Vaginal microbiota as a predictor of preterm birth : an observational cohort study

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Short title: Vaginal microbiota signatures of preterm birth

At a glance

Why was this study conducted?

Despite the growing number of scientific studies highlighting the link between vaginal microbiota composition and adverse pregnancy outcomes, data remain controversial, mostly because studies are relatively small-sized and do not investigate cofounding factors such demographics and medical and obstetrical history. Moreover, microbiota data are difficult to transpose into a real-life perspective where clinical management relies primarily on clinical risk factors and vaginal swab cultures.

Key findings

We included over 2,400 pregnant women in a large longitudinal cohort study and combined culturomics at admission for preterm labor or preterm rupture of membranes and multivariable analyses in which demographic and obstetrical data were studied. We identified lactobacilli depletion and enterobacteria as strong vaginal microbiota signatures of preterm labor and preterm rupture of membranes. Importantly, both signatures constituted independent risk factors for preterm birth.

What does this add to what is known?

These findings highlight the importance of vaginal microbiota together with demographic and clinical features as risk factors for adverse pregnancy outcome and preterm birth. They also underline the need for an accurate characterization of vaginal microbiota at admission for preterm labor and preterm rupture of membranes for a better management of these complications of pregnancy.

Abstract

Background: Preterm birth (birth before 37 weeks of gestational age) is a frequent and severe adverse pregnancy outcome. Despite the growing number of scientific studies highlighting the link between vaginal microbiota composition and adverse pregnancy outcomes, data remain controversial.

Objectives: To identify microbiota signatures of preterm labor and premature rupture of outer membranes; to determine microbiological risk factors for preterm birth.

Study Design: We conducted an observational, prospective, longitudinal cohort study from August 2018 until June 2023 in 3 maternity wards from university hospitals in the Paris, France, area. Women were categorized in 4 groups including a control group, and 3 groups of increasing risk of intrauterine infection and preterm birth: prelabor rupture of membranes at term, preterm labor, and preterm premature rupture of membranes. Demographic, clinical data, past medical and obstetrical history and pregnancy outcome were collected. Vaginal swabs were collected at admission and were analyzed using culturomics. The association between bacterial species and the cohort groups and eventually preterm birth was studied in univariate analyses. Adjusted odds ratio (aOR) and 95% confidence intervals (95% CI) were calculated in multivariable analyses.

Results: A total of 2,476 women were included, of whom 1,068 (43.1%) in the control group, 477 (19.3%) with term premature rupture of outer membranes, 495 (20.0%) with preterm labor, and 436 (17.6%) with preterm premature rupture of outer membranes. Together with demographic features such as ethnicity and obstetrical history, several vaginal microbiota signatures were identified as correlated with pregnancy outcome. In multivariable analysis, prelabor rupture of membranes at term was associated with enterobacteria (aOR 1.97, 95% CI 1.46-2.65) and *Gardnerella vaginalis* (aOR 5.19, 95% CI 2.22-13.78); preterm labor with lactobacilli depletion (aOR 1.49, 95% CI 1.08-2.06), enterobacteria (aOR 1.86, 95% CI 1.36-

2.53) and *G. vaginalis* (aOR 4.62, 95% CI 1.86-13.34); preterm premature rupture of membranes with lactobacilli depletion (aOR 2.04, 95% CI 1.49-2.80) and enterobacteria (aOR 2.38, 95% CI 1.74-3.24). Finally, together with gestational diabetes, lactobacilli depletion and enterobacteria both represented risk factors for preterm birth, especially in singleton pregnancies (aOR 1.54, 95% CI 1.05-2.28 and aOR 1.62, 95% CI 1.11-2.36, respectively).

Conclusions: In this large cohort study, we identified on the one hand, *G. vaginalis* as associated with prelabor rupture of membranes at term and preterm labor, and on the other hand, lactobacilli depletion and enterobacteria as risk factors for preterm labor, preterm premature rupture of outer membranes, and preterm birth, emphasizing the importance of a healthy microbiota in pregnancy outcome. Further studies are needed to address the benefits of adapted antibiotic prophylaxes and probiotics aiming at restoring a healthy microbiota in reducing the risk of preterm birth.

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Introduction

Preterm birth (PTB), defined as birth before 37 weeks of gestation, is a frequent and severe adverse pregnancy outcome which concerns more than 10% pregnancies and accounts for 14 millions annual births worldwide.[1] As the leading causes of death in children under the age of 5 years, PTB and its sequelae represent one of the most prevalent conditions in the Global Burden of Disease analysis and are therefore considered as a major worldwide health issue.[2,3] Up to 40% of PTB are preceded by preterm premature rupture of membranes (PPROM), increasing the risk of intrauterine infection, *in utero* demise and prematurity.[4,5] Nevertheless, causes of PTB and PPROM are still poorly understood. Beside multiples factors including ethnicity, obstetrical history, and lifestyle, perturbations of the vaginal microbiota are increasingly recognized as possible contributing factors.[6,7]

The composition of the vaginal microbiota is highly dynamic, changing with age, hormonal fluctuations, gestational status, ethnicity, sexual behavior, and lifestyle.[8–10] Microbiota studies based on sequencing of vaginal samples from reproductive-age women have identified five types of vaginal community structures designated community state types (CSTs).[10] Four of these are dominated by one *Lactobacillus* species while the last one, designated CST-IV, is characterized by a more diverse microbiota dominated by anaerobic bacteria such as *Gardnerella vaginalis*, *Prevotella* spp, and *Fannyhessea vaginae* – formerly known as *Atopobium vaginae*. Although the distribution of these CSTs largely varies according to ethnicity,[11] it is generally believed that a healthy vaginal microbiota is dominated by lactobacilli with a low bacterial diversity, particularly during pregnancy.[12,13] Conversely, a decrease in lactobacilli abundance is correlated to an increase of microbiota diversity typically exemplified by the CST-IV. Low-lactobacilli microbiota can furthermore be associated to clinical symptoms known as bacterial vaginosis and with higher susceptibility to sexually transmitted or urinary tract infections.[14] In the context of pregnancy, increasing evidence links preterm labor (PTL),

PPROM, and PTB with low-lactobacilli microbiota characterized by a high diversity and an enrichment in *G. vaginalis* and various anaerobes, whereas microbiota dominated by lactobacilli and more specifically by *Lactobacillus crispatus* (CST-I) have been found associated to term birth.[6,15–17]

However, despite the growing number of scientific studies that highlight the link between vaginal microbiota composition and pregnancy outcomes, data remain controversial. Most studies are relatively small-sized and do not investigate cofounding factors such as ethnicity. Moreover, microbiota data are difficult to transpose into a real-life perspective where clinical management relies primarily on clinical risk factors and vaginal swab cultures. Here, we conducted a large prospective observational cohort study involving more than 2,400 pregnant women and used simple culturomics and multivariable analyses that took demographic and clinical data into account to identify microbiota signatures of PTL and PPROM, and eventually to determine microbiological risk factors for PTB.

