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The effect of sympathetic activation on gut microbiota in obstructive sleep apnea

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**THE EFFECT OF SYMPATHETIC ACTIVATION ON GUT
MICROBIOTA IN OBSTRUCTIVE SLEEP APNEA**

by

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THESIS

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Abstract

Background: Patients with obstructive sleep apnea (OSA) suffer from increased mortality because of complications from metabolic and cardiovascular disease. Excess sympathetic nervous activity and catecholamine exposure contribute to the disease associations of OSA, but the underlying mechanism remains enigmatic. Because catecholamines cause overgrowth of bacteria in the class Enterobacteriaceae in the laboratory, this translational study proposed a role for altered gut microbiota in the complications of OSA.

Objectives: We tested the hypothesis that catecholamine excess in disordered sleep alters intestinal microbiota by comparing urinary catecholamines and the fecal microbiome of 24 patients with obstructive sleep apnea and 23 controls.

Results: Next-generation sequencing of the gut microbiome using the Illumina platform provided evidence for a trend toward altered community structure of gut microbiota in patients with sleep apnea. A positive linear relationship was seen in norepinephrine exposure and Enterobacteriaceae in patients with sleep apnea, but no such relationship occurred in controls.

Conclusions: These findings provide preliminary support for a central role of gut microbiota in the complications of sleep.

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Introduction

The function of sleep and its effects on health and disease are an unsolved problem in medicine. The importance of sleep is highlighted by the wide variety of diseases, including obesity, metabolic syndrome, diabetes, and heart disease and stroke, occurring more frequently in sleep disorders and with chronic sleep loss [1]. Sleep disruption is linked to insulin resistance, increased appetite, and weight gain [1,2]. Even short exposures to moderate sleep deprivation (5 hours sleep) caused a significant increase in weight gain as compared to normal sleep (9 hours) [3]. Circadian rhythm disruption, as occurs in night shift work, has been shown to increase the risk of obesity in women; this increased risk is amplified by the number of years spent working nights [4]. These studies demonstrate that sleep is not simply a phase of relative inactivity and rest, but is important in influencing the current epidemic of obesity and related morbidity.

Disordered sleep has been understood to influence risk for metabolic and cardiovascular disease because of increased sympathetic nervous activity with downstream effects on blood pressure, appetite and insulin sensitivity [1]. In addition to increased sympathetic activity, sleep disorders have additional effects on immune function that are important in the progression of many chronic diseases [1]. For instance, one week of sleep disruption resulted in increased numbers of circulating immune cells [5]. Sleep-deprived rats showed an increase in immature neutrophils, pro-inflammatory cytokines, and serum endotoxin, in a pattern of

immune activation that resembles closely systemic inflammatory response syndrome [6]

In a landmark paper, Everson and colleagues (2000) demonstrated that a few days of sleep deprivation in rats caused widespread infection and sepsis leading to death [7]. Bacteria translocating from the gut were identified as the source of fatal septicemia [7]. More recently, sleep deprivation, and severe stress more generally, has been shown to alter the community structure of the gut microbiota in laboratory animals [8]. Housing mice on a grid floor, which is stressful to mice, resulted in an altered microbiota and changes in metabolism [8]. In addition to causing changes in the community structure of gut microbiota, stress also reduces resistance to colonization and infection by gut pathogens [9]. Exposure to stress has been shown to result in increased density of gram-negative pathogens in the murine gut [10]. In addition, stress was shown to increase the luminal adherence of bacteria in mice, a step that precedes tissue invasion by bacterial pathogens [11]. Similarly, evidence suggests that severe stress results in the loss of beneficial commensal microbes in the human gut, favoring overgrowth and invasion by pathogens [12]. These results are in line with a wide variety of laboratory studies showing that disruption of the normal gut flora reduces resistance to infection; reviewed in: [13].

Because stress alters characteristics of microbiota and infection-risk in mammals it should not be surprising that stress also affects cytokines involved in innate immunity and infection fighting [10]. Bailey and colleagues (2011) showed that

monocyte chemoattractant protein-1 and interleukin-6 were increased after stress only when microbiota were present in the gut [10]. Together, these findings suggest that stress and sleep deprivation alter gut microbiota in animals with downstream consequences for inflammation and metabolism. However, the translational implication of these studies has been unclear. In particular, the effect of disordered sleep on the human microbiota had not been investigated prior to the present study. As outlined below, these various effects of stress and sleep on gut microbiota suggest a testable novel hypothesis to explain the health effects of disordered sleep in humans.

How sleep affects the immune system

Sleep duration and quality has profound effects on human health [1]. A variety of prospective studies have shown that sleep loss increases the risk for stroke, heart disease [14], type II diabetes [15], and all cause mortality [16,17]. Sleep disorders have been shown to influence metabolism and inflammation in humans by modulating oxidative stress and hormones involved in appetite regulation [18]. Circadian rhythm disruption also was shown to alter gene expression in human cells, particularly those involved in immune function and the stress response [19]. As a result of these pathways, sleep deprivation increases proinflammatory gene expression in innate immune cells. Sleep loss has wide-ranging effects on inflammation, including increased number of circulating granulocytes [20], activation of oxidative stress [21,22], and increased in TNF-alpha and other proinflammatory cytokines [23].

Altered metabolism and increased inflammation have also been observed in obstructive sleep apnea (OSA) [1], a disorder that occurs in up to 5% of men and 2% of women [24] and carries a doubled risk of all-cause mortality [25]. The inciting cause of OSA is upper airway obstruction resulting in interrupted ventilation and hypoxia during sleep. Hypoxia, in turn, results in nocturnal arousals that fragment the normal architecture of sleep. These arousals are accompanied by adrenal production of stress catecholamines, including norepinephrine, epinephrine and dopamine. Norepinephrine and other catecholamines peak in OSA during sleep but remain elevated even while awake [26]. Hypoxia and catecholamines are associated with pro-inflammatory gene expression, resulting in the production of inflammatory cytokines and biomarkers, such as C-reactive protein and interleukin 6 [22]. Chronic low-grade inflammation accompanies metabolic changes in OSA, resulting in insulin resistance and metabolic syndrome. These features contribute to the increased incidence of hypertension, stroke, atherosclerosis, and diabetic complications in patients with OSA [25].

Although the pathophysiological events outlined above are well documented in OSA and in sleep deprivation, the cascade of events leading to inflammation and metabolic disease remains poorly understood. In particular, it is unknown whether the gut microbiota drives the inflammatory burden of human sleep disorders.

Sleep deprivation and gut bacteria

Sleep deprivation can cause impairment of intestinal barrier function [7]. A few days of sleep deprivation in rats resulted in translocation of intestinal bacteria and

septicemia, culminating in fatal bloodstream infection [7]. Fragmented sleep also activated neutrophils and caused their migration to the visceral organs in rats [27]. Recent work using an animal model of colitis has shown that both acute and chronic sleep deprivation exacerbate colonic inflammation and delay recovery from colonic injury [28]. These studies suggest that sleep disturbances in mammals can exert powerful effects on gut inflammation in tandem with dangerous changes in intestinal bacteria.

Gut microbiota are an attractive suspect for involvement in the metabolic and inflammatory complications of disordered sleep because altered gut microbiota has been convincingly linked to obesity, metabolic syndrome, and intestinal inflammation in animals [29,30] and in humans [31,32]. The present translational study has the aim of testing whether a well-described phenomenon of laboratory animals, altered microbiota caused by stress and sleep disturbance, is also seen in human disorders of sleep.

The effect of catecholamines on the gut microbiota

Norepinephrine is a stress hormone that is increased in obstructive sleep apnea and decreased after treatment with CPAP [26,33]. Plasma and urinary norepinephrine is increased in patients with OSA [34] because of increased sympathetic nerve activity during the awake period and nocturnal surges of sympathetic activity following apneic events [26]. In OSA, plasma norepinephrine is more elevated than other catecholamines [33]. In addition, a greater reduction in norepinephrine than epinephrine has been reported after successful treatment with CPAP [33]. The

prominence of norepinephrine in OSA is notable because norepinephrine also has been shown to have powerful growth promoting effects on gut bacteria in the laboratory [35].

