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The preterm placental microbiome varies in association with excess maternal gestational weight gain

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Abstract

OBJECTIVE—Although a higher maternal body mass index is associated with preterm birth, it is unclear whether excess gestational weight gain (GWG) or obesity drives increased risk. We and others have shown that the placenta harbors microbiota, which is significantly different among preterm births. Our aim in this study was to investigate whether the preterm placental microbiome varies by virtue of obesity or alternately by excess GWG.

STUDY DESIGN—Placentas ($n = 320$) were collected from term and preterm pregnancies. Genomic DNA was extracted and subjected to metagenomic sequencing. Data were analyzed by clinical covariates that included the 2009 Institute of Medicine's GWG guideline and obesity.

RESULTS—Analysis of 16S recombinant RNA-based metagenomics revealed no clustering of the microbiome by virtue of obesity ($P = .161$). Among women who spontaneously delivered preterm, there was again no clustering by obesity ($P = .480$), but there was significant clustering by excess GWG ($P = .022$). Moreover, among preterm births, detailed analysis identified microbial genera (family and genus level) and bacterial metabolic gene pathways that varied among pregnancies with excess GWG. Notably, excess GWG was associated with decreased microbial folate biosynthesis pathways and decreased butanoate metabolism (linear discriminate analysis, >3.0 -fold).

CONCLUSION—Although there were no significant alterations in the microbiome by virtue of obesity per se, excess GWG was associated with an altered microbiome and its metabolic profile among those women who experienced a preterm birth.

Keywords

excess gestational weight gain; maternal obesity; meta-genomics; microbiome; preterm birth

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The human microbiome encompasses the totality of the microbes that live on and within our bodies and is the core of the emerging fields of metagenomic science and medicine (Table 1).^{1,2} In 2012, the *healthy microbiome* in nonpregnant³⁻⁵ and pregnant^{6,7} women was defined across body sites and was recently expanded to now include in-depth characterization of the placental microbiome⁸ and longitudinal profiling of the vaginal (posterior fornix) microbiome throughout pregnancy.⁹⁻¹¹

Contrary to the commonly held paradigm that the upper reproductive tract and placenta are “sterile,” we and others have shown that microbiota is present, even in the absence of any clinical evidence of intraamniotic infection.^{8,12-25}

Using metagenomic approaches, we have observed that the placental microbiome profile significantly varied in association with spontaneous preterm birth⁸ and that the taxonomic profiles that are associated with term or preterm pregnancies were accompanied by variations in bacterial-encoded metabolic pathways. Collectively, these findings are consistent with multiple other published reports over the years that have demonstrated the presence of microbes in previously held “sterile” sites, including the endometrium,¹⁹⁻²¹ placenta,^{8,13-18,22} and chorion/amnion and amniotic fluid.²³⁻²⁵

Although the cause of preterm birth is multifactorial, its association with infection and inflammation has long been suspected.^{22,26-44} The aforementioned variations in commensal microbiota-driven metabolism may result in the placental microbiome emerging as a key mediator in preterm birth. For example, although bacterial species that are present in preterm pregnancies may not be pathogenic necessarily, a relatively altered microbial community structure (dysbiosis) may convey an environment of localized inflammation that results in preterm birth.^{29,30}

Previously, obesity has been shown to be associated with variation in placental^{45,46} and systemic⁴⁷ inflammation. Additionally, obesity is associated with preterm birth.⁴⁸⁻⁵¹ Altogether, these observations have been assumed to suggest that obesity may contribute to preterm birth through increased inflammation. However, excess gestational weight gain (GWG) has also been associated with increased risk of preterm birth^{50,53} and alterations in metabolic markers.⁵⁴ Thus this begs the question, is it obesity or excess GWG that modulates the triad of inflammation, an altered placental microbiome, and spontaneous preterm birth?

We hypothesized that excess GWG (but not obesity per se) would associate with distinct alterations in the placental microbiome and that these alterations would be pronounced significantly among women who spontaneously deliver preterm. The aims of this study were (1) to discriminate whether maternal obesity or GWG was associated with significant variation in the placental microbiome profile, (2) to determine whether this was observed in both the term and preterm interval, and (3) to determine which placental microbial metabolic pathways were affected by excess maternal GWG.

Materials and Methods

Subjects and sample materials

This study was approved by the Institutional Review Board at Baylor College of Medicine (H-26589) and Harris Health System and was performed with the use of specimens from our universal perinatal database and biospecimen repository (PeriBank database, Institutional Review Board H-26364; Baylor College of Medicine). All pregnant women who come to labor and delivery for delivery are recruited for enrollment into PeriBank, which is Baylor College of Medicine's universal perinatal database and biospecimen repository. A detailed description of PeriBank and the selection criteria for this study, including the clinical metadata that are extracted, is available in our previous publication on the placental microbiome.⁸ Subjects for this study were recruited between August 2011 and November 2012 by trained PeriBank study personnel. As part of the consent process, we discussed with participants the potential risks of participation, including the physical risks that are associated with specimen collection and the possibility that protected health information or deidentified project data that are stored in a public repository could be released accidentally. The protocol and consent form described the precautions that are taken to reduce these risks. Additional protections for participants included coding genomic and metagenomic specimens and sequence data, with the use of controlled-access repositories for extracted nucleic acids and deidentified medical and human genome sequence data, and making efforts to remove any human sequence traces from the microbial data that are deposited in open access databases. If a participant withdrew consent after providing specimens, the remaining specimens and extracted nucleic acids were to be destroyed; however, any metagenomic sequence data that were already published in open access databases could not be retracted.

