

RESEARCH ARTICLE

Urinary Neutrophil Gelatinase-Associated Lipocalin (NGAL) in Patients with Obstructive Sleep Apnea

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Abstract

Background

Obstructive sleep apnea (OSA) is a well-established risk factor for hypertension and cardiovascular morbidity and mortality. More recently, OSA has been implicated as an independent risk factor for chronic kidney disease. Urinary neutrophil gelatinase-associated lipocalin (NGAL) is a well-accepted early biomarker of subclinical kidney tubular injury, preceding an increase in serum creatinine. The goal of this study was to determine if an association exists between OSA and increased urinary NGAL levels.

Methods

We prospectively enrolled adult patients from the sleep clinic of an academic medical center. Each underwent polysomnography and submitted a urine specimen upon enrollment. We measured NGAL and creatinine levels on all urine samples before participants received treatment with continuous positive airway pressure (CPAP), and, in a subset of OSA patients, after CPAP therapy. We compared the urinary NGAL/creatinine ratio between untreated participants with and without OSA, and within a subset of 11 OSA patients also after CPAP therapy.

Results

A total of 49 subjects were enrolled: 16 controls based on an apnea-hypopnea index (events with at least 4% oxygen desaturation; AHI-4%) <5 events/hour (mean AHI-4% = 0.59 +/- 0.60); 33 OSA patients based on an AHI-4% >5 events/hour (mean AHI-4% = 43.3 +/- 28.1). OSA patients had a higher mean body-mass index than the control group (36.58 +/- 11.02 kg/m² vs. 26.81 +/- 6.55 kg/m², respectively; p = 0.0005) and were more likely to be treated for hypertension (54.5% vs. 6.25% of group members, respectively; p = 0.0014). The groups were otherwise similar in demographics, and there was no difference in the number of diabetic subjects or in the mean serum creatinine concentration (control = 0.86

+/- 0.15 mg/dl, OSA = 0.87 +/- 0.19 mg/dl; $p = 0.7956$). We found no difference between the urinary NGAL-to-creatinine ratios among untreated OSA patients versus control subjects (median NGAL/creatinine = 6.34 ng/mg vs. 6.41 ng/mg, respectively; $p = 0.4148$). Furthermore, CPAP therapy did not affect the urinary NGAL-to-creatinine ratio ($p = 0.7758$ for two-tailed, paired t-test).

Conclusions

In this prospective case-control study comparing patients with severe, hypoxic OSA to control subjects, all with normal serum creatinine, we found no difference between urinary levels of NGAL. Furthermore, CPAP therapy did not change these levels pre- and post-treatment.

Introduction

Obstructive sleep apnea (OSA) has been estimated to affect 24% of middle-aged men and 9% of middle-aged women, with 9% and 4%, respectively, meeting criteria for moderate-severe OSA [1]. It is characterized by recurrent complete or partial collapse of the upper airway leading to hypoxia and hypercapnea, and results in large fluctuations in intrathoracic pressure and compensatory arousals to restore respiration. The prevalence of OSA is only expected to increase in parallel with the obesity epidemic, since obesity is the greatest risk factor for its development.

OSA is a well-established independent risk factor for hypertension, coronary artery disease, congestive heart failure, stroke, and death [2–4]. Furthermore, significant evidence has accumulated implicating OSA as a risk factor for the initiation and progression of chronic kidney disease (CKD), independent of the frequently occurring co-morbidities between the two including diabetes, hypertension, and obesity [5–10]. In the largest study to evaluate the independent association between OSA and CKD, Lee, *et al* [11] compared 4,674 newly-diagnosed adult OSA patients to 23,370 age- and sex-matched non-OSA patients. The two groups were followed for the occurrence of CKD diagnosis: after adjustment for numerous potentially confounding comorbidities, OSA patients demonstrated a 1.94-fold increase in the incidence of CKD and a 2.2-fold increase in the incidence of end-stage renal disease (ESRD) [11].