The bacterial growth-enhancing effects of norepinephrine depend on the availability of iron. Catecholamines increase the ability of bacterial siderophores to liberate host iron from the proteins transferrin and lactoferrin [35,36]. Iron-dependent growth does not occur in all bacteria but it is a characteristic of many Enterobacteriaceae, a group of bacteria that includes important gram-negative pathogens. In results consistent with this notion, Enterobacteriaceae were increased, and beneficial Lactobacillus was decreased in Cote d'Ivoire children who were randomized to an iron-supplemented diet [37]. Iron also increased fecal calprotectin, a biomarker of inflammation, in Ivoirian children [37]. While these findings suggest that dietary iron can cause adverse changes in microbiota and gut health, it remains unknown whether the iron-liberating effects of catecholamines occurs in sleep disorders and has similar effects on the gut. Differences in iron-dependent growth raise the possibility that exposure to catecholamines may select for Enterobacteriaceae at the expense of other beneficial bacteria.

Norepinephrine and its metabolites are of interest not only because their abundance in OSA, but because of recently characterized adrenoreceptors expressed by gram-negative bacteria [38]. Adrenergic kinases give bacteria, such as *E. coli*, the ability to sense stress in the host and respond to elevated host norepinephrine with by

activating genes involved in replication and virulence factor expression [38]. Gram-negative bacteria have the capacity to sense host catecholamines and immune signals, and can respond with changes virulence gene expression [39]. Although these are *in vitro* observations, they support the notion that catecholamine excess causes adverse changes in pathogenic bacterial species.

Inflammation and catecholamines are reduced with OSA treatment

A recent meta-analysis involving 23 studies has concluded that the treatment for OSA with continuous positive airway pressure (CPAP) decreases inflammation, as measured by C-reactive protein and Interleukin-6. [22]. CPAP has been shown to reduce the urinary excretion of catecholamines [40] and CPAP withdrawal has the opposite effect [34]. Because OSA patients have elevated circulating catecholamines that is reversible with CPAP treatment [26,33], these features make CPAP an innovative experimental probe to test the effects of chronic catecholamine excess on human gut microbiota

Hypothesis

In light of the broad effects of stress catecholamines in human sleep disorders and on microbial growth in the laboratory, this thesis tested the hypothesis that patients with obstructive sleep apnea have catecholamine-driven changes in the community structure of the gut microbiota. OSA patients were expected to have both increased catecholamines and increased density of intestinal Enterobacteriaceae, a bacterial group that contains many important gram-negative pathogens. It was further

expected that successful treatment with CPAP would decrease catecholamine exposure and decreased density of Enterobacteriaceae.

Experimental Design and Methods

In order to test the effects of catecholamine stress on gut microbiota we contrasted intestinal microbiota and catecholamines in untreated OSA versus healthy controls. Recognizing that the gut microbial community structure is subject to large inter-subject variation, we also included a within-subject contrast to determine the effect of OSA treatment on intestinal microbiota and inflammation. We compared the gut microbiota of OSA patients before versus after treatment with CPAP. Both OSA patients and the control group underwent fecal bacteriologic and urinary catecholamine analysis twice, separated by a minimum of four weeks.

Population, sample, and recruitment

Our subject population included New Mexicans with OSA who had not received prior treatment. We recruited subjects referred for evaluation of OSA and meeting eligibility requirements at the University of New Mexico Sleep Disorders Center. Recruitment began in February 2012 and continued through December 2012. Subjects recruited at the UNM Sleep Disorders Center underwent standard polysomnography. When polysomnogram criteria for OSA disease severity were not met (see below) or when study participants were unable to complete the study protocol, additional subjects were recruited at the same site. The Albuquerque VA Research and Development Committee and the University of New Mexico

Institutional Review Board approved the protocol and informed consent was obtained from all subjects.

Inclusion and exclusion criteria

Patient Group: Subject eligibility requirements include a new diagnosis of obstructive sleep apnea with moderate severity or greater, defined by an apnea hypopnea index (AHI, a validated score of OSA severity) > 15 using the American Academy of Sleep Medicine criteria. Subjects were eligible for inclusion if they were 21 years of age or older and were eligible for CPAP treatment. Exclusion criteria included the following: currently or recently pregnant; use of medication (within four weeks) known to affect gut microflora, including antibiotic treatment, probiotic supplements, oral or injectable corticosteroids, iron supplements, proton pump inhibitors; ethanol abuse or ongoing substance abuse (greater than two alcoholic drinks daily, 14 drinks/week). Because patients were required to collect fecal and urine specimens at home, cognitive impairment, lack of transportation, and homelessness were additional exclusion criteria. Each patient received polysomnography as previously ordered, education on specimen collection, and instructions to deliver specimens to the UNM Sleep Disorders Center after home collection. Subjects were provided reimbursement for their time during each visit that a specimen was delivered.

Control Group: We recruited controls without OSA from the UNM Clinical Translational Science Center (CTSC) subject database, which includes individuals who had previously agreed to be contacted for participation in clinical studies. We

attempted to enroll potential controls with gender, body mass index (BMI) and age that were similar to previously enrolled patients in order to reduce intersubject variability as a source of confounding. Exclusion criteria were that same as for patients, except that potential controls were also excluded if they had a diagnosis of sleep apnea or other sleep disorder. Controls were pre-screened over telephone and re-screened at the time of informed consent. For the control group, the CTSC was the site for consent, anthropometric measurements, education on specimen collection, and the delivery site after specimen collection.

After informed consent all subjects (patient and control) underwent measurement of height, weight, and waist circumference. They received a home collection kit for urine and stool and instructions on collection procedures and how to return the collected specimens. Height and weight were measured without shoes on calibrated scales and waist circumference was measured to the nearest 0.1 cm using an inelastic tape. Body mass index was calculated as weight in kilograms divided by height in meters squared.

Sample Collection

Subjects completed a home collection of urine and stool at two separate time intervals. The first collection occurred in the days immediately following initial enrollment. For patients with OSA, second samples were collected after > 4 weeks of confirmed treatment with CPAP. Compliance was defined as >70% nightly CPAP usage with at least 4 hours use per night. For control subjects, second samples were collected 1-2 months after first collections in an attempt to mirror the time elapsed

between first and second patient collections. For each collection, subjects were given instructions to collect all urine produced for a 24-hour period and to provide a single stool sample. Urine specimens were sent to TriCore Reference Laboratories in Albuquerque, NM for catecholamine and creatinine analysis. Stool specimens were delivered to the researchers on the day of collection. Stool specimens were transported to a laboratory at the VA Medical Center and stored in sterile collection tubes before freezing at -80° C.

DNA extraction

DNA extraction of fecal samples was performed using a QIAmp DNA Stool Mini Kit with the pathogen DNA protocol as per manufacturers instructions, Qiagen (Hilden, Germany). Extracted DNA was eluted in AE buffer. A Nanovue Plus spectrophotometer measured the concentration of DNA. Purified DNA samples were stored at - 20° C for quantitative PCR analyses.

Fecal microbiota analysis by quantitative PCR

We employed a culture-independent approach to compare the abundance of Enterobacteriaceae in patients with vs. without OSA, and pre- vs. post-treatment OSA patient groups. In order to characterize the fecal microbiota, PCR primers specific for the 16S rRNA gene were used to target Enterobacteriaceae (phylum Proteobacteria) and Universal (total) bacteria (Table 1). PCR amplification and detection were performed with a Mastercycler Realplex real-time PCR system (Eppendorf AG, Hamburg, Germany). Each plate cell contained a 4 µl reaction mixture which consisted of SYBR Green PCR Master Mix (SuperArray Bioscience,

Foster City, CA), the specific primers, and 100 ng of template DNA. Each PCR reaction was performed in duplicate; the mean of the duplicate cycle threshold (CT) values was used.

Table 1. Primers used in this study

	Primer	Sequence (5'→3')	References
Total bacteria 16S rRNA gene Universal	F1048 (forward)	GTG (GC)TG CA(CT) GG(CT) TGT CGT CA	[53]
	R1119 (reverse)	ACG TC(AG) TCC (AC)CA CCT TCC TC	[53]
Enteric pathogens (Enterobacteriaceae)	Eco1457 (forward)	CATTGACGTTACCCGCAGAAGAAGC	[54]
	Eco1652 (reverse)	CTCTACGAGACTCAAGCTTGC	[54]

Table 1 Legend. PCR amplification for each target was performed using the primers shown above.

