Simultaneous profiling of oral and placenta microbiome in pregnant women with Preeclampsia: a cross-sectional study

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Short Title: Oral and Placental Microbiome in Preeclampsia

Abstract

Objective: Preeclampsia (PE) is a leading cause of morbidity and mortality in pregnancy with elusive etiology. The roles of oral and placental microbiome in PE are poorly understood. Our study is aim to determine the associations between the oral and placental microbiome in women with and without preeclampsia and periodontal disease (PD) and evaluate the systemic immune response in patients with and without PE and PD.

Design: Prospective, observational study

Setting: Multicenter English.

Population: Fifty-four pregnant patients with and without PE and PD were recruited.

Methods: The microbiome profiles of both oral subgingival region and placenta were characterized by V4 region of 16S rRNA gene sequencing. Systemic inflammation markers tumor necrosis factor-alpha (TNF-α), C-reactive protein (CRP), lipopolysaccharide binding protein (LBP), interleukins 6 & 8 (IL-6, IL-8) in blood were measured by ELISA.

Results: PD significantly increased the risk of PE after adjustments for age, preterm delivery and smoking status (OR=2.26, 95% CI=1.14-4.48, p=0.024). A combined group of oral associated bacteria Veilonella, Fusobacterium, Haemophilus, Granulicatella, Streptococcus, Gemella and Neisseria in placenta had significantly higher prevalence in women with PE compared to women without PE (53.8% vs 19.0%, p=0.018), with the highest prevalence in patients with both PE and PD (58.8%). The relative abundances of Haemophilus, Veillonella and Fusobacterium in oral samples were significantly higher in patient with PE than those without PE. The relative abundances of Haemophilus in oral microbiome was associated with increased risk of PE **(**OR=2.11, 95% CI=1.11-4.52, p=0.032). Proinflammation cytokine analysis showed that PE patients with PD had higher blood IL-8 levels than PE patients without PD (p=0.028). CRP, LBP, TNF-alpha showed no statistical difference in patients with and without PE or PD. Blood IL-6 levels were significantly higher in patients with detectable placenta microbiome compared to those without placental microbiome (p= 0.028).

Conclusion: Oral-like microbiome was identified in placenta more frequently in patients with PE than those without PE. Placental microbiome is associated with systemic inflammation. High abundances of Haemophilus in oral cavity is associated with increased risk of PE.

INTRODUCTION

Preeclampsia (PE) is a leading cause of morbidity and mortality in pregnancy, complicating approximately 2-5% of pregnancies [1-2]. The etiology of PE remains unclear. The placenta has been considered as a central organ in the pathogenesis of PE.

Periodontal disease (PD), has been shown to be positively correlated with preeclampsia. The prevalence of periodontal disease among pregnant women is approximately 40% with a higher prevalence of 60-70% among racial and ethnic minorities[3-5]. PD-associated bacteria can potentiate an aggressive inflammatory response, even in extra-oral sites[6, 7]. Hematogenous spread of the oral bacteria to the placenta was theorized as an important source of amniotic fluid and placental microbiome. It is possible that oral pathogens can transmit to the blood and disseminate to placenta where the blood flow is slow. Indeed, oral bacteria *P. gingivalis* and *A. actinomycetemcomitans* have been detected in amniotic fluid of pregnant women with periodontitis[8], and reported to be associated with placental infections in hypertensive women[9]. A study showed patients with PE had higher levels of *Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum ssp., Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythensis, and Treponema denticola* in the placenta[10, 11]. However, the study had a small sample size and detected a limited number of periodontal disease-associated bacteria in placenta using targeted PCR approach. To date, there is a lack of non-targeted global characterization of placental microbiome and oral microbiome simultaneously in same patients in PE. Furthermore, it is unclear about the relationship between placenta microbiome, if there is any, with systemic inflammation in PE.

To have a deep understanding of those unresolved questions, we prospectively enrolled patients according to their maternal diagnosis: preeclampsia/periodontal disease (PE+/PD+), preeclampsia/no periodontal disease (PE+PD-), no preeclampsia/periodontal disease (PE-/PD+), and no preeclampsia/no periodontal disease (PE-/PE-). We hypothesize that the placenta microbiome is derived, at least partially, from oral microbiome, and placenta

microbiome is associated with PE and elevated systemic inflammatory responses. Outcomes from this study, will help strengthen our understanding of the pathogenesis of preeclampsia and offer a novel preventive or treatment strategy for this long-standing significant hypertensive disorder.

