RĪGAS STRADIŅA UNIVERSITĀTE

RĪGA STRADIŅŠ UNIVERSITY

Jana Žodžika

ABNORMAL VAGINAL MICROFLORA: RISK FACTORS, BED-SIDE DIAGNOSTIC METHODS IN PREGNANCY AND EFFICIENCY OF AN ALTERNATIVE NON-ANTIBACTERIAL TREATMENT MODALITY IN PREGNANT AND NON-PREGNANT WOMEN

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Research supervisor:

MD, PhD, Professor **Dace Rezeberga**, Rīga Stradiņš University

Research scientific consultant:

Professor **Gilbert Donders**, University of Antwerp, Belgium

Approved reviewers:

MD, PhD, Professor **Juta Kroiča**, Rīga Stradiņš University, Latvia

MD, PhD, Professor **Aija Žileviča**, University of Latvia

MD, PhD, Professor **Babill Stray Pedersen**, University of Oslo (Norway)

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MD, PhD, Professor **Ingmārs Mikažāns**

CONTENT

TEXT ABBREVIATIONS

AV	aerobic vaginitis
AVF	abnormal vaginal flora
BV	bacterial vaginosis
CI	confidence intervals
CFU	colony forming unit
CN	coagulase negative
E. coli	*Escherichia coli*
HIV	Human immunodeficiency virus
H_2O_2	hydrogen peroxide
hpf	high power field
ITT	intention to treat
KOH	potassium hydroxide
LBG	lactobacillar grade
Mixed AV-BV	mixed aerobic vaginitis and bacterial vaginosis
M. hominis	*Mycoplasma hominis*
OR	odds ratio
PASW	Predictive Analytics Software
PP	per protocol
S. aureus	*Staphylococcus aureus*
Str. agalactiae	*Streptococcus agalactiae*
spp	species
SPSS	Statistical Package for the Social Sciences
STI	sexually transmitted infections
U. urealyticum	*Ureaplasma urealyticum*
vs.	*versus*

1. INTRODUCTION

1.1. Study topicality

Normal vaginal microflora is an important women`s health factor, maintained by high numbers of different *Lactobacillus* species. Decreased amount of *Lactobacillus* is associated with alterations of vaginal flora *[Redondo-Lopez, et al., 1990*]. Abnormal vaginal microflora is linked to such adverse obstetric outcomes as early/late miscarriage, recurrent abortions, premature rupture of membranes, preterm birth and low birth weight in most cohort studies [*Ralph, et al., 1999; Leitich, et al., 2007; Donders, et al., 2009*].

Preterm birth continues to be one of the most challenging problems in perinatology. In Latvia the rate of preterm deliveries increased from 4.8% in 2008 to 5.8% in 2011 [*Statistical Yearbook of Health Care in Latvia, 2012*] and is similar to the rate in neighbouring countries such as Finland (5.5%) and Estonia (5.7%) [*Blencowe, et al., 2012*]. Infection related morbidity in the perinatal period was 39.2 per 1000 live births in 2011, compared to 28.8 in 2010 [*Statistical Yearbook of Health Care in Latvia, 2012*].

Since ascending vaginal infections are an important reason for miscarriage, preterm delivery and neonatal infections, multiple investigators have attempted to identify patients at risk for preterm labor, followed by treatment in a low risk of genital infections population, but the results have not met initial hopes [*Brocklehurst, et al., 2013*].

While antimicrobial agents provide cure of infections, the prevalence of urogenital pathogen drug resistance is increasing. Furthermore they can cause systemic and local side effects and disrupt protective vaginal microflora. Because of the potentially adverse effects on the newborn, many pregnant women are very cautious about taking antibiotics. During pregnancy, treatment

that restores normal vaginal flora and acidity without systemic effects would be preferable to any other [*Othman, et al., 2012*]. Acidification of the vagina with ascorbic acid (vitamin C) is one possible alternative option to reach this goal.

There are only few studies about the efficacy of vaginal vitamin C [*Petersen, et al., 2004; Petersen, et al., 2011*]. The results of these studies support the effective and safe use of vaginal vitamin C in a six day, mono-therapy regimen in the management of bacterial vaginosis, but there are no sufficient data about long term use of vaginal ascorbic acid in pregnancy and its influence on different abnormal microflora types.

Since there is growing evidence that treatment of abnormal vaginal microflora in early pregnancy can prevent some of the infection-related preterm births [*Ugwumadu, et al., 2004; Lamont, 2005*], it can be important to recognize (and if necessary, treat) pregnant women with flora abnormalities early in pregnancy, preferably at the first antenatal visit using "bed-side" diagnostic tests like vaginal pH measurement and wet mount microscopy, which are not extensively used in Latvia. Many gynecologists start antibiotics based solely on culture of vaginal micro-organisms, leading to overtreatment and antibacterial drug resistance.

Proper identification of risk groups, use of rapid, reliable "bed-side" diagnostic tests during the first antenatal visit in order to detect all types of abnormal vaginal flora, and subsequent early treatment with non-antibacterial drug like vaginal vitamin C could improve antenatal care and pregnancy outcome.

1.2. Study aim

The aim of the present study was to investigate abnormal vaginal microflora in pregnancy and the influence of vaginal application of ascorbic acid (vitamin C) on abnormal vaginal microflora in pregnant and non-pregnant women.

1.3. Study objectives

1. Primary objective – to investigate the impact of vaginal ascorbic acid (vitamin C) in a treatment plus maintenance regimen on abnormal vaginal environment, which is characterized by increased vaginal pH and abnormal microflora on wet mounts in pregnancy.
2. Secondary objectives:
    1) to assess risk factors associated with abnormal vaginal microflora (socio-demographic, medical, reproductive and sexual) in the first trimester of pregnancy;
    2) to identify symptoms and microscopy findings on wet mounts associated with increased vaginal pH in pregnancy;
    3) to evaluate the correlation of elevated vaginal pH and abnormal vaginal microflora on wet mounts with vaginal bacteriologic findings in the first trimester of pregnancy;
    4) to compare pregnancy outcome between the groups of women with normal and abnormal vaginal microflora (who were treated and not treated with vaginal vitamin C).

1.4. Study hypothesis statement

1. Vaginal ascorbic acid (vitamin C) in a treatment and maintenance regimen improves the abnormal vaginal environment in the population of pregnant and non-pregnant women.
2. Abnormal vaginal microflora is associated with a range of socio-demographic, medical, reproductive and sexual risk factors in the first trimester of pregnancy.
3. Increased vaginal pH is related to a number of symptoms and abnormal vaginal flora on wet mounts in the first trimester of pregnancy.
4. Elevated vaginal pH and abnormal vaginal microflora on wet mounts are related to growth of aerobic, facultative anaerobic bacteria and genital mycoplasmas on vaginal cultures in the first antenatal visit of pregnancy.
5. Application of vaginal vitamin C can decrease pregnancy complication rate related to abnormal vaginal microflora.

1.5. Novelty of the study

1. The impact of a non-antibacterial acidifying agent – vaginal ascorbic acid, in the new treatment and maintenance regimen on different types of abnormal vagina flora, focused on the population of pregnant and non-pregnant women is evaluated.
2. Abnormal vaginal microflora is defined in cases with both vaginal pH $\geq$ 4.5 and decreased numbers or absent *Lactobacillus* morphotypes on wet mounts.
3. Different types, including BV, AV, intermediate and mixed flora were assessed.
4. The study focuses on the application of simple and bed-side abnormal vaginal flora diagnostic tests.

5. Correlations between vaginal pH, wet mounts, cultures and different types of abnormal vaginal microflora are analysed.

1.6. The structure and volume of the doctoral thesis

The thesis for PhD degree is written in English. The promotion work consists of 8 parts: introduction, review of literature, materials and methods, results, discussion, conclusions, the list of references and the supplements. The volume of the promotion work comprises 150 pages, including 30 tables, 8 figures. The list of references consists of 252 titles. There are 7 publications, 2 oral presentations, 9 poster presentations on the topic of the promotion work thesis.

2. MATERIALS AND METHODS

This study was performed in four outpatient clinics in Riga: "ARS" (private clinic), Dzirciema Clinic (public clinic), "Quartus" (private clinic), Riga Maternity Hospital (municipal owned public hospital). Patients were asked to participate, if they were at least 18 years old, 6 to 14 weeks pregnant, agreed with the study and signed the informed consent. Pregnant woman with vaginal pH $\geq$ 4.5 were enrolled in a prospective intervention trial. Following enrolment of a woman with pH $\geq$ 4.5, the next two pregnant seen at the clinic with vaginal pH < 4.5 were included in the study as controls. Enrolment of women was planned to continue until the required number vitamin C study population had been included.

Assuming Type I error to be 5% and with a standard difference 0.65 of pH values between treatment and control group, and in order to achieve 80% study power, it was calculated that 140 participants with abnormal microflora on microscopy had to be recruited from the cohort with increased pH.

From March 2010 until May 2012 150 pregnant women with vaginal pH $\geq$ 4.5 and 300 with vaginal pH < 4.5 at the first prenatal visit were enrolled. Out of 150 pregnant women cohort with elevated vaginal pH, 85 were eligible for interventional ascorbic acid study. To reach the vaginal vitamin C study power additional 55 non-pregnant women with the same inclusion criteria were enrolled from September 2011 till May 2012.

Summary of inclusion criteria:

- Vaginal pH/abnormal vaginal microflora and related factors/pregnancy outcome study – 150 pregnant women with vaginal pH $\geq$ 4.5 and in those with normal vaginal acidity (n=300) were included at the first prenatal visit.