Illumina whole microbiome sequencing

We also sent a subset of our sample (OSA patients, n=15; controls, n=11) to a commercial laboratory, Second Genome (San Bruno, CA) to perform deep sequencing of the bacterial metagenome. The sequencing used an Illumina platform based on differences in the V4 region of the 16s ribosomal DNA of bacteria (Illumina, San Diego, CA). The V4 variable region of the 16S rRNA gene was amplified with PCR by using fusion primers designed against the surrounding conserved regions. Each sample was PCR amplified with two differently bar-coded V4 fusion primers. Amplified products were concentrated using a solid-phase reversible immobilization method and quantified by electrophoresis using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Samples underwent multiplexing and pyrosequencing and then quality filtering. Pooled samples containing 96 16S V4 enriched, amplified, bar-coded samples were loaded into the reagent cartridge and then onto the instrument. Amplicons were sequenced for 250 cycles with custom primers designed for paired-end sequencing.

This process yielded 5,135,790 16S rRNA gene sequences. Second Genome provided bioinformatics analysis, including identification of operational taxonomic units (OTUs). OTUs are the functional equivalent of species present in the microbiome and were clustered at 97% using closed reference OTU picking, searched against the Greengenes reference database of 16S sequences. The UniFrac distance metric was applied to resulting taxonomies [41] and provided an estimate of the evolutionary

distance between microbiota to assess effects of OSA and other covariates on between-individual variation in gut microbiota community composition.

Data Analysis

Normally distributed variables, including age, BMI, and urine norepinephrine concentration, were subjected to t-tests (two-sample and paired). Simple linear regression was used to determine the influence of norepinephrine and other variables on fecal Enterobacteriaceae abundance. Longitudinal observations that were clustered by subject (pre- and post treatment in OSA patients; and 1st and 2nd specimens in controls) were analyzed using multilevel mixed-effects linear regression (xtmixed procedure). Second Genome used the nonparametric Adonis method to test for relationships between UniFrac distances between microbiomes of individuals. All statistical tests were performed using STATA (version 11.2) except the Adonis testing.

Results

Sample characteristics

Our original recruitment target was a sample size of 25 patients with obstructive sleep apnea and 25 controls without OSA. We recruited a total of 47 subjects who met all inclusion and exclusion criteria, including 24 OSA patients and 23 controls. In order to recruit 24 OSA patients meeting our inclusion criteria, we enrolled 50 patients who were scheduled for a diagnostic polysomnogram. Thirteen OSA patients were treated and had confirmed compliance and provided both pre-treatment and post-treatment fecal and urine specimens (Figure 1).

Figure 1. Recruitment of OSA patients

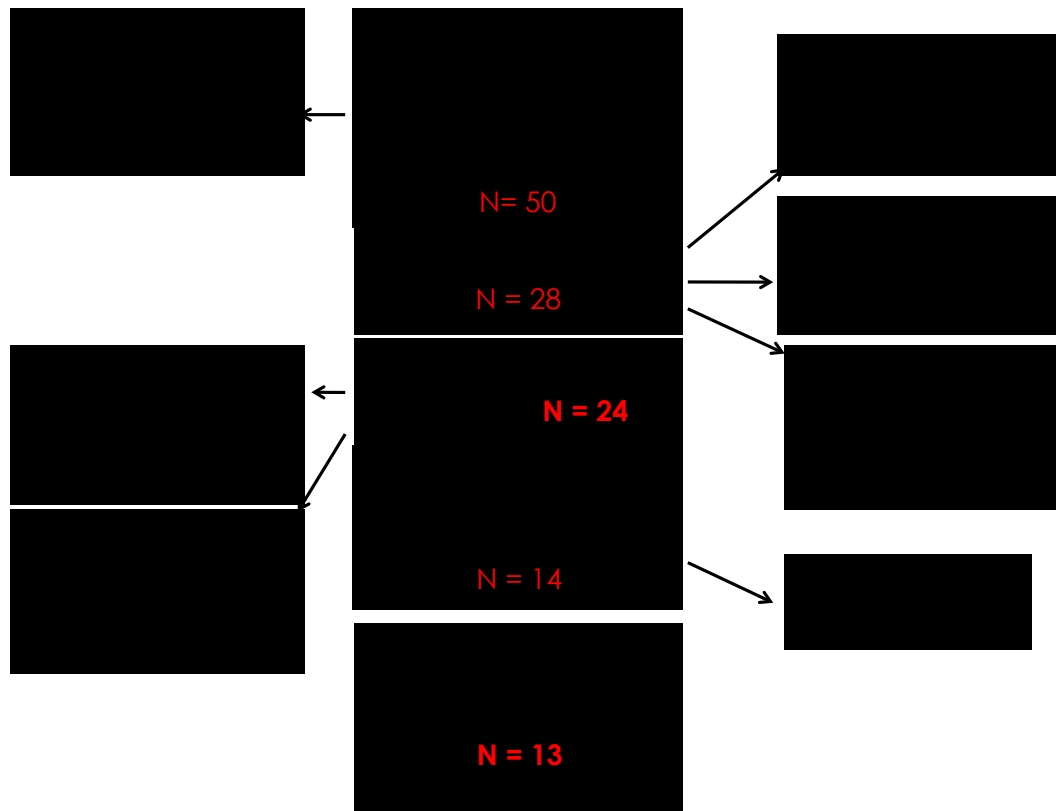


Figure 1 Legend.

The center column shows recruitment and attrition of OSA patients recruited from the UNM Sleep Disorders Center (read from top to bottom). Of 50 subjects enrolled, 28 had confirmed OSA with moderate to severe disease. Of those, 24 met all inclusion and exclusion criteria and provided a first sample of stool and urine. Thirteen enrolled subjects were treated with continuous positive airway pressure, had confirmed treatment compliance, and provided samples for second fecal bacteriological and urinary catecholamine analysis.

Table 2 displays the characteristics of the sample. This adult population is predominantly middle aged, and the age of OSA patients did not differ significantly from that of controls, 47.8 years [43.7-51.9] and 44.3 years [39.3-49.2], mean age [95%CI], respectively. The sample shows a slight bias towards females in both groups. Both groups tended to be obese, though the patient sample was significantly heavier (Table 2). Patients with OSA had a mean of 63 apneic or hypopneic events per hour (apnea hypopnea index, AHI) (Table 2).

Table 2. Sample characteristics

Population Characteristics			
	OSA Patient (n=24)	Control (n=23)	t-test
Age mean [95%CI]	47.8 [43.7-51.9]	44.3 [39.3-49.2]	p = 0.26
BMI mean [95%CI]	43.0 [38.3-49.5]	35.4 [32.2-36.7]	p < 0.05
Sex ratio M:F	10:14 (58% F)	8:15 (68% F)	
AHI mean [95%CI]	64 [48.5-78.8]	n/a	

Urinary catecholamines

Consistent with previous studies showing elevated catecholamines in sleep apnea, patients had significantly higher urinary norepinephrine relative to controls. Of the 24 OSA patients who provided pre-treatment samples, the 24-hour urinary excretion of norepinephrine, corrected for creatinine excretion, was 44.4 $\mu\text{G}/\text{G}$ [36.1-51.3], (mean [95% CI]). Controls had significantly lower 24-hour urinary norepinephrine, corrected for creatinine: 30.35 $\mu\text{G}/\text{G}$ [25.0- 34.8], ($p=0.001$, Table 3a).

For OSA patients, pre- versus post treatment urinary norepinephrine corrected for creatinine trended lower but the difference was not statistically significant (Table 4). Uncorrected norepinephrine excretion was significantly lower for post-treatment compared to pre-treatment values (Table 3b).

Table 3a. 24 hour urine norepinephrine is increased in OSA

24 Hour Urinary Norepinephrine			
	Patient (n=24 pre-treatment samples)	Control (n=40 1 st and 2 nd samples)	t-test
NE µg/g creatinine	44.4 [36.7 - 51.3]	30.35 [25.0- 34.8]	p = 0.001
NE µg/day	79.8 [58.9 – 100.7]	60.5 [47.8-73.2]	p = 0.09

Table 3b. Pre versus Post-treatment urine norepinephrine in OSA

24 Hour Urinary Norepinephrine			
	Pre-treatment n=13	Post-treatment n=13	paired t test
NE µg/g creatinine	44.1 [33.7 – 54.4]	38.0 [27.0 – 49.5]	p =0.14
NE µg/day	81.6 [44.2-119.0]	54.5 [41.1-67.9]	p=0.048

Quantitative PCR

Quantitative PCR performed in our laboratory showed no differences in Enterobacteriaceae abundance in untreated OSA patients compared to controls (Figure 2). Abundance was expressed as a ratio of universal (total) bacteria CT/Enterobacteriaceae CT, where CT is the cycle threshold measured during Realplex quantitative PCR.