MATERIALS AND METHODS

Study participants

We conducted a prospective, cross sectional observational cohort study at two academic medical centers. The study was conducted at John Dempsey Hospital and Hartford Hospital from to October 2019 - December 2020. The protocol was approved by the institutional review boards at both sites. UConn Health was the IRB of record (IRB #: 19-140H-1). Pregnant women admitted in labor were approached regarding participation in the study. Women were eligible if they were age 18-50 years old, and carried a singleton gestation, and gestational age was greater than 28 weeks. Exclusion criteria were: pregestational or gestational diabetes, known or diagnosed HIV-infection in pregnancy, Hepatitis B or C positive status, inflammatory bowel disease, major fetal anomalies, renal disease, chronic antibiotic use in pregnancy, chronic steroid use (≥ 2 months use), and inability to collect specimens within 72 hours after birth. Due to the possibility that labor may influence risk of infection and concern for bacterial contamination of the placenta, all cesarean deliveries for this study were confined to women who underwent elective cesarean delivery prior to undergoing spontaneous labor.

PE was defined as new onset hypertension: BP ≥140 mmHg systolic or ≥90 mmHg diastolic (sustained-on two occasions at least 6 hours apart) in the presence or absence of proteinuria (at least 300 mg per 24 h, a score of 0.3 on protein/creatinine ratio during the study period[12].

Sample Size

This study aimed to establish whether there are microbiota differences of sufficient magnitude in patients with preeclampsia and periodontal disease. This sample size was calculated according

to a previous study that reported the association of microorganisms found in placental tissues obtained from women with pre-eclampsia, and normal pregnant women[10]. Effect size was based on detecting a difference between groups in the microbial β-diversity with varying prevalence and effect sizes[10, 13].

Serum Blood Collection: Blood was collected on admission and prior to the beginning of labor. The samples were centrifuged and serum were stored at −80◦C for cytokines analysis.

Placenta Specimen Collection: At the time of delivery, the placenta was placed in a sterile container and immediately handed off to trained study personnel who wore facial masks and used sterile gloves, scalpel and tissue forceps. Two 1-cm × 1-cm × 1-cm cuboidal sections were circumferentially excised under the surface area of the placenta from specified areas (chorionic plate to basal plate); one area located 4 cm proximal to cord insertion and one area located 4 cm from the placental edge site[14]. The specimens were stored at −80°C for microbiome assessment.

Oral Cavity Specimen Collection. All patients were evaluated for the presence or absence of periodontal disease by a trained periodontist. The dental exam was a full mouth periodontal examination[15]. Measurements were taken at 6 sites around of each tooth on postpartum day one[16]. The following parameters were evaluated: Periodontal Probing Depth (PPD), Bleeding on Probing (Bop), Recession – measured (when present) from cement-enamel junction (CEJ) to free gingival margin, and Clinical Attachment Loss (CAL)[17].

Periodontal disease was defined as the dental exam finding ≥2 non-adjacent interproximal sites with CAL ≥3 mm, and ≥2 non-adjacent interproximal sites with PPD ≥ 4 mm, or ≥1 sites with PPD ≥5 mm[16]. Subgingival dental plaque samples were collected by swiping the tooth surface with a dental explorer, and were placed in DNA Genotek media and stored at −80°C until sequencing.

DNA extraction, PCR amplification, and 16S rRNA gene sequencing

To determine the microbiome profile in placenta and subgingival plaque, we performed V4 regions of 16S rRNA genes sequencing [19]. In brief, a maximum of 0.25g placenta and 200ul subgingival samples were used for DNA extraction using Qiagen DNA mini kit for tissue and blood, according to the manufacturer's protocol[18]. Genomic DNAs were amplified using primers targeting V4 region of 16S rRNA gene, followed by 2x250bp pair-end sequencing using the Illumina Miseq sequencing platform. To control potential microbiome contaminations, negative controls including extraction controls and operation room controls were included for all sample extractions. The 16S rRNA gene sequences were processed with DADA2 pipeline to generate amplicon sequence variants (ASVs) that represent lowest taxonomy unit in 16S rRNA gene sequencing. Data processing included paired end reads merging, denoising, identification and removal of chimeric sequences, assign sequences to bacterial taxonomies. Sequences detected in any negative control were removed from all samples. Bacterial taxonomic identification of amplicon sequence variants (ASVs) was done using the RDP Bayesian classifier against the Silva nr_v119 taxonomy database[20-21]. Sequences that were unidentified at the domain level and those identified as mitochondria were also removed. The generated ASV table, combined with the clinical metadata, were used as input for downstream analysis. We employed rigorous procedures to remove potential placenta microbiome contamination from various sources as illustrated in Figure S1.