- Vaginal culture studies –the first 50 pregnant with vaginal pH ≥4.5 were included as study cases and 50 pregnant women with vaginal pH less than 4.5 were used as a control.
- Vaginal ascorbic acid study – out of 150 pregnant women cohort with elevated vaginal pH, 85 were eligible for interventional ascorbinic acid study (had AVF on native microscopy, were asymptomatic, had no history of miscarriage/preterm birth and agreed to participate in the interventional part of the study). To reach the vitamin C study power additional 55 non-pregnant women with the same inclusion criteria were included. Overall vitamin C study population consisted of 70 women in the interventional group (42 pregnant and 28 non-pregnant) and 70 women in the control group (43 pregnant and 27 non-pregnant). Principles of randomization are described below.

Summary of exclusion criteria: age less than 18 years, less than six and more than 14 weeks of gestation, multiple pregnancy on the first trimester ultrasound scan, systemic diseases, like diabetes, kidney failure, hypertension requiring medication, all women were tested for *Chlamydia*, *gonorrhoea*, syphilis and Human Immunodeficiency virus (HIV) infections according to the basic antenatal care program and were excluded if positive for any of them, did not agree to participate in the study and sign informed consent, additional exclusion criteria for ascorbic acid study – currently/during the previous 2 weeks treated with systemic/local antibiotics, antimycotics and/or *Lactobacillus* preparations, symptomatic vaginal infections, history of late miscarriage and preterm deliveries, in addition for non-pregnant women – postmenopausal status.

Factors related to abnormal vaginal pH/microflora and vaginal bacteriological finding studies were cross sectional, observational. The vaginal vitamin C study was an interventional, randomized study. Pregnancy outcomes were assessed in the prospective cohort.

The study was approved by the Ethics Committee of Riga Stradins University. All participants were informed, asked to sign an informed consent and had rights to withdraw from the study at any time for any reasons and agree only to non-interventional part of the study. Pregnant women continued to receive their antenatal care according to the Regulation of the Cabinet of Ministers Nr 611 "Organization of delivery services." The study was done according to principles deriving from the Helsinki declaration.

There were three principle visits in the study: inclusion, follow-up and post-delivery. There was completed a questionnaire, performed physical examination and collected specimens for microscopic and bacteriologic examinations at the first inclusion visit. Interviews, physical exams and specimen collection were done by the obstetricians/gynecologists. Interviews consisted of questions about demographic, social, medical, reproductive, sexual, recent medication use, genital infection history and current genital tract complaints. During gynecological examination physical findings were documented, two cotton-tipped swabs for cultures and three vaginal smears with cytobrush were taken from the upper vaginal wall: two for wet mount and one for pH measurement, whiff test with 10% potassium hydroxide (KOH). Vaginal pH was measured by pressing a *Machery Nagel* pH strips with a pH range of 3.1–7 into the fluid on the glass slide, allowing it to soak for 10 seconds. These strips were chosen because of their accuracy and ease of use [*Donders (a), et al., 2007*]. Vaginal pH $\geq$ 4.5 was considered abnormal (elevated) [*Amsel, et al., 1983*]. Then a droplet of 10% KOH was added to evaluate "fishy" order of discharges [*Amsel, et al., 1983*]. Specimens for wet mounts were spread on the glass slide, air-dried and then transported to the investigator (Jana Zodzika) for later microscopy after rehydration of the smear with a droplet of saline [*Larsson, et al., 1990*]. A Leica DM1000 microscope (Warburg, Germany) was used, with phase contrast at 400 times magnification.

Results of interviews and physical exams were blinded to the person who performed microscopic examination.

Systematic microscopic examination of wet mounts according to *Donders'* modification of *Schröders'* classification [*Donders, 1999*] was done according the method used at Femicare Research Centre, Belgium.

According to *Donders'* modification of *Schröders'* classification [Donders, 1999]:

- Lactobacillar grade (LBG) I consists of predominant presence of *Lactobacillus* morphotypes, with very few coccoid bacteria presented,
- LBG IIa (intermediate) of lactobacilli outnumbering other microorganisms,
- LBG IIb (intermediate) of other microorganisms outnumbering lactobacillary morphotypes,
- LBG III (completely disturbed flora) has no lactobacilli present. LBG III is further divided in three subgroups: bacterial vaginosis (BV), aerobic vaginitis (AV) and a mixed aerobic vaginitis and bacterial vaginosis (mixed AV-BV) flora.

A predominant granular flora with uncountable bacteria throughout the slide and more than 20% of epithelial cells covered with bacteria (clue cells) were defined as full blown BV, while mixed areas with streaks of BV-like flora or sporadic clue cells combined with other types of microflora were classified as partial BV [*Donders (b), et al, 2007, Donders, et al, 2009*]. AV was diagnosed if short bacilli or cocci, leucocytes and/or parabasal cells were found. The severity of aerobic vaginitis was represented by a composite AV score: slight, moderate and severe (taking into account LBG, number/appearance of leucocytes, presence of parabasal cells and background flora) [*Donders, et al., 2002*].

Patterns with considerably decreased or absent *Lactobacillus* morphotypes (LBG IIb and LBG III) were considered as an abnormal vaginal flora (AVF) [*Donders, 1999*].

From the study population, vaginal cultures were done in the first 50 pregnant women with vaginal pH $\geq$ 4.5 (study group) and in the first 50 participants with vaginal pH < 4.5 (control group). Specimens from the upper vaginal wall were taken with wool cotton-tipped swabs and were immediately placed in universal *Amies* medium and transported within 24 hours to the laboratory of the Infectology Centre of Latvia. Then the samples were inoculated to the following media: Shaedler blood agar, MacConkey agar, egg-salt agar, chocolate and Chromagar Candida agar for the investigation of microorganisms such as *Streptococcus pyogenes (Str. pyogenes), Str. agalactiae*, *Viridans* group streptococci, enterococci, *Staphylococcus aureus (S. aureus), Candida species (spp.),* pathogenic enteric bacteria, *Acinetobacter spp., Haemophilus spp., Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia.* To distinguish between *Str. pyogenes, Str. agalactiae, Viridans* group streptococci, and enterococci, several specific tests were done. Streptococci were cultivated on blood agar to see their degree of hemolysis. If there was β – hemolysis, then susceptibility to bacitracin was tested, if it was positive then it was diagnosed as *Str. pyogenes*, if it was resistant, then CAMP (Christie-Atkins, Munch-Petersen) test was performed and if that was positive then it was diagnosed as *Str. agalactiae*. If on blood agar there was α – hemolysis then a further test for optochin susceptibility was done; if it was negative, then the culture was inoculated on Bile-esculin media [*Mahon, et al., 2000*]. To distinguish between *Haemophilus* species, testing for X and V factor requirements was performed using impregnated strips [*Mahon, et al., 2000*].

Urea-Arginine broth was used for the investigation of *Ureaplasma urealyticum (U. urealyticum)* and *Mycoplasma hominis (M. hominis)* [*Mahon, et al., 2000*]. More than 10^5 CFU/ml (colony forming unit) of *U. urealyticum*

and *M. hominis* was considered elevated concentration and analysed separately [*Rosenstein, et al., 1996*].

In the ascorbic acid study asymptomatic, low risk pregnant and asymptomatic, non-pregnant, premenopausal women with vaginal pH ≥ 4.5 and AVF on wet mounts (LBG IIb and III) were randomized to the **intervention group** (70 participants received 250 mg vitamin C tablets, *Feminella Vagi C*, provided by Polichem S.A., Switzerland, for vaginal insertion at bedtime, for six days, followed one tablet a week, for 12 weeks) and the **control group** (70 participants had no treatment). Randomization was done using statistical package for the social sciences (SPSS) random number generator. Allocation principles were concealed to patients, caregivers and to the person, who performed wet mounts. The intervention group women had additional randomization visit with their gynecologist, when they received the package with study medication, instruction and diary. Intervention group participants made records in the diaries about the use of tablets and complaints.

According to the guidelines of Latvian Association of Gynecologists and Obstetricians [*Latvian Association of Gynecologists and Obstetricians*], pregnant participants with BV and complaints/history of miscarriage, preterm deliveries were treated with clindamycin 2% vaginal cream (*Dalacin*, provided by *Pfizer*) applications for seven days.

Visit 2 (follow-up visit) was at 4 months after randomization (28–32 weeks of gestation), corresponding to 2 to 3 weeks after the last vitamin C tablet insertion for study group. There was questionnaire fulfilled with interviews about sexual, recent medication use, current genital tract complaints, gynecological examination physical findings and vaginal smears with cytobrush were taken from the upper vaginal wall for pH measurement, wet mount and whiff test with 10% KOH, physical, microscopic results at the follow-up visit.

Visit 3 was 6–8 weeks after delivery. Questionnaires about pregnancy outcomes were completed during that visit.

There were different outcomes assessed in the study:

- Demographic, social, medical, reproductive, sexual, recent medication use, genital infection history factors associated with AVF.
- Current genital tract complaints, gynecological and wet mounts examination results related to elevated vaginal pH.
- Vaginal culture results were compared between abnormal vaginal pH/microflora and selected normal vaginal acidity/microflora groups.
- Eficacy endpoint, defined as a composite finding of vaginal pH < 4.5 and normal microflora (LBG I or IIa) on wet mount, as well as mean vaginal pH and microflora patterns were evaluated in women using vitamin C and controls.
- Pregnancy outcomes (rate of miscarriage/preterm births, term deliveries, mean newborns` weight and Apgar score levels, newborn admission to Intensive Care unit and transfer to Children`s hospital) compared between: normal and total AVF groups; normal and non-intervention AVF group; intervention AVF and non-intervention AVF groups; vitamin C and control groups; vitamin C and all non-intervention AVF group.

