OBSTETRICS

A specific bacterial DNA signature in the vagina of Australian women in midpregnancy predicts high risk of spontaneous preterm birth (the Predict1000 study)

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BACKGROUND: Intrauterine infection accounts for a quarter of the cases of spontaneous preterm birth; however, at present, it is not possible to efficiently identify pregnant women at risk to deliver preventative treatments.

OBJECTIVE: This study aimed to establish a vaginal microbial DNA test for Australian women in midpregnancy that will identify those at increased risk of spontaneous preterm birth.

STUDY DESIGN: A total of 1000 women with singleton pregnancies were recruited in Perth, Australia. Midvaginal swabs were collected between 12 and 23 weeks' gestation. DNA was extracted for the detection of 23 risk-related microbial DNA targets by quantitative polymerase chain reaction. Obstetrical history, pregnancy outcome, and demographics were recorded.

RESULTS: After excluding 64 women owing to losses to follow-up and insufficient sample for microbial analyses, the final cohort consisted of 936 women of predominantly white race (74.3%). The overall preterm birth rate was 12.6% (118 births); the spontaneous preterm birth rate at <37 weeks' gestation was 6.2% (2.9% at ≤34 weeks' gestation), whereas the preterm premature rupture of the membranes rate was 4.2%. No single individual microbial target predicted increased spontaneous preterm birth risk. Conversely, women who subsequently delivered at term had higher amounts of *Lactobacillus crispatus*, *Lactobacillus gasseri*, or *Lactobacillus jensenii* DNA in their vaginal swabs (13.8% spontaneous preterm birth vs 31.2% term; *P*=.005). In the remaining women, a specific microbial DNA signature was identified that was strongly predictive of spontaneous preterm birth risk, consisting of DNA from *Gardnerella vaginalis* (clade 4), *Lactobacillus iners*, and *Ureaplasma parvum* (serovars 3 and 6). Risk prediction was improved if *Fusobacterium nucleatum* detection was included in the test algorithm. The final algorithm, which we called the Gardnerella Lactobacillus Ureaplasma (GLU) test, was able to detect women at risk of spontaneous preterm birth at <37 and ≤34 weeks' gestation, with sensitivities of 37.9% and 44.4%, respectively, and likelihood ratios (plus or minus) of 2.22 per 0.75 and 2.52 per 0.67, respectively. Preterm premature rupture of the membranes was more than twice as common in GLU-positive women. Adjusting for maternal demographics, ethnicity, and clinical history did not improve prediction. Only a history of spontaneous preterm birth was more effective at predicting spontaneous preterm birth than a GLU-positive result (odds ratio, 3.6).

CONCLUSION: We have identified a vaginal bacterial DNA signature that identifies women with a singleton pregnancy who are at increased risk of spontaneous preterm birth and may benefit from targeted antimicrobial therapy.

Key words: diagnostic test, *Fusobacterium* spp, *Gardnerella* spp, genotype, *Lactobacillus* spp, preterm birth, preterm premature rupture of the membranes, real-time polymerase chain reaction, *Ureaplasma* spp, vagina

Introduction

Intrauterine infection is a well-established cause of preterm birth (PTB), particularly in deliveries before 32 weeks' gestation where it is responsible for 40% to 80% of cases; these early births are associated with the greatest rates of infant morbidity and mortality.[1,2] Common sites of infection include the amniotic fluid, fetal membranes, and placenta, with a number of pathways established as to how bacteria are able to access these tissues.[3] The most common and well accepted of these pathways is bacteria from the vagina ascending through the cervix into the uterine tissues, resulting in establishment of intrauterine infection and an ensuing inflammatory response in maternal and fetoplacental tissues that culminates in preterm delivery.[1,4]

A wide range of vaginal bacteria have been detected in the amniotic cavity and placenta of preterm deliveries; of these, *Ureaplasma* spp are the organisms most commonly detected.[5] In the context of vaginal colonization, of the 2 *Ureaplasma* spp known to colonize humans, *U parvum* seems to be of most significance for PTB[6–8]; however, approximately 50% of pregnant women are vaginally colonized with this organism, most of whom will not deliver preterm, limiting its utility as an independent diagnostic PTB marker. Other bacteria frequently linked to PTB are commonly associated with the vaginal dysbiotic state known as bacterial vaginosis (BV), including *Mycoplasma* spp and anaerobic organisms such as *Gardnerella vaginalis*, *Mobiluncus* spp, and *Atopobium vaginae*.[9–13] Conversely, a large body of evidence exists suggesting that vaginal *Lactobacillus* spp offer some level of protection against PTB,[14] with *Lactobacillus crispatus* in particular being associated with delivery at term.[15,16] Attempts to exploit this knowledge and assess the effectiveness

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AJOG at a Glance

Why was this study conducted?

A large number of spontaneous preterm births (sPTBs) originate from an infectious etiology; however, at present, there are no suitable diagnostic tests available to predict women at risk.

Key findings

Vaginal swabs from women who delivered at term had higher amounts of *Lactobacillus crispatus*, *L gasseri*, or *L jensenii* DNA. In the remaining women, a specific microbial DNA signature was identified, which was strongly predictive of sPTB risk, consisting of DNA from *Gardnerella vaginalis* (clade 4), *L iners*, and *Ureaplasma parvum* (serovars 3 and 6). Risk prediction was improved if *Fusobacterium nucleatum* detection was included.

What does this add to what is known?

A specific vaginal bacterial DNA signature present in Australian women during midgestation is indicative of sPTB risk. Accurate species- and strain-level identification of microbes is critical for use of vaginal microbiology in predicting the risk of sPTB.

of *Lactobacillus* spp probiotic supplements in preventing PTB are lacking, although Krauss-Silva et al[17] did show a modest, nonsignificant reduction in spontaneous PTB in women with asymptomatic BV and no history of PTB given vaginal *Lactobacillus* sp probiotics. Today, a number of vaginal and oral probiotic supplements are commercially available, with vaginally administered *L plantarum* being 1 of these that has demonstrated efficacy in reducing BV and candidiasis, especially after antibiotic treatment.[18,19]

However, defining which vaginal bacteria are potential pathogens or probiotics in the context of PTB is far from clear cut. One of the greatest limitations of many previous vaginal microbial profiling PTB studies is their lack of taxonomic resolution[10,16,20–22]; this is especially critical for probiotics, where strain-level identification is likely to be paramount to a successful clinical outcome. Short-amplicon 16S ribosomal RNA (rRNA) gene sequencing used by the bulk of these studies results in microbial profiles that are generally limited to genus-level identification at best.[23,24] Considering the large number of species contained within the various human vaginal bacterial genera, only some of which are known pathogens, species- or genotype- or strain-level resolution is likely to be needed for a diagnostic biomarker panel to retain high enough specificity for clinical use. For example, our own research into vaginal *Ureaplasma* spp colonization and PTB[6] found that only 1 species (*Ureaplasma parvum*) was associated with PTB and, within that, 1 particular genotype (SV6) was associated with the highest risk. More recently, Rittenschober-Böhm et al[7,8] confirmed this association for *U parvum* in a >4000 cohort of pregnant women and found that genotypes SV1, SV3, and SV6 were high risk. In addition, the race or ethnic background of women plays a crucial role in documenting associations between vaginal microbes or microbial communities and PTB risk.[12,13,25,26] In general, previous studies have revealed that high-risk profiles in African American women are very different to those in white women[12,13,16] and do not typically involve *Ureaplasma* spp, the organism most commonly associated with PTB. Furthermore, geographic and environmental factors may also influence vaginal microbial communities,[27] and a role for host genetic determinants has also been proposed.[28]

This study aimed to utilize high-resolution molecular methods to document the presence of DNA in vaginal swabs from a range of microorganisms previously linked to spontaneous PTB (sPTB) risk in a large cohort of pregnant Australian women during midgestation and to examine associations between their microbiological characteristics, maternal demographics, and pregnancy outcome. We hypothesized that detection of specific genotypes of *U parvum*, in combination with other microorganisms, may be predictive of increased sPTB risk and that such a predictive vaginal microbial DNA profile may be of use to identify women in future studies who could benefit from antimicrobial intervention to improve pregnancy outcome.

Materials and Methods

Subjects

The study consisted of 1000 women with singleton pregnancies recruited between July 2015 and December 2017 from the antenatal population at King Edward Memorial Hospital (KEMH), Perth, Western Australia. The study was approved by the Human Research Ethics Committee of the Western Australian Department of Health, Women and Newborn Health Service (2015035/EW), and the University of Western Australia Human Research Ethics Committee (RA/4/1/7758).

Inclusion and exclusion criteria

Nulliparous and multiparous women with a singleton pregnancy were eligible for inclusion if they were aged ≥16 years and attending antenatal clinics at KEMH between 12 and 23 weeks' gestation. Recruitment was enriched by preferential selection of women with a history of PTB by targeting antenatal clinics within the hospital more likely to be attended by women with previous PTBs. All participants had to be able to speak and read English.

Women were ineligible for the study if they were unable to provide informed consent, using illegal drugs, HIV or hepatitis C virus positive, taking antibiotic agents, or carrying a multiple pregnancy.

Questionnaires

Upon recruitment to the study, women were invited to complete a medical and lifestyle questionnaire in a private

setting. The questionnaire first inquired about medications currently used (antibiotic, natural, or probiotic) and past diagnoses of urinary tract infections or vaginal thrush. Information regarding current smoking or alcohol use was sought as yes or no. The use of any nicotine replacement therapies was also noted. An additional question addressed the frequency of sexual intercourse.

Pregnancy outcome data

Pregnancy outcome data from the hospital's electronic medical records were accessed by experienced research midwives and coded after completion of the pregnancy. The hospital's Stork database was accessed to confirm the information when necessary.

Sample collection

An overview of the sample collection process is depicted in Figure 1. Participants provided 2 self-collected vaginal samples (Copan Diagnostics, Brescia, Italy) at 12 to 23 weeks' gestation (median, 19.4; range, 12.1−23.9).