Materials and methods

Study design

We conducted an observational, prospective, longitudinal cohort study from August 2018 until June 2023 in 3 maternity wards, of which 2 of level 3 and 1 of level 2b, from university hospitals in the Paris, France, area. The total annual number of births at these 3 centers is approximately 11,000. Women were categorized in 4 groups including a control group and 3 groups of increasing risk of intrauterine infection and PTB. The eligibility criteria for maternal participation in the study included all pregnant women aged >18 years who responded to the following inclusion criteria : *i)* prelabor rupture of membranes at term (TPROM) e.g., rupture of membranes after 37 weeks of gestation and more than 12 hours before labor onset; *ii)* preterm labor (PTL) e.g., hospitalization for threatened PTB or PTL with intact membranes; and *iii)*

PPROM e.g., rupture of membranes before 37 weeks of gestation. The control group was constituted by pregnant women who delivered at term with no complication. In this latter group, women were proposed to be enrolled at the time of *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) antenatal screening between 34 and 38 weeks of gestational age (GA) as recommended by the French guidelines for the prevention of perinatal infectious disease.[18] Women who did not speak or understand French were excluded.

Demographic, clinical data, past medical and obstetrical history, and pregnancy outcome were collected. Notably, maternal place of birth was collected as a reflect of ethnicity, given that reporting ethnicity itself is forbidden in France.

Sampling and laboratory procedures

Vaginal swabs were sampled at admission or at GBS antenatal screening for the control group and sent to the laboratory in Amies transport medium (ESwab™ system, Copan Diagnostics, Italy). Specimens were immediately processed for analysis by conventional culture methods using non-selective rich media incubated at 37°C in aerobic atmosphere with 5% CO_2. In addition, a Columbia agar with 5% horse blood, nalidixic acid and colistin (bioMérieux, Marcy l'Etoile, France) for the selective detection of anaerobes and Gram-positive cocci and a Granada agar (Fisher Scientific, Illkirch, France) for the selective detection of GBS were incubated at 37°C under anaerobic atmosphere. Lastly, a Drigalski and a Candida agar plates (bioMérieux, Marcy l'Etoile, France) for the selective detection of Gram-negative rods and yeasts, respectively, were incubated at 37°C under aerobic atmosphere. All plates were examined after 16h and 44h of incubation. All different morphotypes of bacterial and yeast colonies were identified using matrix assisted laser desorption ionization - time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Billerica, Massachussetts, USA). The density of vaginal colonization was estimated semi-quantitatively as follows: colonies on a single quadrant, low;

colonies on more than two quadrants, medium to high. In addition, the overall vaginal microbiota composition was assessed by direct microscopic examination of the vaginal samples after Gram staining and determination of the Nugent score in 2 out of 3 maternity wards according to routine practices.[19]

Primary outcomes and studied factors

The primary outcomes were PROM at admission and PTB in the PTL and PPROM groups. The studied factors were demographics (country of birth as a reflect of ethnicity, body mass index (BMI)), pregnancy-related risk factors (gestity, parity, history of preterm delivery, multiple pregnancy, gestational diabetes, hypertensive disease, tobacco use), and vaginal microbiota composition assessed by culturomics. A specific attention was paid to bacteria of the *Lactobacillus* genus, and to species potentially associated with adverse outcomes including enterobacteria (*Escherichia coli*, *Klebsiella pneumoniae*, and others), *Staphylococcus aureus*, GBS, *Streptococcus anginosus*, *G. vaginalis*, strict anaerobes (*Prevotella* spp., *Bacteroides* spp., and others), and fungi of the *Candida* genus.

Statistical analyses

All data were collected in a computerized database and analyzed using the GraphPad Software v8.3.0 (Dotmatics, Boston, Massachusetts, USA) and RStudio v1.1.447 (Posit, Boston, Massachusetts, USA). Firstly, we assessed the prevalence of the studied factors according to the cohort groups as a proportion of the total number of participants. Secondly, to study the risk factors associated to each cohort group and the risk factors for PTB, a multivariable logistic regression model was used for prognostic covariates. For univariate statistical analyses, Mann-Whitney tests were used when appropriate, and categorical data were compared using chi-squared or Fisher's exact tests. Odds ratios and 95% CI were calculated in univariate analyses.

Adjusted odds ratios were then calculated using any variables remaining associated with the outcome with a p-value <0.2 from the univariate analysis, excepting microbiological variables which were all included in the analyses. Two models were used to study enterobacteria. In the first one, enterobacteria were analyzed as a single variable. In the second one, the two most represented species e.g., *E. coli* and *K. pneumoniae*, were specifically analyzed. A p-value < 0.05 was considered significant. Missing data were excluded.

Ethics declaration

The reported study was reviewed by the French ethics committee who granted approval on November, 2017 (identifier 2017 – A02755-48; NCT03371056). All individuals enrolled in the cohort provided signed informed consent that included authorization for the collection of medical information and biological samples. All collected data were anonymized and integrated in a secured electronic database.