After written consent was obtained, clinical information was extracted from the medical record and subject interviews. The parent study (H-26589) was a case-cohort study that used 2 case-cohorts. Briefly, for the first case-cohort (preterm birth), the PeriBank database was queried to identify subjects who spontaneously delivered at <37 weeks' gestation by best obstetric estimate. *Spontaneous preterm birth* was defined by standard obstetrics definitions as adequately documented gestational age >24 and <37 weeks' gestation (by last menstrual period with <20-week gestation sonogram or by <14-week sonogram with subsequent second-trimester sonogram) and no evidence of maternal or fetal comorbidity that indicated induction of labor (such as preeclampsia or placenta previa). For the second case-cohort (antenatal infection), the database was queried for women with laboratory evidence of antenatal infection, which included sexually transmitted diseases, urinary tract infections, skin abscesses, appendicitis, periodontal disease, or bacterial meningitis. The control groups for cohort comparison were created by a 1:1 match with previously identified case subjects; women were matched for gestational age (except in the preterm case-cohort), mode of delivery, race/ethnicity, body-mass index, age, and diabetic status. In this manner, we enabled preterm birth cases potentially to carry a diagnosis of a remote history of antenatal infection and vice versa; this is consistent with the principles of a population-based case-cohort study design. Subjects with fatal fetal anomalies, fetal growth restriction, prelabor preeclampsia, placenta previa, and multifetal gestation were excluded because of the

likelihood of indicated preterm birth. Women with intrapartum chorioamnionitis that was suspected clinically and had been treated with intrapartum antibiotics and confirmed by positive maternal or infant culture were excluded from this analysis.

For the present study, extracted and analyzed clinical metadata included self-reported and/or documented prepregnancy weight, documented early pregnancy (defined as <20 weeks' gestation) weight, documented maternal delivery weight, and documented maternal height. Self-reported prepregnancy weight frequently is used in the literature,^{50,55,56} and the use of self-reported prepregnancy weight has been reported to be within a few pounds of actual prepregnancy weight.⁵⁷ The use of first-trimester weight as a surrogate when prepregnancy weight is unknown is also a reasonable substitution when the prepregnancy weight is unknown.⁵⁸ Of the 237 subjects in this study, the prepregnancy weight was used in 130 of the women (54.8%). Of the remaining 107 women, the first-trimester weight was used in 98 of 107 pregnancies (91.6%); weights up to 20 weeks gestation were used for the remaining 9 women. Ergo, of the 237 women who were included in the analysis herein, 228 of 237 women (96.2%) used prepregnancy or first-trimester documented weights, although only 3.8% arose from documented weight in the early midtrimester (>14 and <20 weeks' gestation). Patients who had none of these variables were excluded because data were insufficient to calculate GWG. Other extracted clinical metadata included gravidity, parity, the presence or absence of *Streptococcus agalactiae*, the presence of antepartum infections and antibiotic use (ie, sexually transmitted infection, urinary tract infection, skin abscesses, appendicitis, periodontal disease, or bacterial meningitis), routine demographic variables and maternal health comorbidities (eg, age, race/ethnicity, smoking status, diabetes mellitus, hypertensive, hypothyroid status), and pregnancy outcomes (ie, gestational age at delivery, mode of delivery, presence of gestational hypertensive, and preeclamptic disorders). For the purposes of this study, all classes of diabetes mellitus and all hypertensive disorders were assessed in aggregate.

GWG calculation

GWG was calculated by subtracting the prepregnancy or early pregnancy weight (defined as <20 weeks' gestation) from the delivery weight. Baseline body mass index (BMI) was determined with the use of prepregnancy or early pregnancy (<20 weeks' gestation) weight. As noted previously, 96.2% of study subjects had a documented prepregnancy or first-trimester maternal weight. To calculate whether GWG exceeded recommendations, we used previously validated, BMI-specific formulas that were derived from the Institute of Medicine.^{59,60} *Excess weight gain* was defined as weight gain that was in excess of the maximum recommended weight gain for a given gestational age and prepregnancy BMI. Specifically, the Institute of Medicine recommends a maximum of 4.4 pounds in the first trimester for all women, regardless of BMI; thus, this was used as the first-trimester component and was added to the maximum recommended weekly weight gain, which is BMI-specific. These values are 1.3, 1, 0.7, and 0.6 pounds per week for underweight, normal weight, overweight, and obese women, respectively. This rate was multiplied by the number of weeks of pregnancy from the second trimester through delivery to yield the formulas shown in Table 2, which shows the maximum recommended GWG by BMI^{59,60}; GWG that exceeded the maximum recommended GWG was defined as *excessive GWG*. The women

were then assigned to 2 cohorts: those with excess GWG (excess GWG) and those without excess GWG (no excess GWG).

Placental samples

All samples were collected by placental pathology-trained personnel according to the PeriBank Manual of Procedures. As described in the parent publication, after the placenta is delivered by the obstetrics provider, it is immediately passed off to PeriBank personnel in a clean container.⁸ In a separate room, 4–6 1 cm × 1 cm × 1 cm cuboidal sections are excised circumferentially from separate areas of the placenta; each section was located approximately 4 cm from the cord insertion site by PeriBank personnel who wore facial masks and gloves and used sterile scalpels and tissue forceps.⁸ These clean and sterile conditions are part of the standardized technique that is used in the banking of all PeriBank specimens.⁸ After excision, the placental surfaces, namely the maternal decidua and the fetal chorionamnion, were excised and discarded to avoid potential contamination from maternal, fetal, or environmental surface exposure during delivery (vaginal or cesarean) or at any time during transport.⁸