Figure 2. Enterobacteriaceae abundance in OSA versus controls

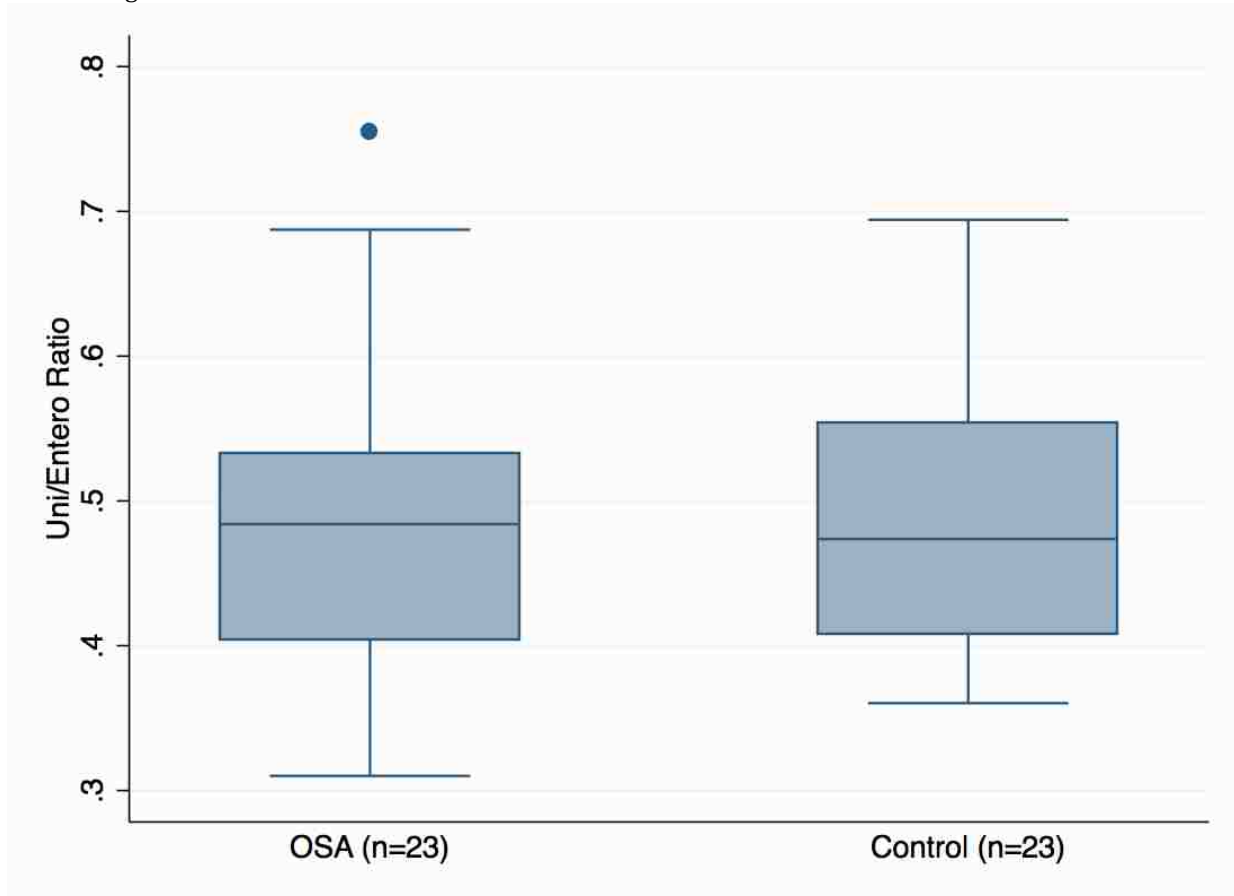


Figure 2 Legend.

Box plot showing median, 25th and 75th percentile values for Enterobacteriaceae relative to total bacteria in pre-treated patients and controls. Abundance is similar in both groups (t-test, $p > 0.05$). Because lower CT values indicate higher density of bacteria, Enterobacteriaceae is expressed as the ratio of Universal (total) bacteria CT/ Enterobacteriaceae CT. One OSA patient sample had a DNA concentration too low to analyze.

In accord with previous studies showing an effect of norepinephrine on bacterial growth, we found a significant effect of norepinephrine on bacterial abundance in the Enterobacteriaceae group (Figure 3). Linear regression demonstrated a significant positive linear relationship in the OSA patient group ($p = 0.011$, $R^2 = 0.18$). No relationship between these variables was seen in controls without sleep disorders ($p = 0.27$; Figure 3). Among OSA patients, Enterobacteriaceae abundance

also did not differ in the pre-treatment vs. post treatment condition ($p=0.4$, linear mixed-effects model).

Figure 3. Enterobacteriaceae density as a function of urine norepinephrine

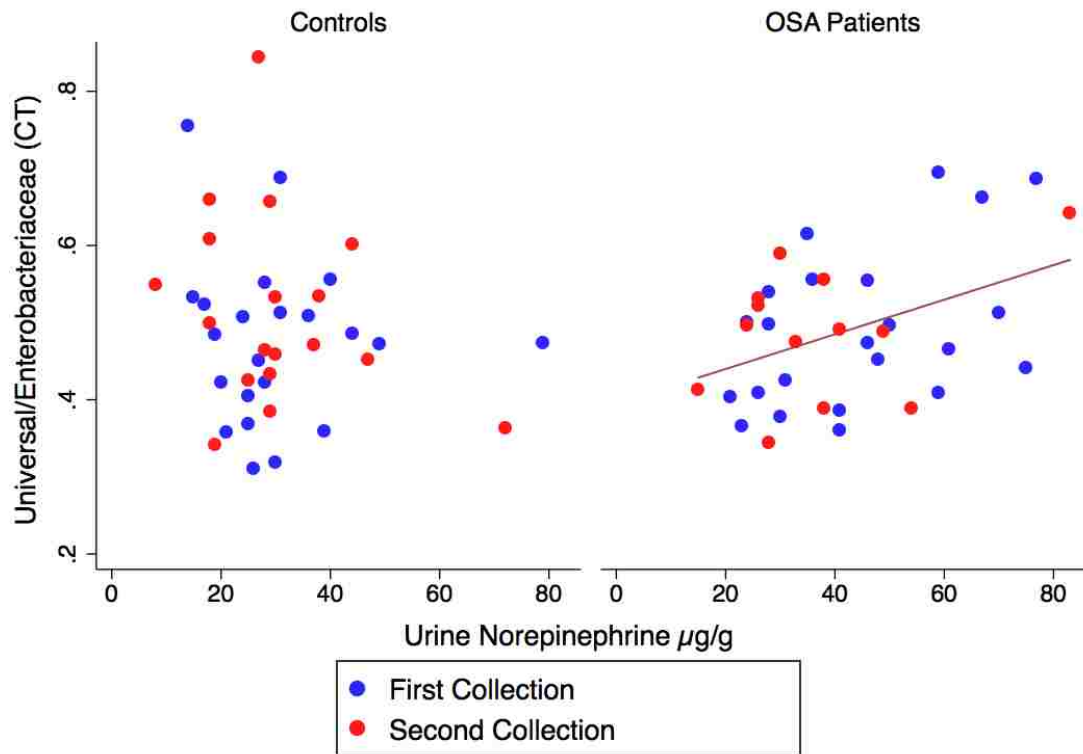


Figure 3 Legend.

The density of Enterobacteriaceae is significantly associated with norepinephrine production in OSA but not in controls. Higher CT values correspond to fewer numbers of bacterial DNA and low CT values correspond to higher number of bacteria. Because of this inverse relationship, we displayed the relative proportion of Enterobacteriaceae as: Universal bacteria CT/Enterobacteriaceae CT. Urine norepinephrine is measured as micrograms/g of urine creatinine from a 24-hour collection performed at the same time as the stool collection.

Eighteen controls without sleep disorders provided two specimens of urine and stool and thirteen OSA patients provided pre- and post-treatment samples, permitting comparison of within-subject changes in Enterobacteriaceae and

norepinephrine (Figure 4). Although controls showed no significant relationship between these variables ($p=0.71$), the within-subject change of Enterobacteriaceae is positively associated to the corresponding change in norepinephrine ($p=0.028$, $R^2= 0.37$, linear regression).