Results

Study population

A total of 2,476 women were included, of whom 1,068 (43.1%) in the control group, 477 (19.3%) in the TPROM group, 495 (20.0%) in the PTL group, and 436 (17.6%) in the PPROM group. The characteristics of the study population are summarized in Table 1. A number of demographical and clinical characteristics varied according to the group at admission. For instance, women born in Sub Saharan Africa were overrepresented in the PTL and PPROM groups. In addition, the PTL and PPROM groups were characterized by a higher proportion of multiple pregnancies, history of preterm deliveries, and obesity. Eventually, 283 (57.6%) and 58 (13.4%) women of the PTL and PPROM groups delivered at term.

Vaginal microbiota signatures of preterm labor and premature rupture of membranes

A Nugent score was determined for 2,018 (81.5%) vaginal samples and indicated that bacterial vaginosis was uncommon, below 5% whatever the cohort group considered (Table S1). However, intermediate scores were overrepresented in the PPROM group (60/330, 18.2% *vs*. 110/851, 12.9% in controls, p=0.02). Culturomics of the vaginal samples revealed significant differences according to the cohort groups, most importantly in the abundance of lactobacilli, enterobacteria, and anaerobes, including *G. vaginalis* (Figure 1 and Table S1). Overall, lactobacilli were less frequently isolated in the PTL (357/493, 72.4%) and PPROM (274/418, 65.6%) groups compared with controls (894/1066, 83.9%). Conversely, vaginal samples positive for enterobacteria and anaerobes were overrepresented in the high-risk groups. For instance, enterobacteria were isolated in more than 25% women of the TPROM, PTL and PPROM groups *vs*. 16% in controls. *E. coli* was the most frequent species and showed the strongest significant differences. Similarly, *G. vaginalis* was cultured in 13.7% (65/474), 16.8% (83/495), and 10.6% (46/434) samples from the TPROM, PTL and PPROM groups, respectively, *vs*. 7.8% (83/1,067) controls. The abundance of *S. aureus*, GBS and *Candida* spp. did not vary significantly according to the cohort groups (Table S1).

Demographical and clinical characteristics such as ethnicity and BMI are linked with pregnancy outcomes and are also presumed to impact microbiota composition. Therefore, we tested their association with the species of the vaginal microbiota and found that the place of birth (Table S2) and the BMI (Table S3) were both correlated with the presence and abundance of lactobacilli.

Demographic, clinical and microbiological factors associated with PTL and PROM

Next, we addressed the association between demographics, clinical factors, and vaginal microbiota with PTL and PROM using univariate (Table S4) then multivariable logistic

regression analyses (Figures 2 and S1). Altogether, the factors the most associated with TPROM were a North African or Asian origin, nulliparity, and a vaginal sample positive for enterobacteria (aOR: 1.97, 95% CI 1.46-2.65) or *G. vaginalis* (aOR: 5.19, 95% CI 2.22-13.78). PTL was strongly associated with the maternal place of birth and most-importantly with birth in French overseas territories and Sub-Saharan Africa, with nulliparity, a multiple pregnancy, a history of preterm delivery, and smoking during pregnancy. Considering vaginal microbiota, the absence of lactobacilli (aOR: 1.49, 95% CI 1.08-2.06) and the presence of enterobacteria (aOR: 1.86, 95% CI 1.36-2.53) or *G. vaginalis* (aOR: 4.62, 95% CI 1.86-13.34) were all associated with PTL. Lastly, women from all areas outside Europe were more frequently represented in the PPROM group in comparison with controls, together with nulliparous women, women with a multiple pregnancy, a history of preterm delivery, and overweight. Considering vaginal microbiota composition, the absence of lactobacilli (aOR 2.04, 95% CI 1.49-2.80) and the presence of enterobacteria (aOR 2.38, 95% CI 1.74-3.24) in vaginal swab cultures were both strongly correlated with PPROM.

Altogether, the absence of lactobacilli in vaginal swab cultures was a risk factor for PTL and PPROM but not for TPROM, enterobacteria were associated with TPROM, PTL, and PPROM, and *G. vaginalis* was a signature of TPROM and PTL but not of PPROM. Similar results were obtained with the second model of multivariable logistic regression where *E. coli* and *K. pneumoniae* were included instead of enterobacteria (Figure S1), showing that vaginal colonization by *E. coli* was strongly associated with TPROM (aOR 1.91, 95%CI 1.37-2.67), PTL (aOR 2.02, 95% CI 1.43-2.86), and PPROM (aOR 2.39, 95% CI 1.69-3.38).

Vaginal microbiota with enterobacteria or lacking lactobacilli are risk factors for preterm birth

We next focused on the PTL and PPROM groups, with the highest risk of adverse pregnancy outcomes, and we sought to determine whether the vaginal microbiota at admission could be predictive of PTB. Firstly, we analyzed in univariate and multivariable analyses the demographical, clinical and microbiological risk factors for spontaneous PTB (510/826, 61.7%, Table S5). This first analysis showed that the outcome was highly correlated with multiple pregnancy (aOR 4.80, 95% CI 2.72-9.03, $p<0.0001$). Whereas vaginal microbiota lacking lactobacilli and including enterobacteria were both associated with PTB in univariate analysis, only enterobacteria remained correlated with PTB in multivariable analysis (aOR 1.64, 95%CI 1.15-2.37, Figure 3A). Similar results were obtained in the second model of multivariable analysis where *E. coli* was associated with preterm birth (aOR 1.52, 95% CI 1.03-2.29, Figure S2A). In a second analysis focusing on singleton pregnancies which represented 86.7% (716/826) of women admitted for PTL or PPROM, the absence of lactobacilli and the presence of enterobacteria both remained significantly correlated with PTB in multivariable analysis (aOR 1.54, 95% CI 1.05-2.28 and 1.62, 95% CI 1.11-2.36, respectively, Figures 3B and S2B, Table S6).

Comment

Principal Findings

In this cohort study of over 2,400 pregnant women from various ethnicities recruited in three French maternity wards, we identified using simple culturomics of vaginal samples distinct microbiological signatures of PTL, TPROM, and PPROM. Notably, we identified, using multivariable logistic regression models, that vaginal microbiota, together with demographic and clinical features, was correlated with these complications of pregnancy. For instance, PTL was strongly associated with the presence of *G. vaginalis* and less significantly with the absence of lactobacilli, TPROM with the presence of *G. vaginalis* and enterobacteria, whereas PPROM

was correlated with a depletion in lactobacilli and with the presence of enterobacteria. Importantly, microbiota composition at admission, and particularly a depletion in lactobacilli and the presence of enterobacteria were predictive of PTB.