Statistical analysis was performed using SPSS version 18.0 (predictive analytics software – PASW). Distribution of socioeconomic factors was obtained from two-way and multi-way frequency tables. The prevalence rates for vaginal microflora and adverse reactions to treatment were also obtained by two-way frequency tables. Statistical significance of the differences in prevalence rates and distribution of risk factors between groups was assessed using chi-square or Fisher's exact test. Statistical significance of the differences in mean values between groups was tested using independent sample t test. The level of statistical significance was chosen at 5% ($p<0.05$). Relations between

pathological vaginal microflora and various risk factors were assessed using univariate and multivariate logistic regression. Variables that showed a significant association at the level 10% ($p<0.1$) in univariate analysis were included in the multiple logistic regression analysis. Risk pathological vaginal microflora depending on presence of various risk factors was also calculated as odds ratios. Vaginal pH sensitivity and specificity were calculated following the appropriate formula [*Riegelman, 2000*]. Ascorbic acid study outcomes were evaluated considering both the *intention to treat population* (*ITT:* all randomized patients) and *per protocol population* (*PP: patients*, who completed the study without major protocol deviations). Patients with no results for any reasons were considered as failures.

3. RESULTS

3.1. Factors associated with abnormal vaginal microflora

AVF group in this study was defined, if participants had combination of vaginal pH ≥4.5 and LBG IIb and LBG III on wet mounts.

The 135 of 150 women with increased vaginal pH had LBG IIb-III and were compared to 256 of 300 participants with normal pH, who had LBG I-IIa on wet mount.

Pregnant women with AVF compared to those with normal vaginal microflora were more often younger than 25 years, less educated, more often unmarried and single or not living with a partner, smoked before and during pregnancy and had BV, but rarely *U. urealyticum*, candidas at least once during the year before pregnancy ($p<0.01$), they also had a trend to be more unemployed/housewives, had ≥ 2 sexual partners during last year and intercourse 48 hours before sampling ($p<0.1$).

AVF was not related to miscarriage/preterm delivery history, number of lifetime sexual partners, frequency of intercourses during last month, a new partner.

Univariate analysis of significant abnormal vaginal pH factors did not show age < 25 years, ≥ 2 sexual partners during last year and intercourse 48 hours before sampling to be significant risk factor for AVF, conversely other factors still increased risks.

There was a trend for more smoking before and during current pregnancy associated with lower education.

Most of participants in all age groups had a higher education, but women with only primary education were more often younger than the age of 25 years ($p=0.05$).

Multivariate logistic regression analysis showed that the highest risk of AVF was associated independently with low level of education, smoking before pregnancy and a history of BV a year before pregnancy, Table 3.1.

Table 3.1.

Multivariate analysis of significant AVF risk factors

Characteristic	Odds ratio (OR) and 95% confidence interval (CI)	P value
Age < 25 years (versus (vs.) ≥ 25 years)	0.9 (0.5–1.8)	0.991
Primary (≤ 9 classes) education (vs. higher)	3.2 (1.1–9.4)	**0.033**
Secondary (12 classes) education (vs. higher)	2.3 (1.4–3.8)	**0.001**
Not married, living with partner (vs. married)	1.4 (0.9–2.2)	0.193
Single/not living with partner (vs. married)	8.1 (0.8–82.1)	0.076
Housewife (vs. employed)	1.8 (0.9–3.7)	0.122
Unemployed (vs. employed)	0.9 (0.4–2.4)	0.935
≥ 2 sexual partners during last year (vs. <2 partners)	1.6 (0.6–4.2)	0.355
Smoking before pregnancy (vs. not smoking)	1.7 (1.0–3.0)	**0.046**
Smoking during pregnancy (vs. not smoking)	1.6 (0.7–3.8)	0.297
BV in year before pregnancy (vs. negative history)	1.8 (0.8–3.9)	**0.044**
U. urealyticum in year before pregnancy (vs. negative history)	0.2 (0.1–0.8)	**0.027**

Some interesting differences of risk factors between specific types of abnormal vaginal flora, such as BV and AV microflora, were noted. Women with a history of BV before pregnancy were more often less educated, smokers and unmarried than women without previous BV, with similar, but less strong associations for AV. Similarly, frequent intercourse and recent intercourse < 48 hours were more frequent in the BV versus the normal group, but now the relation was even stronger in the AV group. Of note, compared to normal flora women, *Candida* infection was found less often in BV cases, while its rate was similar in the AV group, while *U. urealyticum* was more frequently found in normal women and not or rarely in BV or AV women.

3.2. Symptoms and microscopical findings related to increased vaginal pH

The 150 women with increased vaginal pH were compared to 300 participants with normal pH. Most complaints were similar between the two pH groups, but 37% of women with increased pH complained of abundant vaginal discharge, compared with 26% in controls (p=0.023) and 11% of participants with elevated vaginal pH had experienced a bad smell (only 3% in normal vaginal pH group, p=0.001). Upon examination, women with normal vaginal pH more often had normal (74% vs. 24%, p<0.001), less often a thin, homogeneous (5% vs. 48%, p<0.001) and yellow discharge (4% vs. 9%, p=0.044). Positive whiff test was associated with elevated pH (p<0.001), but of all women in the abnormal pH group, only 55% had positive amine test.

There was strong correlation between elevated vaginal pH and AVF on wet mounts. AVF (LBG IIb-III) patterns on wet mounts were more often found in women with elevated vaginal pH (135/150, 90%) than in participants with vaginal pH < 4.5 (44/300, 15%), p<0.001.

The participants with vaginal pH ≥ 4.5 were more likely to have BV (36 women, 23%), AV (22 women, 14%), mixed AV-BV flora (52 women, 34%) and LBG IIb (25 women, 16%). Of 300 participants with pH < 4.5, 44 had abnormal flora patterns (29 had LBG IIb, 8 AV, 6 MF and 1had BV), Table 3.2.

Table 3.2.

Comparison of clinical and microscopic examinations results between pH groups

Characteristic	Total (n=450) N (%)	pH < 4.5 (n=300) N (%)	pH≥4.5 (n=150) N (%)	P value
Complaints:				
increased discharge	134 (30)	78 (26)	56 (37)	**0.023**
burning	15 (3)	12 (4)	3 (2)	0.284
itching	31 (7)	22 (7)	9 (6)	0.546
bad smell	26 (6)	9 (3)	17 (11)	**0.001**
bloody discharge	6 (1)	4 (1)	2 (1)	1.000
low abdominal pain	54 (12)	36 (12)	18 (12)	0.913
others	4 (0.9)	3 (1)	1 (0.7)	1.000
Type of discharge:				
normal	257 (57)	221 (74)	36 (24)	< **0.001**
thin, homogeneous	87 (19)	15 (5)	72 (48)	< **0.001**
“cheese” like	54 (12)	38 (13)	16 (11)	0.515
bloody	6 (1)	4 (1)	2 (1)	1.000
yellow	25 (5)	12 (4)	13 (9)	**0.044**
Positive whiff test	87 (19)	4 (1)	83 (55)	< **0.001**
Clue cells	90 (20)	6 (2)	84 (56)	< **0.001**

Continuation of the Table 3.2.

Characteristic	Total (n=450) N (%)	pH < 4.5 (n=300) N (%)	pH≥4.5 (n=150) N (%)	P value
Lactobacillary grades:				**< 0.001**
I	177 (40)	169 (57)	8 (7)	
IIa	94 (21)	87 (30)	7 (5)	
IIb	54 (11)	29 (8)	25 (16)	
III BV	37 (8)	1 (0.3)	36 (23)	
III AV	30 (7)	8 (3)	22 (14)	
III MF	58 (13)	6 (2)	52 (34)	
Normal microflora patterns (LBG I, Ia)	271 (60)	256 (85)	15 (10)	**< 0.001**
Abnormal microflora patterns (LBG IIb, LBG III)	179 (40)	44 (15)	135 (90)	
BV type**:**				**< 0.001**
Partial	57 (13)	4 (1)	53 (35)	
Full	37 (8)	1 (0.3)	36 (23)	
AV score:				**< 0.001**
No/slight AV	418 (92)	293 (98)	125 (83)	
Moderate	25 (6)	4 (1)	21 (14)	
Severe	7 (2)	3 (1)	4 (3)	
Lactobacillary morphology:				
Normal types	263 (58)	237 (79)	26 (17)	**< 0.001**
Leptosomic	69 (15)	60 (20)	9 (6)	**< 0.001**
Short, course	126 (28)	100 (34)	26 (17)	**< 0.001**
Absent	123 (27)	16 (5)	107 (70)	**< 0.001**
Sperm cells	34 (8)	15 (5)	19 (12)	**0.005**

Continuation of the Table 3.2.

Number of leucocytes:				**0.001**
<10 per high power field (hpf)	177 (39)	108 (36)	69 (46)	
>10 per hpf, <10 per epithelial cell	201 (45)	152 (51)	49 (33)	
>10 per epithelial cell	72 (16)	40 (13)	32 (21)	
Candida:				0.866
none	379 (84)	254 (85)	125 (83)	
spores	33 (7)	23 (8)	10 (7)	
hyphae	8 (2)	5 (1)	3 (2)	
both	30 (7)	18 (6)	12 (8)	

Most cases of full BV were associated with increased pH. pH test sensitivity for full BV was 97%, but less for severe aerobic vaginitis – 60%. Also for partial BV, pH test sensitivity (92%) was better than for moderate aerobic vaginitis (70%). Specificity of pH for all BV cases was 83%, and for total AV cases was 70%.

