Novel Molecular Investigations Into:
(1) Obstructive Sleep Apnea and (2)
De Novo Postpartum Hypertension

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Chapter 1: Introduction

There are two themes of the work herein presented: (1) exploration of the molecular pathophysiology of obstructive sleep apnea (OSA), and (2) the role of angiogenic factors in selected diseases, including OSA, de novo hypertension developing in the postpartum period (de novo PPHTN), and diabetic nephropathy.

Chapter 2 presents our work to date regarding our discovery that levels of circulating Artemin (ART) are elevated among patients with untreated OSA as compared to control subjects. ART is a protein belonging to the glial cell line-derived neurotrophic factor (GDNF) family of growth factors. ART has a role in the development and maintenance of peripheral sympathetic nervous system neurons, as well as in angiogenesis. Among a prospective cohort of patients enrolled from the Sleep Disorders Clinic at Beth Israel Deaconess Medical Center (BIDMC, Boston, MA), we show that serum ART is elevated among patients with OSA as compared to controls, persisting after multivariable adjustment. We further constructed an inducible transgenic mouse model overexpressing human ART, and demonstrate that constitutive overexpression of ART results in >50% mortality among male mice by 13 weeks of life. Through extensive physiologic experiments on these transgenic mice, the mechanism of lethality is most probably cardiac in nature, although further confirmatory cardiac experiments are required (and are currently underway). These data may implicate ART in the increased cardiac morbidity and mortality observed among OSA patients.

Chapter 3 presents work currently under consideration for publication in *PLOS ONE* (submitted on 3/27/2015). We evaluated urine samples collected from our prospective cohort of OSA patients and control subjects for the early renal tubular injury biomarker neutrophil gelatinase-associated lipocalin (NGAL). Our hypothesis was that OSA’s recurrent hemodynamic events on the kidney—resembling renal ischemia-reperfusion injury—would result in elevated urinary
NGAL levels prior to a detectable increase in serum creatinine, as is observed in both clinical and experimental models of kidney ischemia-reperfusion injury. We further posited that such recurrent renal hypoperfusion events may in part explain the recent epidemiologic observation that OSA independently doubles the risk for the development of both chronic kidney disease and end-stage renal disease.

Chapter 4 presents our work currently in revision for publication in *Circulation* (the original submission from 1/21/2015 is presented). This project was born out of the observation that, while antepartum hypertensive disorders have received considerable investigation, only limited data exist regarding the epidemiology of newly-developed hypertension in the postpartum period (*de novo* PPHTN). Furthermore, even less is known about the pathophysiology underlying the development of *de novo* PPHTN. We therefore prospectively studied almost 800 consecutive women delivering a singleton pregnancy at BIDMC. We also evaluated their antepartum admission blood samples for the angiogenic factors soluble fms-like tyrosine kinase 1 (sFlt-1) and placental growth factor (PIGF). We found that the development of *de novo* PPHTN was actually common (9.9%). Furthermore, the clinical risk factors and antepartum angiogenic profile of women developing *de novo* PPHTN strongly resembled those of women with preeclampsia, suggesting that women developing *de novo* PPHTN may represent a group with “subclinical preeclampsia” who do not manifest hypertension until postpartum. This work, to the best of our knowledge, represents the largest prospective study within the literature designed to specifically study PPHTN. Moreover, it provides guidance regarding which women might benefit from closer follow-up in the postpartum period, which often does not first occur until 6 weeks after delivery.

Chapter 5 presents an editorial co-written with Samir M. Parikh (Co-Mentor for Master’s Program) that was published in the *Journal of the American Society of Nephrology* [Volume 25(1): pages 1-3, 2014]. This editorial accompanies an original research article by Dessapt-
Baradez et al. ("Targeted glomerular angiopoietin-1 therapy for early diabetic kidney disease", pages 33-42 of same issue) and contextualizes the group’s findings with regard to angiogenic factors affecting the vascular endothelium in diabetic nephropathy.

Lastly, but perhaps most importantly, I acknowledge those mentors, advisors, and collaborators who have been (and continue to be) instrumental in this work, but also instrumental in my growth as a physician-scientist. In particular, I would like to thank both Drs. S. Ananth Karumanchi and Samir M. Parikh for their daily teaching, guidance, and support over the past several years. I would also like to especially thank Dr. Robert J. Thomas for sharing his expertise in sleep apnea and clinical investigation with me, as well as for his significant time investment in our shared projects. Finally, I would like to especially thank Dr. Chandra C. Ghosh for all of his time spent training me in molecular biology and mouse model techniques, for his significant ongoing contributions to the Artemin work, and for his continued friendship.
Chapter 2: Association of Circulating Artemin with Obstructive Sleep Apnea

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 collapse of the upper airway leading to hypoxia and hypercapnia, 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. Most worrisome, however, is that OSA is a well-established independent risk factor for hypertension, coronary artery disease, congestive heart failure, stroke, bradyarrhythmias, ventricular arrhythmias, and death [2-5].

Despite intensive research into the pathophysiology of OSA over the past 20 years, the molecular mechanisms by which these adverse cardiovascular outcomes occur remain incompletely understood. Our laboratory (Dr. S. Ananth Karumanchi, Principal Investigator) focuses on the role of angiogenic factors in health and disease. We were initially intrigued by a report that the serum from OSA patients was associated with impaired angiogenic activity [6]. Following our area of expertise and these early data, we embarked on a prospective study enrolling newly-diagnosed OSA patients (and control patients whose sleep studies did not qualify them for a diagnosis of OSA) from the BIDMC Sleep Disorders Clinic, to evaluate their blood for circulating mediators of this impaired angiogenic activity.

Upon enrollment of six subjects within each group (OSA and control groups; the “discovery cohort”), we determined the relative amounts of various angiogenic proteins, growth factors, and cytokines between groups via a protein antibody microarray (product number ARY007, R+D Systems, Minneapolis, MN). The clinical characteristics of the discovery cohort are presented in Table 1. As is evident, the OSA group represented patients with severe, hypoxic sleep apnea
as compared to the unaffected control subjects (Apnea-Hypopnea Index-4%= 47.9 events/hour versus 0.73 events/hour, respectively; p=0.0121).

Although the protein array revealed several circulating molecules that were significantly different between groups (data not shown), we were particularly interested in the difference observed for the molecule Artemin (Figure 1). However, before initiating further investigation into Artemin (ART), we needed to confirm the elevation observed among OSA patients via a more quantitative method than afforded by the microarray, as well as confirm the finding among a larger cohort of patients. To these ends, I continued enrollment until achieving ~50 participants, with a 2:1 ratio of OSA patients to control subjects. The clinical characteristics of this “confirmation cohort” are presented in Table 2. 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/m$^2$ vs. 26.81 +/- 6.55 kg/m$^2$, 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), both of which are characteristic of OSA patients. 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. In parallel with enrollment of this confirmation cohort, I developed a sandwich ELISA assay from a commercially available pair of antibodies (product number DY2589, R+D Systems, Minneapolis, MN) to more accurately quantify the concentration of serum ART.

Upon technical optimization of the ART ELISA, the 16 control subjects and 33 untreated OSA patients’ sera samples were assayed. As Figure 2 demonstrates, the circulating ART levels by ELISA were significantly different between groups (OSA > controls), and this difference persisted upon step-wise multivariable adjustment (Table 3).
We were further interested in ART based on other groups’ previous work establishing this molecule’s importance in the development and maintenance of peripheral sympathetic neurons [7, 8]. Artemin knockout mice demonstrated abnormalities in the migration and axonal projection pattern of the entire sympathetic nervous system [8]. ART is expressed by arterial vascular smooth muscle cells from both neonatal and adult rat innervated femoral and tail arteries, and its coreceptor, GFRalpha3, is expressed along with the RET tyrosine kinase on postganglionic sympathetic neurons [9]. Furthermore, ART has been shown to support the survival of sympathetic neurons that compose the rat superior cervical ganglion [10]. Even more interesting, it has been demonstrated in vitro that ARTN-soaked beads cause both superior cervical and lumbar sympathetic ganglia dissected from rats of various developmental ages to extend neurites towards the bead [11].