Figure 4. Change in Enterobacteriaceae density as a function of change in norepinephrine

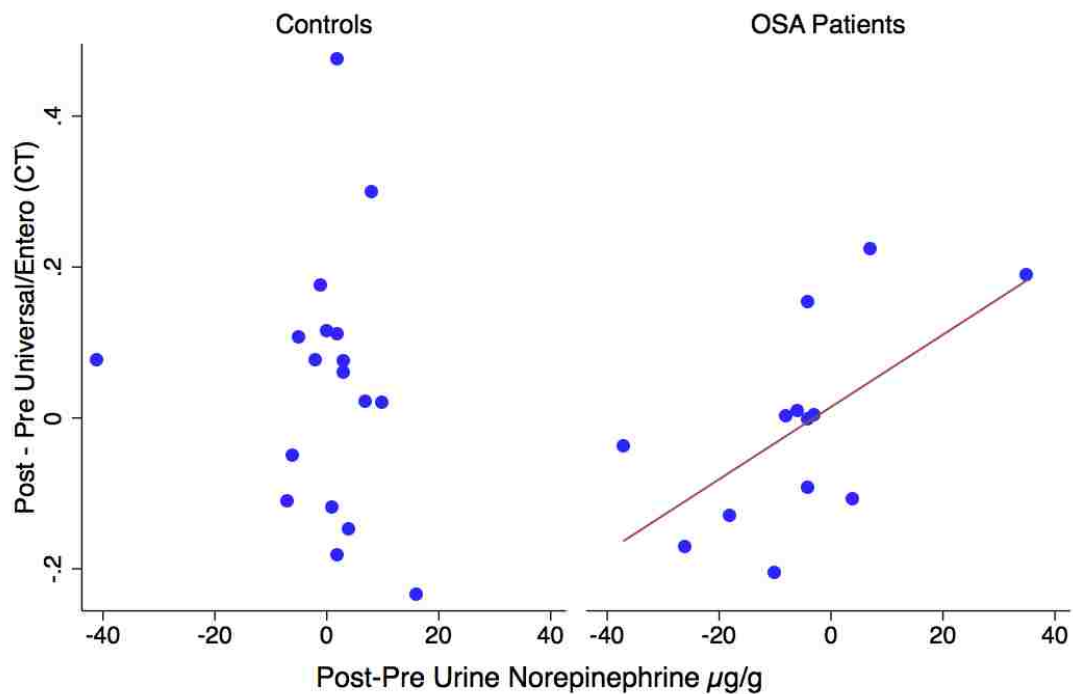


Figure 4 Legend.

This figure plots the within subject change in Enterobacteriaceae abundance and norepinephrine. No relationship occurs in subjects without OSA measured at two time points ($n=18$, $p = 0.27$). A significant linear relationship is seen in OSA patients (treated – untreated, $n= 13$, $p = 0.011$, linear regression).

A linear mixed-effects model tested whether fecal Enterobacteriaceae abundance was influenced by the following variables: creatinine-corrected norepinephrine

(NE), BMI, age, gender, and OSA patient status. A model that included all variables and interaction effects showed a positive association only for the interaction of OSA and NE ($p=0.025$). When stratified by OSA condition, a significant positive association between Enterobacteriaceae and norepinephrine emerged in the OSA patient group ($p=0.007$) and not for controls ($p=0.6$).

Metagenomic analysis with Illumina

Effect of disease status:

Fecal bacterial DNA from 15 OSA patients and 11 controls was analyzed with Illumina sequencing at Second Genome (San Bruno CA). Considerable intersubject variability existed in the community structure of the gut microbiota and no clear pattern differentiated patients from controls at the phylum level (Figure 5). Principal components analysis (PCoA) was used to represent the degree of similarity of the gut microbiota between individual subjects and to compare differences in bacterial taxa in patients and controls. A PCoA plot shows a borderline significant whole microbiome difference between OSA patients and controls ($p = 0.08$, Adonis test, weighted UniFrac distance, Figure 6). Similarly, PCoA revealed a borderline significant microbiome separation between OSA patients and controls using unweighted UniFrac distance ($p = 0.08$, Adonis test; Figure 7). Unweighted UniFrac distance depends on the presence/absence (not abundance) of 1426 taxa present in at least one sample.

Figure 5. OSA patients and controls do not differ at the phylum level

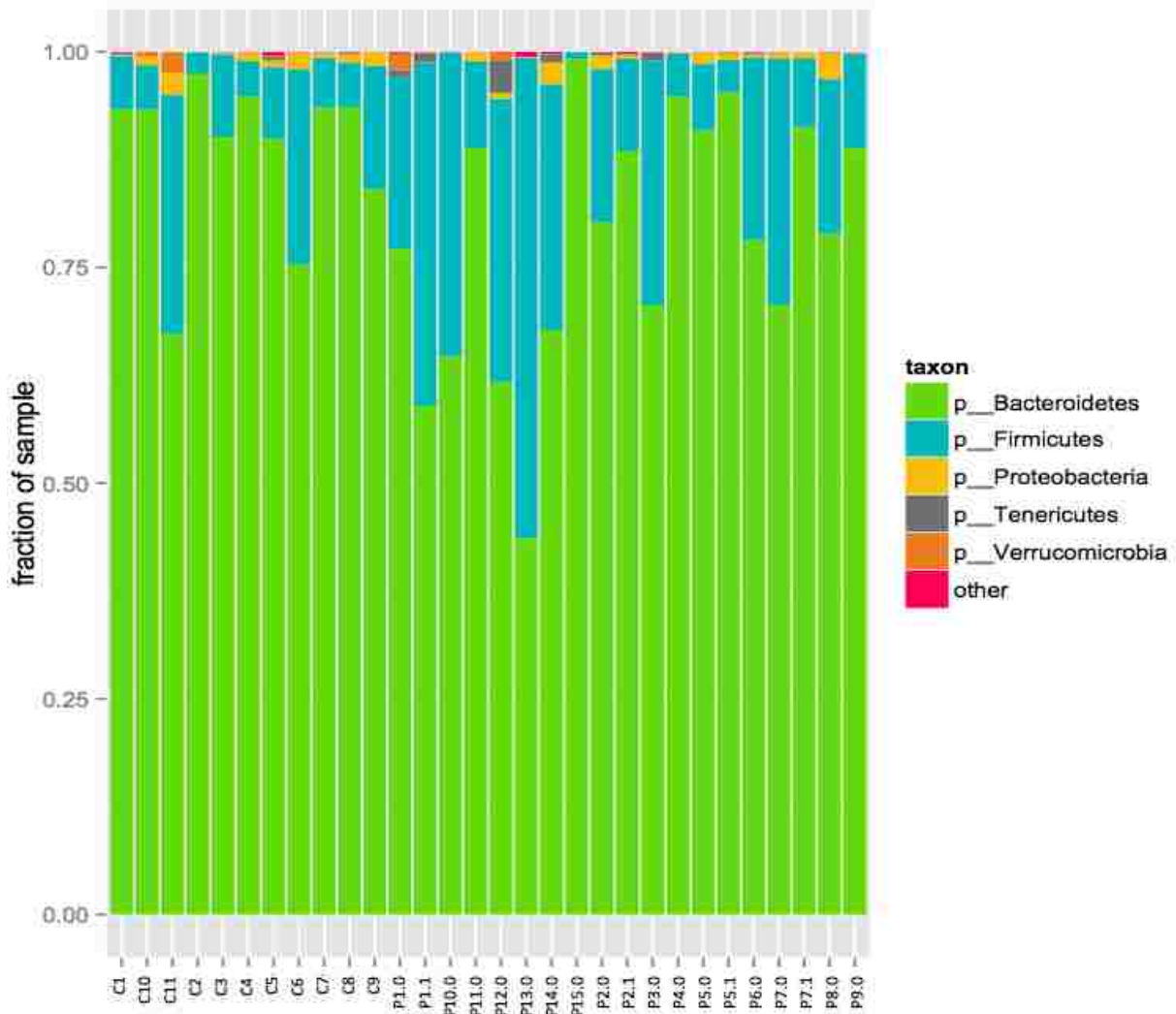


Figure 5 Legend.

Substantial intersubject variation exists and no clear pattern by patient status is seen in this sample. Patient samples have the prefix P, Controls have the prefix C.

