Original Article

An exploratory study of associations with spontaneous preterm birth in primigravid pregnant women with a normal cervical length

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Abstract

Background: Predictors of spontaneous preterm birth in primigravid women remain undetermined.

Aim: We evaluated whether biomarkers in vaginal secretions and/or differences in the dominant bacterium in the vaginal microbiome predicted the risk for spontaneous preterm birth in primigravid women with a cervical length >25mm.

Study design: In a prospective study, 146 second trimester pregnant women with their first conception and a cervix >25mm were enrolled. The vaginal microbiome composition was characterized by analysis of 16S ribosomal RNA gene sequences. The concentrations of D- and L-lactic acid, matrix metalloproteinase (MMP) 2, 8 and 9 and tissue inhibitor of metalloproteinase (TIMP) 1 and 2 in vaginal secretions were measured by ELISA. Cervical length was determined by vaginal ultrasonography. Pregnancy outcome data were subsequently collected. There was a spontaneous preterm birth (SPTB) in 13 women (8.9%) while in an additional 8 women (5.5%) preterm delivery was medically indicated. Lactobacillus iners was the dominant vaginal bacterium in 61.5% of women with a SPTB but only in 31.2% of those who delivered at term (p = 0.0354). The vaginal concentration of TIMP-1 (p = 0.0419) and L-lactic acid (p = 0.0495) was higher in women with a SPTB as compared to those who delivered at term. Lactobacillus iners dominance was associated with elevated levels of TIMP-1 (p = 0.0434) and TIMP-2 (p = 0.0161) and lower levels of D-lactic acid (p < 0.0001) compared to when L. crispatus was dominant.

Conclusion: In this exploratory study of primigravid women, elevations in vaginal TIMP-1 and L-lactic acid and L. iners dominance in the vaginal microbiome are associated with an increased occurrence of SPTB.

Introduction

Preterm birth, defined as delivery before 37 weeks of gestation, remains a major unresolved problem in obstetrics. It is the leading cause of perinatal morbidity and mortality [1] and the second largest direct cause of death in children <5 years old [2]. In 2014, including data from 107 countries, the estimated global rate of preterm birth was 10.6% [3]. There are multiple causes of spontaneous preterm birth (SPTB) including infection, sterile inflammation and uterine distension. In addition, preterm birth may be medically indicated (IPTB) due to preeclampsia, fetal distress or other causes [4]. A history of a premature delivery in a prior pregnancy is currently the best predictor of a subsequent preterm birth. Other major risk factors that have been associated with increased risk for a preterm birth include Black race, low or high maternal weight, a mid-trimester cervical length ≤25mm and smoking [5–8]. Variations in the species composition of the vaginal microbiome have also been shown to be associated with the incidence of preterm birth in different populations [7–11].
In primigravid women, defined as an initial gestation with no known prior early or late pregnancy losses, assessing risk for a premature delivery is more challenging due to the absence of a pregnancy history. We recently demonstrated that variations in the levels of the D- and L-isomers of lactic acid and tissue inhibitor of metalloproteinase-1 (TIMP-1) in vaginal secretions were predictive of cervical length and the dominant bacterium in vaginal secretions from 340 mostly multigravidae pregnant women in their second trimester [12]. In that study we did not evaluate associations with preterm delivery.

**Objective**

The objective of the present study was to evaluate the hypothesis that these same measurements would be associated with risk of spontaneous preterm birth in women with a first conception whose cervix was >25mm. If validated, these findings could then be the basis of a more definitive investigation.

**Materials and methods**

**Subjects**

Eligible participants in this prospective study were 146 pregnant women in their mid-trimester with a first conception who were seen at one of three outpatient maternity units in Brazil: The Federal University of São Paulo, The Federal University of Jundiai, and The Federal University of Ceara. Inclusion criteria were first known conception, scheduled to undergo a routine vaginal ultrasound to assess cervical length and consent to obtain vaginal secretions for research. Measurement of cervical length by experienced physicians followed a uniform procedure at all three centers, and has been detailed previously [12]. Exclusion criteria were a prior conception, vaginal bleeding, obesity (body mass index >35 kg/m²), signs or symptoms of a genital tract infection, antibiotic usage in the previous two weeks, an endocrine or immune disorder, sexual intercourse within the past two weeks or the inability to provide written informed consent. Data from some of the woman from Sao Paulo (65 women) were included in a previous study that only evaluated associations between microbiome composition and compounds in vaginal secretions, but did not examine their influence on pregnancy outcome [12]. The study was approved by the institutional review boards at each hospital and all subjects provided written informed consent.