Several groups have demonstrated chronically elevated circulating catecholamines (norepinephrine and epinephrine, primary neurotransmitters of the sympathetic nervous system) in OSA patients, which decrease with treatment. Increased carotid body sensitivity to apnea-induced hypoxemia leads to augmented sympathetic activity. An attenuated baroreflex also contributes to the increased sympathetic activity characteristic of OSA. In light of the central role the sympathetic nervous system occupies in OSA pathophysiology, we were naturally excited about ART’s potential role in contributing to this excess sympatho-excitation.

We therefore decided to construct a novel transgenic mouse overexpressing human ART in an inducible/suppressible fashion, in order to examine the physiologic effects of excess circulating ART. Our cloning scheme is depicted in Figure 3. We cloned human ART (transcript 4) into the pTRE-tight vector (Clontech Laboratories, Inc., Mountain View, CA) such that it was under the transcriptional regulation of the tetracycline transactivator (tTA) protein. I next confirmed ART protein expression of our vector construct (that was suppressible with addition of tetracycline to the culture media) in a series of transfection experiments using tTA-harboring HEK293 cells. Upon
confirmation of ART expression, we submitted our vector construct to the BIDMC Transgenic Mouse Facility for pronuclear injection of fertilized ova and subsequent uterine implantation. This resulted in the creation of two independent founder mouse lines, which we named “line 1” and “line 39.”

Upon several generations of breeding these two founder lines with Vascular Endothelial Cadherin (VE-Cad)-driven tTA mates (to generate “double transgenic” mice--abbreviated as “DT” or “DTg” henceforth--as depicted in Figure 3), a striking result emerged: the male DT mice of each independent founder line (1 and 39) displayed sudden death beginning at about 50 days of life and continuing forward such that ~50% of male DT mice from either founder line spontaneously died by ~day 100, as compared to both: (1) control mice (tTA gene alone or wild-type), and (2) DT mice (from either founder line) in which ART was suppressed only in utero (via maternal tetracycline consumption in her water source) and subsequently expressed from birth onward (Figure 4).

We explored multiple physiologic parameters in these DT male mice over the next several months, in an attempt to explain the mechanism of lethality. I performed over 50 individual necropsies on DT and control animals, but found no consistent gross anatomic pathologic or histopathologic difference between ART-overexpressing animals and control animals upon evaluation of the brain, lung, liver, kidneys, gastrointestinal organs, or gonads. We collected blood samples from dozens of mice for analysis of liver transaminases, renal function parameters (blood urea nitrogen and creatinine), complete blood count, and coagulation studies including prothrombin time and laser-induced arterial thrombosis time: there were no significant differences in any of these parameters between groups.

Given ART’s effect on the efferent arm of the sympathetic nervous system, we hypothesized that the DT mice may have manifest systemic or pulmonary hypertension. Thus, in collaboration with Dr. Peter Kang’s laboratory (BIDMC), we performed a series of terminal hemodynamic
experiments in which a pressure transducer was inserted into either the carotid artery (for systemic blood pressure measurements) or right ventricle (to measure RV systolic pressure, which corresponds to the pulmonary artery systolic pressure) in isoflurane-anesthetized male DT and control mice. The results of these studies are shown in Figure 5: no systemic hypertension or pulmonary hypertension was measured in DT animals relative to controls, and there was in fact a statistically significant lower systolic blood pressure among the DT animals relative to controls.

We next wanted to biochemically confirm excess sympathetic nervous system efferent activity among the DT mice. In collaboration with Dr. Karel Pacak’s laboratory (National Institutes of Health), we submitted ~40 urine samples from DT and control animals for analysis of urinary catecholamines. The results are shown in Figure 6: the urinary creatinine-corrected urinary levels of DOPA (the precursor of all catecholamines) and DHPG (the stable terminal breakdown product of norepinephrine and epinephrine) were significantly higher among the DT animals relative to control animals. Furthermore, 6 out of 8 of the highest corrected urinary DOPA levels belonged to a DT male who spontaneously died, as did 3 out of 5 of the highest corrected urinary DHPG levels, demonstrating a clustering of spontaneously-dying males at the highest levels of these catecholamine byproducts. These data demonstrate excess biochemical sympathetic efferent activity among DT animals, at least among those who experienced the sudden death phenotype.

In light of the well-established relationship between excess sympathetic nervous system activity and adverse effects on myocardial function, in the context of the whole of the data presented above, we next embarked on pilot echocardiographic and electrophysiologic experiments. In collaboration with Dr. Eliyahu Khankin (BIDMC), we performed serial echocardiography on six mice over a 6-week period spanning 6 to 12 weeks of life. These pilot echo data are presented in Figure 7. We found a significantly increased left ventricular anterior wall depth (in millimeters) among the DT male mice at 12 weeks of age, and this increased wall thickness was accompanied by a significantly reduced diastolic volume (in microliters). Further, we observed a trend toward
increased left ventricular ejection fraction among the DT male mice, that approached but did not meet statistical significant (p=0.0754).

In collaboration with the laboratory of Dr. Jonas Galper at Tufts Medical Center, we very recently commenced continuous telemetric monitoring studies among a cohort of DT male mice and their control littermates. A very exciting event occurred on 4/22/2015, and these data are presented in Figure 8. A 5.1-week-old DT male mouse experienced sudden cardiac death, and the entirety of the progressive cardiac rhythm disturbance was captured. Specifically, over approximately 38 hours of continuous recording, heart rate variability (HRV) significantly diminished at T-31 hours and remained diminished for the remainder of the recording. Furthermore, over a 12-hour period from T-18 to T-6 hours, there is a linear decrease in heart rate from 600 beats per minute (bpm) to 170 bpm, which represents extreme bradycardia for mice. In parallel with this linear decrease in heart rate, a worsening frequency of atrio-ventricular blocks occurred. Ultimately, there is a final deterioration over the period between T-0.5 until T-0.0 (with severe atrio-ventricular blocks), and the animal dies from cardiac arrest.

Summary and Future Directions

Thus, to summarize the main findings to date:

- Circulating Artemin levels are increased in OSA patients and persist after multivariable adjustment;
- Male Artemin transgenic mice exhibit increased mortality, in two independent founder lines;
- The data to date suggest that this sudden death is cardiac in etiology; and
- Excess Artemin may cause altered left ventricular structure/function and arrhythmogenesis.
The larger important question, however, is: does Artemin contribute to the increased risk of cardiac morbidity/mortality observed among OSA patients?

In order to complete this story and ultimately answer this question, we are pursuing the following future directions:

- Confirmation of the arrhythmia/sudden cardiac death phenotype in more transgenic mice via continuous telemetry (in progress);
- Performing additional echocardiographic studies to confirm ventricular structural and/or functional derangements in Artemin mice (in progress);
- Initiating histologic studies on the mouse hearts (e.g., Trichrome stain to evaluate for cardiac fibrosis, IF, IHC; all in progress);
- Investigations into cardiac signal transduction (in progress); and
- Determination of why developmental (i.e., in utero) exposure to Artemin is necessary for the lethal phenotype (in progress).

### Table 1. Clinical Characteristics of Discovery Cohort

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<th>Control</th>
<th>OSA Untreated</th>
<th>P-value</th>
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<td></td>
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<tr>
<td>Hispanic</td>
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<td>BMI (kg/m²)</td>
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<td>DBP (mmHg)</td>
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<td>83.0 (+/- 9.5)</td>
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<td>AHI-4%</td>
<td>0.73 (+/- 0.73)</td>
<td>47.9 (+/- 30.1)</td>
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<td>RDI (events/hr)</td>
<td>14.5 (+/- 8.8)</td>
<td>65.6 (+/- 23.6)</td>
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<td>Lowest O2 Sat %</td>
<td>92.7 (+/- 2.1)</td>
<td>75.3 (+/- 9.2)</td>
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<td>REM%</td>
<td>15.9 (+/- 7.0)</td>
<td>11.3 (+/- 10.9)</td>
<td>0.4066</td>
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*Significant at P<0.05
OSA=obstructive sleep apnea, M=male, F=female, BMI=body mass index, 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.