Figure 6. Principal Components plot of the community structure of microbiota comparing OSA patients and controls, using weighted UniFrac Analysis

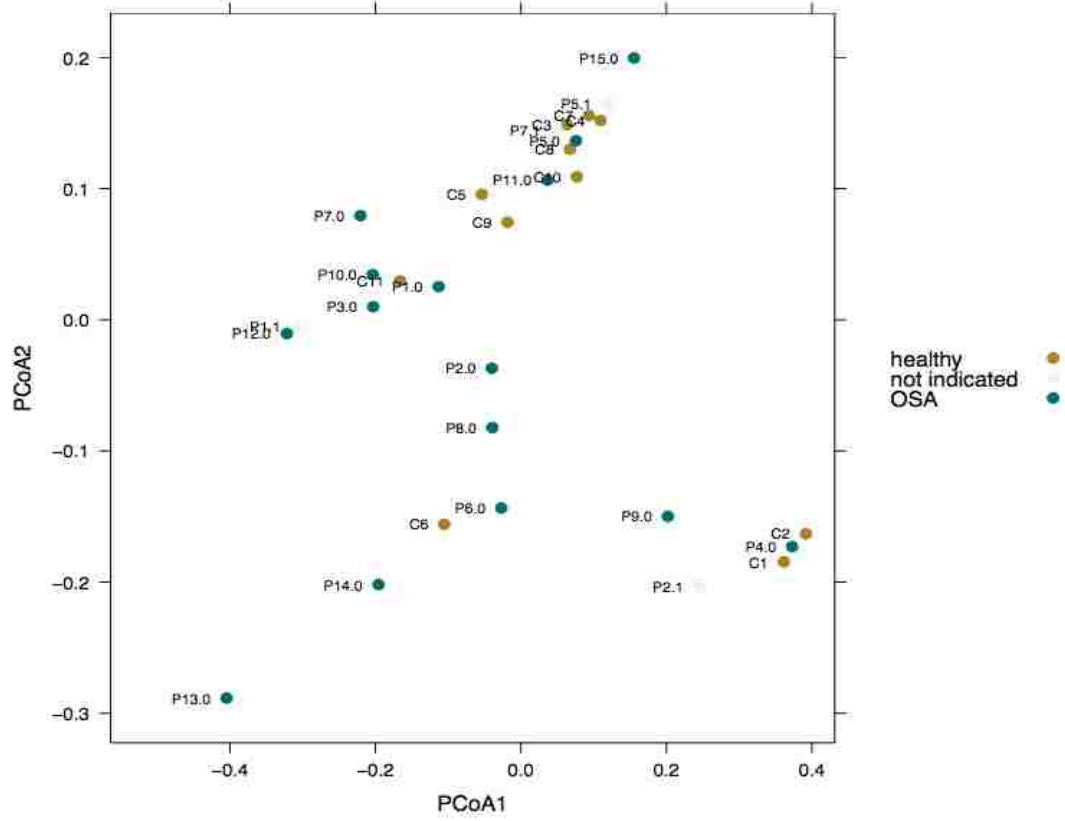


Figure 6 Legend.

Weighted UniFrac Analysis, based on the abundance of 1426 taxa is borderline significantly different ($p=0.08$).

Figure 7. Principal Components plot of the community structure of microbiota comparing OSA patients and controls, using unweighted UniFrac Analysis

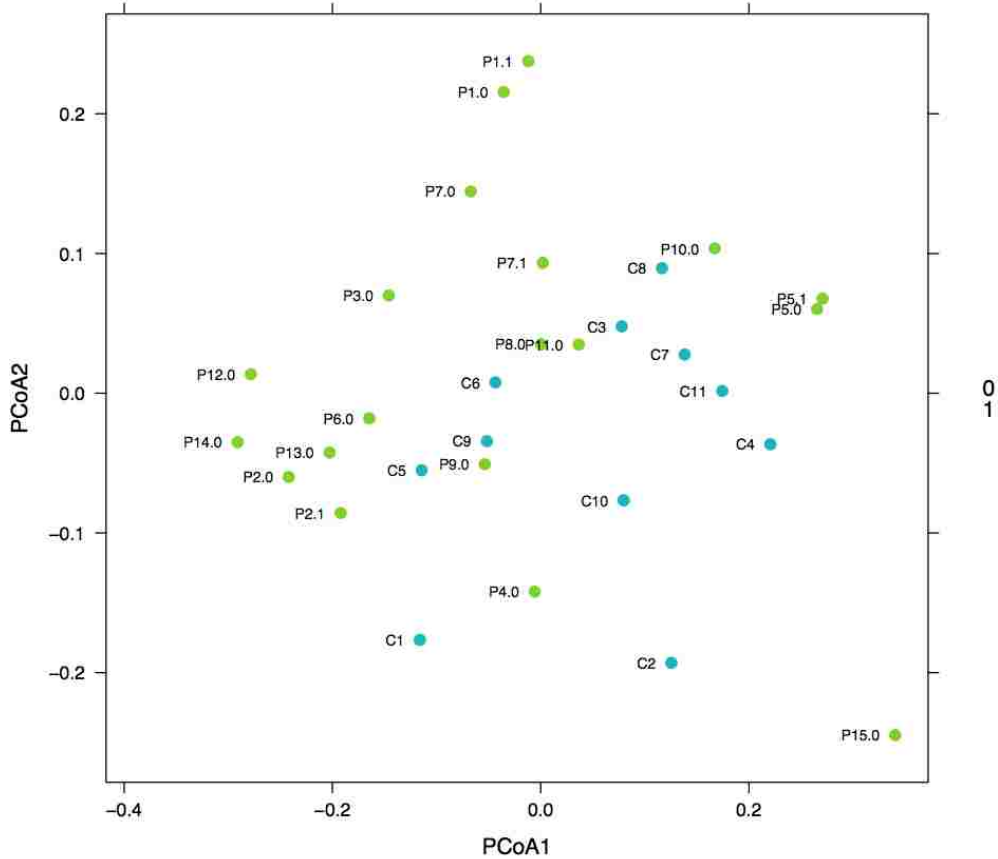


Figure 7 Legend: Unifrac Principal Components analysis revealed a borderline significant separation of microbiota composition between OSA patients and controls. Based on the UniFrac metric on the presence/absence of 1426 taxa present in at least one sample ($p = 0.08$, Adonis test).

In addition, no whole microbiome differences were found between male and females in this sample ($p = 0.11$, Adonis test) based on weighted UniFrac distance, which estimates the abundance of 1426 taxa (Figure 8). A nonsignificant separation of the whole microbiome is seen when we compare four levels of BMI ($p=0.58$; weighted UniFrac distance, Figure 9).

Figure 8. The community structure of the gut microbiota does not differ by gender

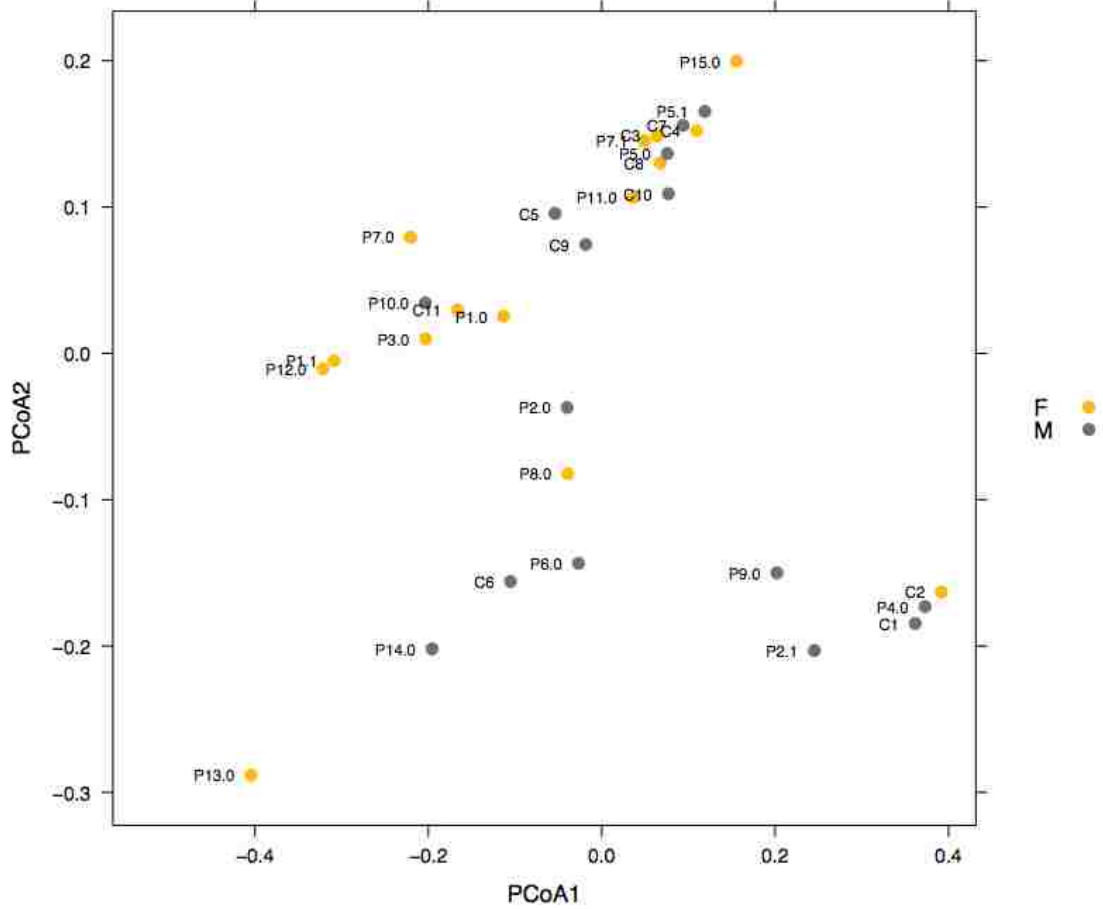


Figure 8 Legend.