Metagenomics sequencing

This study was a subgroup analysis of our initial case-cohort study of the placental microbiome among subjects with antepartum infections or subjects with spontaneous preterm delivery (<37 weeks' gestation).⁸ In the previous study, after we identified subjects with any antepartum infections or spontaneous preterm birth, we then queried the database for 1:1 matches for women without antepartum infection or preterm birth. As described in our previous publication,⁸ the placental samples from 48 women were selected randomly from the overall case-cohort to undergo whole-genome shotgun sequencing. Approximately one-fourth of these samples were from the preterm birth case-cohort; one-fourth of the samples were from the remote antenatal infection case-cohort, and the remaining one-half of the samples were from the control subjects. Of note, the nested 48 subjects did not significantly differ from their derived cohort counterparts by any measure other than their case definition (not for maternal age, mode of delivery, race/ethnicity, BMI, maternal comorbidities, or tobacco use; Student *t* test on continuous variables and chi-squared analysis for categorical variables with lack of significance designated to be $P > .05$; 7). The present study used women ($n = 237$ of 320) for whom pregestational weight or early gestational weight was documented.

Metagenomic DNA extraction and sequencing

All samples were collected under uniform protocol (PeriBank) by placental pathology-trained personnel who wore facial masks and gloves, as described earlier. Approximately 100–150 mg of frozen placental tissue were taken from each sample and were homogenized and heated then processed with the use of PowerSoil DNA Isolation Kits (MO-BIO Laboratories, Carlsbad, CA).^{3,6,8} Briefly, genomic DNA was isolated according to the standard protocol from the MO-BIO PowerSoil DNA Isolation Kit (MO-BIO Laboratories).^{3,6,8} DNA was quantified by Nanodrop assay (Thermo Fisher Scientific, Wilmington, DE) and Picogreen assay (Invitrogen, Carlsbad, CA) and extracted DNA samples were stored at -20°C . Bacterial genomic DNA that had been isolated from placental

samples was sheared to approximately 700 base pairs, and Illumina adapters (Illumina Inc, San Diego, CA) were ligated to the sheared fragments for whole genome shotgun sequencing, and analysis. DNA fragments were then paired-end sequenced (150 base pair [bp] reads) in multiplexed pools on the Illumina HiSeq 2500 platform (Illumina Inc) in the Baylor College of Medicine-Human Genome Sequencing Center, as described in our previous papers,^{3,6,8} including the parent publication on the placental microbiome.⁸ Because of the concern for bias in low-abundance samples, all specimens were thrice extracted and thrice run to enable triplicate extraction and sequencing. Sequences from all 3 extractions were combined for analysis.⁸

16S recombinant DNA-based metagenomic sequencing and data analysis

Variable region amplicons of the bacteria 16S rRNA gene were sequenced as previously described.^{3,6,8} For whole genome shotgun sequencing and analysis, bacterial genomic DNA isolated from placental samples was sheared to approximately 700 bp and Illumina adapters were ligated to the sheared fragments. DNA fragments were then paired-end sequenced (150 bp reads) in multiplexed pools on the Illumina HiSeq 2500 platform. From 30 ng/μL of DNA, variable region amplicons were sequenced as previously described.^{3,6} The variable regions 1–3 of the 16S rRNA gene were amplified by polymerase chain reaction with bar-coded universal primers 27F (F, forward primer) and 534R (R, reverse primer) that contained linker sequences for 454-pyrosequencing.⁸ Samples that contained no template and those that contained known 16S rDNA sequences were used as positive and negative controls in these polymerase chain reaction steps. Variable-region amplicons were quantified by Picogreen assay and pooled in equimolar amounts. Multiplex sequencing was performed at the Baylor College of Medicine-Human Genome Sequencing Center on a 454-FLX Titanium sequencer (454 Life Sciences, Banford, CT). Raw 16S sequences were demultiplexed with quantitative insights into microbial ecology.⁶¹ Taxonomy was assigned with the use of the Ribosomal Database Project (RDP) classifier (version 2.2, re-trained with GreenGenes; Center for Microbial Ecology, Michigan State University, East Lansing, MI).^{62–64}

Concordant with previous work of ours and others, all 16S data were quality filtered to attain high-quality reads.^{3,6,8,61–64} Sequences from each file were preprocessed; thereby, sequences with length <200 nucleotides or >1000 nucleotides and sequences with minimum average quality score <20 and sequences without precisely matched 5' primers were removed. Sequences were assigned thereafter to their sample of origin based on barcode matches and the 5' primer, and the barcodes were excised after the filtering. RDP classifier (version 2.2 [re-trained using the release Feb. 4, 2011, of the GreenGenes taxonomy⁶²]) was used to assign taxonomy for each read at various levels. We used reads with confidence level >50% at lowest level of assignment for further analysis.⁸ The read counts at each taxonomy level (phylum, order, family, genus) were normalized to relative abundance.

For operational taxonomic unit (OTU) analysis, quality, and RDP-filtered reads were analyzed with the quantitative insights into microbial ecology⁶¹ pipeline and AbundantOTU⁶³ and to assign each read to OTUs. Quantitative insights into microbial ecology performed OTU picking, with error correction and OTU centroid choice at 97%

similarity.⁶¹ Chimer-aSlayer⁶⁴ was used to identify and remove chimeric sequences from the following analyses: Artifact caused by sequencing error was removed with the use of AbundantOTU as a denoising approach. OTUs were analyzed by RDP classifier (version 2.2) and well to find out the corresponding taxonomy. The number of reads assigned to each OTU was counted and normalized. The generated OTU table, combined with the clinical metadata, was used as input for follow-up analysis.