All lactobacillary morphotypes were found more often in the pH $\leq$ 4.5 group ($p<0.001$).

Sperm cells were more often detected in the vaginal smears of women with abnormal than in those with normal pH (12% versus 5%, $p=0.005$), but most of them (68% of participants with sperm cells) had abnormal vaginal microflora on microscopy ($p< 0.001$). From the smears with sperm, two women claimed no intercourse during the previous two days.

Elevated vaginal pH was associated with increased numbers of leucocytes (> 10 per epithelial cell): 21% versus 13%, $p=0.001$.

3.3. Vaginal bacteriological study

96% of women with increased pH and 86% of women with normal vaginal pH showed positive cultures (p value non-significant). In total, 19 different microorganisms were recovered from the vagina. Of 100 participants, the most common microorganisms isolated were coagulase negative (CN) *Staphylococcus* in 56, *U. urealyticum* in 34, *Escherichia coli (E. coli)* in 18, *Candida* species in 16 and *M. hominis* in 15 cases.

Increased vaginal pH was significantly associated with positive *M. hominis* ($p<0.001$), *U. urealyticum* ($p=0.017$) *E. coli* ($p=0.018$) and mixed group consisting of Gram positive cocci/Gram negative bacilli ($p=0.015$) cultures, but normal vaginal pH with combined Gram positive cocci group ($p=0.015$). Correlations of abnormal vaginal microflora microscopic patterns with cultures were similar to those with elevated pH.

LBG I was found in 35, LBG IIa in 14, LBG IIb in 17 and LBG III in 34 participants. Of the latter, eight had BV, five AV and 21 mixed BV-AV flora. 43 of 50 participants with elevated vaginal pH and six of 50 pregnant women with normal vaginal acidity had AVF on microscopy ($p<0.001$). *U. urealyticum* and *M. hominis* were found more often in the BV and mixed BV-AV flora ($p<0.05$**)** than in other flora types, and all cases with high numbers of *M. hominis* were encountered in women with LBG III ($p=0.001$). *E. coli* was more often encountered in the AVF group ($p=0.008$), with a trend to be more often cultured in cases with LBG IIb, AV and mixed BV-AV flora ($p=0.072$). Gram positive cocci (including *Str. agalactiae*, $p=0.032$, and *Viridans* group streptococci, $p=0.013$) were more often found in association with normal vaginal microflora patterns.

Combining both vaginal environment parameters – vaginal pH and microflora type on microscopy, 43 participants had a normal pattern (acidic

vaginal pH and LBG I-IIa) and 44 pregnant women had an abnormal pattern (elevated vaginal pH and LBG IIb-III). In the univariate analysis *U. urealyticum* (odds ratio (OR) 3.1, 95% CI 1.2–8.2, p=0.019), *M. hominis* (OR 18, 95% CI 2.2–145.2, p<0.001) and *E. coli* (OR 7.5, 95% CI 1.64–37.6, p=0.008,) were significantly more often found in the abnormal group, OR for high numbers of *M. hominis* were not possible to calculate as all cases were cultured in the abnormal microflora group. The univariate analysis did not find significant association between *Str. agalactiae* and *Viridans* group streptococci with normal microflora and acidity group. Comparison of culture results in the groups with no, mild or heavy leucocytosis, demonstrated, that *E. coli* was significantly associated with increased numbers of leucocytes on native microscopy (p=0.03).Multivariate logistic regression analysis showed the highest risk of abnormal vaginal flora associated with *M. hominis* and *E. coli*, Table 3.3.

Table 3.3.

Association of different bacteria with the abnormal vaginal microflora in the multivariate logistic regression analysis

Cultured microorganisms	OR	Standard deviation error	P value	95%CI
U. urealyticum	2.6	1.7	0.155	0.7–9.5
U. urealyticum (high numbers)	1.2	1.2	0.802	0.2–7.9
M. hominis	14.4	15.8	**0.015**	1.6–124.4
E. coli	8.5	7.3	**0.013**	1.6–45.9

3.4. Vaginal ascorbic acid (vitamin C) study

Eficacy endpoints were analysed in the pregnant, non-pregnant and total study population.

3.4.1. Results in pregnant women population

There were 42 pregnant women in the vitamin C and 43 in the control group. Correspondingly 36 and 37 of in each study arm completed the full protocol and could be analysed as per protocol. Overview of study population is described below in the section 4.2. The baseline characteristics (age, weight, height, education, marital status, sexual and general history, mean vaginal pH, results of native microscopy) of all cases of both groups were comparable.

In the ITT population 29 of 42 (70.7%) of the ascorbic acid and 12 of 43 (29.3%) participants in the control group demonstrated normalization of the abnormal vaginal flora (difference 41.4%, 95% CI 21.8–60.5, $p<0.001$). In the PP population the flora normalized in 25 of 35 (71.4%) of study patients versus 10 of 37 (28.6%) of controls (difference 44.4%, 95% CI 23.7–65.1, $p<0.001$).

Mean vaginal pH on follow-up visit decreased in both treatment and control groups: from 5.05±0.37 to 4.3±0.4 in the intervention group ($p<0.001$) and from 5.02±0.30 to 4.6±0.5 in the control group ($p<0.001$), but the decrease was significantly more marked in the ascorbic acid group, than in the controls ($p<0.0003$). On follow-up visit prevalence of normal vaginal pH and microflora on wet mount was higher in ascorbic acid group ($p<0,001$).

Vaginal ascorbic acid did not reduce the prevalence of pure LBG III BV flora (6/9 normalization in the vitamin C group vs. 6/11 in the control group ($p=0.622$), nor on pure LBG III AV flora (6/10 in vitamin C group vs. 1/4 in controls, $p=0.25$). However, in women with mixed LBG III AV-BV flora, normalization of microflora was more evident in the vitamin C group (11/16)

than in the control group (4/18), p=0.005. Analysing the impact of vitamin C on any kind of AV (combined group of LBG IIb associated with AV, pure LBG III AV and mixed AV-BV) or any kind of BV flora (including LBG IIb associated with BV, LBG III BV and mixed AV-BV), the result was significantly better in the intervention group, Table 3.4.

Table 3.4.

Prevalence of normal vaginal flora in different abnormal vaginal flora groups on follow-up visit

Microflora types on screening visit (number of cases)	Prevalence of LBG I-IIa on follow-up visit			
	Total (n)	Vitamin C group (n = 42)	Control group (n = 43)	P values
PureBV (20)	12	6	6	0.622
PureAV (14)	7	6	1	0.315
Any AV (59)	28	21	7	**0.001**
Mixed AV-BV (34)	15	11	4	**0.005**

3.4.2. Results in total study population and non-pregnant subgroup

There were 28 non-pregnant women in the vitamin C and 27 in the control group. 58 of 70 participants in each study arm completed the full protocol and could be analysed as per protocol. In the intervention group 7 (10%) women prematurely withdrew from the study because of side effects (irritations), 1 had a spontaneous abortion, 3 were lost to follow up and 1 had deviation from protocol because of systemic antibiotic use (due to urinary infection) during the study period. In the control group 2 had spontaneous abortions, 1 went into preterm labor at 29 gestational week, 4 were lost to

follow up and 5 had deviations from study protocol (4 used systemic antibacterial medications due to urinary and respiratory infections and had vaginal antibacterial treatment because of symptoms and 1 used per oral probiotics).

In the ITT population, 36 of 70 (51.4%) of the ascorbic acid and 17 of 70 (24.3%) participants of the control group demonstrated normalization of the abnormal vaginal flora (difference 27.1%, 95% CI 11.7–42.6, $p<0.05$). In the PP population the flora normalized in 31 of 58 (53.5%) of study patients versus 13 of 58 (22.4%) of controls (difference 31%, 95% CI 14.3–47.8, $p<0.05$). Results of ITT population non-pregnant subgroup analysis did not show improvement of the microflora (7 with normal flora of 28 in vitamin C group versus 5 of 27 participants in control group, p value nonsignificant).

On follow-up visit prevalence of normal vaginal pH and microflora on wet mount was higher in ascorbic acid group ($p<0.001$), but not in the subgroup of non-pregnant participants (p value nonsignificant).

Like in the pregnant women subgroup total study population results showed that vaginal ascorbic acid did not reduce the prevalence of pure BV flora (8/36 normalization in the vitamin C group versus 7/36 in the control group ($p=0.5$), pure AV flora (9/26 in vitamin C group vs. 3/26 in controls, $p=0.25$), but in women with mixed AV-BV flora and any kind of AV or BV flora the normalization was more evident in the vitamin C group (p values respectively 0.017, 0.001 and 0.031).

Most common side effects in the vitamin C group were itching and irritation. Itching cause complaints in 11 cases (16%) after treatment and in 13 cases (19%) during the maintenance regimen. Irritation occurred in 16 patients (23%) after the treatment regimen and 7 patients prematurely withdrew from the study due to this reason. Irritation persisted in 6 cases (9%) during the maintenance phase of vitamin C use. There were no significant associations

found between complains and vaginal pH, regimen of treatment, microflora type, number of leucocytes or presence of *Candida* on wet mounts.

3.5. Results of the vaginal clindamycin group

17 pregnant women received treatment with vaginal clindamycin cream. Mean vaginal pH on follow-up visit decreased from 5.02±0.05 to 4.21±0.14 and LBG I-IIa was restored in 80% ($p<0.001$). These results were comparable to vitamin C data (p non-significant).

3.6. Pregnancy outcome

Pregnancy outcome data were available for 102 of 135 women with pH≥4.5/LBG IIb-III and for 217 of 256 with normal and vaginal acidity and microflora. The final data were missing, if participants did not arrive on visit 6–8 weeks after delivery. Drop outs baseline characteristics were similar to those who completed the study.