Principal components analysis plot of fecal microbiota indicating subject gender. F = Female, M = Male. Weighted UniFrac analysis shows that the microbiota does not significantly differ between males and females, $p = 0.105$. Based on the abundance of 1426 taxa.

Figure 9. Community structure of microbiota does not differ by body mass index

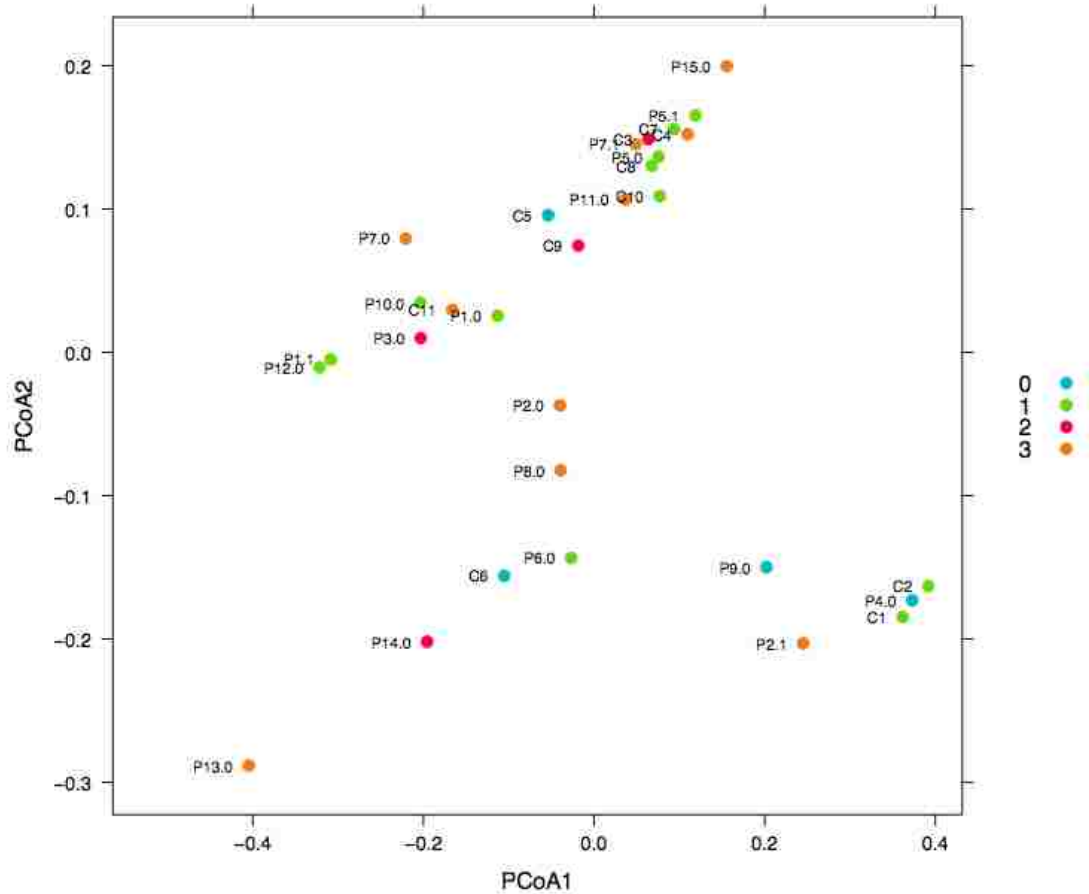


Figure 9 Legend.

Weighted UniFrac Analysis, which measures microbiota similarity based on the abundance of 1426 taxa does not differ based on level of BMI. $P = 0.578$.

Effect of elevated norepinephrine:

Individuals with elevated NE, regardless of disease status, appeared to share similarity in 246 taxa, compared to subjects with NE values that fell in the normal range (Figure 10). The majority of these bacterial taxa were in the Firmicutes phylum.

Figure 10. Heirarchical clustering analysis of microbiota partitioned by urine norepinephrine

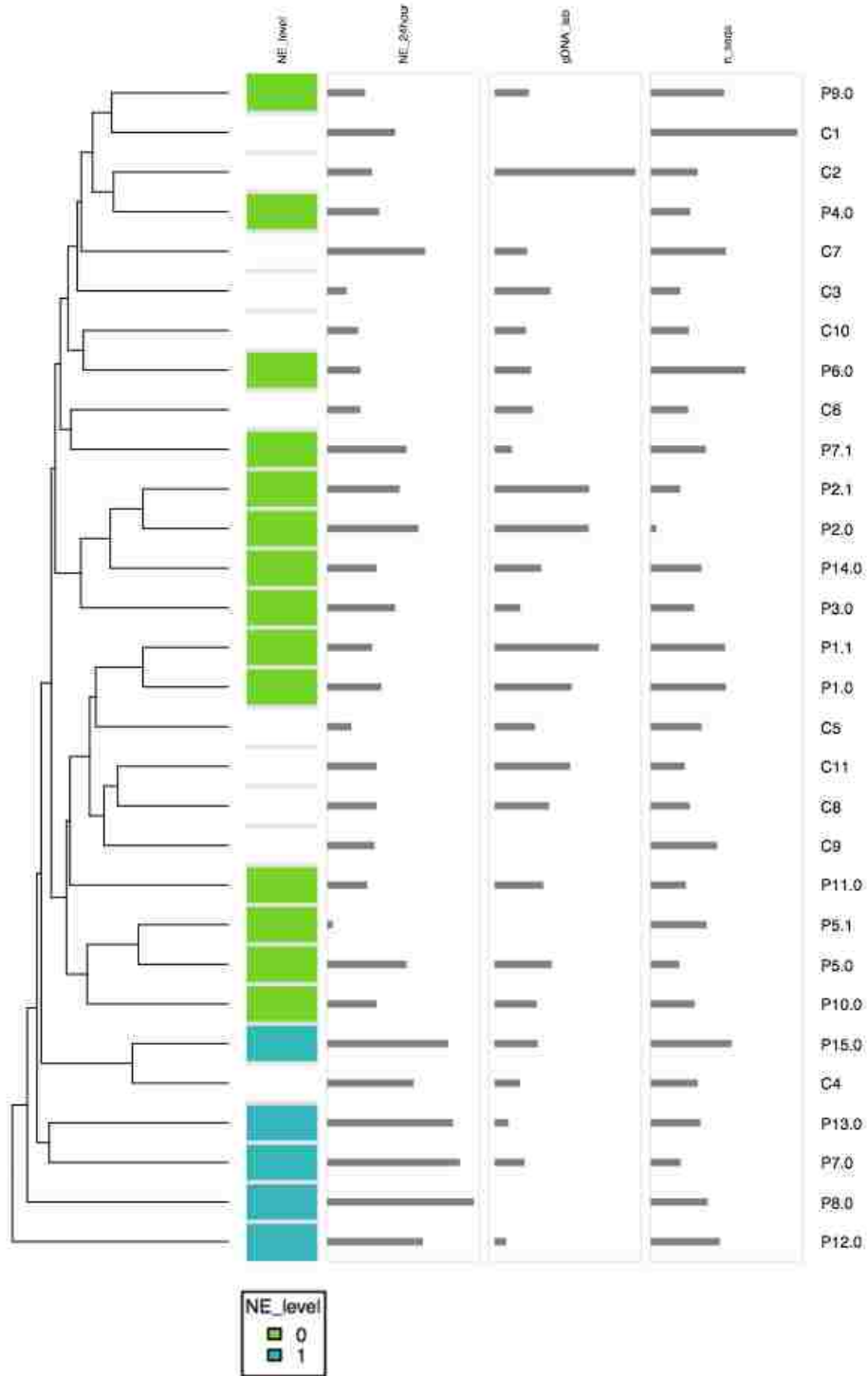


Figure 10 Legend. Hierarchical clustering analysis based on binary metrics of 246 taxa showed samples were clustered into two groups, with NE elevated and without NE elevated, based on the TriCore reference range. Individuals with elevated norepinephrine (NE) levels showed microbiota taxonomic similarity to each other. One individual with elevated NE (P15.0) clustered with the individuals with normal NE.