The mechanisms by which OSA may directly damage the kidneys remain incompletely understood. Clearly, OSA may indirectly worsen renal function via exacerbation of both hypertension [12] and glycemic control [13], two of the best-established risk factors for CKD. However, experimental data suggest OSA may directly produce end-organ injury via mechanisms including excess sympathetic nervous system activity [14,15], increased renin-angiotensin-aldosterone system activity [16,17], hypoxia/reoxygenation-induced formation of reactive oxygen species [18], endothelial dysfunction [19], inflammation [20], and perturbations in renal hemodynamics [5, 21–23].

In particular, hemodynamic studies in OSA patients have demonstrated reduced diurnal renal blood flow that improves after treatment with continuous positive airway pressure (CPAP) [5,22], as well as impaired renal arterial vasodilating capacity [21]. Furthermore, in a porcine model of OSA, marked renal hypoperfusion was observed after the application of each obstructive respiratory event, with a mean drop in renal blood flow of over 60% (from 190 +/- 24 ml/min to 70 +/- 20 ml/min; $P < 0.00001$) [23].

We therefore hypothesized that repetitive renal hypoperfusion events caused by obstructive apneas throughout the night, and perhaps diurnal reduction in renal blood flow, may be responsible for subacute kidney injury that accumulates over time. We reasoned that recurrent

renal hypoperfusion of hypoxemic blood during each obstructive apnea, followed by restoration of perfusion of normoxic blood between apneas, may mimic an “ischemia-reperfusion” type [24] of cumulative renal tubular epithelial cell injury. Moreover, we posited that such renal tubular epithelial cell injury would be present before an increase in the serum creatinine manifests.

To test this hypothesis, we prospectively enrolled newly-diagnosed OSA patients and unaffected subjects from our Sleep Disorders Clinic to assay their urine for neutrophil gelatinase-associated lipocalin (NGAL). NGAL has emerged as one of the most promising urinary biomarkers for the early detection and prediction of kidney injury, generally preceding a detectable increase in the serum creatinine [25]. NGAL is highly upregulated and released into the urine by injured renal tubular epithelial cells soon after experimental ischemia-reperfusion injury in mice [26]. Moreover, in several studies of patients undergoing cardiac surgery—characterized by renal ischemia-reperfusion—elevated urinary NGAL levels both preceded acute kidney injury (AKI) defined as an increase in serum creatinine, and were also associated with poor clinical outcomes [27–29].

Methods

Study Design and Oversight

This prospective, observational study was performed at the Beth Israel Deaconess Medical Center (BIDMC, Boston, MA), in accordance with all institutional policies and with approval of the hospital’s institutional review board, the BIDMC Committee on Clinical Investigations (CCI). The BIDMC CCI first approved this study (under protocol number 2008P000467) on 3/13/2009 and has renewed the protocol annually. All participants provided written informed consent upon enrollment.

Study Participants and Clinical Data

Adult participants were recruited from the BIDMC Sleep Disorders Clinic over the period from 2009–2014. The only exclusion criterion was established stage 3 chronic kidney disease or higher, corresponding to an estimated glomerular filtration rate of less than 60 mL/min/1.73m² by the abbreviated MDRD equation [30]. Each participant’s medical record was reviewed for the following clinical information: gender, age, self-identified race, body mass index (BMI; kg/m²), diagnoses of hypertension, diabetes mellitus, and other chronic medical conditions, current medications, recent clinic blood pressure measurements, and recent serum creatinine measurements.

Standard in-center polysomnography (PSG) was performed on all patients around the time of study enrollment. PSGs were scored using standard American Academy of Sleep Medicine criteria [31] by registered sleep technologists. An *apnea* was defined as cessation of airflow for at least 10 seconds and a *hypopnea* as an abnormal respiratory event lasting at least 10 seconds with at least a 30% reduction in airflow and at least a 4% oxygen desaturation [31]. The apnea-hypopnea index-4% (AHI-4%) was calculated by summing all recorded apneas and hypopneas and dividing by the total hours of sleep recorded, resulting in units of *number of apneas and hypopneas with at least 4% oxygen desaturation per hour*. Additional sleep metrics were available for most participants, including: the respiratory disturbance index (RDI), which accounts for all scored respiratory events regardless of oxygen desaturation, sleep efficiency defined as the percentage of time actually asleep while in bed, and the percentages of total sleep time spent in non-rapid eye movement sleep stages 1 through 3 (Stages N1% through N3%) and in rapid eye movement sleep (REM%). While the control subjects did not qualify for a diagnosis of OSA, most were having sleep-related complaints that prompted their PSG, usually excessive

daytime sleepiness. The average duration of sleep-related symptoms was similar for both the control and the OSA groups at the time of enrollment (approximately 2 years).

