Gut microbiota dysbiosis in preeclampsia patients in the second and third trimesters

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Abstract

Background: Preeclampsia (PE) is a serious complication that affects maternal and perinatal outcomes. However, the mechanisms have not been fully explained. This study was designed to analyze longitudinal gut microbiota alterations in pregnant women with and without PE in the second (T2) and third trimesters (T3).

Methods: In this nested case-control study, which was conducted at Nanjing Maternity and Child Health Care Hospital, fecal samples from 25 PE patients (25 fecal samples obtained in T2 and 15 fecal samples obtained in T3) and 25 matched healthy controls (25 fecal samples obtained in T2 and 22 fecal samples obtained in T3) were collected, and the microbiota were analyzed using 16S rRNA gene sequencing. The diversity and composition of the microbiota of PE cases and controls were compared.

Results: No significant differences in diversity were found between the PE and control groups (P > 0.05). In the control group, from T2 to T3, the relative abundances of Proteobacteria (median [Q1, Q3]: 2.25% [1.24%, 3.30%] vs. 0.64% [0.20%, 1.20%], Z = 3.880, P < 0.05), and Tenericutes (median [Q1, Q3]: 0.12% [0.03%, 3.10%] vs. 0.03% [0.02%, 0.17%], Z = -2.369, P < 0.05) decreased significantly. In T2 was lower than in T3 (median [Q1, Q3]: 18.16% [12.99%, 30.46%] vs. 31.09% [19.89%, 46.06%], Z = -2.417, P < 0.05). However, in T3, the relative abundance of Firmicutes was significantly lower in the PE group than in the control group (median ± standard deviation: 60.62% ± 15.17% vs. 75.57% ± 11.53%, t = 3.405, P < 0.05). The relative abundances of Bacteroides, Proteobacteria, and Enterobacteriaceae were significantly higher in the PE group than in the control group (median [Q1, Q3]: 31.09% [19.89%, 46.06%] vs. 18.24% [12.90%, 32.04%], Z = -2.537, P < 0.05; 1.52% [1.05%, 2.61%] vs. 0.64% [0.20%, 1.20%], Z = -3.310, P < 0.05; 0.75% [0.20%, 1.00%] vs. 0.01% [0.004%, 0.023%], Z = -4.152, P < 0.05). Linear discriminant analysis combined effect size measurements showed that the relative abundances of the phylum Bacteroidetes, class Bacteroidia and order Bacteroidales were increased in the PE group, while those of the phylum Firmicutes, the class Clostridia, the order Clostridiales, and the genus unidentifed Lachnospiraceae were decreased in the PE group; and these differences were identified as taxonomic biomarkers of PE in T3.

Conclusion: From T2 to T3, there was an obvious alteration in the gut microbiota. The gut microbiota of PE patients in T3 was significantly different from that of the control group.

Keywords: Gut microbiota; Preeclampsia; Inflammation; Second trimester; Third trimester

Introduction

Preeclampsia (PE) is a pregnancy-specific multisystemic disorder characterized by hypertension and either proteinuria or other multisystemic complications after 20 weeks of gestation and is one of the major causes of maternal and perinatal mortality worldwide, affecting 3% to 8% of all pregnancies in the world.[1] Currently, there is no known cure other than delivery. Although endothelial dysfunction, disturbed placentation, oxidative stress, and an exaggerated inflammatory response to pregnancy have been suggested to be involved in the development of PE, the precise and specific pathogenesis of PE remains unclear because of its heterogeneity and complexity.[2]