Subsequent to completion of all laboratory analysis, acquisition of patients’ clinical data revealed that 34 of the women, all with a cervical length ≤25mm, were treated with vaginal progesterone either alone or in combination with pessary and/or cerclage placement and/or antibiotics for the duration of their pregnancy. Only 3 (8.8%) of these women subsequently had a preterm delivery. Since these exogenous interventions exert a major influence on pregnancy outcome, all of these treated women were subsequently excluded. Thus, the analysis was confined to primigravidae women with a normal cervix (>25mm).

**Samples**

Collection of vaginal samples for analysis of compounds and microbiome composition were as previously reported [12,13]. Briefly, samples were obtained from the posterior vaginal wall just prior to cervical length measurement, using either a cotton swab or the Copan ESwab sample collection system (Fisher Scientific, Pittsburgh, PA). They were stored at −80°C until shipped on dry ice to the Witkin lab in New York. Samples for microbiome analysis were then forwarded on dry ice to the Forney lab in Idaho.

**Vaginal compound analysis**

The selection of compounds to be analyzed was based on findings from a prior investigation of biomarkers associated with cervical length and vaginal microbiome composition [12]. Concentrations of D- and L-lactic acid were measured by commercial colorimetric kits that were specific for each isomer (BioAssay Systems, Hayward, CA). The TIMP-1, TIMP-2, matrix metalloproteinase (MMP)-2, MMP-8 and MMP-9 level in each vaginal sample was determined by commercial ELISA kits (Thermo-Fisher Scientific, Waltham, MA). Assay values were converted to mM (lactic acid) or ng/ml (TIMP, MMP) by reference to a standard curve that was generated with each assay. Laboratory personnel were blinded to all clinical data.

**Vaginal microbiome analysis**

The composition of the vaginal microbiome was determined by amplification of the V1–V3 region of the gene coding for bacterial 16S ribosomal RNA following our published procedure [12]. To simplify the analysis the initial data were filtered based on determination of dominance in a community. To be considered for analysis a bacterial species had to be in the top 15 of
mean rank abundance, mean relative abundance and total read counts.

**Statistics**

Differences in concentration of compounds in vaginal secretions when *Lactobacillus crispatus* or *Lactobacillus iners* were dominant in the vaginal microbiome as well as differences in the concentration of vaginal compounds between women with a SPTB or term birth were analyzed by the non-parametric Mann-Whitney test since the observed values were not normally distributed. Differences in dominant bacterium between groups were assessed by Fisher’s exact test. A *p* value < .05 was considered significant. All statistical analyses utilized GraphPad Prism (San Diego, CA).

**Results**

Characteristics and pregnancy outcomes of the 13 women with a SPTB, 8 with an IPTB and 125 women with a term delivery are shown in Table 1. Women in all three groups were comparable in age, body mass index, racial distribution, education level, mean cervical length and gestational age at sample collection. The mean gestational age at delivery was 34.1, 31.7 and 38.9 weeks in women with a SPTB, IPTB and term birth, respectively (*p* < .0001, SPTB or IPTB vs. term birth). The mean birthweight was 2235, 1956 and 3186 grams in women with a SPTB, IPTB and term birth, respectively (*p* < .0001 SPTB vs. term, 0.0014 IPTB vs. term). Of the 13 who delivered preterm spontaneously, 3 had preterm premature rupture of membranes. The indications for IPTB were preeclampsia (*N* = 5), gestational diabetes mellitus (*N* = 2) and sepsis (*N* = 1). Subjects from the three sites did not differ in maternal age, percentage of women with a preterm birth or gestational age at delivery.

The dominant vaginal bacteria, defined as >50% of the total bacteria in a sample, are shown in Table 2. *Lactobacillus iners* was the dominant bacterium in the majority (61.5%) of women who had a SPTB and an IPTB (50.0%), In contrast, *L. iners* was dominant in only 31.2% of women who delivered at term (*p* = 0.0354 SPTB vs. term). *Lactobacillus crispatus* was dominant in 30.8%, 25.0% and 39.2% of women who had a SPTB, IPTB or term birth, respectively. *Gardnerella vaginalis* was dominant in only one woman who delivered preterm (IPTB) and in 17 women who delivered at term. The absence of a dominant bacterium was observed in 1 woman with a SPTB (7.7%), 1 with an IPTB (12.5%) and 6 (4.8%) who delivered at term. The distribution of dominant bacteria was similar for women at all three clinical sites.

The association between pregnancy outcome and concentrations of compounds in vaginal secretions for women with a SPTB or term delivery is shown in Table 3. The median concentrations of TIMP-1 (6.3 vs. 1.9 ng/ml, *p* = .0419) and L-lactic acid (1.5 mM vs. 0.9 mM, *p* = .0495) were higher in women with a SPTB than in women with a term birth.