Vaginal swab collection

Detailed verbal, written, and pictorial instructions were provided to all women in an attempt to standardize the swab collection process. Briefly, while wearing gloves, participants inserted the swab 5 cm into their vagina and gently rotated this for 20 seconds, ensuring the walls of the vagina came into contact with the swab. Swabs were then immediately placed into a collection tube containing either 1 mL of universal transport medium (UTM) media (*Ureaplasma* spp, swab 1) or 1 mL of liquid Amies media (e-Swab, swab 2), snapped at the midstem breakpoint, capped, and stored at 4°C. All samples were transported to the laboratory on ice for processing on the same day; most swabs were processed within 6 hours of collection, with the maximum time from collection to processing being 24 hours. The multiple swab collection protocol was validated in 20 samples to examine reproducibility of data between sequentially collected swabs (Supplemental Material S1).

Detection of microbes in vaginal swabs

Universal transport medium swabs (swab 1)

UTM tubes were vortexed for 10 seconds to release cells from swabs. Swabs were subsequently pressed against the tube wall to release remaining free liquid and then discarded. Vaginal swab eluates were transferred to a 2 mL microfuge tube (Sarstedt, Inc, Nümbrecht, Germany) and frozen at −80°C until DNA extraction.

e-Swabs (swab 2)

e-Swab tubes were vortexed for 10 seconds to release cells from swabs. Swabs were subsequently pressed against the tube wall to release remaining free liquid and then discarded. Vaginal swab eluates were transferred to 2 mL microfuge tubes (Sarstedt, Inc) and centrifuged at 10,000 × *g* for 10 minutes at 4°C. Supernatants were carefully removed (leaving the final approximately 50 μL to ensure the cell pellet was not disturbed), and cell pellets were resuspended in 500 μL of sterile 1X phosphate-buffered saline and frozen at −80°C until DNA extraction.

DNA extraction from universal transport medium swab eluates (swab 1)

UTM swab eluates were defrosted at room temperature and centrifuged at 40,000 × *g* for 5 minutes at 4°C. Supernatants were carefully removed (leaving the final approximately 50 μL to ensure the cell pellet was not disturbed), and cell pellets were resuspended in 350 μL of buffer MBL/ribonuclease A (QIAGEN, Hilden, Germany). DNA was extracted from swab eluates using a QIAGEN MagAttract Microbial DNA kit (QIAGEN) on an automated Kingfisher Duo extraction platform (Thermo Fisher Scientific, Waltham, MA) as per manufacturer's instructions, with the exception of the bead-beating procedure. Here, sterile 0.1 mm glass beads were aseptically decanted into 2 mL microfuge tubes (Sarstedt, Inc), swab eluates added, and tubes subjected to bead beating for 45 seconds at 6500 rpm on a Precellys 24 tissue homogenizer (Thermo Fisher Scientific). Purified DNA in buffer EB was stored at 4°C until analysis.

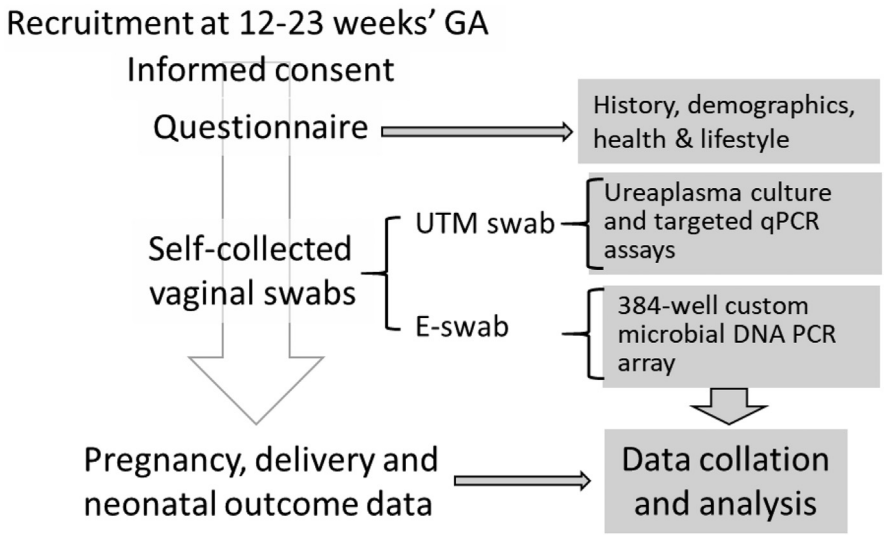
DNA extraction from e-Swab eluates (swab 2)

e-Swab eluates were defrosted at room temperature and DNA was extracted from 200 μL of swab eluate using an InviMAG Universal Kit (STRATEC Molecular, Birkenfeld, Germany) on an automated Kingfisher Duo extraction platform (Thermo Fisher Scientific) as per manufacturer's instructions. This kit is designed for rapid purification of genomic, bacterial, and viral DNA and viral RNA from a variety of clinical samples, including swabs. Purified DNA in elution buffer M was stored at 4°C until analysis. The remaining e-Swab eluates were refrozen at −80°C for long-term storage.

15-target custom 384-well polymerase chain reaction array

On the basis of a combination of our previous original work,[6] a review of the literature on the uterine microbiome in cases of PTB,[29] and the meta-analysis by Mendz et al[5] regarding intrauterine bacterial infection and PTB, we selected 15 bacterial targets indicative of higher or lower PTB risk for inclusion in a customized 384-well quantitative polymerase chain reaction (qPCR) array. The bacterial genera and species, their associated target genes, and limit of detection are presented in Table 1. All assays were designed (with the exception of *Mycoplasma hominis* and *M genitalium* assays), synthesized, and spotted by QIAGEN in a 384-well plate format using 5' FAM-labeled hydrolysis probes (1 target per row). A positive PCR control was included as a 16th target in the final row of the plate to control for any PCR inhibition that may have been introduced from the sample during DNA extraction. Positive (QIAGEN Microbial DNA Positive Control V2; #338135) and negative (nuclease-free water) control templates for each target were included with every run. The positive control template was prepared according to the manufacturer's instructions and included human genomic DNA (EpiTect

FIGURE 1
An overview of the sample collection process in the Predict1000 study



GA, gestational age; *PCR*, polymerase chain reaction; *qPCR*, quantitative polymerase chain reaction; *UTM*, universal transport medium.
Payne et al. Prediction of preterm birth using vaginal microbiology. Am J Obstet Gynecol 2021.

Control DNA; #59568) to more accurately reflect the true composition of a clinical sample. Reaction mixtures (final concentration) consisted of 1X QIAGEN Microbial qPCR Master Mix, primers and probes (prespotted and provided in dehydrated form in 384-well PCR plates) (QIAGEN), 4 μL template, and nuclease-free water (Integrated DNA Technologies, Coralville, IA) to a final volume of 10 μL. PCR cycling conditions consisted of an initial denaturation and Taq activation at 95°C for 10 minutes, followed by 40 quantification cycles of 95°C for 15 seconds and 60°C for 2 minutes (data acquiring). All reactions were conducted on a ViiA7 real-time PCR system and data were analyzed using QuantStudio Real-Time PCR Software v1.3 (Life Technologies, Carlsbad, CA).

Ureaplasma parvum and Ureaplasma urealyticum quantitative polymerase chain reaction assays

U parvum and *U urealyticum* DNA was detected from UTM vaginal swab DNA using real-time PCR targeting the urease gene, as described by Yi et al.[30] Reaction mixtures (final concentration) consisted of 1X Taqman FAST Advanced Master Mix (Life Technologies), 0.9 μM primers UU1613F and UU1524R (Life Technologies), 0.25 μM probes UU-parvo-FAM and UU-T960-VIC (Life Technologies), 5 μL of template DNA, and nuclease-free water (Integrated DNA Technologies) to a final volume of 20 μL. PCR cycling conditions consisted of an initial denaturation or Taq activation at 95°C for 20 seconds, followed by 40 quantification cycles of 95°C for 1 second and 60°C for 20 seconds (data acquiring). All reactions were conducted on a ViiA7 real-time PCR system and data were analyzed using QuantStudio Real-Time PCR Software version 1.3 (Life Technologies).

Ureaplasma parvum genotypes SV1, SV3, and SV6

U parvum genotypes SV1, SV3, and SV6 were detected from UTM vaginal swab DNA using a multiplex real-time PCR assay targeting the multiple-banded antigen gene, as described by Payne et al.[31] Reaction mixtures (final concentration) consisted of 1X Plex Mastermix (SpeeDx), 1X UP primers and probes mix, 5 μL of template DNA, and nuclease-free water (Integrated DNA Technologies) to a final volume of 20 μL. PCR cycling conditions were as follows: initial denaturation at 95°C for 2 minutes; 10 cycles of 95°C for 5 seconds and 61°C for 30 seconds (−0.5/cycle); and 40 cycles of 95°C for 5 seconds and 52°C for 40 seconds (data acquiring). All reactions were conducted on a ViiA7 real-time PCR system, and data were analyzed using QuantStudio Real-Time PCR Software v1.3 (Life Technologies).

Candida albicans quantitative polymerase chain reaction

Candida albicans DNA was detected from UTM vaginal swab DNA using a real-time PCR assay targeting the RNase P RNA gene of *Candida* sp. Reaction mixtures (final concentration) consisted of 1X Taqman FAST Advanced Master Mix (Life Technologies), 0.9 μM primers ALB-F (5' ACACGGAGTTTTAAGGCTGTAGAAG 3'), ALB-R (5' GTAGTAAAGAATTACTCACAGCCAACCA 3'), 0.25 μM probe ALB (FAM-CACGGCGCCATTCCCATACGAAG-TAMRA), 5 μL of template DNA, and nuclease-free water (Integrated DNA Technologies) to a final volume of 20 μL. PCR cycling conditions and data analysis were as described for *Ureaplasma parvum and Ureaplasma urealyticum*.

Atopobium vaginae quantitative polymerase chain reaction

Atopobium vaginae DNA was detected from UTM vaginal swab DNA using a real-time PCR assay targeting the 16S rRNA gene as described by Menard et al.[32] Reaction mixtures (final concentration) consisted of 1X Taqman FAST Advanced Master Mix (Life Technologies), 0.9 μM primers AV-F and AV-R, 0.25 μM probe AV-FAM, 5 μL of template DNA, and nuclease-free water (Integrated DNA Technologies) to a final volume of 20 μL. PCR cycling conditions and data analysis were as described for *Ureaplasma parvum and Ureaplasma urealyticum*.