The gut microbiota, which is a complex and massive community of microorganisms species living in the digestive tract, plays an important role in host metabolism, immunity, and nutrition absorption.[3] Imbalance in the gut microbiota composition is linked to host metabolic abnormalities and systemic inflammation, which contributes to the development of many diseases, such as obesity, type 2 diabetes, atherosclerosis, nonalcoholic fatty liver disease, hypertension, and chronic kidney diseases.[4-9]
PE is characterized by hypertension and multiple organ dysfunction after 20 weeks of gestation with normal blood pressure in the first trimester (T1). However, the factor(s) that cause elevated blood pressure and the specific etiology remain unclear. Kell et al.\(^{12}\) found that dormant microbes, which can become reactivated, shed inflammagens such as lipopolysaccharides (LPS) and thereby initiate inflammatory cascades, played an important etiological role in PE. Currently, only a few studies have investigated the relationship between gut microbiota dysbiosis and PE. Liu et al.\(^{13}\) found that there was a significant structural shift of the gut microbiota in PE patients, which might be associated with the occurrence and development of the disease. One recent study found that PE diagnosed in the third trimester (T3) of pregnancy was associated with a disrupted gut microbiota composition compared with that in women with uncomplicated pregnancies.\(^{14}\) Our recent study also found similar microbiota dysbiosis in patients with PE.\(^{15}\) However, all these studies have focused on the imbalances in the gut microbiota composition at the onset of PE. Given the profound alterations in composition occurring from T1 to T3, only prospective studies that focus on the longitudinal differences occurring during gestation in different trimesters could determine the relationship between gut microbiota dysbiosis and disease progression.\(^{16}\) Because the gut microbiota of T1 is similar in many aspects to that of healthy nonpregnant controls, we mainly focused on gut microbiota alterations in T2 and T3, and we conducted a nested case-control study to compare the composition of the gut microbiota in pregnant women with and without PE using 16S rRNA gene sequencing to investigate the association of PE with disrupted gut microbiota in T2 and T3.

### Methods

#### Ethical approval

This study was conducted at Nanjing Maternity and Child Health Care Hospital from January 2018 to December 2018. This study was reviewed and approved by the ethics review board of Nanjing Maternity and Child Health Care Hospital (approval number, 2017-003). Signed consent was acquired from all subjects for the use of their data and samples for scientific purposes.

#### Patients and groups

Two thousand pregnant women were included in the study at the T1 visit (6–8 weeks’ gestation), and the maternal characteristics such as maternal age, height, weight, and gestational age were recorded. The pre-pregnancy body mass index (BMI) was determined as the weight (kg) divided by the square of the height (meters).

A nested case-control study was conducted. Twenty-five women who subsequently developed PE were included in the case group (PE group), and 25 healthy pregnant women with similar ages and BMIs were chosen as negative controls with a 1:1 match. The inclusion criteria for the PE group matched the diagnostic criteria of the American College of Obstetricians and Gynecologists for PE,\(^{17}\) including a blood pressure \(\geq 140/90\) mmHg for two consecutive measurements at least 4 h apart and proteinuria \(\geq 300\) mg, or in the absence of proteinuria, any of the following conditions: thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema or cerebral or visual symptoms. The exclusion criteria for pregnant women were as follows:

1. multiple pregnancies;
2. diabetes, chronic hypertension, renal disease, or other complications before pregnancy; and
3. the use of antibiotics, glucocorticoids, or immunosuppressive drugs within 1 month of the time of sample collection.

Fecal samples were collected at the T2 and T3 visits at 20–24 and 32–34 weeks’ gestation, respectively. The stool samples of the healthy control group in T2 (SC group, \(n = 25\)) and T3 (TC group, \(n = 22\)) and those of the PE group in T2 (SP group, \(n = 25\)) and T3 (TP group, \(n = 15\)) were collected for gut microbiota analysis [Figure 1].

#### Sample collection, DNA extraction, and microbiota analysis

Fecal samples were collected in tubes by the participants and then frozen at \(-20^\circ\)C. The samples were transferred to the laboratory on dry ice within 24 h of collection and stored at \(-80^\circ\)C until DNA extraction.