Analysis of demographic and clinical data

Descriptive statistics were used for demographic and clinical information. We first summarized women by periodontal disease and preeclampsia status for continuous variables using mean and standard deviation, categorical variables were analyzed using frequencies and percentages. The differences between the groups were tested using two-sample t-tests for continuous variables after proper transformation and Fisher's exact tests for categorical

variables. The prevalence of periodontal disease between women with and without preeclampsia was then determined by Chi-square testing. A logistic regression model was fitted to evaluate the associations between preeclampsia and periodontal disease, adjusting for potential confounding variables.

Statistical analysis of placenta and oral microbiome

Descriptive analysis of placental microbiome includes prevalence and relative abundances of a combination of placental microbiome in each group. Data in two placenta sites was combined to represent placenta microbiome for a given patient. Kruskal–Wallis test or Fisher's exact test and Wilcox-sum rank test were used to test prevalence and relative abundances difference across groups, respectively. Permutational multivariate analysis of variance (PERMANOVA) was performed to evaluate global microbiome difference between two groups after accounting for potential confounding variables such as age, ethnicity, smoking status, and history of preeclampsia[24]. Wilcox-Sum-Rank testing and DESeq2 were performed to identify differential bacteria between groups[25]. A logistic regression model was used to determine the risk of oral microbiome in development of PE. P values from multiple comparisons were adjusted using False Discovery Approach. Adjusted p value <0.05 was considered as statistical significance.

Cytokine Analysis

Cytokine serum levels were determined using commercially available kits according to the manufacturer instructions. The cytokine serum levels, expressed as medium fluorescence intensity (MFI), were log transformed and compared among the four groups (preeclampsia (PE) and periodontal disease (PD) by using Kruskal–Wallis followed by Dunn's post-test where the p-value was adjusted for multiple testing using the Benjamini-Hochberg method.

All above statistical analyses of the microbiome were performed with R 3.3.2 (Vienna, Austria) software.

RESULTS

A total of 126 women were screened from October 2019 to December 2020. Seventy-two patients did not meet inclusion criteria, leaving 54 women eligible for the study (Figure S2). Patient characteristics and demographics by (preeclampsia and periodontal status) are presented in Table 1. All clinical variables were comparable across four groups except that birth weight (p=0.001) and gestational age (p=0.008) were significantly lower in patients with PE. Interestingly, periodontal disease was confirmed in 19 (65.5%) women with preeclampsia compared to 7 (28%) women without preeclampsia (p=0.007). Further logistic regression analysis showed periodontal disease increased the risk of preeclampsia after adjustment for age, preterm delivery and smoking status (OR=2.26, 1.14-4.48, p=0.024).

Ninety-six out of 99 placental samples from 54 patients were successfully sequenced, resulting in 2241 ASVs. DNA extraction and procedure room control accounted for a large proportion of sequenced reads of placenta (81.7%). After contamination removals from DNA extraction controls and procedure room control, 2142 ASVs were left.

To address the potential microbial contamination during delivery, difference of placenta microbiome between cesarean and vaginal delivery were evaluated. The microbiome in placenta after removing extraction and procedure room controls was significantly different between cesarean and vaginal delivery by PERMANOVA ($p<0.05$) (Figure S3) suggesting delivery mode contributes to the microbiota detected in placenta. Wilcox-Sum-Rank test showed vagina-related microbiome represented by Lactobacillus, Prevotella, Gardnerella, Megasphaera and common stool microbiota represented by Bacteroides, Faecalibacterium were significantly higher among vaginal deliveries. Skin-related bacteria Bacilli were significantly higher in women who had cesarean deliveries. In total, ASVs from 67 genera noted to be different between

delivery modes were removed from placenta microbiome, resulting in 1482 ASVs in 96 placental samples.