Principal coordinate analysis (PCoA) was used to measure beta diversity on normalized OTU tables with Unifrac distance. Briefly, these plots demonstrate the degree to which each placenta's microbiome differs from that of other placentas. With Unifrac, all taxa that are identified are placed on a phylogenetic tree. A branch that leads to taxa from both samples is marked as shared, and those taxa that appear only in one sample are marked as unshared. *Distance* in this sense is defined as the sum of unshared branch lengths over the sum of all branch lengths (both shared and unshared). Each sample is then plotted in its own dimension at the appropriate distance for each other point on the plot. The aggregate data from the 2 groups of placentas (obese or non-obese women or women with and without excess GWG, respectively) are used to calculate the overall degree of difference between the 2 groups, which is reflected in the permutational analysis of variance (PERMANOVA). Boruta feature selection and linear discriminate analysis effect size algorithms were used to detect significant features (taxonomy and pathway abundance) that differentiate groups and rank these features by effect size.

Whole genome shotgun metagenomic analysis

As anticipated, there was a large amount of human DNA sequence in our placental metagenomics datasets.^{3-6,8} Thus, to process the whole genome shotgun (WGS) reads, human contamination was identified and removed with Best Match Tagger⁶⁵ according to the steps described in the HMP Human Sequence Removal Standard Operating Procedure³⁻⁵ and elsewhere.^{8,65-69} This ensured quality and privacy. Metabolic pathway reconstruction was inferred directly based on short reads with the HMP Unified Metabolic Analysis Network.⁶⁷ Quality filtered reads were uploaded to metagenomic-RAST server (MG-RAST)⁶⁵ for taxonomy identification with the use of WGS reads. Taxonomy assignment was based on best hit classification, with the use of the M5NR database as an annotation source with a maximum e-value cutoff of 1, minimum percent identity cutoff of 50%, and minimum alignment length cutoff of 15. MG-RAST was used to generate low level Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway abundance for comparison between case and control-cohort samples. MG-RAST-generated taxonomy and pathway abundance were further normalized by the number of reads that were mapped to each sample. HUMAnN (the HMP Unified Metabolic Analysis Network)⁶⁷ was used to KEGG-based community functions for metabolic reconstruction from WGS reads.

Metabolic pathway reconstruction directly inferred the KEGG-based community functions as described elsewhere.^{3-6,8,65-67,69} For linear discriminate analysis effect size analysis, the alpha value that was used for the factorial Kruskal-Wallis test was 0.05. The threshold used on the logarithmic linear discriminate analysis score for discriminative feature is 2.0, as we previously demonstrated.^{6,8}

Statistical analysis

Descriptive findings of clinical data were reported. Where appropriate, χ^2 testing was performed to assess categorical variables; the Student *t* test was performed on continuous variables with normal distribution, and the Wilcoxon rank-sum test was performed on continuous variables without normal distribution. A probability value of $< .05$ was considered statistically significant. Statistical analysis was performed with SPSS software (version 22.0; SPSS Incorporated, IBM Corp, Armonk, NY). The significance of PCoA plots were analyzed with PERMANOVA, which uses distance metrics to confirm the strength and statistical significance of sample groupings.⁶⁸

All sequencing files have been submitted to the Sequence Read Archive.⁸

Results

Of the 320 women in our original data set,⁷ 237 women had sufficiently early prenatal care to calculate GWG; of these 237, 228 women (96.2%) had documented prepregnancy or <14 -week maternal weight values. As shown in Figure 1, we had 16S sequencing data on 237 women (144 women [60.8%] without excess GWG and 93 women [39.2%] with excess GWG) and WGS data on 37 women (24 women [64.9%] without excess GWG and 13 women [35.1%] with excess GWG). As shown in Table 3, most of the women were Hispanic (85.6%) and multiparous (48.9%). Most of the subjects with excess GWG were obese or overweight at baseline ($P < .001$), with an overall higher prepregnancy BMI (27.9 vs 30.2 kg/m²; $P = .007$). However, among women who delivered preterm, there was no significant difference in BMI among subjects with excess GWG (mean BMI, 28.3 vs 30.6 kg/m², respectively; $P = .203$; Table 3). Infant birthweight was statistically higher among women with excess GWG when compared with those without excess GWG by an average of 201.1 g ($P = .015$). There were no statistically significant differences between excess and no excess GWG cohorts by race/ethnicity, mode of delivery, or other comorbidities or demographic variables. In total, 22.8% of women who were included in this study underwent cesarean delivery.

To investigate whether the placental microbiome varied with excess GWG, we compared the phylum-level relative abundance by BMI and then by excess GWG in all women. As shown in Figure 2, A, and delineated in Table 4, among all subjects there are no appreciable significant differences in the relative abundance of Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Cyanobacteria, Chloroflexi, Aquificae, nor Verrucomicrobia between either obese and non-obese subjects nor with and without excess GWG (Figure 2, A; Table 4). However, when we specifically examined phyla level relative taxonomic abundance among preterm women by virtue of maternal weight gain (Figure 2, A), we observed appreciable and significant increased abundance of Firmicutes, Actinobacteria, and Cyanobacteria (Figure 2, A; Table 4) and decreased relative abundance of Proteobacteria (Figure 2, A; Table 4). This was not unique to only phyla level relative taxonomic abundance, but also held true at the family (Figure 2, B) and genus level (*Vibrio*, *Burkholderia*, and *Beijerinckia*; Table 4). Although we could reach species-level resolution with our WGS data, we did not distinguish significantly distinct abundance of any given single species by virtue of GWG nor obesity (data not shown). Collectively, these findings are consistent with composite

genus level and courser distinctions among preterm placental microbiome communities when comparing subjects with and without excess GWG, but not obesity per se.