A subset of OSA patients who were successfully treated with CPAP submitted post-treatment urine samples for analysis. *Successful CPAP treatment* was defined as at least 1 month of CPAP therapy titrated to achieve the lowest possible residual AHI-4%, with documented compliance of at least 4 hours of use per night.

Measurement of Urinary Creatinine and Neutrophil Gelatinase-Associated Lipocalin

Each participant provided a clean-catch urine sample upon enrollment (and for a subset of participants, also after successful treatment with CPAP) between the hours of 9:00AM and 4:00PM. Collected urine samples were immediately transported to the laboratory, aliquoted, and stored at -80 C until analysis. Urinary Neutrophil Gelatinase-Associated Lipocalin (NGAL) concentrations were determined via quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Inc., Minneapolis, MN). Urinary creatinine concentrations were determined by Jaffe reaction using a commercially available assay (R&D Systems, Inc., Minneapolis, MN).

Statistical Analysis

Participant characteristics and clinical data are presented either as means +/- standard deviations or as number of patients (N) falling within a category. Characteristics between OSA and control groups were compared using independent samples t-tests or Fisher's exact tests, as appropriate. Pearson's correlation coefficients were used to determine the associations between mean arterial pressure (MAP) and BMI, and between urinary NGAL per creatinine (NGAL/Cr) and AHI-4%. The Mann-Whitney test was used to compare the urinary NGAL/Cr ratios between control and untreated OSA groups. Paired t-test (two-tailed) was used to compare the urinary NGAL per creatinine ratios before and after treatment with CPAP among 11 OSA patients. Statistical analyses were conducted with the use of GraphPad Prism software, version 6.0d (GraphPad Software, Inc.). Two-tailed P values of less than 0.05 were considered to indicate statistical significance.

Results

Patient Characteristics

A total of 49 subjects were prospectively enrolled in this study: 16 subjects fell into the control group based on their PSG demonstrating fewer than 5 hypoxic events per hour (as defined by AHI-4%); 33 subjects fell into the OSA group based on an AHI-4% of greater than 5 events per hour. The OSA group had a significantly higher mean BMI than the control group (36.58 +/- 11.02 kg/m² versus 26.81 +/- 6.55 kg/m², respectively; $p = 0.0005$) and was much more likely to be on one or more medications for hypertension (54.5% versus 6.25% of group members, respectively; $p = 0.0014$). (Table 1).

The control and OSA groups were otherwise similar in demographic composition, including in the proportions of men and women, the mean age, and by self-identified racial categorization. There was no significant difference in the number of diabetic patients between groups (0 versus 3 in control and OSA groups, respectively; $p = 0.5415$), or in the mean serum creatinine concentration between groups (control = 0.86 +/- 0.15 mg/dl, OSA = 0.87 +/- 0.19 mg/dl; $p = 0.7956$). (Table 1).

Table 1. Patient Characteristics. OSA = obstructive sleep apnea, M = male, F = female, Afr Amer = African American, BMI = body mass index, DM = diabetes mellitus, HTN = hypertension, SBP = systolic blood pressure, DBP = diastolic blood pressure, AHI-4% = apnea-hypopnea index of respiratory events per hour with at least 4% oxygen desaturation, RDI = respiratory disturbance index, O2 sat % = percentage oxygen saturation recorded, Stage N1% = percentage of total sleep in non-REM sleep stage I, Stage N2% = percentage of total sleep in non-REM sleep stage II, Stage N3% = percentage of total sleep in non-REM sleep stage III, REM% = percentage of total sleep in rapid eye movement (REM) sleep. Table shows mean (+/- standard deviation) or N = number of patients falling into category.