To further minimize potential contaminations, we filtered the placental microbiome by keeping ASVs identified in oral samples and with >1% of averaged abundance in oral samples (Figure S1). The relative abundance of 1% was considered a biologically reliable or relevant cut-off for microbiome research in low biomass samples and was used for previous placental microbiome studies[22-23]. This yielded a final set of 23 ASVs from 10 genera including Streptococcus, Haemophilus, Veilonella, Fusobacterium, Neisseria, Granulicatella, Gemella, Actinomyces, Campylobacter and Leptotrichia in placenta. The majority of these microbiota are well-known periodontal disease-associated bacteria. Streptococcus was the most prevalent bacteria in placenta, account for 27.7% of patients in our cohort (13/47 =27.7%), with the rest of 9 genera accounting for 2-8% of total patients (Figure 1a).

Next, we analyzed the placenta microbiome in patients with and without PE (PE+ and PE-) and four subgroups: PE+/PD+, PE+PD-, PE-/PD+ and PE-/PE-. We found 7 bacteria Veilonella, Fusobacterium, Haemophilus, Granulicatella, Streptococcus, Gemella and Neisseria that were present in higher proportion in PE+ group compared to the PE- group. However, none of the individual genus showed statistical difference in prevalence or abundance among PE+ and PE- groups or the four subgroups. This is likely due to different patients having different bacterial compositions in their placenta microbiome. However, because different bacteria may confer similar function and the identified placental microbiome were all associated with periodontal-disease, we combined the prevalence or abundance of the 7 bacteria genera and tested the differences of combined microbiome among groups. Interestingly, the prevalence of combined placental microbiota in PE+ patients (14/26=53.8%) was significantly higher than PE- patients (4/21=19.0%) (p=0.018) (Figure 1b). The combined placental microbiota showed the highest

prevalence of placenta microbiome in PE+/PD+ group (10/17=58.8%), and it was significantly higher than PE-/PD- groups (3/15=20%) (p=0.035). (Figure 1c). The abundances of combined placental microbiota showed significantly difference between two groups (PE+ vs PE) (p=0.021) and four subgroups (p=0.020) (Figure S4). Together, we confirmed our hypothesis that some periodontal disease-related bacteria are high in prevalence and abundance in PE patients.

Thirty-five out of 48 oral samples were successfully sequenced and included in the analysis. PERMANOVA analysis showed that the global oral microbiome profile had marginally significant difference between PE+ and PE- groups (p=0.09) and between PE+PD+ and PE-PD- groups (p=0.07). However, Differential taxa analysis by DESeq2 showed Haemophilus (p=0.15), Veillonella (p=0.020), Fusobacterium (p=0.046) were higher in PE+ than PE- patients (Figure 2a), and higher in PE+PD+ than PE-PD- patients (Figure 2b). However, the three taxa showed similar relative abundances in PE patients with and without PD, suggesting these oral bacteria may be associated with PE independent of PD. Importantly, logistic regression analysis showed Haemophilus but not Veillonella and Fusobacterium in subgingivae was associated with increased risk of PE (OR=2.11, 95% CI=1.11-4.52, p=0.032). This raises the possibility that the oral microbiome may potentially serve as a marker for PE.

The blood levels of CRP, LBP, TNF-α and IL-6 showed no statistical difference in patients with or without PE or PD (Table S1 and S2) even after stratification for term deliveries with Kruskal-Wallis. However, IL-8 levels were significantly higher in PE+/PD+ groups compared to PE+/PD- group (p=0.28,Figure 3a).

To examine the relationship between proinflammatory cytokine and placental microbiome, we compared cytokines levels in patients who had a detected and non-detected placental microbiome. Interestingly, we found there were elevated levels of blood IL-6 in placentas with

297 detected microbiome than placentas without detected microbiome (p= 0.028,Figure 3b),
298 indicating the presence of placental microbiome is associated with elevated systemic
299 inflammation.

300 **Discussion**

301 **Main findings**

302 By simultaneously profiling the oral and placental microbiome in the same pregnant women, we
303 identified oral-like bacteria in placenta from more than half of the patients with PE, compared to
304 less than 20% in patients without PE. The presence of placental microbiome was associated
305 with increased systemic inflammation in PE. High abundances of Haemophilus in oral cavity is
306 associated with increased risk of PE.