Total fecal DNA was extracted using the cetyltrimethylammonium bromide (CTAB)/ sodium dodecylsulfate (SDS) method. Universal primers (515F and 806R) linked to indices and sequencing adaptors were used to amplify the V4 region of the 16S rRNA gene. The sequencing libraries were generated using the Ion Plus Fragment Library Kit (Thermo Fisher Scientific, USA) according to the manufacturer’s recommendations. Finally, the library was sequenced on an Ion S5TM XL platform (Thermo Fisher Scientific, USA), and 400 bp/600 bp single-end reads were generated.

After quality filtering of the raw reads by using the Cutadapt quality control process (V1.9.1, http://cutadapt.readthedocs.io/en/stable/) and removing the chimeric sequences using the UCHIME algorithm (UCHIME Algorithm, http://www. Drive5.com/usearch/manual/uchime_algo.html), the clean reads were finally obtained. Sequence analysis was performed by Uparse software (Uparse v7.0.1001, Robert C. Edgar, USA, http://drive5. com/uparse/). Sequences with \(\geq 97\%\) similarity were assigned to the same operational taxonomic units (OTUs). The Silva Database (https://www.arb-silva.de/) based on the Mothur algorithm was used to annotate the taxonomic information.
Statistical analysis

SPSS (ver. 21.0, SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The Shapiro-Wilk test was used to check the normality of the data distribution. We used the mean ± standard deviation (SD) to represent the data that exhibited a normal distribution and the median and interquartile range (median [Q1, Q3]) to represent the data that showed a skewed distribution. Comparisons between groups were performed with Student’s t test for quantitative variables that showed normal distributions. The Wilcoxon rank sum test was used to compare the abundance distributions of different taxonomic compositions. Alpha and beta diversity metrics were calculated for each sample using the QIIME software (version 1.9.1) based on the rarefied OTU counts. The QIIME workflow begins with raw sequencing data plus metadata describing the samples, and can provide tabular output including diversity measures. The Shannon and Simpson diversity indices were used to estimate the α diversity, which represented the species abundance in a single sample. The β diversity, which was based on the weighted UniFrac metric, was used to evaluate differences in the species complexity in the samples. Based on the UniFrac phylogenetic distance, the test of the significance of the clustering of samples in the study was carried out by one-way analysis of similarities (ANOSIM). Principal coordinate analysis (PCoA) was performed to obtain the principal coordinates and visualize the complex multidimensional data, and PCoA plots based on weighted UniFrac distance analysis were used to evaluate the beta diversity (similarities or differences between individuals in communities). Linear discriminant analysis (LDA) combined effect size measurements (LEfSe) analysis was applied to identify the differentially abundant bacterial taxa among the groups. Only those taxa for which a log LDA score >4.0 was obtained were ultimately included. P < 0.05 was considered to be statistically significant.

Results

Characteristics of the PE patients and the control group

The baseline characteristics of the PE group and the control group are summarized in Table 1. There were no differences in age, BMI, or gestational weeks between the two groups [Table 1]. In the PE group, ten stool samples from the T3 visits were not available for analysis. However, the age and BMI of those ten cases (34.6 ± 4.8 years, 23.5 ± 2.0 kg/m²) were not significantly different...
from those of the remaining 15 cases (31.7 ± 3.5 years, 23.9 ± 1.6 kg/m²) during T3 (P > 0.05).

Gut microbiota in the PE group and the control group
A total of 87 fecal samples were obtained for sequencing. At the phylum level, the majority of the OTUs were found to belong to **Firmicutes**. **Bacteroidetes** was the next most abundant phylum, followed by **Actinobacteria**, **Tenericutes**, and **Proteobacteria**. We calculated the abundances of the five most predominant phyla in the microbial community structure [Figure 2A]. The remaining bacterial population belonged to the other phyla (**Fusobacteria**, **Euryarchaeota**, unidentified Bacteria, **Verrucomicrobia**, and **Melainabacteria**), which had a relative abundance of less than 1% in the four groups [Figure 2A]. At the family level, the top ten taxa that were detected are visualized in Figure 3A. **Lachnospiraceae**, **Bacteroidaceae**, **Veillonellaceae**, **Ruminococcaceae**, and **Prevotellaceae** comprised more than half of the bacterial community.