Results in the Context of What is Known

These results are in perfect agreement with those already published in the literature. Considering PTB, a meta-analysis conducted by Gudnadottir *et al.* in which the vaginal microbiome was assessed before the onset of labor reported an increased risk of preterm delivery in women with low-lactobacilli microbiota.[16] In a recent longitudinal cohort study of 175 pregnant women comprising 40 PTB, the vaginal microbiome of pregnancies that ended preterm was found to be genetically more diverse, a diversity which was driven by *Gardnerella* spp.[20] Lastly, the analysis of the vaginal microbiota at delivery of a subset of 749 women from our cohort study using shotgun metagenomics found that PTB was correlated to the overall diversity of the microbiota.[17] In our study, the role of lactobacilli depletion and the correlated increased microbiota diversity as risk factors for PTB varied according to the studied population. For instance, we identified lactobacilli depletion as a risk factor for PTB only in women with a singleton pregnancy. These divergences are likely due to the specificities of our cohort which included high-risk pregnancies and multiple pregnancies, which appeared as the strongest risk factor for PTB.

Considering PPROM, Brown *et al.* analyzed the vaginal microbiome of 86 women and identified reduced *Lactobacillus* spp. abundance and increasing vaginal bacterial diversity as a risk factor.[21] Similarly, Yan et al. analyzed the vaginal microbiota of 112 women and found an increased abundance of *G. vaginalis* and other anaerobes in women presenting with PPROM. In line with the study of Brown *et al.*, we found that a depletion in lactobacilli was strongly associated with PPROM both in univariate and multivariable analysis. Conversely, *G. vaginalis*

was not identified as a risk factor for PPROM. This discrepancy is likely related to the methodology that was used in our study where microbiota was assessed by culture, which is less sensitive than metagenomics, especially for anaerobes.

Clinical Implications

The role of bacterial vaginosis and *G. vaginalis* in PTL and PTB has been suspected for a long time.[22,23] Growing evidence regarding the direct role of *G. vaginalis* in the pathophysiology of PTL is now accumulating. *G. vaginalis* and other anaerobes such as *Prevotella* spp. are able to degrade the cervical mucus, favoring ascending intra-uterine infections, local inflammation, uterine motility, and PTL.[24,25] It is now generally recognized that an antibiotic treatment, mainly based on metronidazole or clindamycin, should be administered to women presenting with PTL and diagnosed with symptomatic bacterial vaginosis. In many countries, national guidelines also recommend screening for bacterial vaginosis and treatment of women with a history of PTB, although this strategy has not proven to be efficient in reducing the risk of PTB.[26–28] Here we show that *G. vaginalis* is a risk factor for PTL but more importantly that it is the absence of lactobacilli and the presence of enterobacteria that seeme predictive of PTB. These observations, in line with numerous metagenomic studies which underline the protective role of lactobacilli and more especially of *L. crispatus*, *L. gasseri*, and *L. jensenii*,[17,29,30] argue for a comprehensive management of bacterial vaginosis in pregnancy aiming at restoring a healthy microbiota. Such management would presumably associate antibiotics targeting anaerobes to probiotics, as proposed for recurrent vaginosis.[28]

Our results also raise questions about the management of vaginal colonization by enterobacteria during TPROM, PTL and PPROM. We identified *E. coli*, and more generally enterobacteria as strongly associated with TPROM, PTL and PPROM in univariate and multivariable analyses. Both were also identified as risk factors for PTB. However, in contrast to GBS, there is currently

no consensus guidelines for the screening and the administration of antibiotics against enterobacteria during pregnancy, not even in case of PROM. In the absence of signs of infection, strategies largely vary between countries and range from no treatment to large spectrum antibiotics such as 3rd generation cephalosporins.[31] Our observations also argue in favor of administering a prophylaxis adapted to the pathogenic species present in the vaginal microbiota at admission for TPROM, PTL, and PPROM, although further studies are needed to ascertain the benefits of such a strategy.

Research Implications

Interventional studies aiming at assessing the role of antibiotics alone or in combination with probiotics in reducing the risk of PTB and PPROM in high-risk populations and women who experience bacterial vaginosis should be undertaken. These studies should rely on randomized control trials that would take cofounding variables such as ethnicity, BMI, and gestational age at intervention into account. Indeed, a given probiotic strain would not necessarily be protective for all women, a property that might be linked to women's CSTs. In addition, the benefits of personalized targeted antibiotic prophylaxes directed against the specific pathogenic species *e.g.*, GBS, enterobacteria, *Candida* spp. and others, found in the vaginal flora in reducing the risk of PTB especially in case of PPROM should also be investigated.

Strengths and Limitations

This is a large cohort study where over 2,400 women were enrolled, including almost 500 PTL and 500 PPROM. In addition to this major strength, our study also investigated important cofounding variables of the outcome measures including demographical factors such as ethnicity, BMI, history of PTB, and others. Hence, we could demonstrate that the vaginal microbiota is an independent risk factor for TPROM, PTL, and PPROM, and also for PTB.

However, there are limitations that must be taken into account in the interpretation of our results. First of all, microbiota analyses were carried out using simple culturomics and not metagenomics. This leads to three main issues: (i) fastidious species such as *Atopobium* spp., or mollicutes could not be studied; (ii) culturomics being less sensitive than metagenomics, cultured species were most likely the predominant ones and minor species have probably been overlooked; (iii) although a semi-quantitative analysis of the cultured species was initially performed, it rapidly came out because of differences in their growth characteristics, that this analysis was biased and not representative of the microbiota equilibrium. Eventually, only the presence or absence of major species was investigated. The second main limitation is the absence of identification of lactobacilli at the species level. Consequently, the vaginal microbiota were not categorized according to CSTs, and the protective role conferred by the different species could not be addressed.