| | Control | OSA Untreated | P-value |
|--------------------------|-----------------|------------------|----------|
| N | 16 | 33 | |
| Sex (M/F) | 6M / 10F | 20M / 13F | 0.2217 |
| Age (years) | 47.4 (+/-12.8) | 49.6 (+/-11.4) | 0.5465 |
| White | 12 | 21 | 0.5261 |
| Afr Amer | 0 | 4 | 0.2889 |
| Hispanic | 2 | 5 | 1.0000 |
| Other Race | 2 | 3 | 1.0000 |
| BMI (kg/m ²) | 26.81 (+/-6.55) | 36.58 (+/-11.02) | 0.0005* |
| Hx of DM | 0 | 3 | 0.5415 |
| Tx for HTN | 1 | 18 | 0.0014* |
| SBP (mmHg) | 118.2 (+/-14.6) | 130.3 (+/-11.4) | 0.0034* |
| DBP (mmHg) | 75.7 (+/-8.8) | 82.9 (+/-9.2) | 0.0129* |
| AHI-4% | 0.59 (+/-0.60) | 43.3 (+/-28.1) | <0.0001* |
| RDI (events/hr) | 10.87 (+/-1.90) | 58.74 (+/-4.88) | <0.0001* |
| Lowest O2 Sat % | 91.7 (+/-2.5) | 75.4 (+/-8.8) | <0.0001* |
| Sleep Efficiency % | 81.1 (+/-2.9) | 77.7 (+/-2.1) | 0.3490 |
| Stage N1% | 7.96 (+/-1.59) | 15.87 (+/-1.98) | 0.0033* |
| Stage N2% | 60.67 (+/-2.62) | 57.68 (+/-2.29) | 0.4240 |
| Stage N3% | 13.85 (+/-2.64) | 6.84 (+/-1.44) | 0.0146* |
| REM% | 17.34 (+/-2.32) | 14.48 (+/-1.92) | 0.3724 |
| Creatinine (mg/dl) | 0.86 (+/-0.15) | 0.87 (+/-0.19) | 0.7956 |

*Significant at P<0.05

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Sleep Metrics

The difference between the mean AHI-4% of the OSA group versus the control group was highly statistically significant (43.3 +/- 28.1 events/hour versus 0.59 +/-0.60 events/hour, respectively; p<0.0001) (Fig 1A and Table 1). Similarly, the lowest percent oxygen saturation

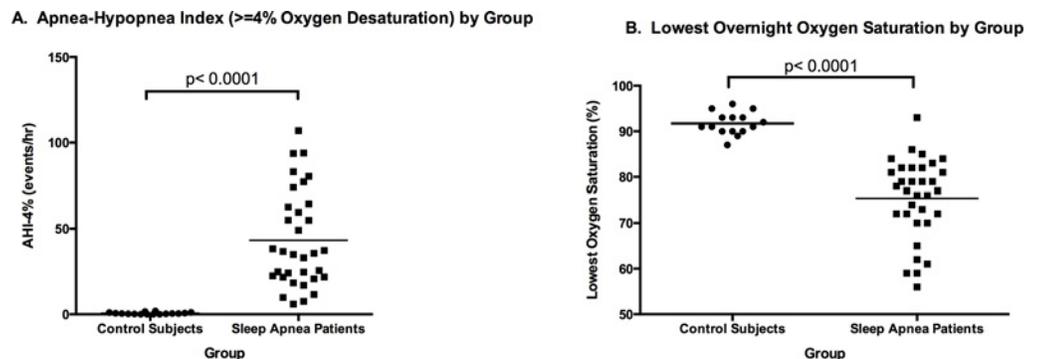


Fig 1. Sleep Apnea Severity between Control Subjects and Affected Patients. A depicts the individual apnea-hypopnea indices (average number of respiratory events with at least 4% oxygen desaturation per hour; AHI-4%) between control versus sleep apneic groups. B depicts the lowest percent oxygen saturation recorded by overnight pulse oximetry between groups. Horizontal lines within each group of data points indicate the group mean.