Table 2. Clinical Characteristics of Confirmation Cohort

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<td>Age (years)</td>
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<td>White</td>
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<td>Other Race</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>26.81 (+/-6.55)</td>
<td>36.58 (+/-11.02)</td>
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<tr>
<td>SBP (mmHg)</td>
<td>118.2 (+/-14.6)</td>
<td>130.3 (+/-11.4)</td>
<td>0.0034*</td>
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<td>DBP (mmHg)</td>
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<td>AHI-4%</td>
<td>0.59 (+/-0.60)</td>
<td>43.3 (+/-28.1)</td>
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<td>RDI (events/hr)</td>
<td>10.87 (+/-1.90)</td>
<td>58.74 (+/-4.88)</td>
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<td>Lowest O2 Sat %</td>
<td>91.7 (+/-2.5)</td>
<td>75.4 (+/-8.8)</td>
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<tr>
<td>Sleep Efficiency %</td>
<td>81.1 (+/-2.9)</td>
<td>77.7 (+/-2.1)</td>
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<td>Stage N1%</td>
<td>7.96 (+/-1.59)</td>
<td>15.87 (+/-1.98)</td>
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<td>Stage N2%</td>
<td>60.67 (+/-2.62)</td>
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<td>Stage N3%</td>
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<td>REM%</td>
<td>17.34 (+/-2.32)</td>
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<td>Creatinine (mg/dl)</td>
<td>0.86 (+/-0.15)</td>
<td>0.87 (+/-0.19)</td>
<td>0.7956</td>
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*Significant at P<0.05
Table 3. Step-wise Multivariable Adjustment of Serum Artemin Levels between Groups

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<th>P-Value</th>
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<tr>
<td>(1) Unadjusted</td>
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<tr>
<td>(2) Adjusted for Age, Race, Gender, BMI</td>
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<tr>
<td>(3) = (2) + Adjustment for Hypertension</td>
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</tr>
<tr>
<td>(4) = (3) + Adjustment for Diabetes Mellitus</td>
<td>0.041</td>
</tr>
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Figure 1. Serum Artemin is Elevated in Untreated OSA Patients as Compared to Control Subjects on Protein Microarray

Figure 2. Natural logarithm of Serum Artemin Concentrations (Measured by Sandwich ELISA) between Untreated OSA Patients versus Control Subjects
A double transgenic mouse positive for both VE-Cad-tTA and tetO-hArtemin allows for overexpression of human Artemin into the circulation that is suppressible with tetracycline in animal’s water supply.

Figure 3. Construction of a “Tetracycline-Off” Mouse Expressing Human Artemin in Vascular Endothelium with Secretion into the Circulation

Figure 4. Survival Curves for Male Control Mice (tTA gene alone or wild-type), Double Transgenic Mice Constitutively Expressing Artemin (DT-Line 1 and DT-Line 39), and Double Transgenic Mice with In Utero-Only Suppression of Artemin (DT-Dox)
Figure 5. Terminal Hemodynamic Studies in Double Transgenic versus Control Males. Please refer to the text for details on the experimental methodology.
Figure 6. Urinary DOPA and DHPG Levels (Corrected for Urinary Creatinine; Cr) Among Double Transgenic and Control Mice
Figure 7. Pilot Echocardiographic Studies on 6 mice. Echocardiographic measurements are shown between groups for ~12-week old male mice.
Tufts Collaboration: Continuous Electrophysiologic Telemetry Monitoring

Double Transgenic Male: Sudden Cardiac Death at 5.1 weeks

Figure 8. Preliminary Electrophysiologic Study (Continuous Telemetry) on a Double Transgenic Mouse Exhibiting Sudden Cardiac Death at 5.1 Weeks of Life

References


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/creatinine ratio 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 NGAL levels. Furthermore, CPAP therapy did not change these levels pre- and post-treatment.
**Question**  
**Response**

### Financial Disclosure

Please describe all sources of funding that have supported your work. A complete funding statement should do the following:

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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.
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Dear Ms. Nancarrow and members of the PLOS Editorial Team,

We are excited to submit the attached original research manuscript, “Urinary Neutrophil Gelatinase-Associated Lipocalin (NGAL) in Patients with Obstructive Sleep Apnea” to PLOS ONE for consideration. This is the initial submission of this work to your journal.

Obstructive Sleep Apnea (OSA) has long been identified as an independent risk factor for hypertension and increased cardiovascular morbidity and mortality. However, it is only recently appreciated that OSA is an independent risk factor for chronic kidney disease, which affects more than 10% of adults in the USA alone. Despite this important new association, the underlying mechanisms by which OSA may directly damage the kidney remain understudied, and even less understood.

We therefore prospectively enrolled newly-diagnosed OSA patients from our sleep disorders clinic to assess for subclinical renal tubular injury (i.e., before an increase in serum creatinine), both compared to an unaffected group of control subjects, and also compared before and after treatment with continuous positive airway pressure (the gold standard therapy for OSA). To assess renal tubular injury, we measured urinary Neutrophil Gelatinase-Associated Lipocalin (NGAL). NGAL is a well-validated early injury marker in a variety of renal disease models—particularly ischemia-reperfusion renal injury, which closely resembles OSA’s hemodynamic effects on the kidneys.

The work herein appears to be the first to evaluate any renal tubular injury biomarker in the urine of OSA patients, and represents an important early contribution to understanding OSA’s effect on the kidney. We have no preference with regard to the PLOS ONE Academic Editor who handles this manuscript.

With regard to potential external reviewers, we offer the following suggestions: (1) Samuel T. Kuna, MD, Associate Professor of Medicine, Perelman School of Medicine at the University of Pennsylvania [skuna@mail.med.upenn.edu | Samuel.Kuna@va.gov], (2) Ronald D. Chervin, MD, Professor of Sleep Medicine and Professor of Neurology, University of Michigan Medical School [chervin@med.umich.edu], (3) Daniel J. Gottlieb, MD, MPH, Associate Professor of Medicine, Harvard Medical School [dgottlieb@partners.org], and/or (4) Susan Redline, MD, MPH, Professor of Sleep Medicine, Harvard Medical School.

Best Regards,

Manish R. Maski, MD
(On behalf of Samir M. Parikh, MD, S. Ananth Karumanchi, MD, and Robert J. Thomas, MD)
Urinary Neutrophil Gelatinase-Associated Lipocalin (NGAL) in Patients with Obstructive Sleep Apnea

Manish R. Maski,¹ Robert J. Thomas,² S. Ananth Karumanchi,¹,³,⁴ Samir M. Parikh¹

¹Division of Nephrology/ Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA
²Division of Pulmonary, Critical Care, & Sleep Medicine/ Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA
³Department of Obstetrics and Gynecology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA
⁴Howard Hughes Medical Institute, Boston, MA, USA

*Corresponding Author:
Manish R. Maski, MD
Email: mmaski@bidmc.harvard.edu (MRM)
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/1.73 m² 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.

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). 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).

Table 1. Patient Characteristics

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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

*Significant at P<0.05
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).

**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 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).
Fig. 1. Sleep Apnea Severity between Control Subjects and Affected Patients
Panel 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. Panel 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.

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).

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.

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).

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.
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)

Figure 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

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 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 was 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 effect of OSA physiology on urinary NGAL levels. Lastly, we found no correlation between urinary NGAL/Cr and the severity of OSA as indicated by the AHI-4%.

Our data point away from renal tubular epithelial cell injury as a primary mechanism of the initiation of kidney injury in OSA. We speculate that the kidney injury associated with OSA is instead primarily glomerular 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].