Discussion

The importance of sleep in immune regulation and metabolism has generated much recent scientific attention, but the harmful effects of disordered sleep have lacked a cohesive physiological explanation. The present translational study examined whether a phenomenon reported in animal models and *in vitro* – alteration of gut bacteria from stress and catecholamines - occurs in the human sleep disorder OSA. We describe a borderline significant fecal microbiome difference between OSA patients and controls. Overlap in the gut microbiome in the two groups suggests that human sleep disorders are not accompanied by log-scale changes in microbial growth that have been reported in laboratory bacteria exposed to catecholamines [35,42]. However, the relatively small numbers of fecal samples that underwent genomic sequencing may have lacked statistical power to clearly discriminate between these groups.

In a novel finding, OSA patients showed a positive association of the abundance of Enterobacteriaceae and urine norepinephrine, a measure of systemic sympathetic nervous activation. In previous animal studies of sleep deprivation, Enterobacteriaceae were the bacteria most often associated with intestinal bacterial overgrowth and translocation to mesenteric lymph nodes [7]. In the present study, fecal Enterobacteriaceae were not significantly increased in OSA patients nor changed with treatment. Nevertheless, we found that the change in norepinephrine that occurred after treatment of OSA with CPAP was a significant predictor of shifts in the abundance of Enterobacteriaceae. These findings are the first to link stress

hormones with altered gut bacteria in a human disease state. Because Enterobacteriaceae are a cause of inflammation in many human diseases [43], our observations provide support for the hypothesis that catecholamine-driven changes in gut microbiota are a source of inflammation and metabolic disturbance in OSA.

The linear relationship between norepinephrine and Enterobacteriaceae occurred in OSA patients but not in controls, suggesting that bacteria may be sensitive to norepinephrine only when patients have a chronic illness. However, it is possible that some other variable, such as apnea or hypoxia, was responsible for the observed relationship. Arguing against this view, the measured apnea hypopnea index was a non-significant predictor of Enterobacteriaceae abundance by simple and multiple regressions (results not shown). Indeed, norepinephrine was the only significant positive predictor of Enterobacteriaceae in this study, which supports the view that catecholamines were a determinant of Enterobacteriaceae abundance in OSA patients. This view is further supported by the significant relationship between the within-subject changes in Enterobacteriaceae (before and after CPAP treatment) with the corresponding shift in norepinephrine in OSA. These results imply that this study's failure to find a group difference in Enterobacteriaceae between before and after CPAP treatment occurred because of substantial overlap of norepinephrine exposure in the two groups.

Previous work has linked increased Enterobacteriaceae with intestinal inflammation in humans after iron supplementation [37] in inflammatory bowel

disease [43] and with pregnancy [32]. The link between catecholamine and Enterobacteriaceae abundance in OSA make it plausible that catecholamine-driven changes in Enterobacteriaceae contribute to the inflammatory burden of OSA. Many bacteria in the Enterobacteriaceae group respond to catecholamines with changes in virulence gene expression [44-47]. The subset of catecholamine-activated Enterobacteriaceae – those that express virulence genes conferring epithelial adherence and toxin production – may be sufficient to induce inflammation [43]. A priority for future work on sleep will be to test whether norepinephrine is linked to virulence expression in OSA.

Limitations

One limitation of this study is that we did not measure catecholamine exposure in the gut itself. Although it is possible to measure fecal catecholamines, we opted for a standard measure of systemic catecholamine exposure, the 24-hour urine catecholamine test. We surmised that because urinary excretion of norepinephrine reflects increased sympathetic nerve traffic in OSA, gut bacteria would have access to norepinephrine because of its presence in the enteric nervous system and enteric circulation. Future work will be needed to confirm that assumption.

Some medications and foods are known to influence catecholamine test results, introducing another potential source of variability of this study. Although patients and controls were provided written instructions to abstain from caffeine, alcohol, and diuretic medications during the collection period, this study did not control for all medications and foods that can potentially affect catecholamine excretion.

An additional limitation of our approach is that sleep apnea was excluded in the control group by self-report, and the controls did not undergo polysomnography to exclude a sleep disorder. Because polysomnography was omitted in the control group, it is possible that some in this group may have had some degree of sleep apnea. Future studies will address this limitation by testing all subjects with a polysomnogram.

Future Directions

Involvement of a catecholamine-Enterobacteriaceae relationship in sleep provides a rich framework for devising testable hypotheses. Here we consider a few examples of these extensions of the model.

The relationship between the catecholamine norepinephrine and Enterobacteriaceae reported here is a first step in understanding the how the microbiome influences health outcomes related to stress and sleep. It remains unclear whether catecholamine driven changes in gut bacteria influence metabolic, immunologic, and cardiovascular outcomes. The immediate next stage is to measure inflammatory biomarkers in blood and stool to understand whether Enterobacteriaceae has a relationship to inflammation in OSA. Because inflammation and metabolism are closely related, we will also explore the interrelationships between insulin resistance, norepinephrine, and gut microbiota. This research area has potential implications for many common conditions of sympathetic overdrive in addition to sleep apnea. For instance, short-term sleep

deprivation has been reported to increase plasma norepinephrine levels [48] and causes changes in metabolism that lead to weight gain [3]. The association of norepinephrine and harmful bacteria seen in OSA makes it plausible that gut microbiota are involved in the metabolic changes of sleep deprivation. Notably, the fragmented sleep that occurs in hospitalized patients resembles the interrupted sleep found in OSA [49]. Because nocturnal arousals increase norepinephrine production [48], changes in gut microbiota may be common in hospitalized patients, with potentially harmful effects on immune and metabolic function.

Treatment and diagnostic implications for sleep disorders

If catecholamine-driven bacterial changes occur in OSA, probiotics and prebiotics may provide a potential treatment. Probiotics are living strains of commensal bacteria that are thought to promote health. Probiotics have been shown to inhibit virulence factor production by some pathogenic species and reduce numbers of Enterobacteriaceae and other pathogens by competitive exclusion [50,51].

Prebiotics are nutrients that are selective growth substrates for probiotic strains.

Prebiotics thus have many of the same potential beneficial effects as probiotics.

Synbiotics, are the combination of the two, usually a strain of Bifidobacteria or Lactobacillus plus galacto-oligosaccharides, have the advantages of both. Future research will test the hypothesis that synbiotics can block catecholamine-induced changes in Enterobacteriaceae in OSA.

The ability of stress catecholamine to modulate microbial and mammalian signaling pathways provides another translational research opportunity in sleep. The recent

discovery of bacterial adrenergic kinases has shown that bacteria sense and respond to changes in catecholamines, including norepinephrine [38,39]. The capacity of pathogenic Enterobacteriaceae to upregulate virulence gene expression when exposed to norepinephrine may make it possible to screen for altered gene transcription in disordered sleep. If the microbial-mammalian transcriptome can discriminate between patients with and without OSA, a meta-transcriptomic signature may be identified that could lead to a noninvasive test for sleep disorders.

The function of sleep

Why organisms sleep is a medical and biological mystery without a satisfactory explanation. Previous work has shown that the sleep phase of the circadian cycle is characterized by changes in immune gene transcription and cytokine production [19,52]. These observations have prompted the hypothesis that mammalian sleep has a critical function in providing host resistance to parasites [52]. If this view is correct, sleep-related immune activity might be important also in managing the composition and activity of the microbiota. It is well established that norepinephrine production is suppressed in healthy sleep and markedly increased in disrupted sleep [48]. We hypothesize that microbiota such as Enterobacteriaceae take advantage of the presence of excess catecholamines in OSA to promote their fitness at the expense of the host. Thus, the origin of sleep may lie in host-microbiome interactions involving stress hormones, with consequences for metabolism and immunity in both health and disease.

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