There was no difference between both study groups regarding pregnancy complications, use of antibiotics (systemic or vaginal) during pregnancy, type or mode of deliveries. Most pregnant participants (90%) had term deliveries.

Analysis of pregnancy outcomes between normal and AVF (including cases with as well as without any intervention), did not show major differences between normal and AVF groups. However, both first and fifth minute Apgar score levels were higher in newborns whose mothers` had both normal vaginal microflora ($p=0.027$ and $p=0.023$).

Analysis of pregnancy outcome between normal and non-intervention AVF group participants (cases without antibacterial or vitamin C treatment) showed higher rates of miscarriage, preterm birth and Apgar score in the non-treated abnormal microflora group, Table 3.5.

Table 3.5.

Pregnancy outcome analysis in the non-intervention population

Characteristic	Total (n=249) N (%)	Vaginal pH <4.5 and LBG I-IIa (n=191) N (%)	Vaginal pH ≥4.5 and LBG IIb-III (n=58) N (%)	P value
Pregnancy outcomes:				**0.019**
early miscarriage	18 (7)	10 (5)	8 (14)	
late miscarriage	4 (2)	4 (2)	0	
preterm delivery at 22–26 weeks of gestation	0	0	0	
preterm delivery at 27–36 weeks of gestation	5 (2)	2 (1)	3 (5)	
term delivery	222 (89)	175 (92)	47 (81)	
Newborn weight		3647±479	3515±553	0.099
Apgar score				
1. minute		7.7±0.8	7.2±1.4	**0.001**
5. minute		8.8±0.8	8.3±1.7	**0.003**

Newborns of pregnant women with AVF, who had received antibacterial treatment (Dalacin, other antibiotics) or vitamin C, had better fifth minute Apgar score (p=0,003), compared to non-intervention participants, but pregnancy outcomes were not significantly different.

Although mean newborn weight was not significantly different between normal and all types abnormal vaginal microflora groups (p=0.1), the sub-group analysis showed lower birth weights in the any AV (p=0.045) and mixed BV-AV groups (p=0.02), Table 3.6.

Table 3.6.

Mean newborn weights in the different vaginal microflora groups

Mean newborn weight in the normal vaginal microflora group	Mean newborn weights in the different abnormal vaginal microflora groups	P value
LBG I-IIa (n=212): 3576±673 g	BV only (n=30): 3571±490 g	0.917
	AV only (n=25): 3554±473 g	0.477
	Any BV (n=76): 3513±450 g	0.091
	Any AV (n=75): 3497±495 g	**0.045**
	Mixed BV-AV (n=44): 3430±477 g	**0.018**

We also analysed the influence of different suspected risk factors (smoking during pregnancy, AVF in the first trimester, hypertension, anemia, fetal abnormalities, abnormal ultrasound scan findings) on newborns` weight in the study. As expected – current smoking had the most negative effect on birth weight in the multivariate analysis (p=0.004).

Bacteriological findings of *M. hominis*, *U. urealyticum* or different types of aerobic bacteria at the first antenatal visit were not associated with poor pregnancy outcome when compared to culture negative pregnant women.

We didn't see statistical difference in either pregnancy outcomes, or newborn mean birth weight and the first minute Apgar score levels in the vitamin C and control group in ITT population. Still, mean fifth minute Apgar score was significantly better in the intervention group (p=0.031). Also, even though numbers were too small for obtaining meaningful statistical differences, there were 3 preterm deliveries in the control group (8%) and none in the vitamin C group.

There was statistical difference found in pregnancy outcomes comparing vitamin C with non-intervention AVF group participants (combined group of

controls and those participants who rejected any treatment): in non – treated population more early miscarriage and preterm deliveries at 27-36 weeks of gestation were observed (p=0.037), Table 3.7.

Table 3.7.

Comparison of pregnancy outcomes in vitamin C and all non-intervention AVF group

Characteristic	Vitamin C group (n=41) N(%)	Non-intervention population (n=58) N (%)	P values
Pregnancy outcomes:			**0.037**
early miscarriage	1 (3)	8 (14)	
late miscarriage	0	0	
preterm delivery at 22–26 weeks of gestation	0	0	
preterm delivery at 27–36 weeks of gestation	0	3 (5)	
term delivery	40 (98)	47 (81)	
Mean newborn weight	3528±422	3515±553	0.906
Apgar score			
1. minute	7.6±0.2	7.2±1.4	0.187
5. minute	8.8±0.1	8.3±1.7	**0.033**

In PP population, all vitamin C had term deliveries, two of controls delivered prematurely (p not significant), but Apgar score levels at the first and fifth minutes of age were better in the vitamin C group (p= 0.032 and p=0.041).

We didn't see statistical difference in either pregnancy outcomes, or newborn mean birth weight and the first minute Apgar score levels compared dalacin and vitamin C or control group.

4. DISCUSSION

4.1. Risk factors of abnormal vaginal microflora in the first trimester of pregnancy

Since AVF is linked to many adverse obstetric outcomes (early/late miscarriage, recurrent abortions, premature rupture of membranes, preterm birth, low birth weight, neonatal infections) in most cohort studies [*Hay, et al., 1994; Ralph, et al., 1999; Leitich, et al., 2007; Donders, et al., 2009*], it is important to recognize who are at risk for AVF as early in pregnancy as possible. This is especially might be important for women who have a history of adverse obstetric outcomes making recommendations for appropriate further management.

In this study AVF was defined in cases with both vaginal pH $\geq$ 4.5 and LBG IIb/LBG III on wet mounts. We believed, that current approach could better select the patients with potentially infections related risk for adverse pregnancy outcomes, because elevated vaginal pH reflected alkaline vaginal environment associated with lower numbers of lactobacilli [*Rönnqvist, et al., 2006*] and women with different types of AVF (not only women with BV, but also AV and mixed/intermediate AVF) had been included. Authors of recent Cochrane Database Systematic review of antibiotics for treating BV in pregnancy had concluded, that it did not reduce the risk of preterm birth before 37 weeks, but when screening criteria were broadened to include women with AVF (intermediate and BV), there was 47% reduction in preterm birth [*Brocklehurst, et al., 2013*].

In the present study, we found low educational level, smoking before pregnancy and history of BV in the year before pregnancy to be the risk factors most strongly associated with abnormal vaginal pH/microflora in the first

trimester of pregnancy, but history of *U. urealyticum* in the year before pregnancy was associated with normal vaginal flora. Less significantly AVF were related to age < 25 years, current smoking, being unmarried, not employed, having ≥2 sexual partners in the year before pregnancy and intercourse 48 hours before sampling, Table 3.2. Our findings are similar to those of a large French population-based study, where low education level, young age and tobacco use during pregnancy were recognized as BV risk factors [*Desseauve, et al., 2012*]. Education promotes increasing awareness, responsibility, knowledge of self-care, healthy lifestyles and behaviours [*Koch, et al., 2007*]. Low education level usually is associated with low incomes, social class and is related to risks such as unhealthy habits (smoking, alcohol, and drug abuse), violence, weak families and others.

Following the publication of the Survey of Reproductive Health of Inhabitants of Latvia in 2011, the impact of lack of education on reproductive health issues in schools and families is an important subject for discussion in society in Latvia. According to the survey, only 52% of women from 15–19 years of age have discussed reproductive health issues in their families [*Survey of Reproductive Health of Inhabitants of Latvia, 2011*]. Unfortunately, health education is not a compulsory subject in schools in Latvia, and, even more, the fraction of students, who have had this subject at schools has decreased from 26.2% in 2008/2009 to 18.4% in 2010/2011 [*Survey of Reproductive Health of Inhabitants of Latvia,2011*]. The leading information sources on reproductive health issues are media and internet, but the information provided is very often incorrect and subjective. Therefore, health education in schools is important. Sexual behaviour and smoking are modifiable (by education) risk factors associated with abnormal vaginal microflora and adverse pregnancy outcomes. Overlap of many risk factors is possible. In the present study it was found that women with primary and secondary education were more likely to have

smoked before pregnancy, but denied (or possibly quit) smoking during pregnancy, and they were more often unmarried.

Unlike other work, this study could not find a strong association between abnormal vaginal microflora and sexual history (number of previous sexual partners, new partner during last six month) and habits [*Rezeberga, et al., 2002; Beigi, et al., 2005; Schwebke, et al., 2005; Vogel (a), et al., 2006; Larsson, et al., 2007; Fethers, et al., 2008*]. Possibly this can be explained by the selection of a slightly different study group: all abnormal vaginal flora types, including intermediate, BV, AV and mixed AV-BV were analysed, but the study excluded sexually transmitted infections (STI). Another possible explanation is that pregnant women did not give honest answers. On the other hand there were more associations observed between full BV cases and sexual habits, such as frequent intercourse and unmarried status, also BV in past history, but recent intercourse was more often associated with clear AV flora.

Smoking is known to be an important health risk factor. Smokers are at increased risk for cancers, chronic lung, oral, cardiovascular and infectious disease [*Huttunen, et al., 2010; Lee, et al., 2012*]. Like in the present study, smoking was recognized by others as a risk factor for abnormal vaginal microflora [*Rezeberga, et al., 2002; Vogel (a), et al., 2006; Larsson, et al., 2007; Desseauve, et al., 2012*]. While in this study the numbers of cigarettes per day was not analysed, it is known, that the risk of AVF and BV is directly proportional to the number of cigarettes smoked [*Smart, et al., 2004*]. A variety of chemicals from tobacco are present not only in airways, but also in cervical mucus [*McCann, et al., 1992*]. They alter the innate immunity of mucosa and adaptive immunity at the systemic level [*Lee, et al.,* 2012] and so add the risk of infections and related complications. Therefore, in young pregnant smokers, vaginal flora has to be evaluated more carefully in order to avoid pregnancy complications related to reproductive tract infections.