307

308 **Interpretation**

309 PE has long been considered as a placental disorder. Despite the lack of cohesive evidence of
310 healthy placenta microbiome[26,27], there is a consensus that the microbiome may be present in
311 placenta and contributes to pathogenesis in maternal or fetal diseases. After rigorous potential
312 contamination removal, we found seven bacterial taxa from more than half of the patients with
313 PE, compared to 20% in patients without PE. Sequencing the oral and placenta microbiome in
314 parallel in our study is advantageous than any previous studies that were only focused on either
315 placenta or oral microbiome. In addition, among the 7 placenta-associated microbiota, the
316 relative abundances of Haemophilus, Veillonella and Fusobacterium were significantly higher in
317 the oral samples of PE+ patients than PE- patients, further supporting an oral origin of these
318 placenta-associated bacteria. However, whole genome shotgun sequencing or other higher
319 taxonomic classification approaches as well as addition of vaginal and gut sampling sites are
320 needed to confirm exact origin of the bacteria.

321

Fusobacterium was previously found to be prevalent in placenta of PE patients by targeted PCR amplification[10,28]. Our results confirmed this finding and additionally revealed other PE-associated placenta microbiota through 16S sequencing. The underlying reasons why these specific taxa can disseminate to placenta merit future investigation. It is possible that both host factors and microbiota can contribute to microbiome seeding in placenta since hormonal changes during pregnancy in women and invading capacity of FadA adhesin from F. nucleatum[29] can increasing endothelial permeability.

Although the relative abundances of Haemophilus, Veillonella and Fusobacterium in the oral samples were significantly higher in PE+ patients than PE- patients, they were similar between PD+ and PD- patients. This suggests that these oral bacteria are potential markers for PE independent of PD status. Interestingly, when analyzing the oral microbiome, we found Haemophilus, but not Veillonella and Fusobacterium in subgingivae was a risk factor for PE. A prior study showed that Haemophilus was considered important in early biofilm formation, but not necessarily more prominent in caries[30]. This suggests in addition to bacteria associated with caries, non-caries associated microbiome may also be important for PE. Because we collected oral samples when PE was diagnosed, we could not conclude whether increased abundances of these oral bacteria are consequence or drivers of PE.

Previous studies have shown that proinflammatory cytokine mediators produced in pregnancy may be responsible for the exacerbated endothelial damage seen in women who develop preeclampsia in pregnancy[31-32]. However, our study did not observe any statistical difference in plasma levels of the cytokine mediators in either of the two groups (PE and PD) even after removing the potential confounder, preterm delivery, and evaluating only the term deliveries. However, subgroup analysis found that within PE+ patients, PE+/PD+ patients had higher IL-8 level in blood than PE+/PD- patients. This may have been a result of being underpowered for

the analysis of plasma levels among the designated inflammatory, as our study was only powered to evaluate the microbiome. We showed that detectable microbiota in placenta was associated with increased plasma levels of IL-6, suggesting presence of placenta microbiome may increase proinflammatory response or vice versa.

STRENGTHS AND LIMITATIONS

This study had several strengths. Special attention was given to the protocol development for collection of placenta specimens, and rigorous bioinformatic pipeline was employed to decrease the risk of contamination. This study is also one of the first studies to investigate the origins of the placental microbiome in women with preeclampsia and periodontal disease by comparing the bacterial taxonomic levels for multiple body sites. Prior to our study, this had not been investigated in detail.

One limitation of this study was the absence of collecting vagina and stool sampling for all women which restricts the analysis of the placental microbial colonization pattern to only oral microbiota. Lastly, our study was mainly an association study, and we were not able to show a causative link between the microbiome in women with periodontal disease and preeclampsia.

Future studies should include early and multiple time points sampling during the whole pregnancy will allow us to determine whether there is a causal role of these oral bacteria in PE in human. If this is confirmed, oral microbiome, with easy sampling in clinic, may be used as an alternative strategy to predict or prevent PE. Future longitudinal studies should shed light on causative factors between the placental microbiome and preeclampsia.

CONCLUSION

Our findings suggest that oral-associated placenta microbiome is prevalent in PE, and is associated with higher systemic inflammation. High abundances of Haemophilus in oral cavity is associated with increased risk of PE.