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recorded by continuous pulse oximetry during PSG was significantly lower in the OSA group versus the control group (mean OSA = 75.4 +/- 8.8% versus mean Control = 91.7 +/- 2.5%; $p < 0.0001$) (Fig 1B and Table 1). The RDI, which includes all scored respiratory events regardless of oxygen desaturation, was likewise highly different between groups (mean OSA = 58.74 +/- 4.88 events/hour versus mean Control = 10.87 +/- 1.90 events/hour; $p < 0.0001$). Only the percentages of total sleep spent in non-rapid eye movement stages 1 (light sleep; N1%) and 3 (deep sleep; N3%) differed between OSA and control groups (mean OSA N1% = 15.87 +/- 1.98% versus mean Control N1% = 7.96 +/- 1.59%, $p = 0.0033$; mean OSA N3% = 6.84 +/- 1.44% versus mean Control N3% = 13.85 +/- 2.64%, $p = 0.0146$). The percentage of total sleep spent in rapid eye movement stage (REM%) was not different between groups (mean OSA REM% = 14.48 +/- 1.92 versus mean Control REM% = 17.34 +/- 2.32; $p = 0.3724$). (Table 1).

Correlation between Mean Arterial Pressure and BMI

For both the OSA and control groups, the mean arterial blood pressure (MAP) positively correlated with the BMI [$r = 0.4532$ ($p = 0.0092$) and $r = 0.5517$ ($p = 0.0330$), respectively] (Fig 2).

Urinary Neutrophil Gelatinase-Associated Lipocalin Levels between OSA and Control Groups and Among Subset of OSA Patients before and after CPAP Therapy

There was no significant difference between the urinary NGAL-to-creatinine ratios (ng NGAL/mg creatinine; NGAL/Cr) among the OSA patients as compared to the control subjects (median NGAL/Cr = 6.34 ng/mg versus 6.41 ng/mg, respectively; $p = 0.4148$) (Fig 3). There was no correlation between NGAL-to-creatinine ratio and AHI-4% ($r = -0.1064$, $p = 0.4669$) (Fig 4). Furthermore, among 11 OSA patients who submitted urine samples both before and after successful treatment with CPAP, treatment did not significantly affect the NGAL-to-creatinine ratio (Fig 5).

Discussion

In this prospective case-control study comparing patients with severe, hypoxic OSA to unaffected but otherwise similar control subjects—all with normal serum creatinine—we found no difference between corrected urinary levels of the early renal tubular injury biomarker NGAL. Furthermore, successful CPAP therapy did not change these levels pre- and post-treatment among a subgroup of OSA patients. This current work appears to be the first to evaluate any well-established urinary renal tubular injury biomarker among OSA patients. Our hypothesis was that OSA's recurrent hemodynamic events on the kidney—resembling renal ischemia-reperfusion injury [23,24]—would result in elevated urinary NGAL levels before any detectable increase in

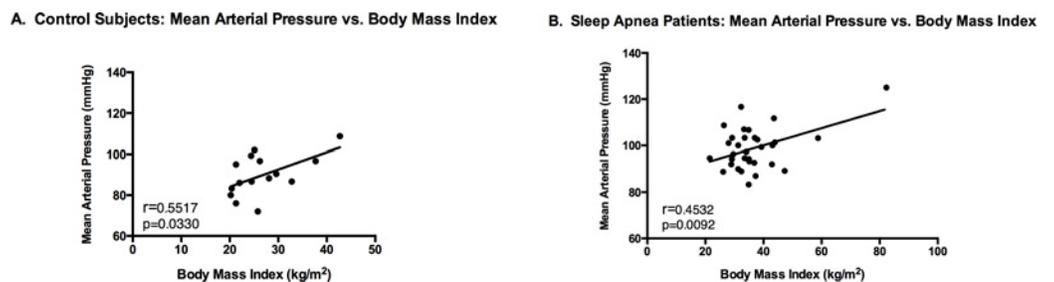


Fig 2. Correlation between Mean Arterial Pressure and Body Mass Index by Group Panel A depicts the individuals within the control group, and Panel B depicts the patients within the sleep apneic group.