We next performed PCoA on all OTUs that were generated by 16S rRNA sequencing that could be assigned to at least phylum level and appeared in a minimum of 5% of samples after chimera removal. Consistent with our findings (Figure 2), when we analyzed by PCoA (Figure 3) and stratified by obesity, there was no significant difference in beta-diversity (nonsignificance by PERMANOVA, $P = .161$ in all subjects and $P = .048$ among preterm subjects; Figure 3). However, when the beta-diversity was interrogated as a variate of GWG, among subjects who delivered preterm, there was a statistically significant clustering by virtue of excess GWG (Figure 3; PERMANOVA, $P = .022$). Clustering was most distinct along the PC1 axis (Wilcoxon rank sum, $P = .02$), which explained most of the variance in the cluster population (14%). When we alternately analyzed alpha diversity among preterm deliveries, excess GWG was also associated with decreased overall species richness and variant abundance (Figure 4, A and B). This is further supported by the data in Table 4, with significant differences to the genus level observed among preterm placental microbial communities.

Furthermore, when we analyzed high-resolution WGS metagenomic data and projected heat maps with Manhattan distance and probability values for significance by Wilcoxon-Mann-Whitney test, we observed distinct, appreciable, and significant variation of genus level taxonomy both between term and preterm (Figure 5, A; p^*) placental communities, and notably among preterm communities with excess GWG (Figure 5, A). When we alternately project the encoded bacterial-metabolic pathways rather than the bacteria themselves (Figure 5, B), we again observed significant variation in benzoate degradation, siderophore group biosynthesis, nucleic and amino acid metabolism and biosynthesis, and DNA replication (Figure 5, B) among pretermplacental microbial communities from women with excess GWG.

Finally, by linking genus level taxonomic differences to their gene carriage patters, we can ascribe the relative significant Figure 2, A, the contribution of specific taxa to perturbations in bacterial metabolic pathways. Figure 6, A, shows the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States-generated functional profile and quantitative insights into microbial ecology-generated genus level bacterial abundance where taxa and pathways represent the positive or negative Kendall correlations (MG-RAST) for preterm subjects with excess GWG ($P = .05$). For example, the significant variation in purine metabolism is attributable to taxa variation from *Burkholderia*, *Prevotella*, and *Coprobacillus*. Figure 6, B, projects these data in significant aggregate and shows that excess GWG was associated with a gene carriage pattern that demonstrated a significant decrease in the abundance of folate biosynthesis pathways and butanoate metabolism, among other pathways (linear discriminate analysis, >3).

Comment

GWG that exceeds the weight gain recommendations of the Institute of Medicine⁶⁰ has been associated with multiple adverse outcomes,^{56,59,70-72} which include preterm birth.⁵⁰⁻⁵³

Excess GWG has also been associated with alterations in metabolic markers in the maternal serum.⁵⁴ We recently demonstrated that the placental microbiome and the bacterial encoded metabolic pathways significantly vary in association with preterm birth.⁸ Notably, these findings were independent of mode of delivery, which means that there were no differences in the placental microbiome of vaginal deliveries compared with cesarean deliveries.⁸ Here we demonstrate that, among women with spontaneous preterm birth, the placental microbiome varies by virtue of excess GWG, but not obesity. Specifically, among preterm births, excess GWG was associated with significant alterations in the composition of the placental microbiome (Figures 2 and 3; Table 4) and its bacterially encoded metabolic pathways (Figures 5 and 6), but obesity was not. Thus the mechanism by which excess GWG results in preterm birth may be through aberrations in the placental microbiome. Of note, based on the results of this study, causality cannot be determined.

The specific changes in the preterm placental microbiome that we observed here pose provocative potential underlying mechanisms with which excess maternal GWG might mediate risk of preterm birth. First, preterm subjects with excess GWG had decreased species richness (Figure 4). In the gut, decreased species richness has been associated with insulin resistance, inflammatory phenotypes, and, notably, infection.^{73,7} In the placenta, we previously demonstrated that decreased richness in the placenta is associated with spontaneous preterm birth.⁸ Together, this suggests that the decreased richness seen in excess GWG may contribute to a risk of preterm birth. The second putative mechanism for modulating preterm birth may occur through variations in the accompanying metagenomic bacterially encoded metabolic pathways. Preterm placentas from women with excess GWG had decreased abundance of folate biosynthesis and butanoate metabolism and increased siderophore biosynthesis (Figures 5, B, and 6). These alterations in the metabolic pathways are mere associations, but their potential significance is provocative. For example, butyrate (or butanoate) is posited to suppress colonic inflammation in the gut.⁷² A decrease could be associated with increased inflammation in other body sites, in this instance, the placenta. With respect to folic acid, which we observed to be increased among preterm placental microbiome communities without excess GWG, the literature in both animal and human studies has been mixed and contradictory.⁷³⁻⁷⁵ Most recently, folic acid supplementation has been shown to increase the risk of preterm birth when initiated in the first trimester.⁷⁵ The increased biosynthesis of siderophore is also intriguing. In response to a paucity of iron in their environment, bacteria manufacture siderophores, which bind scarce iron with high affinity.⁷⁵ The relationship between iron deficiency anemia and preterm birth is well-established in the literature⁷⁶⁻⁸¹; the increased siderophore biosynthesis that is seen in preterm placentas may be a consequence of iron deficiency anemia or may alternatively be causative and worsen the iron status of the mother and fetus and prompt preterm delivery as a response. Additionally, like folate supplementation, iron supplementation has been found to possibly be more harmful than helpful.^{82,83} Combined, our findings raise the notion that the impact of exogenous supplementation may be mitigated by the microbiome-encoded metabolic pathways of the placenta. However, this is speculative, and further research in this area is required.