Gardnerella vaginalis clade detection

Vaginal swab samples found to be positive for *G vaginalis* DNA in the custom 384-well array were subject to 2 additional duplex real-time PCR assays to describe the specific clades of *G vaginalis*

TABLE 1
Overview of the 15 bacterial species and genotype assays in the custom array

Bacterial species/genotype	Target gene/GenBank accession number	Limit of detection (target gene copies)	QIAGEN catalog number
L gasseri	16S rRNA/ NZ_ACGO01000023.1	50	BPID00189A
L crispatus	16S rRNA/ MK601675.1	200	BPID00186A
L jensenii	16S rRNA/ NZ_ACGQ01000014.1	30	BPID00191A
L iners	16S rRNA/ NZ_ACLN01000018.1	50	BPID00190A
F nucleatum	16S rRNA/ FJ471654.1	30	BPID00160A
Le amnionii	16S rRNA/ EU644469.1	20	BPID00204A
S sanguinegens	16S rRNA/AJ344093.1	20	BPID00309A
G vaginalis	16S rRNA/ CP001849.1	40	BPID00163A
Sialidase-producing *G vaginalis*	*Gardnerella vaginalis* glycosyl hydrolase/CP002104	40	BPID00603A
M indolicus (BVAB3)	16S rRNA/ KC311734.1	60	BPID00604A
Mobiluncus spp	16S rRNA/ AJ427624.2	30	BPID00608A
Prevotella spp	16S rRNA/ KP118764.1	90	BBID00606A
Peptostreptococcus spp	16S rRNA/ GU401468.1	30	BPID00607A
My hominis	yidC/GQ294576.1	20	BPID00605A
My genitalium	MgPa/ NC_000908.2	30	BPID00231A

F, Fusobacterium; G, Gardnerella; L, Lactobacillus; Le, Leptotrichia; M, Mageebacillus; My, Mycoplasma; S, Sneathia.
Payne et al. Prediction of preterm birth using vaginal microbiology. Am J Obstet Gynecol 2021.

present in the sample as described by Balashov et al.[33] Reaction mixtures (final concentration) consisted of 1X Taqman FAST Advanced Master Mix (Life Technologies), either 0.9 μM primers Gv1_fuc1_S/Gv1_fuc1_AS/Gv2_hyp_S/Gv2_hyp_AS and 0.25 μM probes Gv1_fuc1_TM-FAM/Gv2_hyp_TM-JOE (reaction mix 1) or 0.9 μM primers Gv3_thi_S/Gv3_thi_AS/Gv4_cic_S/Gv4_cic_AS, 0.25 μM probes Gv3_thi_TM-FAM and Gv4_cic_TM-JOE (reaction mix 2), 5 μL of template DNA, and nuclease-free water (Integrated DNA Technologies) to a final volume of 20 μL. PCR cycling conditions and data analysis were as described for *Ureaplasma parvum and Ureaplasma urealyticum*.

Statistical analysis

Microbial and pregnancy data were summarized using frequency distributions for categorical data and median, interquartile range, and range for continuous data. Categorical outcomes were compared using chi-square and Fisher exact tests and continuous outcomes using the Mann-Whitney U test.

The main objective of the analysis was to develop a microbial biomarker test for the prediction of sPTB risk. Recursive partitioning methods were used to initially identify a combination of such biomarkers for the prediction of sPTB. Internally cross-validated classification trees were constructed to group sPTBs and term births into separate subclasses (or term and non-sPTB births combined) using semiquantitative and presence or absence microbial data. Several classification analyses were performed to predict any sPTB or sPTB at ≤34 weeks' gestation. The recursive partitioning analyses were then further refined to improve the test specificity through an iterative process informed by microbial risk profile.

Univariable and multivariable logistic regression were used to assess the impact of maternal characteristics on the derived microbial biomarker test and the likelihood of sPTB. The covariate effects were summarized using odds ratios (ORs) and their 95% confidence intervals. SPSS version 25.0 (International Business Machines, Armonk, NY) statistical software was used for data analysis. $P<.05$ was considered statistically significant. No adjustment for multiple comparisons was made in the univariate comparisons of demographic, clinical, and microbial characteristics, because these comparisons were only used to describe differences in the data.[34]

Results

Subjects

A total of 2137 women were assessed and approached to participate. Notably, 335 declined and 802 were ineligible; 1000 women in total were recruited to the study. From these, 8 withdrew, 12 were lost to follow-up, and 44 provided samples that were insufficient for microbial analysis. Vaginal samples from the remaining 936 women formed the final study cohort. Demographic, delivery (Table 2), and lifestyle (Table 3) characteristics of these women and rates of associated PTB are provided below. The overall PTB rate (<37 weeks' gestation) was 12.6%, which

TABLE 2
Demographic and birth characteristics of women in the study and associated rates of PTB

Characteristic	sPTB (n=58)	Non-sPTB(n=60)	Term (n=818)	*P* value	*P* value (term vs sPTB)
Maternal age, y					
<25	3 (5.2)	5 (8.3)	81 (9.9)	.566	.503
25–34	38 (65.5)	42 (70.0)	505 (61.9)		
≥35	17 (29.3)	13 (21.7)	230 (28.2)		
Race or ethnicity					
White	46 (79.3)	43 (71.7)	607 (74.2)	.452	.899
Asian	8 (13.8)	4 (6.7)	105 (12.8)		
African	1 (1.7)	4 (6.7)	38 (4.6)		
Indian	1 (1.7)	2 (3.3)	32 (3.9)		
Aboriginal	1 (1.7)	4 (6.7)	17 (2.1)		
Other	1 (1.7)	2 (3.3)	15 (1.8)		
Unknown	—	1 (1.6)	4 (0.5)		
Body mass index, kg/m^2					
<25	18 (32.1)	22 (35.5)	383 (47.0)	.029[a]	.013[a]
25–29.9	20 (35.7)	17 (27.4)	182 (22.3)		
≥30	18 (32.1)	23 (37.1)	250 (30.7)		
Socioeconomic indexes for areas					
<50th percentile	27 (46.6)	24 (38.7)	236 (28.9)	.005[a]	.007[a]
Parity					
Nulliparous	16 (27.6)	24 (40.0)	314 (38.4)	.245	.123
Parous	42 (72.4)	36 (60.0)	504 (61.6)		
History of sPTB[b]	15 (35.7)	3 (8.3)	70 (13.9)	.001[a]	<.001[a]
History of non-sPTB[b]	7 (16.7)	20 (55.6)	98 (19.4)	<.001[a]	.661
Threatened abortion	13 (22.4)	6 (10.0)	89 (10.9)	.027[a]	.012[a]
Antepartum hemorrhage	15 (25.9)	6 (10.0)	55 (6.7)	<.001[a]	<.001[a]
Clinical chorioamnionitis	10 (17.2)	—	2 (0.2)	<.001[a]	<.001[a]
Histologic chorioamnionitis	14 (24.1)	6 (10.2)	39 (4.8)	<.001[a]	<.001[a]
Premature rupture of membranes[c]	39 (67.2)	1 (1.7)	6 (0.7)	<.001[a]	<.001[a]
Threatened preterm labor	46 (79.3)	7 (11.7)	25 (3.1)	<.001[a]	<.001[a]
Preeclampsia	1 (1.7)	21 (35.0)	22 (2.7)	<.001[a]	.733
Gestational diabetes	13 (22.4)	12 (20.3)	122 (15.0)	.199	.134
Gestational age at birth, wk					
≤28	6 (10.3)	7 (11.7)	—		
29–34	21 (36.2)	14 (23.3)	—		
35–36	31 (53.4)	39 (65.0)	—		
≥37	—	—	818 (100)		
Male sex	24 (41.4)	35 (58.3)	396 (48.4)	.174	.342

Payne et al. Prediction of preterm birth using vaginal microbiology. Am J Obstet Gynecol 2021. *(continued)*

TABLE 2
Demographic and birth characteristics of women in the study and associated rates of PTB *(continued)*

Characteristic	sPTB (n=58)	Non-sPTB(n=60)	Term (n=818)	*P* value	*P* value (term vs sPTB)
Birthweight, g					
Median(IQR) [min-max]	2555 (2165–2954) [620–3680]	2444 (1722–3000) [540–4316]	3385 (3100–3700) [2317–5050]	<.001[a]	<.001[a]
<1000	3 (5.2)	5 (8.3)	—	<.001[a]	<.001[a]
<1500	6 (10.3)	12 (20.0)	—	<.001[a]	<.001[a]
<2500	23 (39.7)	31 (51.7)	20 (2.4)	<.001[a]	<.001[a]

Data are expressed as number (percentage) unless indicated otherwise.

IQR, interquartile range; *PTB*, preterm birth; *sPTB*, spontaneous preterm birth.

[a] Significant at $P<.05$; [b] Parous women only (n=582); [c] In all 6 term births (5 at 37^{+0} and 1 at 37^{+1}), premature rupture of membranes occurred at 36 weeks' gestation.

Payne et al. Prediction of preterm birth using vaginal microbiology. Am J Obstet Gynecol 2021.

included 58 sPTB cases (6.2%) and 60 non-sPTB cases that required labor induction or cesarean delivery performed for maternal or fetal indications (6.4%). There were 48 births at ≤34 weeks' gestation (5.2%), comprising 25 sPTB cases (2.9%) and 23 non-sPTB cases (2.2%). The preterm premature rupture of the membranes rate was 4.2%. There were 18 newborns in total with a birthweight of <1500 grams (1.9%), and of these, 6 were sPTB cases (10.3% of the sPTB group). No lifestyle characteristics were predictive of sPTB in their own right (Table 3). Progesterone administration was significantly associated with sPTB (24.4% sPTB vs 8.9% term; $P=.003$); however, it should be noted that in Western Australia progesterone is only prescribed to women at high risk of PTB and will not prevent this outcome in many cases.