Similar to country data that has been reported, in the present study 9% of all enrolled pregnant participants were current smokers, and almost one third smoked before pregnancy. Most of those who had smoked also had abnormal vaginal microflora. Larsson also has found the relations of ex-smokers to AVF: three months before pregnancy 36.4% of the women with BV were smokers compared to 19.4% among the women who had normal vaginal smears (OR 2.4) [*Larsson, et al., 2007*].

Because of the unsafe sexual behaviour of younger people, youth is recognized as an important risk factor for STI [*Larsson, et al., 2007*]. In our study age of <25 years was also associated with abnormal vaginal microflora and younger women were less educated, but multivariate analysis did not show as strong an association with younger age as with low educational level. These results could be explained by the fact that women with STI were excluded from the present study, AVF is not always associated with direct sexual transmission of bacteria and the possibility that sperm can cause biochemical changes in the vaginal environment, as, in our study, AV flora was more often found 48 hours after intercourse.

Although we could not find any associations between AVF and sexual history/habits, single and unmarried pregnant women more often had decreased or absent *Lactobacillus* on wet mount, Table 3.1. According to European Commission data, 43.5% of children in Latvia are born to unmarried women [*European Commission, 2011*]. Our data are similar – only 51% of all participants have registered marriage. Single and unmarried people might indirectly present a risk group of unstable relationships, adverse sexual behaviour, psychological and reproductive health problems and, subsequently, adverse pregnancy outcomes.

Analysing the reproductive history, only previous BV during the year preceding pregnancy was associated with AVF. A past history of BV is a recognized risk factor for recurrent BV [*Bradshaw (b), et al., 2006*].

Conversely, *Candida* and *U.urealyticum* were more often found in the normal vaginal microflora group. This is consistent with data that *U. urealyticum* and *Candida* can exist in normal vaginal flora [*Waits, et al., 2005; Donders (a), 2007*].

It is more likely that data about sexual history and life style during pregnancy is not as reliable as one would suppose. The presence of a current partner, social constraints or the common belief that sexual intercourses during pregnancy are not acceptable or even permitted may all play a role in response accuracy during pregnancy. Probably in pregnant women, the indirect indicators of abnormal vaginal microflora, like low education level, marital status could be more accurate in recognizing the abnormal vaginal flora risk group.

4.2. Bedside diagnostic methods of abnormal vaginal microflora

In many clinical settings, proper diagnostic workups of vulvovaginal symptoms and/or risk assessment of the vaginal flora to prevent gestational complications are inadequate or non-existent [*Msuya, et al., 2009*]. Some obstetricians rely only on syndromic management, while others do vaginal cultures on all pregnant women and (over)treat them with antibiotics.

In Latvia, all pregnant women have upper vaginal smears taken for Gram staining in order to exclude *gonorrhoea* and evaluate vaginal microflora, and also for cytological examinations in the first antenatal visit, but the implications of these tests should be discussed. The aim of cytology is detection of cervical precancerous/cancer lesions, not assessment of vaginal flora. According to the European Cervical Cancer Screening guidelines, cervical cytology tests should be taken in an organized screening programme and postponed in pregnancy with negative screening histories unless the last smear was more than 3–5 years ago and, because of pregnancy associated

changes in the uterine cervix, if a woman has called for routine screening and she is pregnant, the smear should be deferred [*Arbyn, et al., 2008*]. Neither Nugent score, nor lactobacillary grades are detected in Gram stain smears in Latvia. Gram stain recognizes clue cells as a part of BV diagnostic tests and different type of microorganisms, though waiting for results takes additional time and visits. According to the guidelines of the Latvian Association of Gynecologists and Obstetricians, BV should be diagnosed if three out of four *Amsel* criteria are present [*Amsel, et al., 1983*]. To evaluate them, gynecologists are to use vaginal pH strips and take samples for microscopy, bed – side wet mounts or send for Gram stain microscopy. Immediate diagnostic of AVF during the first antenatal visit by using bed-side diagnostic tests – vaginal pH test and wet mount microscopy, could accelerate this process, but in-fact gynecologists in Latvia do not often use vaginal pH strips and are not skilled at performing wet mounts. At the same time, BV diagnosis is, not occasionally, based solely on positive clue cells or even only presence of *Gardnerella vaginalis* on Gram stain laboratory reports, not on *Amsel* criteria.

Another AVF diagnostic method widely used in Latvia is bacteriology. Although there is strong evidence that microscopic findings indicating abnormal vaginal microbial flora are associated with complications in pregnancy such as preterm birth, chorioamnionitis and preterm rupture of the membranes [*Donders, et al., 2008*], there is no consensus regarding use of culture results as a substitute for these findings. This author strongly objects to starting treatment based solely on culture of vaginal microorganisms, "as this leads to overtreatment, exposes mother and fetus to unnecessary toxins, increases the risk of bacterial antimicrobial resistance in both mother and newborn, and enhances the risk of hard to treat, recurrent vulvovaginal candidosis, along with other disturbances of the vaginal ecology". All of the diagnostic methods used should be based on indications and the validity of all

tests should be clear, because unnecessary analysis and controversial results increase stress and anxiety in pregnant women. Pregnant women should receive complete, evidence based information about the tests performed, their specificity, sensitivity and possible influence of results on pregnancy care, risks and benefits.

4.2.1. Value of vaginal pH test in abnormal vaginal microflora diagnostic in the first trimester of pregnancy

We found that elevated vaginal pH in the first trimester of pregnancy is associated with a bad smell, a thin, homogenous or yellowish discharge, and the finding of abnormal vaginal flora, increased number of leucocytes (> 10 per epithelial cell), and presence of sperm cells on wet mount microscopy, Table 3.2.

Our study findings are similar to those of Donders et al. [*Donders (b), et al., 2000*], who confirmed a strong correlation between elevated vaginal pH and abnormal vaginal flora on wet mount. We could not demonstrate a similar association between vaginal acidity and lactobacillary morphology, Table 3.2.

According to *Amsel* [*Amsel, et al., 1983*], increased vaginal pH is one of the four discriminative BV criteria. This study found not only BV, but also aerobic vaginitis and mixed flora changes to be related to abnormal pH. Also, 13% of patients from the normal vaginal pH group had LBG IIb or LBG III (most cases were aerobic or with mixed flora changes), thus making pH test highly sensitive for BV and less sensitive and specific for AV flora diagnosis. However, AV can also be associated with pregnancy complications [*Donders, et al., 2009*]. Therefore, to improve diagnostic accuracy, additional microscopy should be performed. Wet mounts can be done as a rapid "bed-side" test by gynecologists and obstetricians to assess vaginal flora. Wet mount specimens

reflect vaginal lactobacillary flora at least as well as Gram stain and even air-dried rehydrated samples are reliable [*Donders, et al., 1996; Donders (a), et al., 2000*].

Although the finding of abnormal vaginal discharge was a poor predictors of AVF in some studies [*Schwiertz, et al., 2006*], we found that thin, homogenous or yellow discharge was more often associated to elevated vaginal pH/abnormal vaginal microflora, while normal discharge was associated with normal vaginal acidity, Table 3.2.

Donders has found that disturbed LBG and presence of increasing vaginal leucocytosis were correlated with depressed lactate concentration, elevated vaginal pH and increased concentrations of a variety of pro-inflammatory cytokines in vaginal fluid [*Donders (b), et al., 2000*]. We similarly observed that elevated vaginal pH was more often related to increased numbers of leucocytes on wet mounts and also associated with yellow appearance of the vaginal discharge (Table 3.2.). Since data confirms that elevated vaginal pH and neutrophils in early pregnancy are strongly associated with spontaneous preterm births and early third-trimester preterm rupture of membranes, it reflects the importance of infection and/or inflammation in the pathogenesis of this condition [*Simhan, et al., 2003; Simhan, et al., 2005*].

4.2.2. Correlations of bed-side diagnostic tests with vaginal bacteriological findings

According to the data in the current study, it is clear, that the proportion of positive vaginal cultures was above 85% in both normal and abnormal vaginal microflora groups, including all types of aerobic and anaerobic bacteria. Therefore, merely treating any positive culture obtained from the

vagina should never be an option in pregnant women. At the first trimester pregnant women with elevated vaginal pH and abnormal vaginal flora on native microscopy, a recognized risk factor for adverse pregnancy outcome, were more likely to have high concentrations of *M. hominis, U. urealyticum*, and positive cultures of *E. coli*, while *Str. agalactiae* and *Viridans* group streptococci were more related to normal vaginal microflora. Vaginal leucocytosis was significantly associated with *E. coli* colonization.

Although the association between *U. urealyticum, M. hominis* and pregnancy complications, such as late miscarriages, preterm birth, low birth weight and neonatal respiratory diseases is well established [*Donders, (c), et al., 2000; Taylor-Robinson, et al., 2007; Romero, et al., 2008; Donders, et al., 2009*], it is still unclear which pregnant women would benefit from cultures and treatment of these bacteria. *U. urealyticum* commonly inhabits the lower genital tract of sexually active women with colonization rates of up to 80%, and *M. hominis* in 21 to 53% [*Waits, et al., 2005*]. *E. coli* vaginal colonization is observed in 3 to 20%, and *Str. agalactiae* in 6.5 to 36% of pregnant women [*Watt, et al., 2003; Barcaite, et al., 2008*]. Large numbers of *M. hominis* is associated with BV and according to some investigations is an important risk factor for development of preterm labor [*Lamont, et al., 1987; Rosenstein, et al., 1996*].