When a possible interaction between excess GWG (rather than obesity per se) and the placental microbiome with its encoded metabolic pathways in modulating preterm birth is

considered, it is important to reflect on what constituents contribute to the metabolic milieu of the pregnancy. First, there is both the caloric density and source of caloric excess. We previously published our extensive research on the impact of a caloric-dense, high-fat maternal diet in a nonhuman primate model that included our recent demonstration of an altered maternal and offspring microbiome; these effects are diet and not obesity dependent.⁶⁹ Second, different microbiota take up niches in accordance with their ability and optimization of available nutrient sources. These same microbiota in turn contributes to a given niche's metabolic profile, which presumptively over time will further enrich for expansion of such a community. Thus, dysbiosis in a given body site niche is presumed to occur in a cycle of host-microbe-host interactions. It is interesting to speculate that such placental dysbiosis may initiate under conditions of a calorically dense diet (observed clinically as GWG), with ongoing propagation of a dysbiotic community able to co-opt such a localized niche and resulting in inflammation with resultant preterm birth.

Strengths of this study include the rigorously sterile sample collection techniques, data collection, robust analysis, and data validation. The GWG formulas that were used have been validated previously and published.⁵⁹ All samples were processed and sequenced by an experienced team of microbiome investigators, and microbial sequences were isolated successfully and identified from a low-abundance tissue (placenta). Furthermore, we are neither the first to observe nor alone in our finding that the placenta is not sterile.^{8,13-18} This fact was demonstrated previously with culture-based methods. Studies first investigated the placentas of preterm deliveries and cultured bacteria from the placentas of 72% of 56 preterm infants.¹⁵ Later studies isolated bacteria from 67.6% of placentas from clinically infected women who delivered at term.¹⁶ When term noninfected deliveries are studied, early culture-based technology isolated bacteria from 21% of placentas.¹⁷ This rate was lower than infected placentas at term (28%) and preterm (61%), but the bacteria were nevertheless isolated.¹⁷ More recent culture-based studies identified bacteria in 16.4% of placentas from noninfected pregnancies.¹⁴ Previous studies have also used fluorescence-in-situ hybridization or morphologic techniques. One study used a DNA probe that was specific for highly fluorescence-in-situ hybridization conserved regions of the bacterial 16S rRNA sequences and labeled it with fluorescein for fluorescence-in-situ hybridization; they detected organisms in most of fetal membranes (>73%) after term delivery without labor.¹² A later study on biopsy specimens from chorion parenchyma from 1083 placentas that were delivered before the week 28 of gestation demonstrated that, even among placentas that were delivered by cesarean delivery for preeclampsia, the bacterial recovery rate was 25%.¹⁸ Stout et al¹³ similarly found bacteria in the basal plate of placentas, even those who delivered at term and without evidence of infection.

This study also has limitations. There is an overall low-abundance (biomass) of microbiota in the placenta. Based on our recorded extracted specimen weights and resultant read counts, we estimate that ranging from 0.00002–0.002 mg (eg, 20–2000 ng) of bacterial DNA was extracted from every 1 g of placental tissue.⁸ However, we overcame these limitations by using large cohorts, strict adherence to sterile protocol to reduce contamination, and the use of deep massively parallel sequencing with WGS.⁸ A second notable limitation is that, because of the analysis that was performed, we cannot determine whether the bacterial DNA that were identified were from living or dead cells. However, the degree of consistency

among subjects and the demonstrated significance of findings that used robust “big data science” analytic approaches are means of overcoming these potential limitations.

Based on our findings, excess GWG may represent a modifiable risk factor for preterm birth. It is of clinical relevance that patients cannot reasonably or significantly change their obesity status during pregnancy; however, GWG is modifiable, and counseling has been proved to be effective at decreasing GWG.⁷⁶ Because excess GWG is associated with dysbiosis of the placental microbiome in cases of preterm birth, we speculate that appropriate GWG may modulate placental microbial community structure most notably in the preterm interval. Further investigation in this area will elucidate this possibility.

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FIGURE 1. Study design

The *outer circle* represents the numbers for 16S sequencing. The *inner circle* represents the numbers for whole genome shotgun sequencing, all of which also completed 16S. Of the 320 women in our original placental microbiome analysis that used 16S sequencing, 237 women had sufficiently early prenatal care to calculate GWG; of the original 48 nested whole genome shotgun cohort, 37 women had robust GWG data.

GWG, gestational weight gain; WGS, whole genome shotgun.

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FIGURE 2. Differential placental microbial abundance plots

A, The phylum-level relative abundance by body mass index, excess GWG in all subjects, and by excess GWG among preterm women. Among preterm women, there are statistically significant differences in the relative abundance of Proteobacteria, Firmicutes, Actinobacteria, and Cyanobacteria between subjects with and without excess GWG. **B**, Family-level relative abundance (y axis) is represented by *stacked bar plots* that indicate the dominant family for each of the cohorts.

BMI, body mass index; *GWG*, gestational weight gain.

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FIGURE 3. PCoA plots of beta diversity by body mass index

The placental microbiome does not vary with obesity. Principle coordinate analysis of all operational taxonomic units generated by 16S recombinant RNA sequencing is shown on **the left**. There was no significant difference in between-subject beta-diversity by virtue of obesity (permutational multivariate analysis of variance, $P = .161$). Among preterm placentas, the placental microbiome does not vary with obesity. Principle coordinate analysis of all operational taxonomic units generated by 16S recombinant RNA sequencing of only the preterm women is shown on **the right**. There was no significant difference in between-subject beta-diversity by virtue of obesity (permutational multivariate analysis of variance, $P = .48$). Among all women, the placental microbiome does not vary with GWG. There was no significant difference in between-subject beta-diversity by virtue of excess GWG (permutational multivariate analysis of variance, $P = .186$). The microbiome of preterm placentas does vary with GWG. There was statistically significant clustering by virtue of excess GWG (permutational multivariate analysis of variance, $P = .022$) that was most apparent along the PC1 axis (Wilcoxon rank sum, $P = .02$).