Some 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 points away from kidney tubular injury as the etiology of OSA’s independent association with the initiation and progression of CKD. Further investigation is needed to clarify the mechanisms by which OSA may directly injure the kidneys.

Acknowledgments

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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.

**S1 Table. NGAL/Cr Ratios by Gender and OSA Status**

<table>
<thead>
<tr>
<th>Gender (N)</th>
<th>Mean NGAL/Cr (ng/mg)</th>
<th>Median NGAL/Cr (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Men (26)</td>
<td>8.41 (+/-13.73)</td>
<td>4.78 (2.41, 8.57)</td>
</tr>
<tr>
<td>OSA Men (20)</td>
<td>9.39 (+/-15.56)</td>
<td>4.49 (2.01, 9.10)</td>
</tr>
<tr>
<td>Control Men (6)</td>
<td>5.14 (+/-2.56)</td>
<td>5.31 (2.71, 7.25)</td>
</tr>
<tr>
<td>All Women (23)</td>
<td>13.77 (+/-14.73)</td>
<td>8.64 (5.79, 17.02)</td>
</tr>
<tr>
<td>OSA Women (13)</td>
<td>10.98 (+/-9.74)</td>
<td>8.49 (5.05, 11.46)</td>
</tr>
<tr>
<td>Control Women (10)</td>
<td>17.39 (+/-19.44)</td>
<td>9.47 (5.71, 23.44)</td>
</tr>
<tr>
<td>All Subjects (49)</td>
<td>10.93 (+/-14.31)</td>
<td>6.34 (3.21, 10.42)</td>
</tr>
</tbody>
</table>

Table shows mean (+/- standard deviation), median (25%ile, 75%ile), or N=number of patients falling into category.
Figure 1A: Apnea-Hypopnea Index (>=4% Oxygen Desaturation) by Group

- Control Subjects
- Sleep Apnea Patients

AHI-4% (events/hr) vs Group

p < 0.0001
B. Lowest Overnight Oxygen Saturation by Group

\[ p < 0.0001 \]
A. Control Subjects: Mean Arterial Pressure vs. Body Mass Index

![Graph showing the relationship between Mean Arterial Pressure (mmHg) and Body Mass Index (kg/m²). The correlation coefficient (r) is 0.5517 and the p-value is 0.0330.](Fig2A.tiff)
B. Sleep Apnea Patients: Mean Arterial Pressure vs. Body Mass Index

\[ r = 0.4532, \quad p = 0.0092 \]
Urine NGAL/Creatinine Ratio by Group

p = 0.4148

Controls

OSA Patients

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

\[ r = -0.1064 \]
\[ p = 0.4669 \]
Urinary NGAL/Creatinine Ratio Pre- and Post-Treatment

\[ p = 0.7758 \]
Uncorrected Urine NGAL Levels by Group

Urine NGAL (ng/ml)

Controls

OSA Patients

p=0.5226
Disclaimer: The manuscript and its contents are confidential, intended for journal review purposes only, and not to be further disclosed.

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Author(s): S. Ananth Karumanchi, Beth Israel Deaconess Medical Center
Arvind Goel, Beth Israel Deaconess Medical Center
Manish Maski, Beth Israel Deaconess Medical Center
Surichhya Bajracharya, Beth Israel Deaconess Medical Center
Julia Wenger, Massachusetts General Hospital
Dong Zhang, Beth Israel Deaconess Medical Center
Saira salahuddin, Beth Israel Deaconess Medical Center
Sajid Shahul, Beth Israel Deaconess Medical Center
Ravi Thadhani, Harvard Medical School
Ellen Seely, Brigham and Women's Hospital
Epidemiology and Mechanisms of *De Novo* Hypertension in the Postpartum Period

*Arvind Goel,¹ Manish R. Maski,¹ Surichhya Bajracharya,² Julia B. Wenger,³ Dongsheng Zhang,¹ Saira Salahuddin,¹,² Sajid S Shahul,⁴ Ravi Thadhani,³ Ellen W Seely,⁵ S. Ananth Karumanchi,¹,²,⁶ Sarosh Rana²,⁷*

¹Division of Nephrology/Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA
²Division of Maternal-Fetal Medicine/Department of Obstetrics and Gynecology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA
³Division of Nephrology/Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA,
⁴Division of Critical Care/Department of Anesthesiology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA
⁵Division of Endocrinology, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA
⁶Howard Hughes Medical Institute, Boston, MA
⁷Section of Maternal-Fetal Medicine/Department of Obstetrics and Gynecology, University of Chicago, Chicago, IL

**Corresponding Author:** S. Ananth Karumanchi, MD - sananth@bidmc.harvard.edu OR Sarosh Rana, MD - srana2@bsd.uchicago.edu
ABSTRACT

Background

Hypertensive disorders of pregnancy cause significant maternal morbidity and mortality worldwide during pregnancy and in the immediate postpartum period. While antepartum hypertensive disorders have been extensively studied, only limited data exist regarding the epidemiology of postpartum hypertension, and even less is known about its possible pathophysiology.

Methods

We prospectively studied 774 consecutive normotensive women admitted to a single tertiary medical center over an 18-month period for cesarean section of a singleton pregnancy. We collected detailed demographic, clinical, and laboratory data on all women prior to delivery and during the immediate postpartum period. Anti-angiogenic soluble fms-like tyrosine kinase 1 (sFlt1) and the proangiogenic placental growth factor (PIGF), both biomarkers associated with preeclampsia, were measured on antepartum blood samples. Postpartum hypertension was defined as new onset of hypertension, as defined by any single systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg, at least 48 hours after delivery and up to 6 weeks postpartum. We then performed univariate and multivariable analyses to determine factors associated with the risk of developing postpartum hypertension.

Results

Of the 774 women normotensive prior to delivery, 77 (9.9%) developed de novo postpartum hypertension. A higher body mass index at the time of delivery, African-American race and history of diabetes mellitus were associated with development of postpartum hypertension. The antepartum sFlt1/PIGF ratio positively correlated with both
the highest postpartum systolic (r=0.17; p<0.001) and diastolic (r=0.18; p<0.001) blood pressures. Moreover, the highest tertile of the antepartum sFlt1/PIGF ratio was independently associated with de novo postpartum hypertension [OR: 2.75 (1.41, 5.39), P=0.003] in multivariable analysis. Women developing postpartum hypertension had longer hospitalization than those who remained normotensive (days admitted: 6.2 ± 3.9 versus 5.6 ± 3.6 days; p=0.048).

**Conclusions**

*De novo* hypertension in the postpartum period is relatively common and is associated with prolonged hospitalization. Women with postpartum hypertension share similar clinical risk factors for preeclampsia, as well as a similar antepartum plasma angiogenic profile as found in women with preeclampsia. These data suggest that *de novo* postpartum hypertension may represent a group of women with subclinical preeclampsia who do not manifest hypertension until postpartum.
INTRODUCTION

The hypertensive disorders of pregnancy (HDP) are a well-recognized cause of maternal morbidity and mortality worldwide. The World Health Organization, in a systematic review of global data, recently identified HDP as a dominant cause of maternal death in developing countries--accounting for approximately 18% of all maternal deaths worldwide. Even among western countries, characterized by very low rates of maternal death in the peri- and postpartum periods, HDP remain significantly associated with the future development of maternal hypertension (HTN), ischemic heart disease, and stroke.

Development of de novo hypertension in the immediate postpartum period (postpartum hypertension; PPHTN) is an infrequently studied clinical syndrome, that is usually defined as new onset of hypertension appearing post-delivery through 6 weeks postpartum. De novo PPHTN following a normotensive pregnancy may herald the development of seizures (postpartum eclampsia), which is associated with significant morbidity. A recent large retrospective study indeed found that 63.2% of patients readmitted with delayed postpartum preeclampsia had no antecedent diagnosis of a HDP. Furthermore, PPHTN increases the risk of intracerebral hemorrhage, which is compounded by generally decreased medical surveillance in the postpartum period after hospital discharge.