Several studies have evaluated the role of antibiotics to prevent preterm birth in BV cases. Clindamycin administered early in the second trimester to women who test positive for BV seemed to be more effective than metronidazole in reducing preterm birth rate [*Carey, et al., 2000; Ugwumadu, et al., 2004;Lamont, et al., 2005*], probably because it has a larger scale antibacterial activity –against anaerobic gram-negative, aerobic gram-positive bacteria and also *M. hominis* as compared to metronidazole, which is only active against anaerobic bacteria [*Mylonas, 2010; Taylor-Robinson, et al., 2011*]. In our study, high numbers of *M. hominis* was found only in cases with

AVF and had the strongest association with presence of a pathological vaginal environment (Table 3.3.). An association of *U.urealyticum* with decreased lactobacilli and elevated vaginal pH was far weaker than that for *M. hominis*. Hence, we postulate that the mere fact of vaginal colonization with *U. urealyticum* and/or *M. hominis per se* is a poor predictor of an abnormal pregnancy outcome, but that high density vaginal mycoplasma colonization and its associated flora abnormalities should be considered a risk factor for late miscarriage, chorioamnionitis and preterm birth. Other studies have confirmed, that if any effect is present, only high loads of ureaplasmas are related to adverse pregnancy outcomes [*Kasper, et al., 2010*].

In the present study, abnormal vaginal pH was associated not only with BV, but also with AV flora, while *M. hominis* and *U. urealyticum* both were more often found in women with BV and with mixed AV-BV flora. *E. coli* was more typically found in LBG IIb and AV flora and, furthermore, associated with increased vaginal leucocytosis. These results are in concordance with another study, in which *E. coli* growth was inhibited by various *Lactobacillus* strains [*Juarez-Tomas, et al., 2003*]. Our findings are also in line with those of Donders et al. [*Donders (b), et al., 2000*], who demonstrated that aerobic vaginitis has to be considered as an independent risk factor for preterm delivery [*Donders, et al., 2009*].

Not only BV, but also aerobic vaginitis in early pregnancy is linked to preterm delivery and chorioamnionitis [*Rezeberga, et al., 2008; Donders, et al., 2008; Donders, et al., 2009*]. Since the extent of the inflammatory reaction has a particularly important role in the pathogenesis of preterm delivery [*Jacobson, et al., 2003*], the finding of a significant association of *E. coli* in the presence of leucocytosis with AVF found in our study could be important. Increased vaginal leucocytosis correlates with higher concentrations of pro-inflammatory cytokines present in the vagina and with enzymatic activity leading to preterm contractions and intrauterine infections [*Donders, et al., 2002; Romero, et al.,*

2002; Larsson, et al., 2006]. Carey and Klebanoff, in their metronidazole treatment studies of abnormal vaginal microflora in pregnancy, could not show any benefit – even worse – rates of preterm birth went up in the metronidazole group [*Klebanoff, et al., 2001;* Carey, *et al., 2005*]. This was explained by increased *E. coli* and *Klebsiella pneumoniae* concentrations in the vagina at delivery [*Carey, et al., 2005*].Besides their association with prematurity, *Str. agalactiae* and *E. coli* are also a major cause of early neonatal infection [*Stoll, et al., 2011*]. Many authors have recognized the increasing importance of the potential role of *E. coli* in the development of early neonatal disease and sepsis, especially in preterm babies [*Lin, et al., 2011*]. Although, in the present study, *E. coli* and *M. hominis* colonization was strongly associated with decreased or absent *Lactobacillus* morphotypes on microscopy and increased vaginal pH, the association could be weaker in a larger population, due to the wide confidence intervals found.

Performing vaginal pH measurement and wet mounts during the first antenatal visit, if not in all women, at least in women with signs or symptoms of genital infections, history of miscarriages or preterm deliveries are recommended for rapid, early diagnosis of abnormal vaginal microflora. We hypothesize, that treatment should be based on antibiotics covering the abnormal vaginal microflora type, taken into account the susceptibility to *M. ho*minis in BV and to *E. coli* in AV associated cases, or, alternatively, non-antibacterial, broad spectrum antimicrobial medications or probiotics could be used. Prospective studies to confirm the role of *E. coli*, *M. hominis* and *U. urealyticum* to risk assessment of preterm birth and neonatal sepsis in women with abnormal vaginal flora patterns and increased pH, are needed to confirm the hypothesis that targeted treatment can reduce preterm birth.

As vaginal pH and microflora can be normal in *Str. agalactiae* colonization cases, it has to be clear that such bed-side tests are not meant to

replace *Str. agalactiae* detection by cultures in the general population of pregnant women.

4.3. Impact of vaginal ascorbic acid on abnormal vaginal microflora

The lak of solid evidence about the efficacy of antibiotics in preventing infection related preterm deliveries, concerns about safety issues and risk of increasing antibacterial resistance, has encouraged the study of new non-antibacterial treatment options. A treatment that restores normal vaginal microflora and acidity without causing systemic effects or bacterial resistance problems could be preferable [*Othman, et al., 2012*]. Acidification of the vagina with ascorbic acid (vitamin C) is one of these alternative approaches.

There are few studies about the efficacy of vaginal vitamin C [*Petersen, et al., 2004; Petersen, et al., 2011*]. The results of these studies support an effective and safe use of vaginal vitamin C in a six day mono-therapy regimen in women with BV. Petersen et al. showed in 2011, those 8–14 days after treatment, the presence of all 4 *Amsel* criteria was significantly less frequent in the vitamin C group. Since in the previous study results 14 days after insertion of vaginal vitamin C had not been as significant as after 6 days, the six days therapy regimen might be too short and had an insufficient long lasting effect. There are no data about long term use of vaginal ascorbic acid in pregnancy and its influence on different abnormal microflora types.

According to our data, long-term use of vaginal ascorbic acid improves AVF, especially in pregnant women, but did not show a significant improvement on pure AV or BV microflora. Our results are consistent with the findings of Petersen`s double-blind, placebo-controlled study [*Petersen, et al., 2011*]. However, the improvement of the microflora due to vitamin C in our

study was less significant than in earlier study, partly because of a larger number of participants in Petersen's trial, but maybe also due to slightly different study design (included only BV patients), another treatment regimen (6 days mono-therapy) and assessed different clinical outcomes. In addition, our study had a vast proportion of pregnant women. Surprisingly, efficacy endpoints in pregnant women showed better results than the total study population with cure rates of 70.7% (vs. 29.3% in controls), which is comparable to antibiotic treatment trials for AVF in pregnancy, using vaginal clindamycin or oral metronidazole [*Carey, et al., 2000; Lamont, et al., 2003*], but lower compared to oral clindamycin [*Ugwumadu, et al., 2004*]. In non-pregnant patients the improvement of the microflora due to vitamin C could not be demonstrated. The low number of non-pregnant women included in the study can partly responsible for this unexpected finding. Another possible explanation is the change of sexual behaviour during pregnancy and production of high levels of estriol and other steroid hormones by the placenta. It is well known that estrogens improve lactobacilli colonization by enhancing vaginal epithelial cell production of glycogen, which breaks down into glucose and acts as a substrate for the bacteria [*Eckert, 2006*]. High levels of estriol could give additional benefit to acidic pH caused by ascorbic acid in re-establishing normal vaginal environment. This may also explain the spontaneous, partial improvement in vaginal pH in the control group, albeit less pronounced that in the treatment group. This mechanism is also supported by results of vaginal probiotic/estriol regimen studies, in which the microbiological cure of BV cases was better in the study than in placebo group [*Parent, et al., 1996*].

In order to evaluate long-term effects of vaginal vitamin C use, a trial with a treatment and a maintenance regimen was conducted. Previous studies showed gradual increase of vaginal pH one and two weeks after 6 days mono-therapy treatment with vaginal vitamin C [*Petersen, et al., 2004*], but in the present study normal vaginal pH was maintained in a greater proportion of

participants using vitamin C in a maintenance regimen than in controls. As in other studies [*Ugwumadu, et al., 2004*], the prevalence of abnormal vaginal microflora and pH also somewhat decreased without treatment. Still, the results were significantly better in the intervention group.

Although there were no significant associations between side effects and vaginal pH, regimen of treatment, microflora type, number of leucocytes or presence of *Candida* on wet mounts, those who prematurely withdrew from the study generally had *Candida*, increased number of leucocytes and non-BV flora. Probably such women with vaginal inflammation or candida colonisation have greater tendency to develop side effects like irritation due to vitamin C. On the other hand, the likelihood of having such irritation seems less pronounced in BV cases, although larger numbers of participants are required to demonstrate this unequivocally.

Vaginal acidification caused the largest improvement in the LBG III mixed AV-BV group, but not in the pure BV or AV cases. As the effect on normalization was not only significant in the combined AVF group, but also in the groups with BV (with or without another mixed infection) and AV (with or without another mixed infection), the failure to show a significant normalization in the groups with AV and BV may have been due to low numbers in these groups. Hence, we concluded that despite these negative findings, lowering of vaginal pH with vitamin C can most likely be achieved not only in BV, but as well as in AV and cases with intermediate or mixed flora.

