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Alterations of the vaginal microbiota in the third trimester of pregnancy and pPROM

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Abstract. – OBJECTIVE: Preterm premature rupture of membranes (pPROM) is a significant issue in obstetric practice. One of the risk factors for pPROM are vaginal infections in the third trimester of pregnancy.

PATIENTS AND METHODS: We performed an observational study on 600 pregnant women, analyzing the lactobacillary grade (LBG) and the presence of any pathogenic bacteria and/or *Candida* at weeks 28 and 32 of pregnancy and recording any pPROM events at delivery. At week 28, in the case of vaginal infection, the patients were treated for 6 days with a topical association of metronidazole+clotrimazole.

RESULTS: At week 28 of pregnancy 54.2% of women had vaginal infection (32.6% bacterial vaginitis, 33.8% candidiasis and 32.4% mixed infection) and/or abnormal vaginal microbiota (67.4% LBG 2a/2b, 32.6% LBG 3). The total number of pPROM was 8 out of 600 (1.3%). The treatment of vaginal infection at week 28 with the topical association of metronidazole+clotrimazole, led to both the eradication of vaginal infections and the restoration of the vaginal microbiota in 72% of the cases, bringing the level of risk of pPROM similar to that of women without vaginal infection at week 28. In addition, the results showed that women with vaginal infections and/or alteration of vaginal microbiota at week 32 of pregnancy had a higher prevalence of pPROM in comparison to the women without vaginal infection at week 32 ($p<0.001$).

CONCLUSIONS: This observational study showed the high prevalence of vaginal infections in the third trimester of pregnancy and its association with pPROM. Furthermore, data suggested the possible benefits of the topical treatment with metronidazole+clotrimazole in pregnancy to eradicate infections, restore the normal microbiota and reduce the risk of pPROM.

Key Words
Vaginal infections, Vaginal microbiota, Pregnancy, Premature rupture of membrane, Metronidazole, Clotrimazole.

Introduction

According to recent epidemiological studies, premature rupture of membranes (PROM) is an event that affects 8%-10% of all pregnancies[1]. In particular, PROM at term (after 37 weeks of gestation) occurs very frequently, and in about 85% of cases women go into labor spontaneously within 48 hours of the event[1]. Particularly delicate, due to its more serious consequences for both mother and newborn, is preterm PROM (pPROM), defined as premature rupture of membranes before 37 weeks of gestation[1,2]. It is estimated that pPROM is associated with 20-40% of preterm births (PTB)[3,4]. pPROM complicates 2-4% of pregnancies[1,2]. The complications include foetal-neonatal infection (chorioamnionitis and sepsis), morbidity (including pulmonary hypoplasia, skeletal malformations, intraventricular hemorrhage, necrotizing enterocolitis) and neonatal mortality due to prematurity[5]. Neonatal morbidity and mortality are strongly related to the gestational age at which PROM and delivery occur[5,6]; among those born before 30 weeks of gestation, only 25% is free from disability at the age of five years[7].

Management of pregnant women with or at risk of PROM is still one of the most significant issues in obstetric practice. Even though management of both mother and newborn in the event of PROM is codified by international guidelines[8,9], PROM prevention management still appears to be controversial both as regards the definition of the risk factors and the validity of a preventive treatment[10].

In this context, the presence of vaginal infections (whether bacterial or fungal) in the third trimester of pregnancy is a known risk factor for PROM and pPROM[11-15]. Since alterations of the microbiota in the genital tract in the early months of pregnancy may be a predictor for late miscarriage and preterm birth (PTB)[16,17], the detection

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of alterations of the vaginal microbiota can be considered a marker of vaginal infection.

Although some single studies have shown that antibiotic treatments can help prevent pPROM and PTB, in the meta-analyses these effects failed to be shown[18-20]. The reason could be that these studies have focused on bacterial vaginosis as the unique cause of PTB[20], without considering, for example, the role of *Candida* in the vaginal microbiota. Some evidence suggests that screening for the eradication of *Candida* during pregnancy can reduce the risk of premature birth[21-23].