Conclusions

In this large multicentric cohort study, we underlined using culturomics distinct vaginal microbiota signatures of adverse pregnancy outcomes. We identified *G. vaginalis* and lactobacilli depletion as independent and strong risk factors for TPROM and PTL, and of PPROM, respectively. Besides, enterobacteria were identified as risk factors for these three situations and for PTB. Further studies are needed to address the potential of interventional studies aiming at restoring a healthy microbiota in reducing these outcomes.

Transparency declaration

Ethics statement

The reported study was reviewed by the French ethics committee who granted approval on November, 2017 (identifier 2017 – A02755-48; NCT03371056). All individuals enrolled in the

cohort provided signed informed consent that included authorization for the collection of medical information and biological samples. All collected data were anonymized and integrated in an secured electronic database.

Conflicts of interest

The authors have no conflicts of interest to disclose.

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Access to data

All authors had full access to data. Data will be made available upon requests directed to the corresponding author.

Authors' contributions

Conceptualization: C.Po., L.M., and A.T. Methodology: J.R., P.-Y.A., L.M., and A.T. Formal analysis: L.Le., J.R., P.-Y.A., L.M., and A.T. Investigation: L.Le., J.R., and A.T. Resources: C.Pl., L.La, N.G., F.G., and L.M. Data curation: L.Le, J.R., C.Pl, L.La, N.G., and A.T. Writing – Original draft: L.Le and A.T. Writing – Review and Editing: all authors. Supervision: C.Pa, C.Po., L.M. and A.T. Funding acquisition: C.Pa, C.Po and L.M.

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Figure legends.

Figure 1. Vaginal microbiota composition at admission for prelabor rupture of membranes at term (TPROM), threatened preterm birth and preterm labor (PTL), and preterm rupture of membranes (PPROM).

Vaginal microbiota composition was characterized by culturomics and compared between controls (n=1,068) and women admitted for TPROM (n=477), PTL (n=495), or PPROM (n=436). The percentage of vaginal samples positive for each bacterial group or species are represented. Error bars indicate the upper limit of the 95% confidence interval. * $p<0.05$; *** $p<0.001$.

Figure 2. Multivariable analysis of risk factors for prelabor rupture of membranes at term (TPROM), threatened preterm birth and preterm labor (PTL), and preterm premature rupture of membranes (PPROM).

Adjusted odds ratios were calculated using any variables associated with the outcome with a p-value <0.2 from the univariate analysis, excepting microbiological variables which were all included. *S. aureus*, *S. anginosus* and *Candida* spp. did not show significant associations with the outcomes and are not represented. aOR: adjusted odds ratio; CI: confidence interval.

Figure 1.

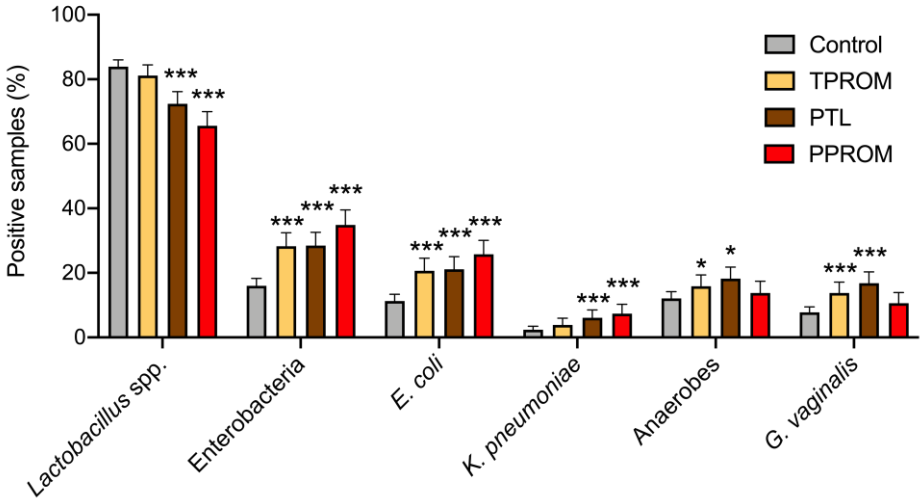

Figure 1. Vaginal microbiota composition at admission for prelabor rupture of membranes at term (TPROM), threatened preterm birth and preterm labor (PTL), and preterm rupture of membranes (PPROM).

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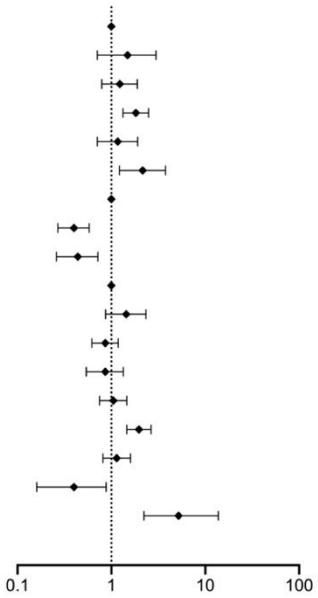
Figure 2.