Despite the potential danger that PPHTN poses to women, only limited data exist in the literature describing its incidence and risk factors. This is again partly due to infrequent medical visits for most women during the immediate post-partum period, often not first occurring until 6 weeks after delivery. Furthermore, the often asymptomatic nature of PPHTN limits capture of these patients for study. Most data in the literature are
retrospective in nature and usually represent only the most severe cases, such as patients readmitted with hypertensive crisis or seizures/eclampsia. Even less is known regarding the possible underlying pathobiology of PPHTN. While iatrogenic causes such as excess intravenous fluid administration and certain medications (eg. non-steroidal anti-inflammatory drugs, ergot alkaloids, etc.) have been associated with PPHTN, very little is known about the pathophysiology of PPHTN.

We therefore prospectively studied ~1000 women with singleton pregnancies for antepartum risk factors that may lead to the development of de novo PPHTN in the immediate postpartum period. In addition to collecting detailed antepartum demographic, clinical, and laboratory data, we measured the antepartum circulating levels of angiogenic factors--the antiangiogenic soluble fms-like tyrosine kinase 1 (sFlt1) and the proangiogenic placental growth factor (PlGF)--that have been linked to the pathogenesis of preeclampsia.

METHODS

Study Design and Oversight

This prospective, observational study was performed at Beth Israel Deaconess Medical Center (Boston, MA) in accordance with all institutional policies and with approval of the hospital’s institutional review board.

Study Participants and Clinical Data

All women (N=988) admitted for cesarean section (C-section) of a singleton pregnancy during the period from October, 2012 through March, 2014 at BIDMC were eligible for the study. 214 subjects were excluded from this study because their pregnancies were complicated by hypertension during the antepartum period due to preeclampsia, chronic
hypertension, or transient hypertension during pregnancy. The remaining 774 subjects who were normotensive during pregnancy were included in this study. We recorded a number of antepartum characteristics such as maternal age, body mass index (BMI; kg/m²) upon admission, gestational age on admission, gestational age at delivery, parity, smoking status, race, history of any hypertensive disorder and/or diabetes in a previous pregnancy, highest systolic and diastolic blood pressures during the antepartum period, and laboratory values such as alanine aminotransferase (ALT) level, serum creatinine and uric acid levels, and platelet count. Each patient’s clinical course was subsequently followed, with collection of additional clinical data such as infant birth weight, maternal estimated blood loss (EBL) during delivery, sequential postpartum systolic and diastolic blood pressures, and intraoperative plus postpartum intravenous fluid therapy. The length of hospital admission, number of patients requiring readmission, and number of patients requiring antihypertensive medication were also recorded for all participants. De novo PPHTN was defined as the new onset of hypertension, as defined by any systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg, at least 48 hours after delivery and up to 6 weeks postpartum.

**Measurement of Antepartum Circulating Angiogenic Proteins**

Discarded venous EDTA blood samples collected prior to C-section (between 12 to 96 hours before C-section), were collected from the hospital laboratory, plasma aliquoted, and stored at -70°C. Blood samples were missing in 5 subjects. A single operator performed quantitative sandwich enzyme-linked immunosorbent assay (ELISA) for both sFlt1 and PIGF biomarkers on these discarded plasma samples from the remaining subjects (N=769) in duplicate using commercially available ELISA kits (R&D Systems, Inc., Minneapolis, MN) as described elsewhere. The interassay coefficient for sFlt1 and PIGF were 7% and 11%, respectively. The operator was blinded to the clinical
information. The clinicians treating the patients did not have any knowledge of the biomarker levels as the assays were done in one batch after the outcomes had occurred.

**Statistical Analysis**

Antepartum, delivery, and postpartum characteristics of normotensive patients are presented overall and by postpartum hypertensive status as means ± standard deviations, medians (25th percentile, 75th percentile), or numbers and percentages. Characteristics were compared between postpartum groups using independent samples t-tests or chi-square tests, as appropriate. Angiogenic factors were natural log transformed to meet the assumptions of parametric testing. Pearson’s correlation coefficients were used to determine the associations between natural log-transformed antepartum angiogenic factors and highest recorded antepartum and postpartum systolic blood pressure (SBP) and diastolic blood pressure (DBP). Separate univariate and multivariable logistic regression models were utilized to predict risk of PPHTN by the overall tertile of each angiogenic factor among normotensive patients. Adjustment was made for variables significant in univariate models as well as clinically known confounders: (1) gestational age, (2) body mass index, (3) nulliparous, (4) race, (5) history of chronic hypertension, and (6) history of diabetes. Odds ratios and 95% confidence intervals were used to summarize the results of logistic regression models.

The average days of hospitalization were compared between normotensive and hypertensive groups using independent samples t-tests (natural log transformed average days of hospitalization) and chi-square tests. Statistical analyses were conducted with the use of SAS software, version 9.4 (SAS Institute). Two-tailed P values of less than 0.05 were considered to indicate statistical significance.

**Results**
Patient Characteristics Associated with PPHTN

Among 774 normotensive pregnancies, 9.9% (n=77) of these women went on to develop \textit{de novo} PPHTN (Table 1). African-American women (20.8\% vs. 7.0\%), women with a higher BMI at delivery (mean ± SD: 34.1 ± 7.3 vs. 30.0 ± 5.2 kg/m$^2$), and women with a history of diabetes mellitus (13.0\% vs. 3.9\%) demonstrated increased risk of developing \textit{de novo} PPHTN as compared to normotensive women (all $P<0.001$). The highest recorded antepartum SBP and DBP, while still in the normotensive range, were significantly higher in those women developing \textit{de novo} PPHTN (SBP 127 ± 8 versus 119 ± 11; DBP 78 ± 7 versus 74 ± 8 [all $p<0.001$]; Table 1). There was no difference in intraoperative and postoperative intravenous fluid volumes administered between groups. More than 90\% of all patients received a nonsteroidal medication, and there was no difference in doses between the two groups.

Angiogenic Factors and Prediction of De Novo PPHTN

Women who developed \textit{de novo} PPHTN had significantly higher antepartum sFlt1 levels as compared to women who remained normotensive postpartum (median $[25^{th}$-$75^{th}$]: 10189 pg/ml [5655-16650] versus 7721 pg/ml [5088-12199]) and a higher sFlt1/PlGF ratio (52.1 [22.0, 84.6] versus 33.1 [14.1, 65.9]) (all $p<0.05$; Table 1).

Those in the highest tertile of antepartum circulating sFlt1 concentration [2.38 (1.32, 4.28); $p=0.004$], PlGF concentration [0.38 (0.20, 0.72); $p=0.003$], and sFlt1/PlGF ratio [2.80 (1.50, 5.23); $p=0.001$] were at a significantly increased risk of developing \textit{de novo} PPHTN in univariate analyses, and these effects persisted after multivariable adjustment: sFlt1: [2.80 (1.45, 5.39); $p=0.002$]; PlGF: [0.43 (0.22, 0.84); $p=0.01$]; and sFlt1/PlGF ratio: [2.75 (1.41, 5.39); $p=0.003$] (Table 2).
The antepartum ln (sFlt1) positively correlated with highest postpartum systolic (r=0.15; p<0.001) and diastolic (r=0.16; p<0.001) blood pressures, the ln (PIGF) negatively correlated with highest post-partum systolic (r=-0.13; p<0.001) and diastolic (r=-0.14; p<0.001) blood pressures, and ln (sFlt1/PIGF ratio) positively correlated with highest post-partum systolic (r=0.17; p<0.001) and diastolic (r=0.18; p<0.001) blood pressures. The predictive risk of de novo PPHTN increased with rising levels of sFlt1/PIGF ratio among all women (Figure 1).

Women developing de novo PPHTN also experienced a significantly longer hospitalization than those who did not (days admitted: 6.2 ± 3.9 versus 5.6 ± 3.6 days; p=0.048).