TABLE 3
Lifestyle characteristics of women in the study and associated rates of PTB[a]

Characteristic	sPTB(n=51)	Non-sPTB (n=59)	Term (n=794)	*P* value	*P* value (sPTB vs term)
Medication use (n=904)					
Antibiotic agents (n=904)	2 (3.9)	2 (3.4)	22 (2.8)	.903	.651
Antifungal agents (n=904)	—	4 (6.8)	28 (3.5)	.126	.252
Probiotic supplements (n=904)	5 (9.8)	3 (5.1)	58 (7.3)	.646	.578
Vitamin supplements (n=904)	28 (54.9)	33 (55.9)	511 (64.4)	.191	.179
Other natural remedies (n=904)	—	2 (3.4)	10 (1.3)	.255	.653
Progesterone (n=757)	11 (24.4)	5 (9.6)	59 (8.9)	.005[b]	.003[b]
Any smoking before 20 wk gestation (n=936)	12 (20.7)	11 (18.3)	103 (12.6)	.113	.078
Current alcohol (n=934)	4 (7.1)	3 (5.0)	37 (4.5)	.684	.509
Vaginal intercourse/wk (n=872)					
None	13 (27.7)	11 (19.6)	183 (23.8)	.754	.740
1–2	26 (55.3)	40 (71.4)	486 (63.2)		
3–4	6 (12.8)	3 (5.4)	75 (9.8)		
5≥	2 (4.3)	2 (3.6)	25 (3.3)		

Data are expressed as number (percentage) unless indicated otherwise.

PTB, preterm birth; *sPTB*, spontaneous preterm birth.

[a] A total of 904 women completed the study questionnaire to some extent (percentages out of 904 unless otherwise stated); smoking and alcohol (yes or no) data were supplemented with obstetrical data from participant medical records; [b] Significant at $P<.05$.

Payne et al. Prediction of preterm birth using vaginal microbiology. Am J Obstet Gynecol 2021.

Association between detection of vaginal microbial targets and spontaneous preterm birth

Semiquantitative analysis

Custom 15-target microbial real-time polymerase chain reaction array. A standard curve was generated in duplicate from the microbial DNA V2 positive control at concentrations of 20,000, 2000, 200, and 20 target gene copies. Using the mean cycle threshold (Ct) values generated for each target from this as a reference, amplified targets for each sample were grouped for semiquantitative analyses as follows: high, >20,000 gene copies; mid, 2000 to 19,999 gene copies; low, <2000 gene copies. Using these criteria, no association between the abundance of any single target and increased risk of sPTB was identified (data not indicated). However, upon collective analysis of "high" gene copy numbers of *L crispatus*, *L gasseri*, or *L jensenii*, a significant negative association was identified between this and risk of sPTB (8.2% low copy vs 3.0% high copy; P=.005). Similar associations were seen for risk of premature rupture of membranes (PROM) (6.1% [n=40] low copy vs 2.1% [n=6] high copy; P=.009).

Presence and absence analysis

All microbial targets. In total, 23 microbial targets were assessed in this study. Of these, 21 were detected in at least 1 sample (Table 4). All samples were negative for *Prevotella* spp and *Mobiluncus* spp. The presence of any individual microbial target was not indicative of a significantly increased sPTB risk. Conversely, the detection of *L gasseri* was significantly negatively associated with sPTB risk (P=.017).

Discrimination of* Gardnerella vaginalis *clades. *G vaginalis* can be separated into 4 distinct clades through the analysis of 4 different genes.[33] Because >50% of our samples were positive for *G vaginalis* DNA, additional analyses were conducted to discriminate these at the clade level.[33] The most frequently detected clade was clade 1, followed by clades 4, 2, and 3. Clades were unable to be resolved in 27 samples (Table 5). No association between any clade and sPTB risk was detected.

Identification of a specific microbial deoxyribonucleic acid signature predictive of spontaneous preterm birth. Considering that our underlying hypothesis was to create a microbial DNA signature that was predictive of women at risk of infection-mediated PTB, data from all non-sPTB cases (n=60) were excluded when conducting analyses to identify a microbial DNA signature to predict the risk of sPTB. Data from 876 women (sPTB, 58; term, 818) were used to identify the most suitable combination of microbial biomarkers for PTB risk prediction. Through a combination of recursive partitioning analyses (Supplemental Material S2, Supplemental Figure 1) and iterative refinement, we arrived at a predictive microbial algorithm that we named the Gardnerella, Lactobacillus, Ureaplasma (GLU) test (Table 6, Figure 2). Additional recursive partitioning analyses were then conducted, combining the GLU test with other known obstetrical risk factors for PTB to further enhance the ability for the prediction of sPTB risk (Supplemental Material S2, Supplemental Figure 2). However, these analyses failed to identify any improvements.

The microbial signature comprising the GLU test was 100% bacterial and consisted of initial discrimination into low- or high-risk categories, respectively, based on the presence or absence of high levels of *L crispatus*, *L gasseri*, or *L jensenii* DNA. After this, in high-risk women, either a combination of *G vaginalis* (clade 4), *L iners*, and *U parvum* (serovars 3 and 6) or detection of *Fusobacterium nucleatum* (in the absence of *U parvum* serovars 3 and 6, irrespective of detection of other bacterial DNA) resulted in a GLU-positive result (Table 6, Figure 2). The final GLU test algorithm predicted 38% of sPTB cases and 44% of sPTB cases at ≤34 weeks' gestation, with ORs of 3.3 and 4.6, respectively (Table 7).

Maternal characteristics and risk of spontaneous preterm birth at <37 and ≤34 weeks' gestation

After adjustment for known obstetrical risk factors, a number of significant associations were identified between specific maternal characteristics and sPTB at <37 weeks' gestation, including having a body mass index (BMI) of 25 to 29 kg/m^2 (P=.02), being in the socioeconomic indexes for areas category at <50 (P=.018), a history of sPTB (P<.001), or a GLU-positive vaginal swab result (P<.001). For cases of sPTB at ≤34 weeks' gestation, only a history of sPTB or a GLU-positive vaginal swab result was significantly associated (P=<.001 in both cases). The only obstetrical risk factor that was more predictive of sPTB risk in its own right than a GLU-positive vaginal swab result was having a history of PTB, both at <37 weeks' gestation (OR, 3.6 vs 3.3) and ≤34 weeks' gestation (OR, 8.5 vs 4.6) (Table 8).

Maternal characteristics and pregnancy or neonatal outcomes in GLU-positive and GLU-negative women

For the analysis of the relationship between maternal characteristics and pregnancy outcomes relative to GLU status, all birth outcomes (including non-sPTB cases) were included (Table 9), because if the GLU test were to be used for the diagnosis of sPTB risk, then treatment decisions would be made prospectively blinded to birth outcome.

Of those maternal characteristics known to be PTB risk factors, women who smoked during pregnancy were significantly more likely to be GLU-positive (P=.02), as were women with a BMI of <25 kg/m^2 (P=.03). Ethnicity did not seem to have a marked impact on GLU status; however, the cohort was predominately of white women, which limited our power to explore this. Interestingly, Indigenous (Aboriginal) women were more than twice as likely to be GLU-positive; however, the numbers (n=22) were too low for any meaningful statistical analysis of this association. A history of sPTB was not associated with GLU status.

In terms of pregnancy outcome, rates of PROM and threatened preterm labor

TABLE 4
Detection of microbial targets in vaginal swab DNA and association with sPTB

Genus/species/genotype	sPTB	Non-sPTB n (%)	Term	*P* value[a]	*P* value sPTB vs Term[a]
L gasseri	17 (29.3)	33 (55.0)	372 (45.5)	.016[b]	.017[b]
L crispatus	25 (43.1)	28 (47.5)	347 (42.4)	.751	.919
L jensenii	17 (29.3)	15 (25.9)	257 (31.4)	.651	.738
L gasseri/L crispatus/L jensenii ≥20,000 16S rRNA gene copies	8 (13.8)	22 (36.7)	255 (31.2)	.012[b]	.005[b]
L iners	32 (55.2)	32 (54.2)	416 (50.9)	.735	.525
F nucleatum	15 (25.9)	17 (28.3)	162 (19.8)	.177	.267
Le amnionii	6 (10.3)	7 (11.7)	82 (10.0)	.919	.937
S sanguinegens	2 (3.4)	5 (8.3)	48 (5.9)	.539	.571
G vaginalis	31 (53.4)	34 (56.7)	438 (53.5)	.895	.989
Sialidase-producing *G vaginalis*	19 (32.8)	26 (43.3)	306 (37.4)	.489	.479
M indolicus (BVAB3)	1 (1.7)	2 (3.3)	12 (1.5)	.566	1.000
Mobiluncus spp	—	—	—	—	—
Prevotella spp	—	—	—	—	—
Peptostreptococcus spp	17 (29.3)	25 (41.7)	219 (26.8)	.044[b]	1.000
My hominis	2 (3.4)	7 (11.7)	20 (2.4)	.003[b]	.652
My genitalium	0 (0)	1 (1.7)	2 (0.2)	.333	1.000
Ureaplasma spp	1 (1.7)	3 (5.0)	24 (2.9)	.567	.723
U parvum	27 (46.6)	29 (48.3)	352 (43.0)	.651	.601
U parvum genotype SV1	7 (12.1)	5 (8.3)	76 (9.3)	.750	.485
U parvum genotype SV3	13 (22.4)	18 (30.0)	166 (20.3)	.195	.699
U parvum genotype SV6	13 (22.4)	11 (18.3)	152 (18.6)	.767	.471
U parvum genotype SV3 and/or SV6	25 (43.1)	28 (46.7)	308 (37.7)	.293	.409
U urealyticum	5 (8.6)	9 (15.0)	77 (9.4)	.355	.841
A vaginae	39 (67.2)	45 (75.0)	616 (75.3)	.393	.172
C albicans	7 (12.1)	10 (16.7)	132 (16.1)	.706	.413

Data are expressed as number (percentage) unless indicated otherwise.

A, Atopobium; *C, Candida*; *F, Fusobacterium*; *G, Gardnerella*; *L, Lactobacillus*; *Le, Leptotrichia*; *M, Mageebacillus*; *My, Mycoplasma*; *rRNA*, ribosomal ribonucleic acid; *S*, Sneathia; *sPTB*, spontaneous preterm birth; *U, Ureaplasma.*

[a] Once the false discovery rate (at 5% or 10%) was controlled for using the Benjamini-Hochberg adjustment, none of the univariately significant associations found without adjustment remained significant after adjustment for multiple testing; [b] Significant at $P<.05$.