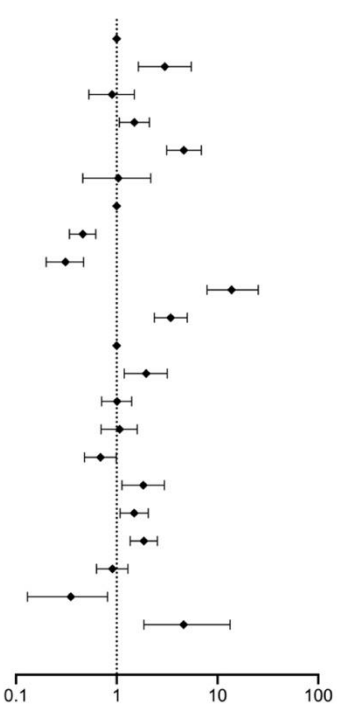
A - TPROM

		aOR (95% CI; p-value)
Place of birth	France	1
	France, overseas territories	1.49 (0.71-2.99; 0.27)
	Europe	1.23 (0.79-1.89; 0.36)
	North Africa	1.82 (1.33-2.49; 0.0002)
	Sub Saharan Africa	1.17 (0.71-1.91; 0.52)
	Asia	2.15 (1.22-3.78; 0.008)
Parity	0	1
	1	0.40 (0.27-0.58; <0.0001)
	≥ 2	0.44 (0.26-0.72; 0.001)
Body mass index	18.5 – 24.9	1
	< 18.5	1.44 (0.87-2.34; 0.15)
	25.0 – 29.9	0.86 (0.62-1.18; 0.35)
	≥ 30	0.86 (0.54-1.34; 0.52)
Vaginal microbiological cultures	Absence of lactobacilli	1.05 (0.75-1.46; 0.78)
	Enterobacteria	1.97 (1.46-2.65; <0.0001)
	Group B *Streptococcus*	1.14 (0.81-1.60; 0.44)
	Anaerobes	0.40 (0.16-0.88; 0.03)
	G. vaginalis	5.19 (2.22-13.78; 0.0003)



B - PTL

		aOR (95% CI; p-value)
Place of birth	France	1
	France, overseas territories	3.00 (1.64-5.49; 0.0003)
	Europe	0.90 (0.53-1.50; 0.70)
	North Africa	1.50 (1.06-2.11; 0.02)
	Sub Saharan Africa	4.64 (3.13-6.93; <0.0001)
	Asia	1.04 (0.46-2.18; 0.91)
Parity	0	1
	1	0.46 (0.34-0.62; <0.0001)
	≥ 2	0.31 (0.20-0.47; <0.0001)
Pregnancy with multiples		13.80 (7.89-25.38; <0.0001)
History of preterm delivery		3.44 (2.36-5.02; <0.0001)
Body mass index	18.5 – 24.9	1
	< 18.5	1.96 (1.19-3.18; 0.007)
	25.0 – 29.9	1.01 (0.71-1.41; 0.98)
	≥ 30	1.07 (0.70-1.60; 0.74)
Gestational diabetes		0.69 (0.48-0.99; 0.048)
Smoking		1.84 (1.13-2.97; 0.01)
Vaginal microbiological cultures	Absence of lactobacilli	1.49 (1.08-2.06; 0.02)
	Enterobacteria	1.86 (1.36-2.53; <0.0001)
	Group B *Streptococcus*	0.91 (0.63-1.29; 0.59)
	Anaerobes	0.35 (0.13-0.81; 0.02)
	G. vaginalis	4.62 (1.86-13.34; 0.002)



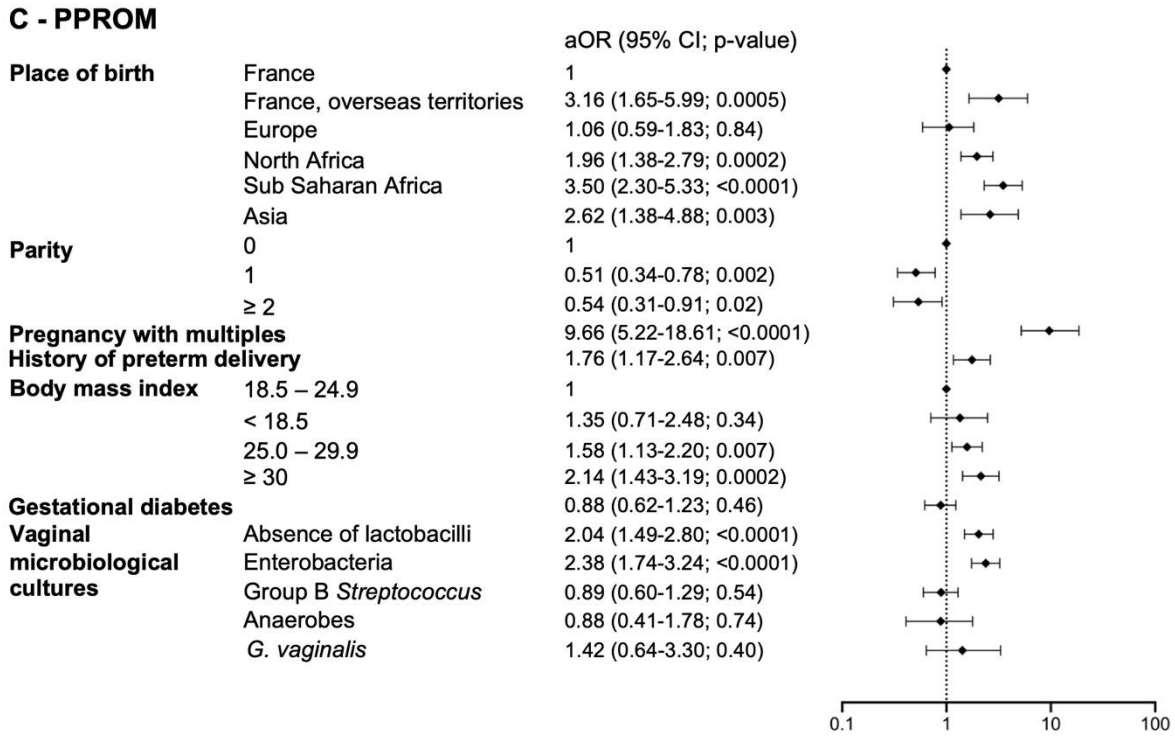
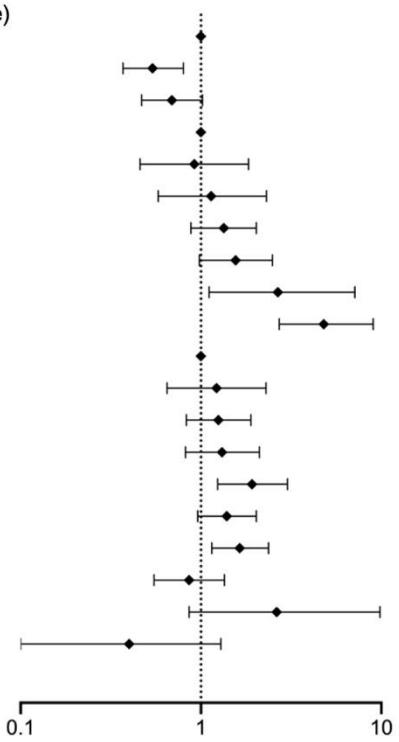


Figure 2. Multivariable analysis of risk factors for prelabor rupture of membranes at term (TPROM), threatened preterm birth and preterm labor (PTL), and preterm premature rupture of membranes (PPROM).