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Urine NGAL/Creatinine Ratio by Group

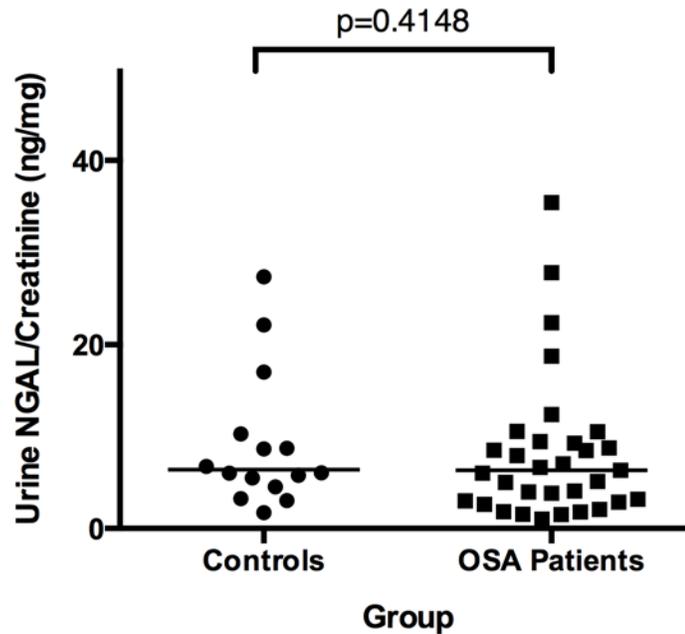


Fig 3. Individual Urinary NGAL-to-Creatinine Ratios (ng/mg) between Control versus Untreated Sleep Apneic (OSA) Groups. Horizontal lines within each group of data points indicate the group median.

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All Subjects: NGAL/Cr vs. AHI-4%

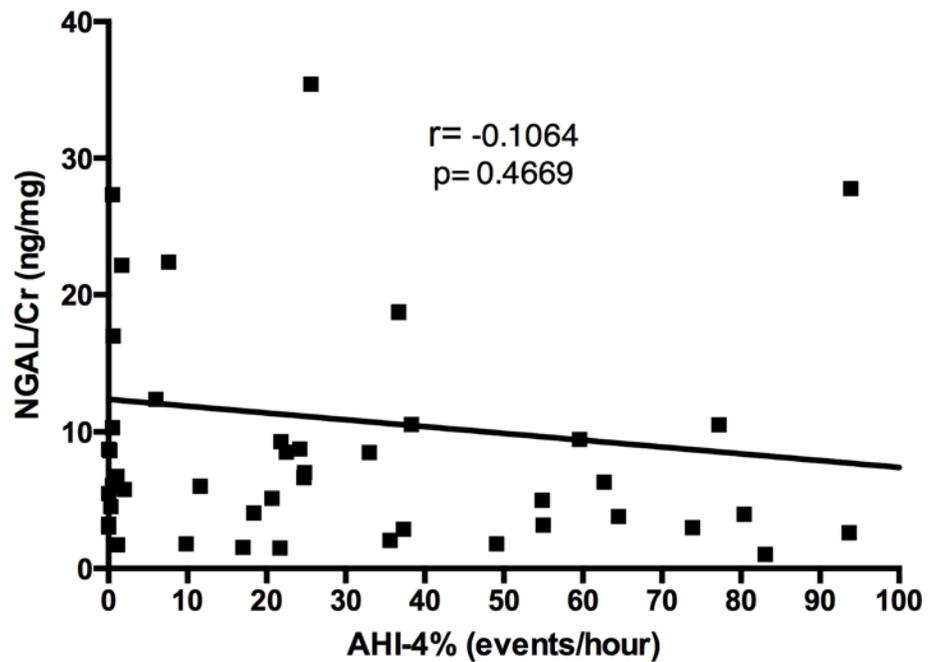


Fig 4. Individual NGAL-to-Creatinine Ratios (NGAL/Cr; ng/mg) versus Apnea-Hypopnea Indices (AHI-4%; events/hour) among All Subjects (Untreated Sleep Apnea and Control).