Alters in gut microbiota from T2 to T3
In the control group, from T2 to T3, at the phylum level, the relative abundances of **Proteobacteria** (median [Q1, Q3]: 2.25% [1.24%, 3.30%] vs. 0.64% [0.20%, 1.20%], Z = −3.880, P < 0.05, Figure 2B) and **Tenericutes** (median [Q1, Q3]: 0.12% [0.03%, 3.10%] vs. 0.03% [0.02%, 0.17%], Z = −2.369, P < 0.05, Figure 2B) significantly decreased. In the control group, at the family level, the top 10 OTUs belonged to **Lachnospiraceae**, **Bacteroidaceae**, **Veillonellaceae**, **Ruminococcaceae**, **Prevotellaceae**, **Bifidobacteriaceae**, **Erysipelotrichaceae**, **Enterobacteriaceae**, **Peptostreptococcaceae**, and **Streptococcaceae**. The relative abundances of **Lachnospiraceae**, **Veillonellaceae**, **Prevotellaceae**, **Erysipelotrichaceae**, **Streptococcaceae**, and **Peptostreptococcaceae** increased, but with no significant difference (P > 0.05) from T2 to T3. The relative abundances of **Ruminococcaceae**, **Bacteroidaceae**, **Bifidobacteriaceae**, and **Enterobacteriaceae** decreased, with no significant difference except for **Enterobacteriaceae** (median [Q1, Q3]: 0.95% [0.25%, 1.64%] vs. 0.01% [0.004%, 0.023%], Z = −5.685, P < 0.05) from T2 to T3 [Figure 3B].

The PE group showed different variations of gut microbiota from T2 to T3. In the PE group, at the phylum level, the relative abundance of **Bacteroidetes** in T2 was lower than that of T3 (median [Q1, Q3]: 18.16% [12.99%, 30.46%] vs. 31.09% [19.89%, 46.06%], Z = −2.417, P < 0.05). At the family level, the top 10 OTUs were the same as that of the control group and there were no significant differences between T2 and T3 [Figure 3B].

Gut microbiota dysbiosis in PE patients
In T2, at the phylum level, the relative abundances of **Firmicutes**, **Bacteroidetes**, **Actinobacteria**, **Proteobacteria**, **Fusobacteria**, **Euryarchaeota**, unidentified Bacteria, **Verrucomicrobia**, and **Melainabacteria** had a relative abundance of less than 1% in the four groups [Figure 2A]. At the family level, the top ten taxa that were detected are visualized in Figure 3A. **Lachnospiraceae**, **Bacteroidaceae**, **Veillonellaceae**, **Ruminococcaceae**, and **Prevotellaceae** comprised more than half of the bacterial community.
and Tenericutes showed no significant differences between the PE group and the control group. Similarly, at the family level, the relative abundances of the top ten taxa showed no significant differences between the two groups.

But in T3, profoundly altered gut microbial compositions were observed between the two groups. At the phylum level, the relative abundance of Firmicutes was significantly lower in the PE group than in the control group (mean ± SD: 60.62% ± 15.17% vs. 75.57% ± 11.53%, \( t = -3.405, P < 0.05 \)). The relative abundances of Bacteroidetes and Proteobacteria were significantly higher in the PE group than in the control group (median [Q1, Q3]: 31.09% [19.89%, 46.06%] vs. 18.24% [12.90%, 32.04%], \( Z = -2.537, P < 0.05 \); median [Q1, Q3]: 1.52% [1.05%, 2.61%] vs. 0.64% [0.20%, 1.20%], \( Z = -3.310, P < 0.05 \)). There were no statistically significant differences in the abundances of the other taxa at the family level between the two groups [Figure 3B].

The \( \alpha \) diversity and \( \beta \) diversity

The Shannon and Simpson index of the PE group were slightly lower than those in the control group in T2 and T3, but there was no statistical significance [Figure 4].