Adjusted odds ratios were calculated using any variables associated with the outcome with a p-value <0.2 from the univariate analysis, excepting microbiological variables which were all included. *S. aureus*, *S. anginosus* and *Candida* spp. did not show significant associations with the outcomes and are not represented. aOR: adjusted odds ratio; CI: confidence interval.

A

		aOR (95% CI; p-value)
Gestational age at admission (weeks)	≥ 32	1
	28-31	0.54 (0.37-0.80; 0.002)
	<28	0.69 (0.47-1.02; 0.06)
Place of birth	France	1
	France, overseas territories	0.92 (0.46-1.84; 0.82)
	Europe	1.14 (0.58-2.31; 0.70)
	North Africa	1.34 (0.88-2.03; 0.17)
	Sub Saharan Africa	1.56 (0.98-2.49; 0.06)
	Asia	2.67 (1.11-7.14; 0.04)
Pregnancy with multiples		4.80 (2.72-9.03; <0.0001)
Body mass index	18.5 – 24.9	1
	< 18.5	1.22 (0.65-2.30; 0.54)
	25.0 – 29.9	1.25 (0.83-1.89; 0.28)
	≥ 30	1.31 (0.82-2.11; 0.26)
Gestational diabetes		1.92 (1.24-3.02; 0.004)
Vaginal microbiological cultures	Absence of lactobacilli	1.39 (0.96-2.03; 0.08)
	Enterobacteria	1.64 (1.15-2.37; 0.007)
	Group B *Streptococcus*	0.86 (0.55-1.35; 0.52)
	Anaerobes	2.63 (0.86-9.83; 0.11)
	G. vaginalis	0.40 (0.10-1.29; 0.15)



B – Singleton pregnancies

		aOR (95% CI; p-value)
Gestational age at admission (weeks)	≥ 32	1
	28-31	0.51 (0.34-0.77; 0.001)
	<28	0.71 (0.47-1.06; 0.09)
Place of birth	France	1
	France, overseas territories	0.82 (0.40-1.67; 0.58)
	Europe	1.27 (0.62-2.67; 0.52)
	North Africa	1.32 (0.85-2.04; 0.22)
	Sub Saharan Africa	1.43 (0.88-2.34; 0.16)
	Asia	2.64 (1.09-7.13; 0.04)
Body mass index	18.5 – 24.9	1
	< 18.5	1.09 (0.57-2.12; 0.79)
	25.0 – 29.9	1.24 (0.81-1.90; 0.32)
	≥ 30	1.27 (0.78-2.08; 0.33)
Gestational diabetes		1.78 (1.14-2.83; 0.01)
Vaginal microbiological cultures	Absence of lactobacilli	1.54 (1.05-2.28; 0.03)
	Enterobacteria	1.62 (1.11-2.36; 0.01)
	Group B *Streptococcus*	0.88 (0.55-1.41; 0.59)
	Anaerobes	2.58 (0.84-9.72; 0.12)
	G. vaginalis	0.37 (0.10-1.22; 0.12)

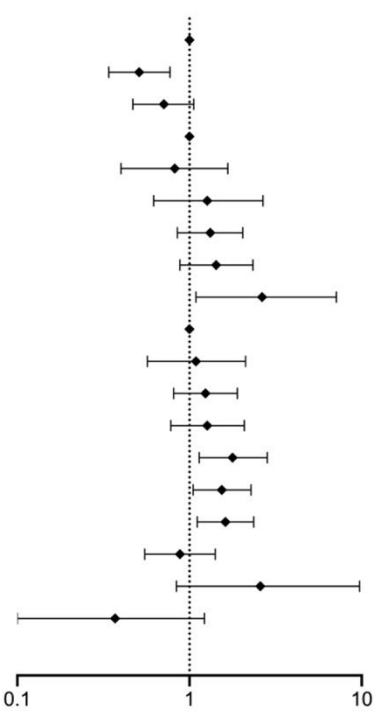


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Figure 3. Multivariable analysis of risk factors for preterm birth in women admitted for threatened preterm birth, preterm labor, and preterm premature rupture of membranes for (A) all pregnancies and (B) singleton pregnancies.

Adjusted odds ratios were calculated using any variables associated with the outcome with a p-value <0.2 from the univariate analysis, excepting microbiological variables which were all included. *S. aureus*, *S. anginosus* and *Candida* spp. did not show significant associations with the outcomes and are not represented. aOR: adjusted odds ratio; CI: confidence interval.

Table 1. Cohort demographic and clinical characteristics.