In the presence of vaginal infections and/or alterations of the vaginal microbiota, the use of a broad-spectrum antimicrobial treatment to promote the restoration of normal vaginal microbiota could, therefore, have a favorable impact on the prevalence of pPROM. Among the drugs available, a possible option is the use of two active substances well known for their efficacy against vaginal infections, such as metronidazole (antibiotic) and clotrimazole (antifungal)[24,25]. The topical association of metronidazole + clotrimazole is a drug with good tolerability and efficacy in vaginal infections and which has been shown to have no effect on lactobacilli, the main components of the normal vaginal microbiota[26-30].

To better understand how to improve the management of vaginal infections, alterations of the vaginal microbiota and pPROM events in the third trimester of pregnancy, an observational study was performed by analyzing in the clinical practice the lactobacillary grade (LBG) and the presence of any pathogenic bacteria and/or *Candida* at weeks 28 and 32 of pregnancy and by recording any pPROM events at delivery. Furthermore, it was observed the effects of topical treatment with metronidazole + clotrimazole in women at week 28 of pregnancy with vaginal infection, in reducing the infections, in restoring the vaginal microbiota and in preventing pPROM events.

Patients and methods

Study Design and Patients

From January 2015 to February 2016, in 2 Italian centers (ASP Catania, Gynaecology and Obstetrics Unit, Bronte Hospital and E. Falcidia Nursing Home, Catania) an observational study was performed on 600 pregnant women at week 28 of gestation, subjected to a vaginal swab routine to search for *Streptococcus agalactiae* (SGB).

The study was conducted in accordance with Good Clinical Practice (GCP) guidelines, regulations concerning clinical trials and the Declaration of Helsinki. Each subject consented to be enrolled in the study and gave their consent to the processing of personal data.

At the same time as the swab for SGB, all of the women underwent a vaginal smear test, and the sample was sent to the Biomedical and Biotechnological Sciences Department of the University of Catania to assess the lactobacillary grade (LBG) and any presence of pathogenic bacteria and/or *Candida*.

The women were assessed at week 28 of pregnancy (T0=baseline), at week 32 of pregnancy (T1) and at delivery (T2). The results of the medical examination, swab, smear and microbiological analysis were recorded at weeks 28 and 32 of pregnancy, while pPROM was recorded at delivery.

At the baseline visit (week 28 of pregnancy), if the lactobacillary grade was altered (LBG 2a/2b or LBG 3) or there was a vaginal infection, patients were treated to eradicate the infection and to restore a normal vaginal microbiota. The treatment was a topical association of metronidazole + clotrimazole (MC) (MC pessary = metronidazole 500 mg + clotrimazole 100 mg per pessary; or MC cream = 20% metronidazole + 4% clotrimazole), one pessary or 5 g of cream once a day for 6 days.

During the observational analysis, the women were evaluated retrospectively. They were divided into 2 groups: the group with vaginal infection and abnormal lactobacillary grade (LBG 2a/2b or LBG 3) at week 28 of pregnancy; and the group that at week 28 of pregnancy had a normal lactobacillary grade (LBG 1) and no vaginal infection.

Microbiological Tests

Lactobacillary grade (LBG) can be considered a variation on Schroder's classification. Grade 1 (LBG 1) corresponds to normal microbiota with a predominant presence of *Lactobacillus* morphotypes. Grade 2 corresponds to intermediate, mixed microbiota; in particular, LBG 2a is near-normal with more lactobacilli than other microorganisms, and LBG 2b has other microorganisms outnumbering lactobacilli. Grade 3 (LBG 3) corresponds to completely disrupted microbiota with only bacteria other than *Lactobacillus* morphotypes[17].