GWG, gestational weight gain; PCoA, principal coordinate analysis; PERMANOVA, permutational multivariate analysis of variance.

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FIGURE 4. Alpha diversity of the placental microbiome

A, Among preterm women with excess GWG, decreased species richness was observed with rarefaction alpha diversity metrics. **B**, Renyi alpha diversity metrics indicates varying diversity among preterm women with excess GWG.

GWG, gestational weight gain.

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FIGURE 5. Heatmap of the placental microbiome and maternal weight gain

A, Heat map demonstrates the genera relative abundance of the placental microbiome among term women without excess GWG (*left*), preterm subjects without excess GWG (*middle*) and preterm subjects with excess GWG (*right*). Stronger intensity of blue indicates higher relative abundance. The probability value was generated by Wilcoxon-Mann-Whitney test. *Red color* indicates significant difference between groups ($P < .05$). **B**, Heat map demonstrates the metabolic activity of the placental microbiome among term women without excess GWG (*left*), preterm women without excess GWG (*middle*), and preterm women with excess GWG (*right*). Stronger intensity of *blue color* indicates higher pathway activity. The probability value was generated by Wilcoxon-Mann-Whitney test. *Red color* indicates significant difference between groups ($P < .05$). *GWG*, gestational weight gain.

P^* indicates the significance of the differences between term with no excess GWG vs preterm with no excess GWG; P^{**} indicates the significance of the differences between

preterm with no excess GWG vs preterm with excess GWG; P^{***} indicates the significance of the difference between term with no excess GWG vs preterm with excess GWG.

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FIGURE 6. Metagenomics of the preterm placental microbiome by maternal weight gain
A, The Kendall correlation of metagenomics RAST server-generated Kyoto Encyclopedia of Genes and Genomes functional profile and genus level bacterial abundance were calculated and plotted. The taxa and pathways shown represent the positive (*red*) or negative (*blue*) correlations for preterm women with excess GWG. The *red square* indicates correlation probability value < .05. **B**, Among preterm placentas, excess GWG is associated with alterations in metabolic function. Detailed analysis on whole genome shotgun data

show that excess GWG has decreased abundance of folate biosynthesis and butanoate metabolism.

GWG, gestational weight gain; LDA, linear discriminate analysis.

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TABLE 1

Terms and definitions

Term	Definition
Microbiota	Microbial population present in different ecosystems (niches) of the body
Microbiome	The totality of the microbes (and their genomes) who share our body space ¹ ; can also refer to the totality of microbes (and their genomes) in a given body site or niche
Metagenome	Entire community genomic repertoire and metabolic profile.
Community	The entirety of microbes in a given body site or niche
Dysbiosis	Microbial imbalance; altered community structure
16S recombinant RNA sequencing	Highly conserved gene in different species of bacteria that enables its use as a universal primer
Whole genome shotgun sequencing	<i>WGS</i> : used for metagenomic analysis
Operational taxonomic units	<i>OUT</i> : a distinct sequence
Diversity	Difference in the bacteria, either within a group of samples (alpha-diversity) or between a group of samples (beta-diversity)
Richness	Number of OTUs (or species) in a sample: richness reflects the abundance of microbes across multiple taxa
Alpha-diversity	Biodiversity within a group of samples ⁶
Beta-diversity	Biodiversity between groups of samples ⁶
Principle Coordinate Analysis	<i>PCoA</i> : used to examine beta-diversity between 2 groups of samples
UniFrac	Used to calculate a distance measure between organismal communities with the use of phylogenetic information; with the use of UniFrac, all taxa that are found are placed on a phylogenetic tree. A branch leading to taxa from both samples is marked as “shared”; branches that lead to taxa that appear in only 1 sample are “unshared.”
Phylogenetic distance	Uses UniFrac: <i>distance</i> is defined as the sum of unshared branch lengths divided by the sum of all shared and unshared branches
Permutational multivariate analysis of variance	<i>PERMANOVA</i> : does not assume a normal distribution.

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TABLE 2

Maximum gestational weight gain calculations^{59,60}

Body mass index	Calculation
Underweight prepregnancy	= 4.4 + 1.3 (GADelivery – 13)
Normal prepregnancy	= 4.4 + 1 (GADelivery – 13)
Overweight prepregnancy	= 4.4 + 0.7 (GADelivery – 13)
Obese prepregnancy	= 4.4 + 0.6 (GADelivery – 13)

GADelivery, gestational age at delivery.