The present study showed that both study groups were closer together in terms of ordination, and a separation between the samples from the PE group and the control group could be observed according to the PC1 and PC2 scores, which accounted for 34.39% and 15.01% of the total variation, respectively [Figure 5]. PCoA could discriminate the TP samples from the TC samples, which revealed a distinct clustering of the microbiota composition between the PE group and the control group in T3, and the ordination axis (PC1 and PC2) explained
approximately 50% of the variability. However, there was a substantial overlap between the SP samples and the SC samples, which suggested that there was no significant difference between the PE group and the control group in T2.

ANOSIM analysis showed that the bacterial microflora compositions of the SP group and the SC group, as well as those of the TP group and the TC group, were significantly different ($P = 0.040$ and $P = 0.016$, respectively).

**Taxonomic biomarkers**

To further investigate which taxa served as biomarkers among the groups, we applied LEfSe to explore the significant changes and relative richness of the bacterial community. We identified no taxonomic biomarkers of PE in T2 between the PE group and the control group. However, we found that the relative abundances of the phylum *Bacteroidetes*, class *Bacteroidia* and order *Bacteroidales* were increased in the PE group, while those of the phylum *Firmicutes*, class *Clostridia*, order *Clostridiales*, and genus unidentified *Lachnospiraceae* were decreased in the PE group in T3, and these differences were identified as taxonomic biomarkers of PE in T3. The results are presented in green and red, which indicate an increase and a decrease in the abundance in the PE group, respectively ($\text{LDA} > 4.0$) [Figure 6A and 6B].

**Discussion**

In this study, we analyzed the shift in the gut microbiota of pregnant women between T2 and T3, and then we compared the changes in the gut microbiota in PE patients and the control group. Our study demonstrated that there were obvious alterations in the composition of the gut microbiota between T2 and T3 in normal pregnant women, and there was no significant difference in the gut microbiota in T2 between the PE group and the control group, while the gut microbiota in T3 in PE patients remarkably differed from that in normal pregnant women.
PE, which is characterized by hypertension and proteinuria after 20 weeks of gestation, is a troublesome disease for clinicians because of its high heterogeneity and unclear etiology. PE shares conventional risk factors with cardiovascular diseases, such as hypertension, obesity, dyslipidemia, and insulin resistance. The pathogenesis of PE has not been fully elucidated, but much progress has been made in recent decades. To date, a few studies have investigated gut microbiota dysbiosis in PE patients. Lv et al.\[14\] demonstrated that disrupted gut microbiota in PE patients was associated with maternal clinical features, and these alterations in the gut microbiota persisted 6 weeks postpartum. Studies of the reduction of the risk of PE through probiotic supplementation confirmed that PE is associated with gut microbes.\[21\] These findings suggested that regulating the intestinal microbiota through probiotics may play a role in the prevention of PE.

Previous studies found that there was a dramatic remodeling of the gut microbiota over the course of pregnancy, with no changes in the gut microbiota in T1 but a substantial shift in the phylogenetic composition and structure in T3.\[16\] In this nested case-control study, fecal sampling in T2 and T3 was used to analyze the gut microbiota to investigate the longitudinal differences between PE patients and healthy controls. To the best of our knowledge, this is the first clinical study that revealed the longitudinal shift in the gut microbiota in women with and without PE from T2 to T3. In our study, we found that from T2 to T3, the relative abundances of Proteobacteria, Tenericutes (at the phylum level), and Enterobacteriaceae (at the family level) were significantly different between the SC group and the TC group. We observed no significant differences in the alterations of gut microbial patterns in T2 between patients with and without PE, but we did observe a substantial difference in the alterations in the gut microbial pattern in T3 between the TP group and the TC group.

In our study, the α diversity indices (richness and diversity) of the fecal microbiota in patients with PE were lower than those of the fecal microbiota in the control group in T2 and T3, but there was no statistical significance, which was similar to the results of a study by Liu et al.\[13\] Koren et al.\[16\] reported that low diversity in microbiomes may be an underlying cause of increased inflammation. Moreover, the β diversity index in patients with PE differed significantly from that in the control group in T3.