Disclosure of interest
There are no conflicts of interest associated with this article.
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Contribution to Authorship
SMC: Work conception, study design, sample collection and writing. **AB**: Sample collection and guidance. **HY**: Microbiome data analysis. **HP**: Sample preparation. **JR**: Microbiome data processing. **ST**: Dental exam. **EH**: Sample collection. **AH**: Dental exam. **CK**: Statistical analysis. **NB**: Dental exam. **KM**: microbiome sequencing. **WC**: Work conception and study design. **YZ**: Work conception, study design, microbiome data analysis and writing.

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Table/Figure Caption List

Figure 1. Placental microbiome in different groups. Ten bacterial genera identified in placenta after rigorous bioinformatic processing. Proportions of women who have a given detectable placental microbiota are illustrated by barplots (1a). Prevalence of placental microbiota among PE+ vs PE- women (p=0.018, Fisher exact test) (1b). Prevalence of placental microbiota among four subgroups (p=0.035, Fisher exact test) (1c).

Figure 2: Relative abundance of three microbiota in subgingival samples that are significantly different between PE+ and PE- patients (2a), and among four subgroups (2b).

Figure 3: Comparison of inflammatory cytokines in four subgroups and in patients with and without detectable placental microbiota. Il-8 levels in blood are significantly higher in PE+/PD+ patients than PE+/PD- patients (p=0.028, Wilcox sum rank) (3a). Il-6 levels in blood are significantly higher in patients with detectable placental microbiota than those without (p=0.028, Wilcox sum rank test) (3b).

Fig S1: Bioinformatic workflow for placental microbiome identification. The protocol covers each data processing steps to remove potential microbial contamination from extraction controls, operation room control, delivery mode, as well as additional filtering step to remove relatively low abundant taxa.

Figure S2. Flow Diagram of participants and samples.

Figure S3. Principal component analysis (PCA) of placental microbiome by mode of delivery. The placental microbiome obtained from C-section (red) and vaginal delivery (blue) is separated clearly in the first and second component of PCA analysis.

Figure S4. Relative abundances of seven placental microbiome across four groups.

593 **Table 1: Clinical characteristics of the 54 participants by subgroups (PE+/PD+, PE+/PD-,**
594 **PE-/PD+, PE-/PD-)**

Variable	PE+/PD+ (n=19)	PE+/PD- (n=10)	PE-/PD+ (n=7)	PE-/PE- (n=18)	P-Value
Age	29.32 ± 5.39	29.2 ± 5.71	31.57 ± 7.79	27.67 ± 5.77	0.517
Birth Weight (grams)	2587.53 ± 764.4	2333 ± 836.24	3206.29 ± 442.91	3376.28 ± 563.29	**0.001**
Ethnicity					0.712
Hispanic	7 (36.8%)	2 (20%)	3 (42.9%)	8 (44.4%)	
Non-Hispanic	12 (63.1%)	8 (80%)	4 (57.1%)	10 (55.6%)	
Gestational Age (weeks)	35.84 ± 2.71	35.5 ± 3.5	38.14 ± 1.68	38.11 ± 1.37	**0.008**
Mode of Delivery					0.247
C-Section	10 (52.6%)	5 (50%)	3 (42.9%)	4 (22.2%)	
Vaginal	9 (47.4%)	5 (50%)	4 (57.1%)	14 (77.8%)	
NICU Admission					0.151
No	12 (63.2%)	8 (80%)	7 (100%)	16 (88.9%)	
Yes	7 (36.8%)	2 (20%)	0 (0%)	2 (11.1%)	
PrePregnancy BMI	31.53 ± 4.4	30.4 ± 7.71	32.43 ± 7.79	32.28 ± 7.09	0.885
Preterm					0.073
No	10 (52.6%)	8 (80%)	6 (85.7%)	16 (88.9%)	
Yes	9 (47.4%)	2 (20%)	1 (14.3%)	2 (11.1%)	
Race					0.650
Asian	0 (0%)	2 (20%)	0 (0%)	0 (0%)	
Black	7 (36.8%)	4 (40%)	2 (28.6%)	5 (27.8%)	
Caucasian	5 (26.3%)	2 (20%)	2 (28.6%)	5 (27.8%)	
Hispanic	7 (36.8%)	2 (20%)	3 (42.9%)	8 (44.4%)	
Smoking					0.291
Former	5 (26.3%)	1 (10%)	2 (28.6%)	1 (5.6%)	
No	13 (68.4%)	9 (90%)	5 (71.4%)	17 (94.4%)	
Yes	1 (5.3%)	0 (0%)	0 (0%)	0 (0%)	

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