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Urinary NGAL/Creatinine Ratio Pre- and Post-Treatment

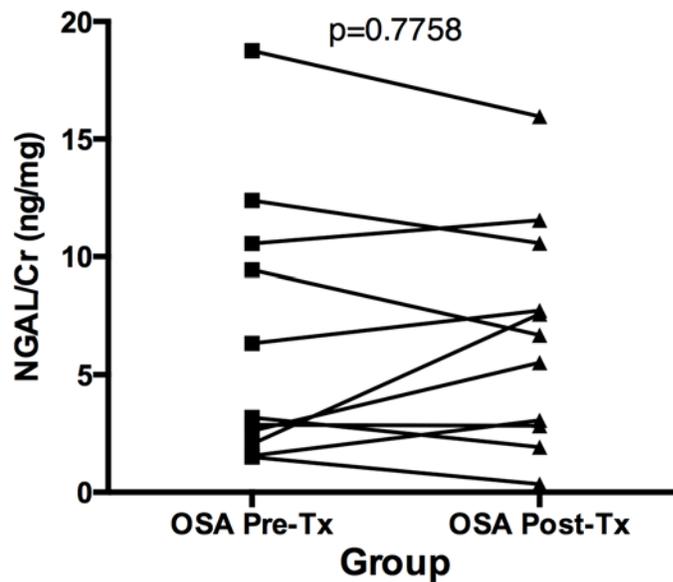


Fig 5. Urinary NGAL-to-Creatinine Ratios (NGAL/Cr; ng/mg) among 11 Sleep Apneic Patients (OSA) before (Pre-Tx) and after (Post-Tx) Successful Treatment with Continuous Positive Airway Pressure.

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serum creatinine, as is observed in both experimental [25,26] and clinical [27–29] models of kidney ischemia-reperfusion injury. That we observed no elevation in corrected urinary NGAL levels (or in uncorrected levels; [S1 Fig](#)) among severe, untreated OSA patients was unexpected.

Our OSA group was representative of patients affected by this condition. For example, they had a significantly higher BMI and were much more likely to be treated for hypertension relative to controls. Moreover, their mean AHI-4% of greater than 40 events per hour classifies their OSA as severe. The OSA group further had evidence of sleep fragmentation, with double the percentage of time spent in light sleep (stage N1) and half the percentage of time spent in deep sleep (stage N3), as compared to controls. In other important respects, however, including gender distribution, age, self-identified race, diabetic status, and serum creatinine level, the OSA group was similar to control participants. Furthermore, the OSA group and the control group similarly demonstrated increasing MAP with increasing BMI, despite 18 of 33 members of the OSA group being treated with one or more antihypertensive medications.

Thus, our cohort appears sufficiently representative to detect a difference in urinary NGAL levels between groups if it existed. Furthermore, with 16 control participants and 33 untreated OSA patients, our power calculation revealed a >80% ability to detect a difference of at least 30% in NGAL/Cr between groups (at $\alpha = 0.05$). Additionally, our pre- and post-CPAP results among a subgroup of successfully-treated OSA patients (with treatment strictly documented), in whom each patient acted as his/her own control, are consistent with no observed effect of OSA physiology on urinary NGAL levels. With regard to a potential difference in NGAL/Cr between OSA patients versus non-OSA subjects stratified by gender, we still found no effect of OSA: median female OSA versus non-OSA = 8.49 vs. 9.47 ng/mg ($p = 0.5981$ by Mann-Whitney test), and median male OSA versus non-OSA = 4.49 vs. 5.31 ng/mg ($p = 0.9757$ by Mann-Whitney test). Lastly, we found no correlation between urinary NGAL/Cr and the severity of OSA as indicated by the AHI-4%.