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TABLE 3

Demographic characteristics by gestational weight gain

Demographic	Overall cohort (n = 237)	Gestational weight gain		P value
		Not excess (n = 144; 60.76%)	Excess (n = 93; 39.24%)	
Age, y ^a	27.2 ± 6.0	27.3 ± 6.1	27.0 ± 5.9	.640
Race/ethnicity, n (%) ^b				
Hispanic	202 (85.6)	126 (88.1)	76 (81.7)	
Black	26 (11.0)	14 (9.8)	12 (12.9)	
White	4 (1.7)	1 (0.7)	3 (3.2)	
Other	4 (1.7)	2 (1.4)	2 (2.2)	.380
Prepregnancy body mass index ^{a,c}	28.8 ± 6.4	27.9 ± 6.4	30.2 ± 6.3	.007 ^d
Underweight	3 ± 1.3	3 ± 2.1	0	
Normal	66 ± 27.8	53 ± 36.8	13 ± 14.0	
Overweight	84 ± 35.4	46 ± 31.9	38 ± 40.9	
Obese	84 ± 35.4	42 ± 29.2	42 ± 45.2	< .001 ^d
Parity ^a	1.6 ± 1.4	1.61 ± 1.4	1.48 ± 1.3	.488
0, n (%)	66 (27.8)	37 (25.7)	29 (31.2)	
1, n (%)	55 (23.2)	36 (25.0)	19 (20.4)	
2, n (%)	116 (48.9)	71 (49.3)	45 (48.4)	.569
Antenatal infection, n (%)	78 (32.9)	42 (29.2)	36 (38.7)	.127
Sexually transmitted infection	45 (19.0)	24 (16.7)	21 (22.6)	.257
Urinary tract infection	42 (17.7)	20 (13.9)	22 (23.7)	.055
Other	5 (2.1)	3 (2.1)	2 (2.2)	.972
<i>Streptococcus agalactiae</i> positive, n (%)	47 (21.9)	30 (22.9)	17 (20.2)	.645
Delivery				
Infant birthweight, g ^a	3173.8 ± 618.0	3095.6 ± 597.7	3296.7 ± 632.6	.015 ^d
Gestational age at delivery, wk ^a	38.2 ± 2.4	38.1 ± 2.5	38.5 ± 2.4	.21
Preterm delivery, n (%)	62 (26.2)	42 (29.2)	20 (21.5)	.190
Cesarean delivery, n (%)	54 (22.8)	33 (22.9)	21 (22.6)	.952
Maternal comorbidities/outcomes, n (%)				
Diabetes mellitus	58 (24.5)	38 (26.4)	20 (21.5)	.393
Uncontrolled diabetes mellitus	10 (4.2)	5 (3.5)	5 (5.4)	.476
Hypertensive disorder	7 (3.0)	5 (3.5)	2 (2.2)	.557

^aData are given as mean ± SD;^bOne woman did not report her race/ethnicity;^cWhen the prepregnancy body mass index was examined among preterm deliveries, there was no difference in the prevalence of obesity among women with excess gestational weight gain; additionally, the mean body mass index was 28.3 ± 7.233 kg/m² vs 30.64 ± 5.355 kg/m² (*P* = .203);

^d Denotes significance.

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Most abundant phyla and genera by gestational weight gain and gestational age at delivery

TABLE 4

Variable	Term gestational weight gain abundance ^a		Preterm gestational weight gain abundance ^a		P value ^b
	No excess	Excess	No excess	Excess	
Phyla	Proteobacteria	0.529345	Proteobacteria	0.587677	0.51827 ^c
	Actinobacteria	0.176771	Actinobacteria	0.17258	0.198641 ^c
	Firmicutes	0.12791	Firmicutes	0.103855	0.146677 ^c
	Bacteroidetes	0.089904	Bacteroidetes	0.079576	0.07039
	Cyanobacteria	0.030468	Cyanobacteria	0.015286	0.02311 ^c
	Escherichia	0.067054	Escherichia	0.117615	0.090976
Genus	Vibrio	0.051607	Vibrio	0.098668	0.073517 ^c
	Burkholderia	0.04352	Streptomyces	0.038562	0.049689
	Streptomyces	0.042644	Burkholderia	0.049596	0.04134 ^c
	Bacteroides	0.040631	Bacteroides	0.035619	0.027274
	Prevotella	0.025092	Prevotella	0.027245	0.026016
	Coprobacillus	0.02033	Neisseria	0.023128	0.020863
	Mycobacterium	0.017375	Shigella	0.017131	0.019918
	Neisseria	0.016759	Coprobacillus	0.01678	0.018003
	Corynebacterium	0.016255	Mycobacterium	0.016083	0.01687 ^c
	Escherichia coli	0.062505	Escherichia coli	0.110747	0.086405
Species	Vibrio sp AND4	0.044276	Vibrio sp AND4	0.089599	0.06796
	Escherichia coli	0.062505	Escherichia coli	0.110747	0.086405

Variable	Term gestational weight gain abundance ^a		Preterm gestational weight gain abundance ^a		P value ^b
	No excess	Excess	No excess	Excess	
<i>Coprobacillus</i> sp D7	0.020276	<i>Prevotella tanneriae</i>	0.020934	<i>Strep. Avermitilis</i>	.910
<i>Prevotella tanneriae</i>	0.019398	<i>Coprobacillus</i> sp D7	0.018469	<i>Bacteroides</i> sp. D20	.648
<i>Bacteroides</i> sp D20	0.016667	<i>Bacteroides</i> sp D20	0.017344	<i>Prevotella tanneriae</i>	.885
<i>Beijerinckia indica</i>	0.014912	<i>Beijerinckia indica</i>	0.015506	<i>Beijerinckia indic</i>	.265
<i>Strep. Avermitilis</i>	0.014021	<i>Strep. Avermitilis</i>	0.015452	<i>Burk. Cepacia</i>	.795
<i>Salmonella enterica</i>	0.010835	<i>Salmonella enterica</i>	0.013655	<i>Burk. Pseudomallei</i>	.807
<i>Burk. Pseudomallei</i>	0.010509	<i>Steno Maltophilia</i>	0.01165	<i>Coprobacillus</i> sp D7	.450
<i>Bacteroides helcogenes</i>	0.009771	<i>Burk. Pseudomallei</i>	0.010584	<i>Prop Acnes</i>	.358
			0.013009	<i>Salmonella enterica</i>	

Only taxa (phyla, genus or species) with any relative abundance > 1% are listed. There was no significant difference among any taxa when comparing obese to non-obese subjects (data not shown).⁷

^a Abundance defined as the relative abundance;

^b Probability value is calculated as the significance of difference of comparing preterm placental microbial relative abundance from women with no excess gestational weight gain with women with excess gestational weight gain;

^c A significantly different abundance compared with term women without excess gestational weight gain.

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