Payne et al. Prediction of preterm birth using vaginal microbiology. Am J Obstet Gynecol 2021.

were both significantly associated with a GLU-positive result ($P=.004$ and $P=.049$, respectively). There was no association between GLU status and either clinical or histologic chorioamnionitis; however, the number of cases was low. For neonatal outcomes, significant associations were identified between GLU-positive status and an Apgar score of <7 at 1 minute ($P=.039$) and intraventricular hemorrhage (IVH; $P=.002$); however, the numbers in the IVH group were very low (n=4). The development of neonatal sepsis (any sepsis diagnosis before discharge) was not associated with GLU status.

Discussion

Principal findings

This study reports the successful development of an assay, namely, the GLU test, that utilizes vaginal microbial biomarkers for PTB prediction. Ascending intrauterine infection has been identified for more than 30 years as a leading cause of sPTB,[1,3] and links between the vaginal microbiota and PTB have been reported for well over a decade[35]; despite this there are still no commercially available, effective diagnostic tests that predict the risk of infection-associated PTB. Our test's design employed high resolution,

TABLE 5
Detection of *Gardnerella vaginalis* clades in *Gardnerella vaginalis*–positive vaginal swab DNA extracts and association with sPTB

Gardnerella vaginalis clade	sPTB	non-sPTB	Term	*P* value	*P* value(sPTB vs term)
1	22 (37.9)	21 (35.0)	286 (35.0)	.900	.647
2	10 (17.2)	15 (25.0)	154 (18.8)	.468	.765
3	3 (5.2)	4 (6.7)	28 (3.4)	.407	.715
4	22 (37.9)	20 (33.3)	258 (18.8)	.587	.313
Unresolved	2 (3.4)	3 (5.0)	22 (2.7)	.649	1.000

Values are expressed as number (percentage) unless indicated otherwise.

sPTB, spontaneous preterm birth.

Payne et al. Prediction of preterm birth using vaginal microbiology. Am J Obstet Gynecol 2021.

targeted molecular methods, based on previously reported associations between microbial intrauterine pathogens and negative pregnancy outcomes.[5] The final microbial DNA signature algorithm encompasses the well-known protective properties of 3 common *Lactobacillus* spp,[36–39] and the 2 microorganisms most commonly detected in the amniotic cavity and/or placental tissues of infection-related deliveries: *Ureaplasma* spp and *F nucleatum*.[5] The algorithm also includes the presence of *L iners*, an organism that has been garnering increasing interest of late in regard to its potential association with PTB,[15,21,40] and *G vaginalis*, 1 of the primary organisms linked to BV[41,42] and previously associated with PTB.[10,16,22] Hence, the test is both novel and yet entirely consistent with the current understanding regarding vaginal microbiota and the pathogenesis of sPTB. Importantly, the test allows the discrimination of women who are positive for *Ureaplasma* sp and who are or are not at risk of PTB. Although we and others have previously shown significant associations between vaginal *Ureaplasma* spp and PTB,[6–8,43] as much as 66% of pregnant women are vaginally colonized with these organisms,[44] rendering detection alone of limited use in terms of guiding treatment decisions. Because the test employs self-collected midgestation vaginal swabs, it could be easily incorporated into routine antenatal care, with a high rate of patient compliance expected; the resultant data could be used to administer appropriate antimicrobial treatments in a timely fashion to attempt to prevent infection-associated PTB. Hence, the practical

TABLE 6
Rates of preterm and term birth stratified according to microbial parameters employed in the GLU test

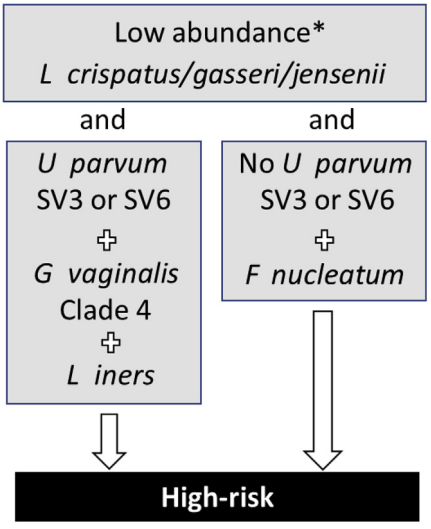
Parameter	N	sPTB at <37 wk GA	sPTB at ≤34 wk GA	Term
Low risk				
High LC/LG/LJ	263	3.0 (8)	2.3 (6)	97.0 (255)
Low LC/LG/LJ, UPSV3/SV6=0, *F nucleatum*=0	306	6.5 (20)	2.3 (7)	93.5 (286)
Low LC/LG/LJ, UPSV3/SV6=1, *L iners*=0	54	5.6 (3)	1.9 (1)	94.4 (51)
Low LC/LG/LJ, UPSV3/SV6=1, *L iners*=1 and *G vaginalis* clade 4=0	91	5.5 (5)	1.1 (1)	94.5 (86)
High risk				
Low LC/LG/LJ, UPSV3/SV6=1, *L iners*=1 and *G vaginalis* clade 4=1	102	11.8 (12)	8.8 (9)	88.2 (90)
Low LC/LG/LJ, UPSV3/SV=0, *F nucleatum*=1	60	16.7 (10)	5.0 (3)	83.3 (50)

Data are presented as number or percentage (number).

F, *Fusobacterium*; *G*, *Gardnerella*; *GA*, gestational age; *L*, *Lactobacillus*; *sPTB*, spontaneous preterm birth; *UPSV3/SV6*, *Ureaplasma parvum* genotype SV3 or SV6.

Payne et al. Prediction of preterm birth using vaginal microbiology. Am J Obstet Gynecol 2021.

FIGURE 2
The GLU test algorithm



F nucleatum, *Fusobacterium nucleatum*; *G vaginalis*, *Gardnerella vaginalis*; *L crispatus*, *Lactobacillus crispatus*; *L gasseri*, *Lactobacillus gasseri*; *L iners*, *Lactobacillus iners*; *L jensenii*, *Lactobacillus jensenii*; *U parvum*, *Ureaplasma parvum*.

Payne et al. Prediction of preterm birth using vaginal microbiology. Am J Obstet Gynecol 2021.

clinical applications and benefits of the test are potentially highly significant.

Results

Microbial characteristics of the test

Although we hypothesized that women with specific genotypes of *Ureaplasma* spp might be at increased risk of sPTB, possibly in combination with other bacteria, our study did not identify associations between any single microbial DNA target and increased risk of sPTB. Vaginal *Ureaplasma* colonization, specifically by *U parvum*, has been associated with PTB in most previous studies where this has been investigated,[43] including our own which identified SV3 and SV6 as being particularly high risk.[6] Perhaps the most convincing of these is the Austrian study by Rittenschober-Böhm et al,[8] which reported a significant association between vaginal *U parvum* and PTB among 4330 pregnant women sampled at 12 to 14 weeks' gestation. Furthermore, these same authors reported that this association remained when discriminating by *U parvum* genotype for genotypes SV1, SV3, and SV6.[7] In light of the clear importance of *U parvum* and up to 3 of its 4 genotypes in determining sPTB risk, it will be interesting to assess the comparable prevalence and risk associations in women of different ethnicities and country of origin.

Similarly, we applied clade-level resolution to refine the predictive properties of detection of *G vaginalis*. *G vaginalis* is 1 of the primary organisms linked to BV,[41,42] and numerous studies have reported significant associations between both BV and asymptomatic vaginal colonization by *G vaginalis* and PTB.[9,10,16,22] However, since the separation of *G vaginalis* isolates into 4 clades in 2012,[45] only 1 study has examined whether this association with PTB is clade-specific, with Callahan et al[16] reporting this for clade 2. Although we did not see any association between individual *G vaginalis* clades and PTB on their own, we found that the specificity of the GLU algorithm was improved when *G vaginalis* clade 4, as opposed to *G vaginalis*, was included. Although this contrasts with the findings of Callahan et al[16] and previous evidence of an association between clades 1 and 3 of *G vaginalis* and the presence of BV,[33] our findings could be a reflection of the ethnic and geographic characteristics of our cohort and the low rate of BV. This is supported by data from Janulaitiene et al[46] who reported that clade 4 was the most commonly detected clade among 75 vaginal samples and was not associated with BV. Somewhat unexpectedly, we did not see an association between the presence of the *G vaginalis* sialidase gene and PTB, despite previous studies finding that vaginal sialidase levels are strongly associated with PTB risk.[47–49] Because the measurement of sialidase is utilized as a proxy for BV in the commonly used OSOM BV Blue commercial diagnostic test (Sekisui Diagnostics, Burlington, MA), sialidase positivity has previously been associated with PTB.[9,50,51] Because BV is particularly common in cohorts of African American women,[52] perhaps the generally low rates of BV seen in white women may help to explain this observation.

Our microbial risk algorithm was significantly enhanced by the inclusion of *F nucleatum* in samples in which *U parvum* genotypes SV3 and SV6 were absent. *F nucleatum* has previously been reported as the second most common bacteria likely to be isolated from infected amniotic fluid in cases of PTB,[5] so its inclusion in the test is a significant strength and is consistent with pathophysiological predictions. Although *F nucleatum* is commonly associated with the oral cavity and conditions such as periodontal disease,[53] it has also been

TABLE 7
Diagnostic performance characteristics of the GLU test

Characteristic	%Positive	Sensitivity	Specificity	PPV	NPV	PLR	NLR	Accuracy
sPTB at <37 wk gestation	18.7	37.9	82.9	13.6	95.0	2.22	0.75	79.9
95% CI		25.5–51.6	80.1–85.4	9.9–18.4	93.9–95.9	1.54–3.18	0.61–0.92	77.1–82.5
sPTB at ≤34 wk gestation	18.7	44.4	82.2	7.4	98.0	2.52	0.67	81.1
95% CI		25.5–64.7	79.6–84.8	4.9–11.1	97.1–98.5	1.61–3.93	0.48–0.95	78.4–98.5

Data are presented as percentage or 95% CI.