	Control n=1,068	**TPROM n=477**	**PTL n=495**	**PPROM n=436**	**p-value**
Maternity ward, n (%)					
Center 1	682 (63.9)	262 (54.9)	246 (49.7)	163 (37.4)	NT
Center 2	386 (36.1)	215 (45.1)	144 (29.1)	216 (49.5)	NT
Center 3	0	0	105 (21.2)	57 (13.1)	NT
Age, median (IQR), years	33 (30-36)	33 (30-36)	32 (29-35)	33 (30-36)	<0.0001
Gestational age at inclusion, median (IQR), weeks	36 (35-36)	39 (38-40)	29 (26-32)	32 (27-35)	<0.0001
Maternal place of birth, n (%)					
France	565 (53.1)	211 (46.2)	185 (38.4)	142 (33.2)	<0.0001
France, overseas territories[a]	29 (2.7)	13 (2.9)	30 (6.2)	25 (5.8)	0.001
Europe, other countries	81 (7.6)	44 (9.6)	26 (5.4)	24 (5.6)	0.04
North Africa	206 (19.3)	111 (24.3)	95 (19.7)	115 (26.9)	0.004
Sub-Saharan Africa	89 (8.4)	28 (6.1)	106 (22.0)	84 (19.6)	<0.0001
Asia	35 (3.3)	29 (6.3)	12 (2.5)	20 (4.7)	0.01
Others	60 (5.6)	21 (4.6)	28 (5.8)	18 (4.2)	0.58
Missing	*3 (0.3)*	*20 (4.2)*	*13 (2.6)*	*8 (1.8)*	NT
Gestity, n (%)					
1	317 (29.7)	215 (45.1)	156 (31.5)	148 (33.9)	<0.0001
2	341 (32.0)	127 (26.6)	146 (29.5)	102 (23.4)	0.006
≥3	409 (38.3)	135 (28.3)	193 (39.0)	186 (42.7)	<0.0001
Missing	*1 (0.1)*	*0*	*0*	*0*	NT
Parity, n (%)					
0	477 (44.7)	317 (66.5)	267 (53.9)	223 (51.1)	<0.0001
1	385 (36.1)	105 (22.0)	150 (30.3)	115 (26.4)	<0.0001
≥2	205 (19.2)	55 (11.5)	78 (15.8)	98 (22.5)	<0.0001
Missing	*1 (0.1)*	*0*	*0*	*0*	NT
Multiple pregnancy, n (%)	19 (1.8)	1 (0.2)	78 (15.8)	53 (12.2)	<0.0001
History of preterm delivery, n (%)	111 (10.4)	18 (3.8)	100 (20.2)	73 (16.7)	<0.0001
Missing	*3 (0.3)*	*0*	*0*	*0*	NT
Body mass index, median (IQR)	22.4 (20.4 - 25.7)	22.4 (20.2 - 25.2)	22.9 (20.3 - 26.6)	24.5 (21.5 - 28.8)	<0.0001
Underweight (<18.5), n (%)	57 (5.3)	36 (7.5)	42 (8.6)	23 (5.4)	0.05
Normal weight (18.5-24.9), n (%)	685 (64.2)	314 (66.0)	278 (56.7)	204 (47.8)	<0.0001
Overweight (25-29.9), n (%)	213 (20.0)	88 (18.5)	103 (21.0)	112 (26.2)	0.02
Obese (≥30), n (%)	112 (10.5)	38 (8.0)	67 (13.7)	88 (20.6)	<0.0001
Missing	*1 (0.1)*	*1 (0.2)*	*5 (1.0)*	*9 (2.1)*	NT
Gestational diabetes, n (%)	197 (18.5)	79 (16.6)	78 (15.8)	94 (21.6)	0.10
Missing	*1 (0.1)*	*0*	*0*	*0*	NT

	Control n=1,068	TPROM n=477	PTL n=495	PPROM n=436	p-value
Hypertensive disease, n (%)	41 (3.8)	6 (1.3)	22 (4.4)	5 (1.1)	0.001
Missing	*1 (0.1)*	*0*	*0*	*0*	NT
Smoking during pregnancy, n (%)	57 (5.4)	27 (5.7)	37 (7.5)	29 (6.7)	0.36
Missing	*3 (0.3)*	*0*	*4 (0.8)*	*3 (0.7)*	NT
Fetal growth restriction, n (%)	29 (2.7)	5 (1.0)	46 (9.3)	39 (8.9)	<0.0001
Missing	*1 (0.1)*	*0*	*0*	*0*	NT
Gestational age at delivery, median (IQR), weeks	40.0 (39.0-40.7)	39.7 (39.0-40.6)	37.7 (34.3-39.1)	35.0 (30.6-36.4)	<0.0001
Extremely preterm (<28 weeks), n (%)	0	0	37 (7.6)	65 (15.0)	<0.0001
Very preterm (28-31 weeks), n (%)	0	0	34 (6.9)	76 (17.5)	<0.0001
Moderately preterm (32-36 weeks), n (%)	0	0	137 (27.9)	235 (54.1)	<0.0001
Term (≥ 37 weeks), n (%)	1066 (100)	477 (100)	283 (57.6)	58 (13.4)	<0.0001
Missing	*2 (0.2)*	*0*	*4 (0.8)*	*2 (0.5)*	NT
Mode of delivery, n (%)					
Vaginal, spontaneous	764 (71.7)	324 (68.0)	305 (62.1)	246 (56.8)	<0.0001
Vaginal, induced	118 (11.1)	86 (18.0)	87 (17.7)	56 (12.9)	0.0002
Planned Caesarean	61 (5.7)	5 (1.0)	38 (7.8)	60 (13.9)	<0.0001
Caesarean in labor	123 (11.5)	62 (13.0)	61 (12.4)	71 (16.4)	0.09
Missing	*2 (0.2)*	*0*	*4 (0.8)*	*3 (0.7)*	NT
Interval between ROM and delivery, median (IQR), days	0 (0-1)	1 (1-2)	0 (0-1)	4 (1-17)	<0.0001
Missing	*21 (2.0)*	*3 (0.6)*	*42 (8.5)*	*13 (2.9)*	NT
Antibiotics between inclusion and delivery[b], n (%)	139 (13.0)	209 (43.8)	146 (29.6)	353 (81.0)	<0.0001
Missing	*0*	*0*	*2 (0.4)*	*0*	NT
Live births, n (%)	1083 (100)	478 (100)	561 (98.9)	481 (99.0)	NT
Missing	*3 (0.3)*	*0*	*6 (1.2)*	*2 (0.4)*	NT

[a] Including territories in South America (French Guiana), the Atlantic Ocean (Guadeloupe, Martinique, Saint-Martin, Saint-Barthélémy, Saint-Pierre-et-Miquelon), the Indian Ocean (Reunion Island, Mayotte), the Pacific Ocean (New Caledonia, French Polynesia, the Wallis and Futuna Islands), and the French Southern and Antarctic Territories.

[b] Including intrapartum antibiotic prophylaxis and excluding non-systemic antibiotic therapy.

IQR: interquartile range; NT: not tested; PPROM: preterm premature rupture of membranes; PTL: threatened preterm birth or preterm labor; ROM: rupture of membranes; TPROM: prelabor rupture of membranes at term.