4.4. Pregnancy outcome

We found that elevated vaginal pH and abnormal vaginal microflora during the first trimester of pregnancy were associated with early miscarriage and preterm deliveries in the non-intervention population, compared to normal

vaginal microflora group, Table 3.5. Although early miscarriage was the most common adverse pregnancy outcome in both normal and AVF study groups, it was lower in pregnant women with normal vaginal microflora and acidity. Hobel and Ralph also found BV associated with the first trimester miscarriage [*Hobel, et al., 1999*; *Ralph, et al., 1999*]. These results might emphasize early (< 20 weeks of gestation) treatment of AVF, suggested by clindamycin treatment studies [*Ugwumadu, et al., 2003*], although in the present study women with vaginal clindamycin treatment did not show any benefits, compared to vitamin C or control group, which can be explained also by small numbers of cases. Unlike findings of other studies [*Leitich, et al., 2007; Donders, et al., 2009*], an association between AVF and late miscarriage was not found, which also could be explained by the small number of participants. Nevertheless, the present results are consistent with others' data demonstrating increased preterm birth risk in the pregnant women with AVF [*Hauth, et al., 2003; Leitich, et al., 2007; Donders, et al., 2009*].

Although outcomes in all AVF intervention group (all participants with AVF, who had received vaginal vitamin C or vaginal clindamycin or systemic antibiotics) were not better compared to non-treated AVF group, pregnant women, who received vitamin C, had statistically significant better results - less miscarriage and no preterm deliveries, compared to non-intervention AVF group, Table 3.7. Same results were not achieved, analysing solely vitamin C and control group, probably because of few numbers.

The role of abnormal vaginal microflora on fetal growth is controversial, because many factors can negatively influence it. There are studies that do not support the impact of genital infections on fetal growth [*Carey, et al., 1991*], but many other authors have shown the opposite [*Svare, et al., 2006; Vogel (b), et al., 2006; Donders, et al., 2008, Vedmedovska, et al., 2010*]. In most studies, BV was associated with fetal growth restriction [*Svare, et al., 2008, Vedmedovska, et al., 2010*], but this study found the presence of aerobic

vaginitis, not pure BV, associated flora and mixed AV-BV flora related to lower birth weight (Table 3.6.) A major reason for this discrepancy could be that in others (and olders) studies AV was not addressed separately, and may have been mixed up with the diagnosis of 'BV'. *U. urealyticum*, *Fusobacterium* spp. and *M. hominis* are the bacterial species most commonly isolated from the amniotic cavity of women with preterm labor and intact membranes, such aerobic bacteria as *Str. agalactiae*, *S. aureus*, *Str. viridians*, *E. coli*, *Enterococcus faecalis* can be present as well [*Zhou, et al., 2010*]. Of importance, aerobic bacteria and the finding of aerobic vaginitis at first prenatal visit were associated with funisitis at birth [*Rezeberga, et al., 2008*], which may lead to fetal inflammatory response syndrome, and most likely also impairment of fetal growth. Although multivariate analysis in the present study showed smoking during pregnancy to be the most relevant low newborn` weight risk factor, the role of AVF, particularly associated with aerobic vaginitis, in the fetal growth deserves further investigation.

The higher Apgar scores in the normal vaginal microflora compared to AVF was also observed. In 1952, Virginia Apgar proposed the Apgar score as a means of evaluating the physical condition of infants shortly after delivery [*Apgar, 1953*]. The Apgar scoring system remains relevant for the prediction of neonatal survival today [*Casey, et al., 2001*].

Others have found that low Apgar score is associated with BV positive mothers [*Laxmi, et al., 2012*], while some authors have showed Gram-positive cocci, *Str. agalactiae*, and Gram-negative bacilli [*Wojkovska-Mach, et al., 2012*] related to low Apgar score as well as maternal chorioamnionitis and early-onset septicemia and early-onset pneumonia in newborns. As vaginal environment normalization may prevent abnormal vaginal microflora to ascend in the uterus, by vaginal ascorbic acid application it may explain, why infant's Apgar score at five minutes improved in both ITT and PP populations.

However, small number of cases prevented us to find significant differences in neonatal infectious diseases.

In order to decrease complication rates of AVF, treatment should most likely be started early in pregnancy and be focused on the specific infections related risk groups. There are data showing that risk assessment should be based not only on history of miscarriage/preterm delivery, presence of abnormal vaginal flora, but ideally also on pro- inflammatory cytokines and genotype [*Gomez, et al., 2010; Lamont (b), et al., 2011*]. Despite the fact that the present study was not designed to investigate the impact of AVF treatment on pregnancy outcome, the findings probably indicate that the treatment of AVF in early pregnancy with vaginal vitamin C in the treatment and maintenance regimen can improve vaginal microflora and subsequently also pregnancy outcome, but this should be verified in further trials.

4.5. Limitations of the study

Lack of placebo in the vitamin C study could increase the influence of confounding factors, like pH levels measured by gynecologists, although microscopic evaluation was blinded.

The small number of participants limited evaluation of AVF, colonization of different bacteria and vaginal ascorbic acid treatment impact on pregnancy outcome.

Additionally bacteria identification using standard culture techniques can limit investigation of other microbes, which can be identified only by molecular – based methods. Nevertheless it had practical implications, as advanced techniques are not widely available and suitable for everyday practice, yet identification of abnormal flora might be crucial during the first antenatal visit.

5. CONCLUSIONS

1. Vaginal ascorbic acid in long term – treatment/maintenance regimen improves abnormal vaginal microflora in pregnancy, in non-pregnant women the effect is less pronounced.
2. Although overlap of different risk factors is possible, low level of education, smoking and history of BV during the year before pregnancy are the most important risk factors of abnormal vaginal microflora in the first trimester of pregnancy.
3. Increased vaginal pH is strongly associated with abnormal vaginal microflora (aerobic vaginitis, bacterial vaginosis and mixed flora), with complaints of increased, bad smell and thin, homogenous, yellow discharge on examination in the first trimester of pregnancy. Vaginal pH measurement and wet mount microscopy are an ideal combination for a reliable, rapid "bed-side" risk assessment of the vaginal environment.
4. Abnormal vaginal flora and elevated vaginal pH in the first trimester of pregnancy correlates with *M. hominis* and *E. coli* overgrowth in cultures.
5. Abnormal vaginal microflora in the first trimester of pregnancy is associated with early miscarriage, preterm birth and lower Apgar score of newborns. Treatment with vaginal ascorbic acid in long term – treatment/maintenance regimen is related to better Apgar scores in newborns.

6. CLINICAL IMPLICATIONS AND FUTURE ASPECTS

- As it is important to increase educational level and knowledge of important women`s health issues in Latvia; health education in schools should be compulsory
- Vaginal pH measurements should be always used for routine gynecology examination
- For rapid, cheap AVF diagnostic bed side tests – pH and wet mounts are recommended
- Vaginal ascorbic acid in long term – treatment/maintenance regimen can be used to restore abnormal vaginal microflora and vaginal pH in pregnancy, probably cases with symptomatic vaginal inflammation should be excluded
- Prospective studies to confirm *E. coli*, *M. hominis* and perhaps *U. urealyticum* relation to risk assessment of preterm birth and neonatal sepsis in women with abnormal vaginal flora patterns and increased pH, and effect of abnormal vaginal flora type specific treatment on preterm birth reduction are mandatory
- Randomized, double blind, placebo-controlled studies of vaginal vitamin C prevention of preterm delivery/late miscarriage and neonatal infectious complications associated with abnormal vaginal microflora could be extremely interesting

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8. PUBLICATIONS AND CONFERENCE THESIS

1. Publications

- Zodzika J., Rezeberga D., Donders G.G., Vedmedovska N., Vasina O., Bite R., Pundure I., Silberga I., Socenova J., Melngaile O. Impact of the vaginal ascorbic acid in the treatment and maintenance regimen on the abnormal vaginal environment. Arch Gynecol Obstet, 2013; 288: 1039–1044.
- Zodzika J., Rezeberga D., Vasina O., Bite R., Pundure I., Donders G.G. Composition of vaginal flora in relation to vaginal pH and wet mount diagnostic tests of the first trimester of pregnancy. Proceedings of the Latvian Academy of Sciences. Section B, 2013; 67, 6 (687): 20–30.
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2. Conference thesis

2.1. Oral presentations

- Zodzika J., Rezeberga D., Vasina O., Bite R., Pundure I., Vidnere I., Matule D., Zile O., Pavlova Z. Effect of the vaginal Vitamin C on the abnormal vaginal environment. 22nd European Congress of Obstetrics and Gynecology. Tallinn, Estonia, 2012.
- Zodzika J., Rezeberga D., Vasina O., Bite R., Pundure I., Zīle O., Pavlova Z., Vidnere I., Kampara I., Krumina S. Influence of *Escherichia coli* on vaginal flora in pregnancy. VIth Latvian congress in Obstetrics and Gynecology, 2011.

2.1. Poster presentations:

- Zodzika J., Rezeberga D., Vasina O., Jermakova I., Baranovska D., Dresmane A. Impact of abnormal vaginal microflora on pregnancy outcome. World Congress on Building Consensus out of Controversies in Gynecology, Infertility and Perinatology. Istanbul, Turkey, 2013.
- Žodžika J., Rezeberga D., Vasina O. Patoloģiskas maksts mikrofloras riska faktori grūtniecēm Latvijā. 2012. gada RSU Zinātniskā konference.
- Zodzika J., Rezeberga D., Vasina O., Bite R., Pundure I., Vidnere I., Matule D., Zile O., Pavlova Z., Donders G.G. Effect of vaginal vitamin C on the vaginal pH in pregnancy. International Scientific Conference on Probiotics and Prebiotics. Kosice, Slovakia, 2011.

- Žodžika J., Rezeberga D., Vasina O. Maksts pH diagnostiskā testa ticamība maksts floras izmaiņu noteikšanai grūtniecēm pirmajā trimestrī. 2011. gada RSU Zinātniskā konference.
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