CI, confidence interval; *NLR*, negative likelihood ratio; *NPV*, negative predictive value; *PLR*, positive likelihood ratio; *PPV*, positive predictive value; *sPTB*, spontaneous preterm birth.

Payne et al. Prediction of preterm birth using vaginal microbiology. Am J Obstet Gynecol 2021.

TABLE 8
Maternal characteristics and risk of sPTB

Characteristic	OR	95% CI	*P* value	aOR	95% CI	*P* value
sPTB at ≤37 wk GA						
Maternal age						
<30	1.00					
30–40	0.80	0.46–1.42	.450			
≥40	1.65	0.59–4.61	.240			
Body mass index, kg/m^2						
<25	1.00			1.00		
25–29	2.56	1.34–4.89	.004[a]	2.24	1.13–3.60	.020[a]
≥30	1.53	0.78–3.00	.214	1.14	0.56–2.33	.718
White race	1.33	0.69–2.56	.390			
SEIFA at <50th percentile	2.13	1.24–3.66	.006[a]	2.02	1.13–3.60	.018[a]
Smoking in pregnancy	1.81	0.93–3.53	.081			
Alcohol in pregnancy	1.62	0.56–4.73	.374			
History of sPTB						
P1+, no	1.00			1.00		
P1+, yes	3.44	1.75–6.78	<.001[a]	3.61	1.75–7.42	<.001[a]
P0	0.82	0.43–1.55	.538	0.91	0.47–1.75	.773
GLU positive	2.96	1.70–5.19	<.001[a]	3.28	1.81–5.95	<.001[a]
sPTB at ≤34 wk GA						
Maternal age						
<30	1.00					
30–40	0.78	0.35–1.73	.545			
≥40	0.62	0.08–4.94	.653			
Body mass index, kg/m^2						
<25	1.00					
25–29	2.52	0.98–6.48	.055			
≥30	1.71	0.65–4.48	.278			
White race	1.20	0.48–3.02	.696			
SEIFA at <50th percentile	2.14	0.98–4.61	.053	2.09	0.93–4.66	.073
Smoking in pregnancy	1.53	0.56–4.12	.403			
Alcohol in pregnancy	0.81	0.11–6.11	.836			
History of sPTB						
P1+, no	1.00			1.00		
P1+, yes	6.70	2.63–17.03	<.001[a]	8.45	3.21–22.29	<.001[a]
P0	1.25	0.48–3.27	.652	1.32	0.49–3.54	.578
GLU positive	3.73	1.71–8.13	.001[a]	4.55	2.00–10.34	<.001[a]

aOR, odds ratios simultaneously adjusted for all predictors (maternal age, white race, smoking, and alcohol during pregnancy removed from the adjusted model); *GA*, gestational age; *OR*, odds ratio; *SEIFA*, socioeconomic indexes for areas; *sPTB*, spontaneous preterm birth.

[a] Significant at *P*<.05.

Payne et al. Prediction of preterm birth using vaginal microbiology. Am J Obstet Gynecol 2021.

TABLE 9
Demographics, pregnancy, and neonatal outcomes according to GLU test status

Characteristic	GLU negative (n=761)	GLU positive (n=175)	*P* value
Maternal characteristics			
Race or ethnicity (n=935)			
White	569 (74.9)	127 (72.6)	.102
Indigenous	14 (1.8)	8 (4.6)	
Other	177 (23.3)	40 (22.9)	
Parity=0			
No	483 (63.5)	99 (56.6)	.090
Yes	278 (36.5)	76 (43.4)	
History of sPTB (parity>0) (n=582)			
No	409 (84.7)	85 (85.9)	.765
Yes	74 (15.3)	14 (14.1)	
Body mass index, kg/m^2 (n=934)			
<25	355 (46.8)	68 (38.9)	.030[a]
25–29	182 (24.0)	38 (21.7)	
30+	222 (29.2)	69 (39.4)	
Smoking in current pregnancy			
No	668 (97.8)	142 (81.1)	.020[a]
Yes	93 (12.2)	33 (18.9)	
Alcohol in current pregnancy (n=934)			
No	719 (94.7)	171 (97.7)	.093
Yes	40 (5.3)	4 (2.3)	
Socioeconomic indexes for areas at <50th percentile (n=910)			
No	510 (68.9)	113 (66.5)	.536
Yes	230 (31.1)	57 (33.5)	
Pregnancy outcomes			
Premature rupture of membranes (n=935)			
No	730 (96.1)	159 (90.9)	.004[a]
Yes	30 (3.9)	16 (9.1)	
Threatened preterm labor (n=935)			
No	704 (92.5)	153 (87.9)	.049[a]
Yes	57 (7.5)	21 (12.1)	
Clinical chorioamnionitis (n=935)			
No	752 (98.9)	171 (98.3)	.707
Yes	9 (1.2)	3 (1.7)	
Histologic chorioamnionitis (n=927)			
No	703 (93.4)	165 (94.8)	.475
Yes	50 (6.6)	9 (5.2)	

Payne et al. Prediction of preterm birth using vaginal microbiology. Am J Obstet Gynecol 2021. *(continued)*

TABLE 9
Demographics, pregnancy, and neonatal outcomes according to GLU test status *(continued)*

Characteristic	GLU negative (n=761)	GLU positive (n=175)	*P* value
Maternal sepsis after delivery (n=930)			
No	629 (83.2)	147 (84.5)	0.682
Yes	127 (16.8)	27 (15.5)	
Apgar score at 1 min of <7 (n=929)			
No	654 (86.5)	139 (80.3)	.039[a]
Yes	102 (13.5)	34 (19.7)	
Apgar score at 5 min of <7 (n=929)			
No	736 (97.4)	169 (97.7)	1.000
Yes	20 (2.6)	4 (2.3)	
Special care nursery admission (n=935)			
No	541 (71.1)	115 (66.1)	.194
Yes	220 (28.9)	59 (33.9)	
Neonatal sepsis			
No	681 (89.5)	155 (88.6)	.724
Yes	80 (10.5)	20 (11.4)	
Intraventricular hemorrhage			
No	760 (99.9)	172 (98.3)	.002[a]
Yes	1 (0.1)	3 (1.7)	

Data are expressed as number (percentage) unless indicated otherwise; n=936.

[a] Significant at $P<.05$.

Payne et al. Prediction of preterm birth using vaginal microbiology. Am J Obstet Gynecol 2021.

associated with PTB in multiple studies[54–58]; however, its presence in the vaginal microbiota in the context of PTB risk has until now remained largely unexplored. Previous studies have suggested that transfer to the uterus occurs by means of vaginal ascension[1] or hematogenous spread arising from the oral cavity.[54,55] Unfortunately, we did not collect data on *F nucleatum* oral colonization in the women or their partners and did not employ subspecies or strain-level detection for this organism. All of these factors should be considered in future studies to help elucidate whether *F nucleatum* is a true vaginal or oral microbe, whether it can exist at either site independently, and, most importantly, if vaginal or oral strains are predominately associated with sPTB.

L iners, despite being a common vaginal commensal bacteria, has attracted substantial interest of late in regard to PTB, with Kindinger et al[15] reporting a significant association between vaginal colonization and a short cervix. This species of *Lactobacillus* sp has also been linked to "intermediate" vaginal community states (a vaginal microbiota that may indicate a shift toward BV) and BV.[59–61] *L iners* is also frequently seen to cooccur with *G vaginalis*, unlike other "protective" *Lactobacillus* spp such as *L crispatus*,[62] and has also been previously associated with an increased risk of vaginal infection with *Chlamydia trachomatis*.[63] Of note, *L iners* is the only vaginal *Lactobacillus* spp capable of producing a cytolytic enzyme, inerolysin, which it uses to lyse erythrocytes[64]; this may also explain reported increases in vaginal *L iners* levels during menses.[59,65] Future studies should examine vaginal levels of this enzyme, coupled with the ability of associated strains to produce it *in vitro*, and any association with pregnancy outcome. It should also be noted that although *L iners* is capable of producing lactic acid, it produces little of the D-lactic acid isomer, which is more important in terms of acidification and antimicrobial properties.[66]

Comparison with previous studies

Studies with a similar interest as ours that have employed holistic, vaginal microbial community profiling approaches have shown mixed success, with PTB associations typically being ethnicity and/or population specific. Associations between vaginal *Mycoplasma* spp and PTB have been reported by Wen et al[13] and Foxman et al[12] in cohorts of African American and Hispanic women, whereas DiGiulio et al[10] reported associations between low abundance of *Lactobacillus* spp, high abundance of *Gardnerella* spp, and high abundance of *Ureaplasma* spp and PTB. Callahan et al[16] replicated these data to

some extent in another cohort of white women, again finding a significant link between PTB and low or high *Lactobacillus*/*Gardnerella* spp, and a nonsignificant trend toward high *Ureaplasma* spp and PTB. In contrast, these authors also included a cohort of African American women in their study and reported that the same observations were not useful for PTB prediction.

Most recently, Hočevar et al[22] reported that Slovenian women were at higher risk of sPTB when vaginal *Lactobacillus* spp abundance was decreased and levels of *G vaginalis* and other BV organisms were increased; however, they did not report any association between *Ureaplasma* spp and sPTB. In 1 of the largest studies conducted to date, Elovitz et al[21] described 7 bacterial taxa associated with sPTB in a nested case-control study; however, the risk effect was predominantly seen in African American women, which comprised 75% of the cohort. Here, *Mobiluncus curtisii, M. mulieris*, *Mageebacillus indolicus*, *Sneathia sanguinegens*, *Porphyromonas asaccharolytica,* and *Megasphaera* spp were associated with an increased risk of sPTB (in descending order). In white women, higher rates of sPTB were seen where *L iners* and *A vaginae* were detected, although with much more modest effect sizes than that seen for *M. curtisii, M. mulieris,* and *M. indolicus* in African American women. Of note, this study did not describe any association between *Ureaplasma* sp and the risk of sPTB in white women, unlike that seen in our own previous work,[6] and that of others.[7,8] However, the authors only utilized V3-V4 short-amplicon 16S rRNA gene sequencing for microbial profiling purposes; hence, they would have lacked accurate species-level resolution, because the homology between *U parvum* and *U urealyticum* is identical in this region of the 16S rRNA gene. Collectively, these results reinforce the importance of personalizing vaginal microbial risk prediction assays toward the ethnicity of the population being studied at the very least (geographic and environmental factors may also be important[27]), because our study did not identify any *Mobiluncus* spp DNA and only 15 instances of *M indolicus*, which were the top 2 predictive targets for African American women in the Elovitz et al[21] study.