This analysis, together with the isolation of pathogenic microorganisms and clinical gynecological examination, can be used to diagnose

vaginal infection. The presence and the isolation of pathogenic bacteria and/or *Candida* from the vaginal swab and the lactobacillary grade assessment were performed as reported already in Furneri et al[29].

Statistical Analysis

The demographic, clinical and pPROM data were summarized in frequency (absolute frequency, relative percentage frequency) and distribution tables for the individual variables (mean, standard deviation).

Analysis of the association between prevalence of pPROM events and possible risk factors (vaginal infections and/or alterations of the vaginal microbiota) was performed using Fisher's exact test with 95% confidence intervals. *p*-values <0.05 were considered significant. Statistical analysis was performed using SPSS Statistical Package, ver. 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Vaginal Infections

Table I shows the demographic and clinical characteristics of pregnant women at week 28 of gestation observed during the baseline visit (T0). Of the 600 women, 325 (54.2%) had a vaginal disease: 32.6% of these had bacterial vaginitis, 33.8% candidiasis, while 32.4% had a mixed infection. The women suffering from vaginal infection had abnormal lactobacillary grade, with 67.4% having LBG 2a/2b and 32.6% having LBG 3.

Table II shows the microbes isolated from the vaginal swab in pregnant women at week 28 (baseline visit = T0), in order of frequency. In the 325 women observed, the most frequent pathogen was *Candida albicans*, either on its own (27.7%) or in combination with other microorganisms, making up 54.4% of the pathogens isolated. Thereafter, in order, *Enterococcus sp, Escherichia coli, Gardnerella vaginalis, Peptococcus sp* and *Candida non albicans*, were respectively present in 28.6%, 25.5%, 22.8%, 21.8% and 11.7% of cases, mainly in association with other microbial species.

Changes in the frequency of vaginal infections or changes in the vaginal microbiota between weeks 28 and 32 of pregnancy are shown in Figure 1. In the group of women with a vaginal infection at week 28, topical treatment with an association of metronidazole + clotrimazole eliminated the infection in 235 cases out of 325 (72.3%), while 39 women out of 325 (12%) were resistant to treatment; 51 women out of 325 (15.7%) only retained abnormal vaginal microbiota. In the group of women with no vaginal infection at week 28 of pregnancy, infections were recorded at week 32 of pregnancy in 2 cases out of 275 (0.7%) and abnormal vaginal microbiota in 128 women out of 275 (45.8%).

pPROM Events

The total number of pPROM observed at the end of the study was 8 out of 600 (1.3%).

Table III shows the frequency of pPROM events compared to vaginal microbiota at week 28 of pregnancy. We recorded 3 events (0.9%) in

Table I. Demographic and clinical characteristics observed at the baseline visit (week 28 of pregnancy).

	Vaginal infection and/or abnormal vaginal microbiota at week 28 of pregnancy n=600	
	Present n=325 (54.2%)	Absent n=275 (45.8%)
Age (years), mean ± SD	30.7±4.5	31.0±4.2
Week of pregnancy, mean ± SD	28.4±1.0	27.4±1.0
Vaginal disease, No (%)		
Absent	–	275 (100%)
Pathogenic bacteria (no symptoms)	4 (1.2%)	–
Vaginitis	106 (32.6%)	–
Candidiasis	110 (33.8%)	–
Mixed	105 (32.4%)	–
Vaginal microbiota, No (%)		
LBG – Grade 1	–	275 (100%)
LBG – Grade 2a	72 (22.2%)	–
LBG – Grade 2b	147 (45.2%)	–
LBG – Grade 3	106 (32.6%)	–

Table II. Microbial isolations observed at the baseline visit (week 28 of pregnancy)

