in MMP3 in OSA patients is beneficial to wound healing and cardiovascular health, a favorable effect seems unlikely, when the levels are elevated three-fold above the levels found in healthy control individuals (Figure 1B). Dramatic decreases in MMP3 were obtained by the immunosuppression of TNF-Alpha with the monoclonal infliximab in patients with chronic disabling inflammatory bowel diseases produced.[49] However, infliximab also produced significant reductions in MMP2, which might have adverse effects on OSA patients.

TWEAK has both membrane and soluble isoforms expressed in endothelial cells, fibroblasts, and immune cells.[50] Soluble TWEAK ligand signals via the TNF-related receptor Fn14 (*TNFRSF12A*) to regulate the expression of the pro-inflammatory cytokine and transcription factor NF-κB. Normal TWEAK/Fn14 signaling appears to play beneficial roles in tissue repair after acute injury relevant to CVD, atherosclerosis, and stroke.[51] Reductions in the levels of soluble TWEAK, are associated with the increased risk of CVD, kidney disease, and mortality.[52] Conversely, small increases in soluble TWEAK are also associated with the disease.

The reduced level of TWEAK expression we observed in OSA patients may represent a compensatory mechanism working against a chronic state of inflammation resulting from months or years of intermittent hypoxia. Our data suggest that airways therapy partially or fully restored the levels of TWEAK in most OSA patients. TWEAK preconditioning protects rats from loss of heart function following myocardial ischemia.[53] However, overexpression of recombinant soluble murine TWEAK in mice results in severe cardiac dysfunction.[54] Perhaps the carefully controlled delivery of low levels of recombinant TWEAK protein would be an effective supplement to airways therapy for OSA associated CVD and kidney disease.

CD163 producing macrophages promote angiogenesis and vascular permeability, which result in increased vascular and glomerular inflammation.[55,56] Hence it is not surprising that CD163 is implicated in cardiovascular and kidney disease. Macrophage CD163 is thought to mediate the uptake and clearing of hemoglobin-haptoglobin complexes, which protects tissues from hemoglobin-mediated oxidative damage.[57] Herein, the median level of CD163 was low in OSA patients relative to control or airways treated subjects. CD163 levels may be low enough in OSA patients that this cytokine cannot perform its normal

functions of preventing oxidative damage or promoting normal vascularization. Therapeutic immunosuppression of CD163 has been proposed to treat cancers and autoimmune disorders, wherein patients have elevated CD163 levels. By contrast, therapeutic supplementation with CD163 might be considered for OSA patients, for whom its levels are highly repressed.

In conclusion, altered serum levels of soluble MMP2, MMP3, TWEAK, and CD163 are all risk factors for CVD and renal disease. We presented well-quantified data to show that all four cytokines were expressed at aberrant levels in the serum of OSA patients relative to controls. Most OSA patients receiving airways therapy had cytokine levels near to or statistically equivalent to the levels found in healthy control individuals. These data suggest chronic intermittent hypoxia is one cause for their altered expression in OSA patients. Because acute hypoxia in vitro produces increased levels of all four cytokines, it is possible that chronic hypoxia experienced over months and years attenuates this response in OSA patients. Furthermore, we cannot rule out that other factors such as fragmented sleep or daytime sleepiness were causal to the miss expression of these cytokines.

Code Availability (Software Application or Custom Code)

See Materials and Methods.

Data Sharing Statement

Supplementary Data Files SD1 and SD2.

Ethics Approval (Include Appropriate Approvals or Waivers)

The research presented in this manuscript met all the ethical standards outlined in the US National Research Act of 1974 and the Declaration of Helsinki and were approved by the University of Georgia's Institutional Review Board.

Consent to Participate (Include Appropriate Statements)

See Materials and Methods.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data, took part in drafting the article or revising it for accurate intellectual content, agreed to submit to the Nat Sci Sleep, gave final approval of the version to be published, and agree to be accountable for all aspects of the work. RBM and BGP obtained funding for the project.

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Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported herein.

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