DISCUSSION

In this large prospective clinical study at a single tertiary care hospital, we have demonstrated that the development of de novo PPHTN following normotensive pregnancies was common (9.9%). The clinical risk factors for the eventual appearance of de novo PPHTN strongly resembled those for preeclampsia. Moreover, the antepartum angiogenic factor levels central to the pathogenesis of preeclampsia - the antiangiogenic sFlt1 and the proangiogenic PIGF - followed the same pattern (higher sFlt1, lower PIGF, and higher sFlt1/PIGF ratio) and independently predicted the development of de novo postpartum hypertension. Among all affected patients in our study, the highest tertile of the sFlt1/PIGF ratio increased the odds of developing de novo PPHTN in univariate and multivariate analyses. Finally, women developing PPHTN had longer hospitalization.
Limited data describing the overall incidence and risk factors for PPHTN exist in the literature. Most studies have been retrospective or have been of small cohort size. To our knowledge, this current work represents one of the largest prospective study designed to specifically study PPHTN, within the literature. Our finding that the development of de novo PPHTN was common with an incidence of nearly 10% in our cohort—was quite notable. Considering the associated maternal morbidity (and potential mortality) of unrecognized (and therefore untreated) de novo PPHTN, this higher-than-anticipated frequency suggests that the initial postpartum medical visit may need to occur sooner than is typically performed, particularly for those women with risk factors. From our data, these high risk features appear to be identical to many of those of preeclampsia: elevated BMI, African-American race, history of diabetes mellitus, and antepartum blood pressure elevation in the “prehypertensive” range.

Indeed, evaluation of antepartum circulating angiogenic factors revealed an identical pattern to that observed in preeclampsia, in these women. These data therefore suggest that development of de novo PPHTN may actually represent a group of women with subclinical preeclampsia that manifests as hypertension postpartum. Our findings also extend the biology of these factors - sFlt1 and PlGF - beyond preeclampsia and into another hypertensive disorder of pregnancy. Antepartum measurement of these angiogenic factors, in combination with a woman’s other clinical risk factors for PPHTN, may better enable clinicians in the future to determine which patients require closer surveillance after delivery, and closer follow up after hospital discharge.

Some limitations of our study require mention. First, our cohort was limited to women undergoing C-section only, which may introduce bias and limit the generalizability of our
findings. However, this study design was chosen to increase the time period over which we were able to collect data on postpartum blood pressure measurements, since women undergoing C-section are typically hospitalized longer than those delivering vaginally. Also, all consecutive singleton pregnancies undergoing C-section were included in our study—regardless of the indication for C-section—to minimize this potential bias. Furthermore, we would have preferred to have access to longitudinal blood pressure measurements beyond one week post-delivery in all women, in order to determine the duration of postpartum hypertension among the affected women in our cohort. However, ambulatory blood pressure monitoring is not routinely used to monitor for postpartum eclampsia, and hence this data was not available. Furthermore, data were available only for a limited number of patients at 6 weeks follow-up in the outpatient clinic, as only a fraction of our cohort delivering at our center had their postpartum care there also. Finally, in our study we did not find significant overall morbidity other than a modest increase in the duration of hospitalization. We hypothesize that the majority of the morbidity in the postpartum period may occur in subjects who also had an antepartum hypertensive disorder; however, this was not evaluated, as our primary focus in this initial study was to study the pathogenesis of de novo hypertension among normotensive pregnancies. Future studies should determine if the antepartum angiogenic profile among high risk patients correlates with postpartum morbidity, such as postpartum eclampsia or postpartum HELLP syndrome. It would also be important to evaluate whether women with de novo PPHTN are at risk for the development of cardiovascular disease in the long term.

In conclusion, we prospectively identified the major clinical risk factors for the development of de novo PPHTN—and for the first time, implicated plasma levels of the angiogenic factors sFlt1 and PlGF in the pathogenesis of this disorder. Moreover, our
description of the striking clinical similarities between the factors predicting the development of de novo PPHTN and those predicting preeclampsia, coupled with the similarity of the angiogenic profile which precedes de novo PPHTN and also accompanies preeclampsia, suggest that the development of de novo PPHTN may in fact represent subclinical preeclampsia.

Declaration of Interest

All other authors report no conflict. R. T. and S. A. K are co-inventors on patents related to preeclampsia biomarkers that are held at Massachusetts General Hospital and Beth Israel Deaconess Medical Center. R.T and S.A.K. have financial interest in Aggamin LLC. R.T. reports serving as a consultant to Roche Diagnostics. S.A.K reports serving as a consultant to Siemens Diagnostics. All other authors report no conflict.

Funding

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Table 1. Clinical and Laboratory Characteristics of Initially Normotensive Group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All NL (N=774)</th>
<th>NL/NL (N=697)</th>
<th>NL/PPHTN (N=77)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All prior to delivery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational Age (weeks)</td>
<td>39 ± 2</td>
<td>39 ± 2</td>
<td>39 ± 3</td>
<td>0.70</td>
</tr>
<tr>
<td>Age (years)</td>
<td>33 ± 5</td>
<td>33 ± 5</td>
<td>32 ± 6</td>
<td>0.12</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>30.4 ± 5.6</td>
<td>30.0 ± 5.2</td>
<td>34.1 ± 7.3</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Nulliparous</td>
<td>337 (43.5)</td>
<td>303 (43.5)</td>
<td>34 (44.2)</td>
<td>0.91</td>
</tr>
<tr>
<td>Smoker</td>
<td>14 (1.8)</td>
<td>12 (1.7)</td>
<td>2 (2.6)</td>
<td>0.57</td>
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<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>White/Caucasian</td>
<td>426 (55.0)</td>
<td>383 (55.0)</td>
<td>43 (55.8)</td>
<td></td>
</tr>
<tr>
<td>Black/African American</td>
<td>65 (8.4)</td>
<td>49 (7.0)</td>
<td>16 (20.8)</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>119 (15.4)</td>
<td>113 (16.2)</td>
<td>6 (7.8)</td>
<td></td>
</tr>
<tr>
<td>History of hypertension</td>
<td>13 (1.7)</td>
<td>11 (1.6)</td>
<td>2 (2.6)</td>
<td>0.51</td>
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<tr>
<td>History of Diabetes</td>
<td>37 (4.8)</td>
<td>27 (3.9)</td>
<td>10 (13.0)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>IVF this Gestation</td>
<td>36 (4.7)</td>
<td>31 (4.5)</td>
<td>5 (6.5)</td>
<td>0.42</td>
</tr>
<tr>
<td>Antepartum Characteristics</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Highest SBP (mmHg)</td>
<td>120 ± 11</td>
<td>119 ± 11</td>
<td>127 ± 8</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Highest DBP (mmHg)</td>
<td>74 ± 8</td>
<td>74 ± 8</td>
<td>78 ± 7</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>ALT (U/L)†</td>
<td>18.9 ± 15.1</td>
<td>21.2 ± 18.3</td>
<td>15.0 ± 5.7</td>
<td>0.15</td>
</tr>
<tr>
<td>Creatinine (mg/dL)†</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.97</td>
</tr>
<tr>
<td>Uric Acid (mg/dL)†</td>
<td>4.6 ± 0.9</td>
<td>4.8 ± 0.9</td>
<td>4.5 ± 0.8</td>
<td>0.31</td>
</tr>
<tr>
<td>Platelet Count (K/uL)</td>
<td>218 ± 57</td>
<td>218 ± 56</td>
<td>224 ± 64</td>
<td>0.33</td>
</tr>
<tr>
<td>sFlt1 (pg/ml)‡</td>
<td>7895 (5117, 12619)</td>
<td>7721 (5088, 12199)</td>
<td>10189 (5655, 16650)</td>
<td>0.004*</td>
</tr>
<tr>
<td>PIGF (pg/ml)‡</td>
<td>243 (159, 399)</td>
<td>249 (162, 409)</td>
<td>214 (139, 295)</td>
<td>0.06</td>
</tr>
<tr>
<td>sFlt1/PIGF Ratio‡</td>
<td>34.2 (15.0, 68.0)</td>
<td>33.1 (14.1, 65.9)</td>
<td>52.1 (22.0, 84.6)</td>
<td>0.002*</td>
</tr>
<tr>
<td>Delivery Characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>3355 ± 659</td>
<td>3348 ± 642</td>
<td>3416 ± 793</td>
<td>0.48</td>
</tr>
<tr>
<td>Postpartum Characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estimated Blood Loss (ml)</td>
<td>787 ± 215</td>
<td>787 ± 217</td>
<td>788 ± 188</td>
<td>0.99</td>
</tr>
<tr>
<td>Total IV Fluids‡</td>
<td>2027 ± 674</td>
<td>2036 ± 681</td>
<td>1942 ± 610</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Table shows mean ± SD, median (25th %ile, 75th %ile) or n (%)