Since our data do not demonstrate an elevation of urinary NGAL among untreated sleep apneics with still-normal renal function (as per serum creatinine), we speculate that the kidney

injury associated with OSA may be glomerular (as opposed to tubular) in nature. Indeed, microalbuminuria and frank proteinuria—the hallmarks of glomerular injury—have been observed in OSA patients by independent investigators [32–34]. Furthermore, glomerulomegaly and focal segmental glomerulosclerosis have been well-described on renal biopsy of OSA patients [35–37]. We must, however, acknowledge other possible conclusions that can be drawn from these data. It is possible, for example, that we were unable to detect a brief and/or low-magnitude increase in urinary NGAL based on our study design utilizing daytime urine collections. Furthermore, it may be that the renal insults of untreated OSA were sufficiently chronic among our study participants such that any increase in urinary NGAL eventually normalized over time.

Some additional limitations of our study require mention. First, our total cohort of 49 participants is not large. However, the controls and OSA patients were at such opposite poles of the sleep apnea spectrum that a difference in urinary NGAL levels, if one existed, should have emerged. Still, we cannot exclude the possibility that we were underpowered to detect a difference in urinary NGAL of less than 30% between groups. Second, it is possible that patients referred for evaluation of sleep complaints as a whole (regardless of OSA status and severity) have similarly elevated urinary NGAL levels as compared to subjects not experiencing sleep-related symptoms, which could obscure our ability to detect a difference between groups. A previous study designed to establish a normal reference range for urinary NGAL, derived from urine samples of healthy adults without CKD and using the same assay as we used, found a mean NGAL/Cr of 12.8 ng/mg and 25.0 ng/mg for men and women aged 41–50, respectively [38]. A separate study of non-healthy patients with cardiovascular disease (but normal pre-operative serum creatinine) undergoing cardiac surgery, also using the same NGAL assay, found a pre-operative urinary NGAL/Cr median value of 7.37 ng/mg (interquartile range = 3.11 to 22.24) among a group of 83 subjects [39]. Thus, considering our mean NGAL/Cr of 8.41 ng/mg among all male participants, mean of 13.77 ng/mg among all female participants, and median of 6.34 ng/mg among all 49 study participants (S1 Table), it does not appear sleep clinic patients as a whole have elevated NGAL/Cr. Third, since over 50% of the OSA patients were treated for hypertension, predominantly with an angiotensin-converting enzyme inhibitor (ACE-I) with or without diuretic, we considered the possibility that these medications may reduce urinary NGAL excretion and thereby obscure a difference between groups. However, review of the literature reveals that ACE-I and diuretics have not been shown to affect urinary NGAL levels [40,41]. Lastly, we evaluated only one renal tubular cell injury marker, although several others have been studied for the early detection of kidney injury, for example kidney injury molecule 1 (KIM-1) and *N*-Acetyl beta glucosaminidase (NAG) [42]. Nonetheless, review of the urinary biomarker literature reveals urinary NGAL performs as well, if not better, than these other biomarkers with regard to area under the receiver operating curve values in studies of renal ischemia-reperfusion injury, with values generally between 0.7 to 0.9 [25–29,42].

In conclusion, we prospectively studied a well-validated renal tubular epithelial injury molecule in the urine of OSA patients. The lack of urinary NGAL elevation among patients with severe OSA—and lack of change in these levels before and after CPAP therapy—that we observed potentially points away from kidney tubular injury as a mechanism of renal insult in OSA, within the limitations described. Further investigation is needed to clarify the mechanisms by which OSA may directly injure the kidneys.

Supporting Information

S1 Fig. Individual Uncorrected Urinary NGAL Levels (ng/ml) between Control versus Untreated Sleep Apneic (OSA) Groups. Horizontal lines within each group of data points

indicate the group median.
(TIFF)

S1 Table. NGAL/Cr Ratios by Gender and OSA Status.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MRM SMP. Performed the experiments: MRM. Analyzed the data: MRM RJT SMP SAK. Contributed reagents/materials/analysis tools: SAK SMP RJT MRM. Wrote the paper: MRM RJT SAK SMP.

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