Although the vaginal microbiome studies described earlier have investigated associations between different microbial profiles and PTB risk, differences in the study design and prediction outcomes make it difficult to directly compare the predictive performance of their findings with our data. For example, several of the studies employed a nested case-control design, preventing comparison of predictive statistics.[16,21,37,38] The study by Foxman et al[12] in a clinical trial population (n=499) generated ORs for sPTB ranging from <1 to 2.81 for a variety of bacterial taxa, depending on ethnicity. The performance gains from employing multiple combinations of microbiota were not assessed. In a predominantly white European population (n=759), Donders et al[9] previously reported ORs for PTB of 2.4 and 3.2 based on the presence of BV or aerobic vaginitis, respectively. However, the data from the Vienna Screen and Treat program of Kiss et al[67,68] suggest that BV as a predictor of PTB is much weaker than this. Compared with these studies, the predictive performance of our test is superior. Interestingly, Haque et al[69] employed a microbiome diversity metric, independent of any specific microbial signatures, to assess the risk of preterm delivery in 303 women sampled at multiple times in pregnancy. Their results suggest that increased diversity alone is highly predictive of early delivery (sensitivity, 95%; specificity, 96%; positive predictive value, 0.92; negative predictive value, 0.98), although it is worth noting that the women were derived from several different studies, the outcome was not specific to spontaneous PTB, and early gestation samples showed greater differences in diversity between groups. However, in both practical and diagnostic contexts, diversity is not a readily translatable measure.

Clinical implications

In this study, the GLU test performed with a sensitivity for sPTB of approximately 40%, which might seem to be a relatively modest predictive performance. However, PTB is a heterogenous condition with multiple causes, whereas estimates vary between studies, and according to population, gestational age, and methodology, intrauterine infection at most accounts for approximately 40% of all PTBs. Therefore, a highly specific test, based around the premise that the identification of specific microbiota in the vagina indicates a high risk of ascending infection-driven PTB, might reasonably be expected to identify at most 40% of PTBs. In this light, the GLU test's performance seems more impressive. However, it remains to be seen whether or not antimicrobial treatment based on a GLU-positive diagnosis would reduce the rates of infection-associated PTB as predicted.

Although our findings are likely to be of substantial clinical interest, it remains to be determined how the microbial risk profile (GLU test) we have identified actually interacts with PTB causation. Because *Ureaplasma* spp, *F nucleatum*, and *G vaginalis* have all been frequently identified in infected amniotic fluid of preterm deliveries[5] and have been implicated in the pathogenesis of chorioamnionitis,[70] the most obvious explanation is that there are interactions between the organisms that increase the likelihood of ascending intrauterine infection.[4] Surprisingly, we did not see a significant association between clinical or histologic chorioamnionitis and sPTB in GLU-positive women; this may, in part, be caused by a lack of power. Our data would suggest that *U parvum* (genotype SV3 or SV6) is the most common isolate of preterm deliveries. Interestingly, although many older studies of infected amniotic fluid samples collected in preterm labor reported detection of *U urealyticum*, these results should be interpreted with extreme caution, because despite being separated into 2 biovars (Parvo, *U parvum*; T960, *U urealyticum*) in 1990[71] and then formally named as separate species in 2001,[72] studies occurring well into the early 2000s and even the present day continue to incorrectly classify *U parvum* and *U urealyticum* collectively as *U urealyticum*.[43] This is despite our findings,[6] and those of others,[8,73,74] of low rates of

vaginal colonization by *U urealyticum* and minimal associations between colonization and PTB risk. One notable exception exists in a recent study by Kayem et al[75] who identified *U urealyticum* more often than *U parvum* in infected amniotic fluid samples. Data on vaginal detection were not presented. This is a conundrum that requires additional investigation to resolve.

Despite our understanding of the role of bacteria in driving sPTB, previous attempts to prevent this outcome by treating women with antibiotic agents have met with limited success; this is in part caused by the lack of effective diagnostic approaches for identifying women at specific risk of infection-driven PTB who would benefit from antimicrobial therapy.[76,77] Most previous studies and trials have recruited women based on the diagnosis of BV, which does not account for the presence of vaginal *Ureaplasma* spp. We included a number of BV-associated bacteria in our qPCR array, including those previously reported in PTB-associated amniotic fluid, but surprisingly did not find significant associations between any of these organisms individually or in combination and the risk of sPTB. This may in part be caused by the heterogenous nature of the vaginal microbiome among ethnic populations,[27] a phenomenon described in detail by Ravel et al,[52] among others.

Links between BV-associated organisms and PTB have been reported in a number of studies[9,50,51]; however, BV is far more prevalent in African American populations[52] and as such is unlikely to be as useful for PTB prediction in white populations where the incidence is lower. Nevertheless, some studies have shown considerable success in applying antimicrobial therapies to women with BV to lower PTB rates, despite its relatively poor prognostic profile.[67,68,78,79] We anticipate even greater successes should be achievable using the GLU test in our population, meaning that fewer women are unnecessarily treated, thus reducing inappropriate antibiotic exposure.

Research implications

As previously mentioned, our study revealed that high levels of "protective" *Lactobacillus* spp are associated with reduced risk of PTB, in line with previous findings.[36–39] It is unknown whether this effect is simply caused by bacterial cell numbers or the amount of lactic acid being produced (preventing colonization by pathogens). O'Hanlon et al[80] found that lactic acid was highly efficacious at reducing the levels of 17 different BV-associated organisms *in vitro*; specifically, it is the protonated form of this compound that is effective, as opposed to the lactate anion. Furthermore, these authors showed that this antimicrobial capacity was not purely caused by a reduction in pH, because the results could not be replicated through the acidification of the *in vitro* environment through the use of hydrochloric acid or acetic acid. This potential role of lactic acid in the context of *Lactobacillus* spp and vaginal health was recently thoroughly reviewed by Tachedjian et al.[66] Future studies should aim to measure D-lactic acid levels in the vagina during midgestation and relate these to the *Lactobacillus* sp present, their cell titers, and pregnancy outcome. Although the administration of *Lactobacillus* spp probiotics could prove to be a useful protective approach, it may be that a vaginal lactic acid supplement could be equally efficacious at promoting vaginal health.

The extent to which a maternal inflammatory response is involved in explaining the causal association between the GLU test profile and sPTB risk remains to be determined. Indeed, it is possible that it is the maternal immunologic response that actually predisposes to risk, and the microbial signature we observe is just a reflection of some aspect of immunologic selectivity. It has been reported that the maternal serologic response to *Ureaplasma* sp colonization of the amniotic fluid may be a strong predictor of PTB risk[81]; however, there are also a number of other variables that also need to be considered. Discussion of these is beyond the scope of this study but has been reviewed by Ireland and Keelan.[82] A previous exposure to *U parvum* in terms of vaginal colonization does not seem to predispose pregnant women to increased risk of PTB based on specific T-cell[83] or monocyte[84] responses in the blood; however, power was limited in both of these studies.

Considering a significant proportion of PTBs occur as a result of sterile inflammation,[85–87] it is possible that the mere presence of certain bacteria in the vagina can drive an intraamniotic inflammatory response, irrespective of whether or not the cervical barrier is breached. Alternatively, there may be microbial-host interactions that promote cervical evasion and ascension. Racicot et al[88] showed in a murine model that viral infection of the cervix predisposes to ascending vaginal *Ureaplasma* spp infection. Relating this to humans, previous studies have found associations between human papillomavirus (HPV) and PTB,[89–92] and most recently, a report was published documenting a decline in the rate of PTB after HPV vaccine implementation.[93] Whether cervical HPV infection, or the presence of other commensal microorganisms in the cervicovaginal mucosa, allows certain bacteria greater access to the uterine cavity is unknown and warrants further research.

Strengths and limitations

Our study has several notable differences and strengths compared to the aforementioned studies: its large size (nearly 1000 women), recruitment of an unselected low- to medium-risk cohort (women most likely to benefit from the development of a microbial risk profile screening test), a relatively homogenous white-enriched population, and the collection of complete obstetrical and demographic variables on all women. We also employed molecular microbiological techniques to quantify species-level DNA (unless genus-level was more relevant) and achieved serovar- or clade-specific resolution where such discrimination was likely to be beneficial.

One potential limitation of our study was the targeted microbial DNA detection approach we took. Even though our targets were based on organisms most frequently implicated in cases of PTB or, in the case of certain *Lactobacillus* spp, term birth, other microorganisms would have almost certainly been present in samples and these could have been

associated with positive or negative pregnancy outcomes. An additional limitation lies in the lack of placental histology on all sPTB cases, because this is only routinely performed at <34 weeks' gestation at KEMH. Another potential limitation is that although we excluded women who were currently taking antibiotics, we did not inquire about recent antibiotic use.

Conclusion

This study describes a novel vaginal bacterial DNA test that was effective at the prediction of sPTB in a cohort of predominantly white, low- to medium-risk Australian women during midgestation. The study utilized self-collected vaginal swabs obtained during standard antenatal screening visits between 12 and 23 weeks' gestation, meaning that the GLU test could be readily integrated into current obstetrical practice. Targeted, appropriately controlled qPCR assays were used, ensuring that species- and genotype- or clade-level (where appropriate) bacterial identification was attained with confidence; by utilizing qPCR as the method of detection, result turnaround times were fast (hours) and no specialized equipment beyond that present in the average pathology laboratory was required. Finally, and perhaps most importantly, in our study, a GLU-positive result was the second most predictive factor of sPTB risk and was only marginally weaker than the most well-documented global risk factor—a history of PTB. Additional studies are required to determine whether the GLU test is an effective risk screening test in other populations and ethnicities. ■

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The authors declare that a provisional patent for an Infection-Related Preterm Birth Diagnostic Method has been filed. This patent includes all information contained within this manuscript in relation to the GLU test or algorithm. A contract has been signed with a commercial partner to produce this test if the result of a future randomized clinical trial is favorable, and in this case, M.S.P., J.A.K., D.A.D., and J.P.N. are financial beneficiaries. The other authors report no conflict of interest.