NL=normal, PPHTN=postpartum hypertension, NL/NL= Systolic blood pressure (SBP) <140 and diastolic blood pressure (DBP) <90 prior to delivery and SBP<140 and DBP<90 at 48 hours-6 weeks postpartum after C-section, NL/PPHTN= SBP<140 and DBP<90 prior to delivery and SBP≥140 and/or DBP≥90 at 48 hours-6 weeks postpartum after C-section, IVF=in vitro fertilization, ALT=alanine transaminase, sFlt1=soluble fms-like tyrosine kinase-1, PIGF=placenta growth factor, IV=intravenous

†ALT = 35 subjects, creatinine = 40 subjects, uric acid = 28 subjects; ‡N = 769 subjects for sFlt1, PIGF and sFlt1/PIGF assays

*Significant at P<0.05
### Table 2. Increasing Risk of Postpartum Hypertension with Increasing Level of sFlt1/PIGF Ratio among Women who were Initially Normotensive.

<table>
<thead>
<tr>
<th>Tertile 1</th>
<th>PPHTN N (%)</th>
<th>Odds Ratio (95% CI)</th>
<th>P-value</th>
<th>Tertile 2</th>
<th>PPHTN N (%)</th>
<th>Odds Ratio (95% CI)</th>
<th>P-value</th>
<th>Tertile 3</th>
<th>PPHTN N (%)</th>
<th>Odds Ratio (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univariate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sFlt1</td>
<td>18 (7.0)</td>
<td>1.0 Ref</td>
<td>0.94 (0.47, 1.86)</td>
<td>0.85</td>
<td>38 (14.8)</td>
<td>2.80 (1.50, 5.23)</td>
<td>0.003*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIGF</td>
<td>34 (13.2)</td>
<td>1.0 Ref</td>
<td>0.75 (0.43, 1.28)</td>
<td>0.29</td>
<td>14 (5.5)</td>
<td>0.38 (0.20, 0.72)</td>
<td>0.003*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sFlt1/PIGF Ratio</td>
<td>15 (5.9)</td>
<td>1.0 Ref</td>
<td>1.43 (0.72, 2.84)</td>
<td>0.31</td>
<td>38 (14.8)</td>
<td>2.80 (1.50, 5.23)</td>
<td>0.001*</td>
<td></td>
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</tr>
<tr>
<td>Multivariable†</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sFlt1</td>
<td>18 (7.0)</td>
<td>1.0 Ref</td>
<td>1.12 (0.54, 2.33)</td>
<td>0.76</td>
<td>39 (15.2)</td>
<td>2.80 (1.45, 5.39)</td>
<td>0.002*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIGF</td>
<td>34 (13.2)</td>
<td>1.0 Ref</td>
<td>0.85 (0.48, 1.50)</td>
<td>0.57</td>
<td>14 (5.5)</td>
<td>0.43 (0.22, 0.84)</td>
<td>0.01*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sFlt1/PIGF Ratio</td>
<td>15 (5.9)</td>
<td>1.0 Ref</td>
<td>1.53 (0.74, 3.15)</td>
<td>0.25</td>
<td>38 (14.8)</td>
<td>2.75 (1.41, 5.39)</td>
<td>0.003*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PPHTN=postpartum hypertension, CI=confidence interval, sFlt1=soluble fms-like tyrosine kinase-1, PIGF=placenta growth factor
*Significant at P<0.05
†Adjusted for gestational age, age, body mass index, nulliparous, race, history of chronic hypertension, and history of diabetes. sFlt1, PIGF, and sFlt1/PIGF ratio were analyzed in separate models.
Figure 1. sFlt1/PlGF Ratio and Predictive Risk of Postpartum Hypertension. The cumulative predicted risk of postpartum hypertension at different levels of natural log transformed sFlt-1/PIGF ratio. The sample sizes shown below the figure represent the number of patients at risk for each 2 unit increment of ln sFlt-1/PIGF ratio.
The Vasculature in Diabetic Nephropathy: All Tied Up?

Manish R. Maski and Samir M. Parikh
Division of Nephrology and Center for Vascular Biology Research, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts


Diabetic nephropathy (DN) is the leading cause of ESRD in the United States, and its global occurrence is rising rapidly. As evidenced by the wide spectrum of debilitating macro- and microvascular complications that patients eventually experience, the vascular endothelium is a prominent target of longstanding diabetes. In the hunt for breakthrough discoveries that could someday transform the treatment of DN, signaling pathways that regulate the form and functions of blood vessels have recently garnered significant attention. In this issue of JASN, Dessapt-Baradez et al. have deployed a combination of conditionally transgenic mice and human DN biopsy studies to advance the proposition that therapies targeting the vasculature may ameliorate DN.1

In 2001, the National Institutes of Health initiated the Animal Models of Diabetic Complications Consortium to tackle the well known resistance of inbred mouse strains to DN and other diabetic manifestations, a limitation that had severely impeded investigation of the molecular pathogenesis underlying progressive diabetes. The field of vascular research in DN received a substantial boost in 2006 and 2007 when two groups reported in JASN that diabetic mice null for endothelial nitric oxide synthase (eNOS−/−) recapitulated major structural and functional features of advanced human DN.2,3 The enzyme eNOS is highly specifically expressed in the endothelium and is responsible for producing the vasodilator nitric oxide, which is thought to contribute to vascular homeostasis. Loss of eNOS-derived nitric oxide activity has been observed in diabetic patients well before the onset of severe end-organ complications (reviewed by De Vriese et al.4).

Although the regulation of eNOS is complex,5 two important growth factors upstream of eNOS have been implicated in DN—vascular endothelial growth factor-A (VEGF-A) and, more recently, angiopoietins-1 and -2 (Angpt-1 and -2). VEGF-A and Angpt-1 are constantly secreted by healthy podocytes and signal distinct receptors expressed on the surface of endothelial cells, VEGFR1/2 and Tie-2, respectively. As a potentially useful oversimplification, VEGF-A signals through VEGFR2 to induce angiogenesis and to attenuate barrier function, the latter activity accounting for VEGF’s original name of “vascular permeability factor.”6 Angpt-1 activates Tie-2 to stabilize newly sprouted vessels, and, importantly, it prevents vascular leakage and enhances basal microvascular barrier function.7–9 Systemic administration of the anti-VEGF antibody bevacizumab can produce glomerular endotheliosis, proteinuria, and thrombotic microangiopathy, indicating an essential role for VEGF in the maintenance of glomerular architecture and health.10 No comparable clinical data regarding Angpt-1 inhibition exist because drugs targeting this pathway are investigational. But unlike with VEGF-A, conditional knockout mice suggest that Angpt-1 is dispensable in the mature glomerulus.11

The available data for VEGF-A in DN appear to be conflicting. For example, VEGF-A has been reported to be upregulated12 or downregulated13 in human DN biopsies. Deletion of VEGF from podocytes has been shown to exacerbate DN in the streptozotocin (STZ) model of type 1 diabetes by Sivaskandarajah et al.,14 whereas Veron et al.15 reported that inducible overexpression of VEGF in the podocyte causes severe nodular glomerulosclerosis in the STZ model. Similar to the results of Veron et al., the Giuni laboratory showed that podocyte overexpression of a naturally occurring VEGF inhibitor called sFlt-1 improves DN.16 An attempt to synthesize the VEGF literature in DN is beyond the current scope of this editorial, but in contrast, the current report from Dessapt-Baradez et al. adds to two prior independent experimental studies that collectively demonstrate a renoprotective role for Angpt-1 in DN.