The relationships between the concentrations of compounds in vaginal secretions and *L. crispatus* or *L. iners* dominance in the vagina are shown in Table 4. The D-lactic level was markedly higher when *L. crispatus* was dominant (*p* < .0001). In contrast, the levels of TIMP-1 (*p* = .0434) and TIMP-2 (*p* = .0161) were higher when *L. iners* predominated.

**Discussion**

In women with a first conception (primigravid) and a normal cervical length, elevated levels of L-lactic acid...
TIMP-1 0.8 (0.2 – 0.28) has been observed [12,14]. The present results on women with a shortened cervix and an elevated rate of preterm delivery, has previously been observed [7,9,12]. The present study now suggests that L. iners dominance may also be a risk factor for preterm birth even in women who do not have a shortened cervix. Unlike L. crispatus, whose dominance in the vaginal microbiome is associated with a marked decrease in levels of other bacteria, multiple bacterial species coexist in the vagina when L. iners predominates [8,12]. A possibility is that L. crispatus possesses mechanisms to limit proliferation of other bacterial species that are lacking in L. iners. When introduced into the vaginal environment, microorganisms potentially involved in the triggering of preterm birth may, therefore, be more likely to persist if L. iners is dominant.

Table 2. Dominant bacterium in the vagina in women with a first conception and a normal cervical length.

| Dominant bacterium       | SPTB (13) | IPTB (8) | Term birth (125) |
|--------------------------|-----------|----------|------------------|
| Lactobacillus crispatus  | 4 (30.8%) | 2 (25.0%)| 49 (39.2%)       |
| Lactobacillus iners      | 8 (61.5%) | 0 (0%)   | 39 (31.2%)       |
| Lactobacillus jensenii   | 0 (0%)    | 0 (0%)   | 6 (4.8%)         |
| Lactobacillus gasseri    | 0 (0%)    | 0 (0%)   | 3 (2.4%)         |
| Other lactobacilli‡      | 0 (0%)    | 0 (0%)   | 2 (1.6%)         |
| Gardnerella vaginalis    | 0 (0%)    | 1 (12.5%)| 17 (13.6%)       |
| Other non-lactobacilli§   | 0 (0%)    | 0 (0%)   | 2 (1.6%)         |
| No. dominant bacterium   | 1 (7.7%)  | 1 (12.5%)| 6 (4.8%)         |

Dominance indicates more than 50% of the bacteria detected in the vagina.

*a* *p* = 0.0354 vs. term birth; *b*L. delbrueckii, L. vaginalis; *c*Lachnospiraceae, Shuttleworthia. None of the other differences reached statistical significance.

Table 3. Association between pregnancy outcome and compounds in vaginal secretions.

| Compound       | SPTB       | Term birth | *p* value |
|----------------|------------|------------|-----------|
| TIMP-1         | 6.3 (1.3–10.6) | 1.9 (0.3–6.0) | 0.419     |
| TIMP-2         | 9.4 (3.3–14.1) | 4.7 (2.6–8.7) | NS        |
| MMP-2          | 2.7 (1.6–5.3)  | 2.8 (1.2–4.8) | NS        |
| MMP-8          | 45.4 (17.7–63.2) | 29.1 (12.9–52.3) | NS        |
| MMP-9          | 21.2 (17.5–32.5) | 20.0 (13.6–27.2) | NS        |
| D-lactic acid  | 0.4 (0.09–2.5) | 0.7 (0.08–2.1) | NS        |
| L-lactic acid  | 1.5 (0.8–3.4)  | 0.9 (0.3–1.9)  | 0.0495    |

TIMP: tissue inhibitor of metalloproteinase; MMP: matrix metalloproteinase; NS: not significant.

Table 4. Association between dominant bacterium and compounds in vaginal secretions.

| Compound       | L. crispatus | L. iners | *p* value |
|----------------|--------------|----------|-----------|
| TIMP-1         | 0.8 (0.2–2.8) | 1.9 (0.4–6.3) | 0.0434    |
| TIMP-2         | 3.7 (1.6–5.5) | 4.7 (2.6–9.8) | 0.0161    |
| MMP-2          | 1.2 (0.1–3.7) | 1.4 (0.1–3.8) | NS        |
| MMP-8          | 22.1 (13.6–45.8) | 34.2 (7.8–48.8) | NS        |
| MMP-9          | 18.0 (9.8–24.6) | 20.0 (13.8–27.1) | NS        |
| D-lactic acid  | 2.2 (1.1–3.3)  | 0.1 (0.04–0.8) | <0.0001   |
| L-lactic acid  | 1.1 (0.5–2.1)  | 1.4 (0.8–2.4)  | NS        |

TIMP: tissue inhibitor of metalloproteinase; MMP: metalloproteinase; NS: not significant.

and TIMP-1 in their vaginal secretions were associated with a subsequent SPTB. A vaginal microbiome in which L. iners was the dominant bacterium was also more prevalent in women who spontaneously delivered preterm. The data, therefore, are consistent with our prior findings on populations of pregnant women with mixed parity and gravidity [12] and provide supportive evidence that elevations in L-lactic acid and TIMP-1, coupled with L. iners dominance in vaginal microbiota, may be useful in the identification of primigravid women at risk for a preterm birth.