Strains isolated	Women with vaginal infection n=325 No. (%)
Candida albicans	90 (27.7%)
G. vaginalis - Peptococcus sp	44 (13.5%)
Candida albicans - Enterococcus sp.	32 (9.8%)
E. coli - Enterococcus sp.	31 (9.5%)
G. vaginalis - Peptococcus sp - Candida albicans	27 (8.3%)
Candida non albicans	20 (6.2%)
Enterococcus sp.	18 (5.5%)
E. coli - Candida albicans	17 (5.2%)
E. coli - Candida non albicans	13 (4.0%)
E. coli - Enterococcus sp - Candida albicans	9 (2.8%)
E. coli	7 (2.2%)
Enterobacter sp - E.coli	6 (1.8%)
Enterobacter sp	2 (0.6%)
Enterococcus sp - Candida non albicans	2 (0.6%)
G. vaginalis - Candida non albicans	2 (0.6%)
Candida albicans - G.vaginalis	1 (0.3%)
Enterobacter sp – Candida non albicans	1 (0.3%)
Enterococcus sp - Enterobacter sp	1 (0.3%)
Group F Streptococci	1 (0.3%)
Streptococcus agalactiae - Candida albicans	1 (0.3%)

the group of women with vaginal infection and/or abnormal vaginal microbiota at week 28 of pregnancy, and treated with a topical association of metronidazole + clotrimazole. In the group of women without vaginal infection at week 28 and who therefore had not been treated, there were 5 events (1.8%) with a not significant difference in the prevalence of pPROM events between the two groups (RR=0.51; 95% CI: 0.12-2.11).

Table IV shows that the number of pPROM events was significantly higher in the group of women with a vaginal infection and/or abnormal vaginal microbiota at week 32 of pregnancy than in women who that week had no vaginal infection and/or no abnormal vaginal microbiota. We observed 8 pPROM events (3.7%) in the former group versus none ($p<0.001$) in the latter. Analysis of these 8 women revealed that 5 of them were suffering from vaginitis and that the pathogens isolated were respectively *E. coli* + *Enterococcus* sp. in 2 cases, *E. coli* + *Peptococcus* sp. in 1 case, *E. coli* + *Enterobacter* sp. in 1 case, *G. vaginalis* + *Peptococcus* sp. in 1 case. Furthermore, abnormal lactobacillary grade were present in all 8 women (LBG 2a in 1 case, grade LBG 2b in 5 cases and grade LBG 3 in 2 cases).

Adverse Events

During the study, no adverse events were observed related to the topical metronidazole + clotrimazole drug treatment.

Discussion

The management of PROM in pregnant women is still one of the most relevant issues in obstetric practice, especially in the case of pPROM, due to the more severe consequences both for the woman and for the newborn, even in the long term[1,2,6].