The fecal microbiota in patients with PE in T3 showed a significant increase in the abundance of Bacteroidetes. Members of Bacteroidetes have been reported to be associated with immunity and metabolic processes.\[22\] Bacteroidetes, a type of gram-negative bacteria, is the main contributor to LPS biosynthesis. Therefore, high abundances of Bacteroidetes may induce increased inflammation during pregnancy. There are a number of animal models in which LPS (also known as “endotoxin”) was used experimentally to induce a condition resembling PE.\[23\] LPS can activate inflammation mediated by the Toll-like receptor 4 (TLR4) signaling pathway in PE.\[24\] In PE patients, levels of inflammatory factors such as tumour necrosis factor (TNF)-α and interleukin (IL)-6 are increased in the circulation and in trophoblast cells of the placenta, while levels of anti-inflammatory factors such as IL-10 and IL-4 are decreased.\[25\] In our previous study, we found that in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, microbial gene functioning related to LPS biosynthesis was higher in the fecal microbiome of the PE group than that in the control group, and the fecal and plasma LPS concentrations in PE patients were higher than those in the healthy controls.\[15\] This chronic peripheral and placental inflammation plays an important role in the pathogenesis of PE.\[26\]

The fecal microbiota in T3 in patients with PE showed a significant reduction in the abundance of Firmicutes.
**Firmicutes**, most of which are gram-positive, are capable of producing several SCFAs, including lactate, acetate, butyrate, and propionate, which may impact renal sensory nerves and blood pressure. SCFAs are fermentation byproducts of carbohydrates and proteins that help maintain the integrity of the intestinal brush border, reduce systolic blood pressure and serum cholesterol, and improve insulin sensitivity. In our study, the fecal microbiota in patients with PE showed a significant reduction in **Clostridia** and **Clostridiales** (members of **Firmicutes**), which has also been found in other inflammatory diseases, such as inflammatory bowel diseases and Behçet syndrome.

The fecal microbiota in T3 in patients with PE showed a significant increase in the abundance of **Proteobacteria**. **Proteobacteria**, rather than **Bacteroidetes** or **Firmicutes**, emerge as a major source of variable genes (e.g., LPS), the abundance of **Proteobacteria** may capture more of the functional variation. **Proteobacteria** are believed to be important contributors to inflammation associated with metabolic diseases in adults and have been found to be increased in patients with chronic intestinal inflammation. A study by Litvak et al. found that a dysbiotic expansion of **Proteobacteria** was a potential diagnostic microbial signature of epithelial dysfunction in the colon. A recent study found that **Enterobacteriaceae** species (**Enterococcus gallinarum**) could activate pro-inflammatory pathways and alter gut barrier-related molecules in small intestinal tissue during translocation into the internal organs. In our study, we also found that a profoundly altered gut microbiota was associated with increased inflammation in T3 in patients with PE, and these alterations occurred before the onset of PE, which indicated the possible potential role of disrupted gut microbiota in the development of PE; and the underlying mechanisms need to be clarified and precisely verified in an animal model.

The limitations of the present study should also be considered. First, the sample size was limited, and further studies with larger sample sizes will be needed to confirm our results. Second, there was no information obtained from the diet questionnaire on nutrient intake in the study groups. Third, the study only described the phenomenon of gut microbiota dysbiosis in T3 in patients with PE without further investigation of the underlying mechanisms.

In conclusion, in PE patients, from T2 to T3, there was a profound alteration of the gut microbiota. The gut microbiota in T3 in PE patients was different from that in the control group. Gut microbiota dysbiosis in T3 in PE patients was related to inflammation, which might play an important role in the onset and development of PE. We hypothesize that the immune-inflammatory axis may act as the bridge between the gut microbiota and the development of PE.

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**Conflicts of interest**

None.

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