In 2007, a group led by Park used a systemic viral gene therapy approach to achieve excess circulating Angpt-1 for 8 weeks starting during young adulthood in db/db type 2 diabetic mice.17 They found that renal levels of inflammatory adhesion proteins and profibrotic signaling molecules were reduced by Angpt-1 treatment. Urinary albumin excretion was reduced from approximately 150 μg/d to approximately 100 μg/d, and histopathologic changes, namely mesangial matrix expansion and glomerular basement membrane thickness, were similarly reduced by Angpt-1. The study was somewhat confounded because the Angpt-1 group also exhibited less severe elevation of fasting blood glucose levels and less visceral adiposity. In 2011, the Quaggin laboratory genetically deleted Angpt-1 at the end of in utero development and administered STZ 1–3 weeks after weaning.11 Unlike Lee et al. they observed no Angpt-1-dependent effect.
on glucose metabolism, as assessed by the percentage glycosylated hemoglobin. Twenty weeks after STZ administration, diabetic Angpt-1 knockouts had a urine albumin-to-creatinine ratio of 0.25 compared with 0.06 in wild-type diabetic controls. Although not scored, mesangial matrix expansion was much more prominent in the diabetic Angpt-1 knockouts than wild-type diabetic controls.

In the current report, the group led by Gnudi used a gain-of-function system (as did Park et al.), and they studied type 1 diabetes as modeled by STZ (as did Quaggin et al.). They overexpressed Angpt-1 in the podocyte beginning shortly after the induction of hyperglycemia, and they studied outcomes after 10 weeks of excess podocyte-expressed Angpt-1. They noted that excess Angpt-1 did not alter baseline renal physiology or structure. They showed that expression of the Angpt-1 receptor, Tie-2, falls in experimental diabetes, and that their “therapy” of locally expressed Angpt-1 enhances Tie-2 activation. Diabetic Angpt-1 transgenic mice had urinary albumin of 508 μg/d compared with 2101 μg/d in diabetic wild-type controls. Unlike in the prior reports, the authors did not observe any appreciable rescue of mesangial matrix expansion or of GBM thickening by Angpt-1, leaving the physical mechanisms by which diabetic proteinuria improved downstream of Angpt-1 unclear. Finally, using quantitative PCR to compare isolated glomeruli from 12 subjects with DN and 32 living donor controls, they found that DN was associated with induction of the endogenous antagonist of Tie-2, Angpt-2.18

To summarize, experiments from three independent groups that added Angpt-1 to the diabetic milieu or genetically removed it suggest that Angpt-1 confers renoprotection in experimental diabetes. Because the basis of proteinuria in DN is incompletely understood, there is no consensus on how Angpt-1 is attenuating renal damage in diabetes. To wit, Dessapt-Baradez et al. examined glomerular cell proliferation, VEGFR2 phosphorylation, eNOS activation, and nephrin expression in their model. Cross-talk between the VEGF and Angpt-1 signaling axes19,20 and, indeed, the larger network of pathways among endothelia and vascular smooth muscle cells may confound efforts to implicate a single linear cascade. It also remains unclear whether Angpt-1 actually “heals” DN because the interventional studies in mice have commenced at an early, preproteincuria stage of diabetes.

Because only a minority of patients with diabetes develop DN, it will be interesting to test whether polymorphisms in genes comprising vascular pathways or protein levels in the blood or urine can help identify at-risk patients. Conversely, long-term patients with diabetes who do not develop complications may possess a unique vascular-protective profile. If diabetes is fundamentally a metabolic disturbance, we should ask what triggers the dysregulation of vascular pathways21 and why there is a decades-long delay from hyperglycemia to overt complications. Finally, more studies are needed to explore how blood vessel destabilization may contribute to the final common pathway of fibrosis in diabetes as well as other forms of CKD. The study from Dessapt-Baradez et al. provides new evidence implicating Angpt-1 as a renoprotective factor in DN and, more broadly, reminds us that pathogenic molecular events with persistent functional consequences may be unfolding months to years before standard measures of chronic disease are manifest.

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DISCLOSURES

None.

REFERENCES

Antifibrotic Therapy: Is an Antioxidative Regimen the Answer?

Frank Strutz

Department of Nephrology, Stiftung Deutsche Klinik für Diagnostik, Wiesbaden, Germany


CKD is a major burden for the individual patient as well as for society in general. Whereas only 2% of all patients with CKD eventually reach the stage of dialysis dependency, cardiovascular risk rises steadily with the decline in kidney function, resulting in a robust increase in morbidity and mortality. Morphologically, chronic renal failure is most often characterized by glomerulosclerosis, tubular atrophy, and tubulointerstitial fibrosis, the latter often being the most prominent feature.1 Tubulointerstitial fibrosis consists of proliferation and activation of various cells to so-called myofibroblasts and synthesis of extracellular matrix by these cells. A long-standing controversy surrounds which cell type is the main precursor of these matrix-producing cells, including resident fibroblasts, fibrocytes, pericytes, endothelial cells, and epithelial cells. However, the individual contribution of these various cell types to myofibroblast formation may vary according to the method and model examined, pointing to a certain heterogeneity of these cells.2

However, once matrix-synthesizing myofibroblasts have been formed, renal interstitial fibrosis represents a final common pathway for a plethora of CKDs. Thus, renal interstitial fibrosis is a worthwhile target because antifibrotic therapy would make almost all patients with CKD suitable candidates for therapy. Moreover, antifibrotic therapy may also be of clinical importance in other organs, such as the lung and the liver. Initial antifibrotic strategies focused mainly on the neutralization of profibrotic cytokines or the application of antifibrotic cytokines.3 However, in recent years the focus of antifibrotic therapy has shifted to antioxidant therapy. Although oxidation was thought to be of primary importance for inflammatory processes, recent evidence has shown that it is also critical for organ fibrosis in general and the liver and the kidney in particular.4 Many of these potential therapeutical agents have been tested experimentally and clinically in diabetic kidney disease.5 One of the more interesting substances in this regard is pirfenidone, which has antifibrotic, anti-inflammatory, and antioxidant properties. The drug was used successfully to treat many fibrotic disorders, not only in the kidney but also in the lung and the liver. Furthermore, the drug has already been approved for use in patients with idiopathic pulmonary fibrosis; it is in clinical use in Europe but not the United States (the Food and Drug Administration has withdrawn approval in the United States). However, one clinical trial in patients with diabetic nephropathy gave only mixed results,6 whereas a second trial in patients with FSGS is still ongoing.7 Another interesting antioxidant and again antifibrotic agent is tranilast. This synthetic compound attenuates the induction of thioredoxin-interacting protein and oxidative stress. It is used as an antifibrotic agent in Southeast Asia for the treatment of keloid formation. In addition, the drug has been evaluated experimentally8 as well as clinically in diabetic nephropathy,9 although clinical use was confined to only a few patients. Pentoxifylline is a methylxanthine phosphodiesterase inhibitor that has been evaluated in more than 20 studies, mostly in patients with diabetic nephropathy. Unfortunately, most of these studies included only a handful of individuals and were of short duration.10 There was a tendency toward decreased serum creatinine values but no significant effects on proteinuria, and the current evidence does not support the use of the drug in patients with diabetic kidney disease.5

Antioxidative Regimen the Answer?
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