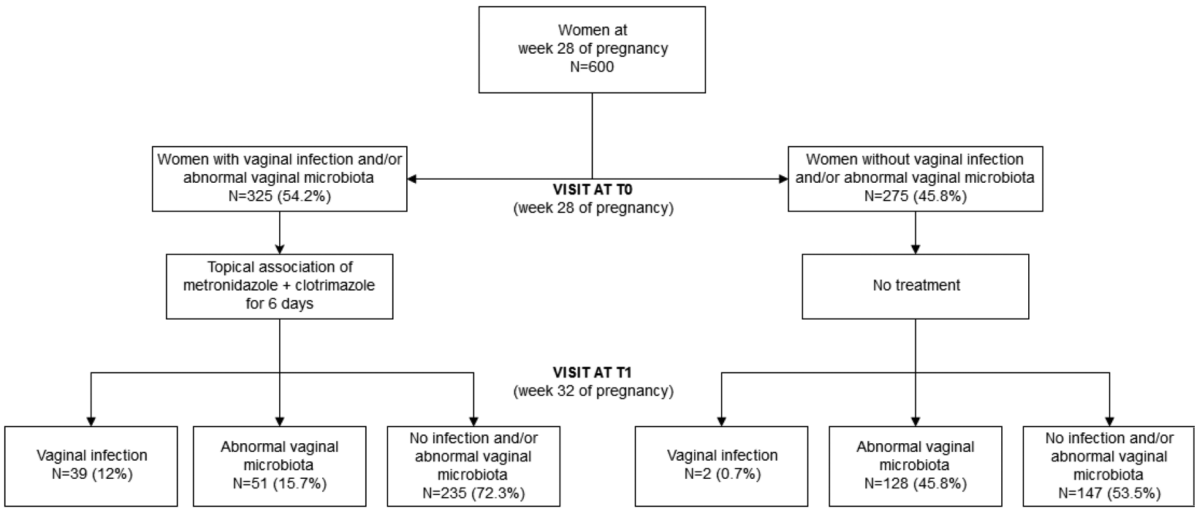

Figure 1. Results of treating vaginal infections with a topical association of metronidazole + clotrimazole (MC). Treatment with MC was performed for 6 days from week 28 of pregnancy and assessed at week 32 of pregnancy.

Table III. pPROM events observed compared to vaginal microbiota at the baseline visit (week 28 of pregnancy)

	Vaginal infection and/or abnormal vaginal microbiota at week 28 of pregnancy n=600	
	Present n=325 (54.2%)	Absent n=275 (45.8%)
Treatment with a topical association of metronidazole + clotrimazole	YES	NO
Delivery		
At term delivery, No. (%)	322 (99.1%)	270 (98.2%)
pPROM, No. (%)	3 (0.9%)	5 (1.8%)

One of the risk factors for PROM and pPROM events now recognized in the literature is the presence of bacterial, fungal, and mixed vaginal infections in the third trimester of pregnancy[11-15]. Indeed, pathogenic microbial contamination of the vaginal environment can spread through the cervix and reach the amniotic cavity, triggering a local inflammatory state and/or proteolytic process that lead to membrane lesions[1,3,4,31]. The finding that the bacterial species isolated in the uterine cavity are very common in the genital tract supports this pathogenetic mechanism[32].

In the present study, of 600 women observed at week 28 of pregnancy 54.2% had a vaginal infection (32.6% vaginitis, 33.8% candidiasis and 32.4% mixed etiology), as shown in Table I.

The prevalence of bacterial vaginitis varies in different countries and with the demographic characteristics of the population studied, reaching frequency rates up to 60%[11,32]. It is estimated that bacterial vaginitis is present in 15-42% of pregnant women, and can lead to a two- to four-fold of increase in the risk of preterm birth and pPROM[33].

Vaginal infections are characterized not only by the presence of pathogenic microorganisms, but also by alterations of the vaginal microbiota, with reduced amount of lactobacilli[11,12,33,34]. This was confirmed in our study: in the sample observed, 100% of the women with vaginal infection had decreased lactobacilli, as demonstrated by the alteration of lactobacillary grade (Table I). Research over the past 20 years has focused mainly on studying the vaginal microbiota, its changes during pregnancy and the potential obstetric and gynecological consequences of such changes[32,33,35-37].

Since alterations of the vaginal microbiota in pregnancy can be considered as a predictor for late miscarriage and preterm birth[16,17,32,34], the finding of an abnormal vaginal microbiota can be considered as a marker of vaginal infection.

Based on these considerations, some authors have evaluated antibiotic therapy as a preventive treatment for pPROM, with conflicting results and no conclusive evidence regarding risk reduction[18]. Similar findings have also been reported for antibiotic therapy for the prevention of PTB[19,20]. The inefficacy of a treatment carried out with an antibiotic monotherapy to prevent pPROM could be explained by the complexity of the vaginal microbiota. In this perspective, we should consider the role of *Candida* that is in the vaginal microbiota up to 40% of pregnant women as about twice the frequency of the non-pregnant women[23,38].