The association between an elevated TIMP-1 level and a shortened cervical length, as well as an increased rate of preterm delivery, has previously been observed [12,14]. The present results on women with a normal cervical length extends these observations and suggest that TIMP-1 may participate in processes that lead to preterm birth independent of cervical remodeling. Mechanisms by which TIMP-1 involvement may contribute to the initiation of pathological events distinct from its MMP-binding activity have recently been reviewed [15]. A likely mechanism by which TIMP-1 contributes to induction of SPTB in the absence of a shortened cervix may be by facilitating bacterial migration to the uterine cavity and induction of infection-mediated premature contractions. The identity and mechanism(s) of bacterial and host cell responsible for TIMP-1 production remain to be determined.

A number of studies have shown that the dominance of L. iners in vaginal bacterial communities is associated with a shortened cervix and an elevated frequency of preterm delivery [7,9,12]. The present study now suggests that L. iners dominance may also be a risk factor for preterm birth even in women who do not have a shortened cervix. Unlike L. crispatus, whose dominance in the vaginal microbiome is associated with a marked decrease in levels of other bacteria, multiple bacterial species coexist in the vagina when L. iners predominates [8,12]. A possibility is that L. crispatus possesses mechanisms to limit proliferation of other bacterial species that are lacking in L. iners. When introduced into the vaginal environment, microorganisms potentially involved in the triggering of preterm birth may, therefore, be more likely to persist if L. iners is dominant.

While all four Lactobacillus species that are typically present in the vagina – L. crispatus, L. iners, L. jensenii, L. gasseri – produce the L-lactic acid chiral isomer levels appear to be highest when L. iners predominates. In addition, L. iners is the only member of this group that does not also produce D-lactic acid [12,16,17]. Therefore, our present findings are consistent with previous investigations that showed elevated levels of L-lactic acid in the concurrent presence of low levels of D-lactic acid is an indication of L. iners vaginal dominance. Potential differences between the D- and L-lactic acid isomers on vaginal immunity have been previously discussed [18,19].

A number of studies have reported associations between G. vaginalis dominance in the vaginal microbiota and preterm birth [8,11]. However, in the present study this bacterium was identified in no women with a SPTB and in only one woman with an IPTB, as opposed to 17 women who delivered at term. G. vaginalis is also a frequent component of the vaginal microbiome in pregnant women with an uneventful gestation and who deliver a healthy infant at term [20–23]. Perhaps, as recently suggested the presence
of specific strains of G. vaginalis determine the consequences of its dominance [24,25]. In any event, the present findings reiterate that G. vaginalis dominance in the vaginal microbiota in primigravidae pregnant women is not necessarily associated with pathogenesis.

Strengths of the present study include its prospective design, collaboration by a group of investigators with unique and complementary areas of expertise and inclusion in the analysis of women from different races at three independent locations. There are limitations to our study. Women with a cervical length ≤ 25 mm received progesterone therapy. This precluded them from being included in the study and, thus, limited our analysis to women with a normal cervical length. In addition, the number of women with a SPTB in the present study was low. The findings, therefore, must be regarded as exploratory and further investigations on larger numbers of women are necessary to validate the reproducibility of the reported observations. The ancestry of people in Brazil is complex and skin color has been shown to be a poor indicator of race in this population [26,27]. Therefore, it is possible that self-declared race may have been inaccurate in an unknown percentage of our subjects.

Summary

In conclusion, the present study extends to women with a first conception our previous finding [2] that measurement of concentrations of TIMP-1 and L-lactic acid in vaginal secretions indicate the likelihood of having a microbiota in which L. iners is dominant. It additionally suggests for the first time that these measurements may have value in determining risk for SPTB in primigravid women, a group that is more difficult to predict pregnancy outcome than in multiparous women. Lastly, development of a low cost, point of care protocol to measure vaginal L-lactic acid and TIMP-1 may have clinical utility, especially, in regions of the world lacking in facilities to examine vaginal secretions for specific bacteria.

Disclosure statement

None of the authors has any conflict of interest to report.

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