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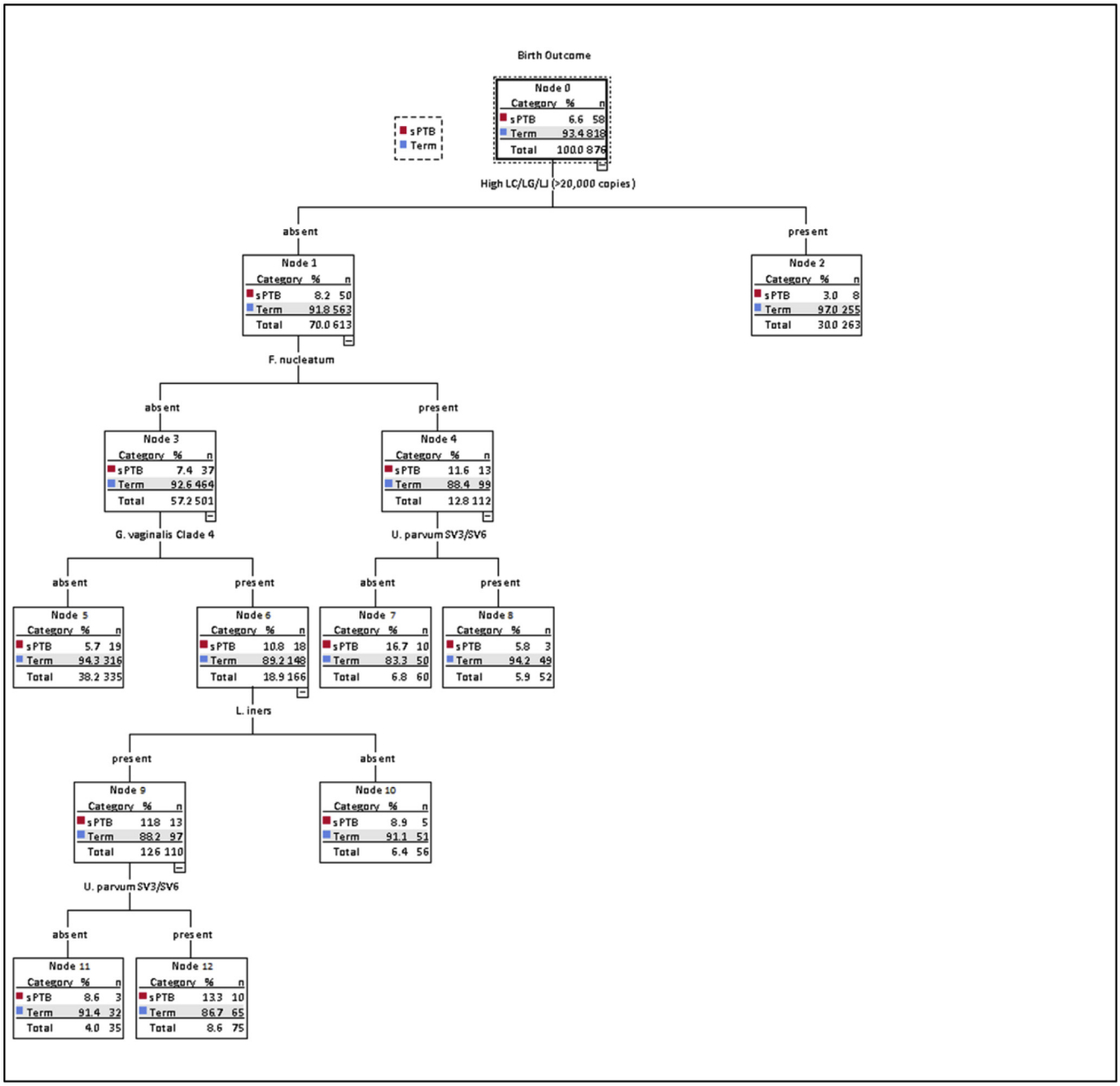
Supplemental Material

S1: Multiple swab protocol validation

To assess whether the collection of sequential swabs from the same site may impact on the vaginal microbial profiles detected using our target-specific assays, we processed 20 vaginal e-Swabs collected at the same time point in a sequential manner. DNA was extracted and used as template in the 15-target custom 384-well array. A total of 59 microbial targets were detected from the 20 samples. From a presence and absence perspective, identical microbial profiles were recorded for 17 of 20 samples (85%); the 3 instances of discrepant results (15%) were all cases of a target being detected in 1 sample at a Ct of $\geq$38 and not being detected in the other. In all of these cases, target detection occurred in the second swab only. Ct variation for detection of the same target within sequential swab samples was $<$1 cycle, between 1-2 cycles, and $>$2 cycles for 61%, 14% and 20% of cases, respectively (Supplemental Table). In cases with Ct variation $>$1 between sequential samples, there were 14 cases (70%) where the first swab yielded better target detection and 6 cases (30%) where this was the case for the second swab.

S2: Recursive partitioning analyses used in construction of the GLU test

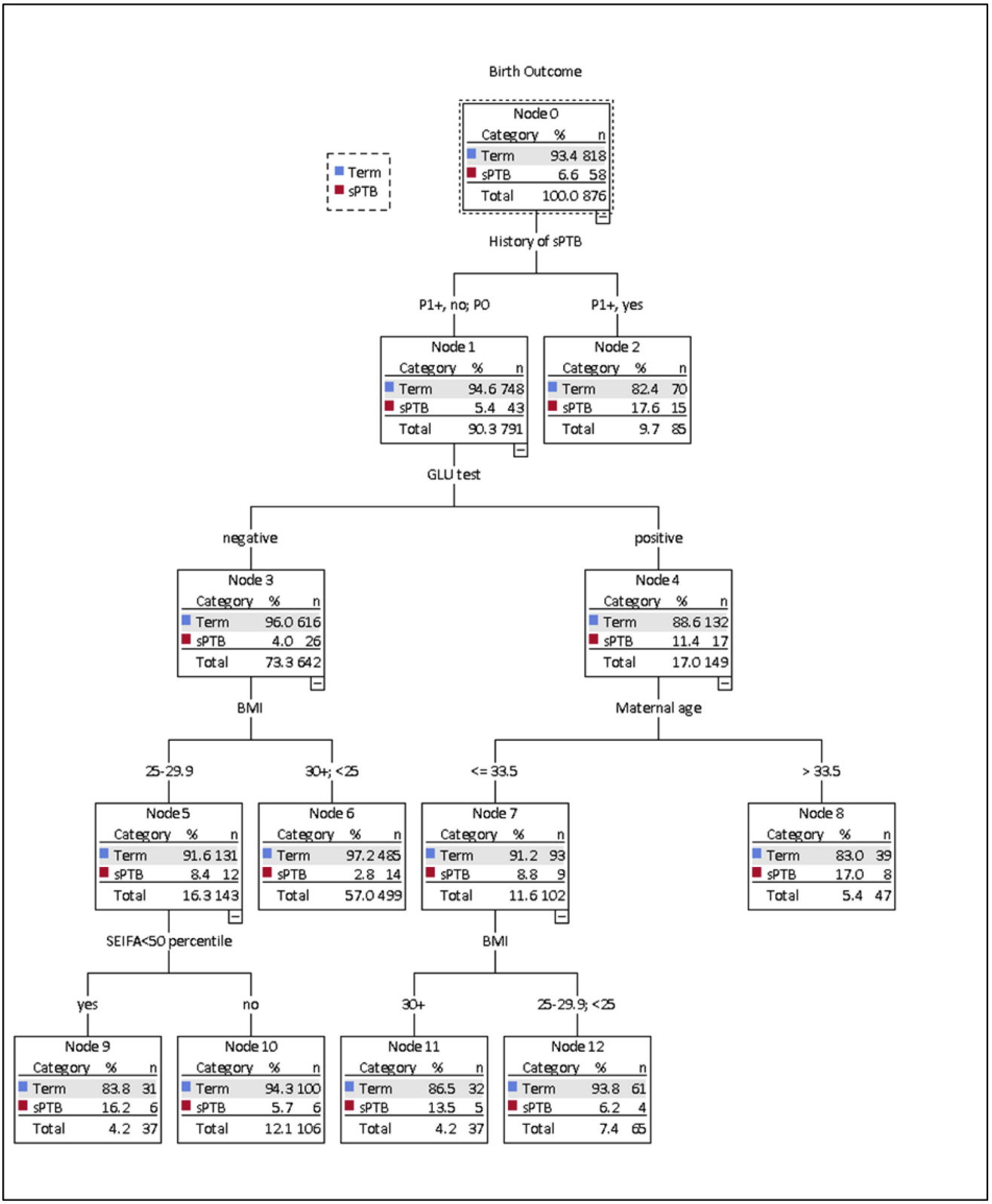
SUPPLEMENTAL FIGURE 1
Recursive partitioning tree most predictive of sPTB risk using microbial biomarkers



F nucleatum, *Fusobacterium nucleatum*; *G vaginalis*, *Gardnerella vaginalis*; *L iners*, *Lactobacillus iners*; *sPTB*, spontaneous preterm birth; *U parvum*, *Ureaplasma parvum*.

Payne et al. Prediction of preterm birth using vaginal microbiology. Am J Obstet Gynecol 2021.

SUPPLEMENTAL FIGURE 2
Recursive partitioning tree showing the impact of additional obstetrical risk factors



BMI, body mass index; *SEIFA*, socioeconomic indexes for areas; *sPTB*, spontaneous preterm birth.

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SUPPLEMENTAL TABLE

An overview of differences in microbial qPCR target detection between sequentially collected vaginal swabs

Target	Number (cycle range)
Ct difference of >2 cycles	12 (2.4–6.0)
Ct difference of 1–2 cycles	8 (1–1.9)
Ct difference <1 cycle	36 (0.01–0.99)

Ct, cycle threshold; *qPCR*, quantitative polymerase chain reaction.

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