In the present study, we observed 66.2% of women with candidiasis or mixed infection (Table I), where *Candida albicans* being the most frequently identified pathogen, comprising the 54.4% of the isolations (Table II). This is important since some evidence suggests that screening for the eradication of *Candida* during pregnancy can reduce the risk of preterm birth[21-23,38]. Also, it was reported that during delivery *Candida* in the vagina may be transmitted to the newborn, giving rise to congenital infections[38]. For all these reasons, an appropriate treatment of vaginal candidiasis during pregnancy could improve the clinical condition of mother and newborn[38]. Indeed recent studies report the effectiveness of treating *Candida* with clotrimazole in reducing preterm birth rates, probably due to the restoration of the vaginal microbiota and to the molecule's antifungal, and in part also antibacterial, properties[23,39,40].

This evidence could explain the efficacy observed for the therapy used in this study, i.e. the association of an antibiotic (metronidazole) with an antimycotic (clotrimazole). Metronidazole and clotrimazole are recommended for the treatment of bacterial and fungal vaginal infections in pregnancy because of their efficacy, safety and tolerability profile[24,25] and, furthermore, both drugs are not active against lactobacillary microbio-

Table IV. pPROM events observed compared to vaginal microbiota at week 32 of pregnancy.

	Vaginal infection and/or abnormal vaginal microbiota at week 32 of pregnancy n=600	
	Present n=218 (36.3%)	Absent n=382 (63.7%)
At term delivery, No. (%)	210 (96.3%)	382 (100%)
pPROM, No. (%)	8 (3.7%)*	0

*Significant difference ($p<0.001$).

ta[26,29]. This last feature is of remarkable importance because of the protective role of lactobacilli and vaginal microbiota in pregnancy[11,12,17,34].

The global results of this observational study allow us to make some considerations about the treatment of vaginal infections and pPROM events. The study showed the effectiveness of broad-spectrum topical treatment with metronidazole + clotrimazole used at week 28 of pregnancy, which eliminated the vaginal infection, not only the bacterial one, and restored the microbiota in 72.3% of the cases observed (Figure 1). The pPROM events frequency in the group of women who had a vaginal infection at week 28 and who were treated with a topical association of metronidazole + clotrimazole were no higher than that observed in the group of women without vaginal infection at week 28, i.e. 0.9% *versus* 1.8% (Table III). This would suggest that by reducing both bacterial and fungal infections and alterations of the vaginal microbiota, treatment with metronidazole + clotrimazole could lower the risk of pPROM in women with vaginal infection up to a level similar to that of women without infection.

Besides, it should be underlined that the use of a topical treatment rather than a systemic administration of metronidazole and clotrimazole is in agreement with the most recent international guidelines: topical therapy in pregnancy is recommended both for its equivalent efficacy compared to the oral route and for its lower incidence of adverse effects[24,25].

Finally, other interesting results were observed at week 32 of pregnancy. The prevalence of pPROM was significantly higher in the group of women who had an infection at week 32 of pregnancy compared to the group that had no vaginal infection at week 32 of pregnancy, 3.7% (8 cases out of 218) of pPROM events compared with no events (Table IV). This finding confirmed the association between vaginal infections at the third trimester of pregnancy and pPROM[11-15].

Conclusions

Waiting for further confirmatory trials, the present investigation provides preliminary information to improve our understanding both of the roles played by alterations in the vaginal microbiota in the third trimester of pregnancy and of how treating these alterations can help prevent pPROM.

Firstly, the study confirmed a high prevalence of vaginal infections among women at week 28 of pregnancy. Secondly, the study found a higher prevalence of pPROM in women with vaginal infections and/or abnormal vaginal microbiota at week 32 of pregnancy compared to women who had no vaginal infections and/or abnormal vaginal microbiota in the same week. Thus, it confirmed an association between vaginal infection in the third trimester of pregnancy and pPROM. Finally, in women with vaginal infections and/or abnormal vaginal microbiota at week 28 of pregnancy, this work showed that a 6-days topical treatment with metronidazole + clotrimazole was useful to eradicate vaginal infections and restore the vaginal microbiota, lowering the risk of pPROM in this group of women.

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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