



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

For obtaining the degree of a Doctor of Medicine

Speciality – Obstetrics and Gynaecology

Research supervisor:

MD, PhD, Professor **Dace Rezeberga**, Rīga Stradiņš University (Latvia)

Research scientific consultant:

MD, PhD, Professor **Gilbert Donders**, University of Antwerp (Belgium)



IEGULDĪJUMS TAVĀ NĀKOTNĒ



Rīga, 2014

ANNOTATION

Normal vaginal microflora is an important women`s health factor, maintained by high numbers of different *Lactobacillus* species.

Abnormal vaginal microflora and infections ascending from the lower urogenital tract represent an important reason for abortions, preterm delivery and neonatal infections. Multiple investigators have attempted to identify the patients at risk for preterm labor, followed by the treatment in a low risk population of genital infections, but the results did not meet initial hopes. Still there is growing evidence that treatment of abnormal vaginal microflora with adequate antibiotics in early pregnancy can prevent at least some of the infections related to preterm birth. While antimicrobial agents cure infections, they can cause side effects. Furthermore, urogenital pathogen drug resistance is on the increase and disrupt protective vaginal microflora. Many pregnant women are very anxious about taking antibiotics because of potentially adverse effects on the newborn. During pregnancy treatment that restores normal vaginal flora and acidity without systemic effects would be preferable to any other treatment.

The aim of the present study is to investigate the influence of vaginal application of ascorbic acid (vitamin C) on abnormal vaginal microflora during pregnancy, and also to identify risk group and assess the validity of the “bed-side” diagnostic tests during the first antenatal visit in order to detect different types of abnormal vaginal flora. There are 450 pregnant (150 with elevated and 300 with normal vaginal pH) and 55 non-pregnant (with elevated vaginal pH and abnormal microflora) women included in the study. Different socio-demographic, medical, reproductive and sexual risk factors of abnormal vaginal microflora in the first trimester of pregnancy are assessed. The results of vaginal pH, wet mounts and bacteriologic examinations are correlated. The impact of vaginal vitamin C in the treatment and then maintenance regimen on different types of abnormal vaginal microflora is analysed in the present study. The major risk factors for abnormal vaginal microflora in the first trimester of pregnancy are low education level, smoking and history of abnormal vaginal microflora before pregnancy. Increased vaginal pH is associated with both aerobic and anaerobic abnormal vaginal microflora on wet mounts and overgrowth of *Mycoplasma hominis* and *Escherichia coli*. Vaginal vitamin C treatment followed by a maintenance regimen improves vaginal microflora in pregnancy. The study is supported by European Social Fund project “Support of doctoral and postdoctoral investigations in Riga Stradins University”.

ANOTĀCIJA

Normāla maksts mikroflora ir svarīgs sievietes veselības faktors, kuru nodrošina pienskābo baktēriju pārsvars. Izmainīta maksts mikroflora var būt par iemeslu spontāniem abortiem, priekšlaicīgām dzemdībām un jaundzimušo infekcijām. Daudzi pētnieki ir mēģinājuši samazināt šo faktoru izraisītos sarežģījumus, identificējot un ārstējot vispārējā populācijā tās grūtnieces, kurām varētu būt paaugstināts, ar infekcijām saistīts, priekšlaicīgu dzemdību risks, taču iegūtie rezultāti nebija efektīvi. Arvien vairāk ir pierādījumi, ka, uzsākot adekvātu antibakteriālo terapiju agrīnā grūtniecības laikā, tiek novērsta vismaz daļa no infekciju izraisītiem sarežģījumiem. Lai gan antibakteriālie līdzekļi nodrošina izārstēšanos no infekcijām, to lietošana rada uroģenitālo mikrobu rezistenci, var izraisīt blaknes un izjaukt maksts normālo ekosistēmu. Daudzas grūtnieces, baidoties no nelabvēlīga iespaida uz bērnu, nevēlas lietot antibiotikas. Ideāli grūtniecības laikā būtu izmantot tādu maksts mikroflu uzlabojošu terapiju, kas saudzē aizsargājošo maksts vidi un neizraisa vispārējas blakus parādības.

Šī pētījuma mērķis ir izpētīt alternatīvas, “ne-antibakteriālas” terapijas – vaginālā vitamīna C, ietekmi uz izmainītu maksts vidi grūtniecēm un sievietēm, kas nav grūtnieces, kā arī izanalizēt riska grupas un izvērtēt klātienē diagnostikas testu pielietojamību dažādu maksts mikrofloras izmaiņu diagnostikā grūtniecēm pirmajā trimestrī. Pētījumā ir iekļautas 150 grūtnieces ar palielinātu un 300 ar normālu maksts pH, kā arī 55 sievietes ar izmainītu maksts mikroflu, kas nav grūtnieces. Šajā pētījumā ir izanalizēti dažādi sociāli, demogrāfiski, reproduktīvie, seksuālās uzvedības izmainītas maksts mikrofloras riska faktori grūtniecēm pirmajā trimestrī, kā arī korelācijas starp maksts pH, natīvo mikroskopiju un uzsējumu rezultātiem. Tika arī izpētīta vaginālā vitamīna C ietekme uz maksts vidi, nozīmējot to agrīnā grūtniecības laikā ārstēšanas, kā arī uzturošā režīmā. Galvenie izmainītas maksts vides riska faktori grūtniecēm pirmajā trimestrī ir zems izglītības līmenis, smēķēšana un izmainīta maksts mikroflora pirms grūtniecības. Palielināts maksts pH ir saistīts ar izmainītu maksts mikroflu – gan aerobisko, gan anaerobisko, kā arī ar *Mycoplasma hominis*, *Escherichia coli* savairošanos makstī. Vaginālais vitamīns C ārstnieciskā, kā arī uzturošā režīmā uzlabo maksts vidi grūtniecēm, taču nav efektīvs sievietēm, kas nav grūtnieces. Pētījums ir veikts ar Eiropas Sociālā fonda programmas “Atbalsts doktorantūras un pēcdoktorantūras pētījumiem medicīnas zinātnēs” atbalstu.

CONTENT

INTRODUCTION	9
STUDY AIM	11
STUDY OBJECTIVES	12
STUDY HYPOTHESIS STATEMENT	13
1. REVIEW OF THE LITERATURE	
1.1. Vaginal microflora types	
1.1.1. Normal vaginal microflora	14
1.1.2. Bacterial vaginosis	16
1.1.3. Aerobic vaginitis	17
1.2. Role of the abnormal vaginal microflora in pregnancy and other non-obstetrical complications	17
1.2.1. Adverse pregnancy outcome	17
1.2.2. Non-obstetrical complications	21
1.3. Risk factors of abnormal vaginal microflora	21
1.3.1. Sexual risk factors	21
1.3.2. Socioeconomic risk factors	22
1.3.3. Smoking	22
1.3.4. Other risk factors	22
1.4. Abnormal vaginal microflora diagnostic methods	23
1.4.1. Signs and symptoms	23
1.4.2. Clinical diagnosis	23
1.4.3. Vaginal pH test	24
1.4.4. Microscopic examinations	25
1.4.5. Cultures based examinations	30
1.4.6. Molecular diagnostics	32
1.4.7. Definition of abnormal vaginal microflora	32
1.5. Treatment of abnormal vaginal microflora	33
1.5.1. Standard treatment with antibiotics	34

1.5.2. Recurrence of bacterial vaginosis	35
1.5.3. Alternative treatment with probiotics	36
1.5.4. Alternative treatment with antiseptics	37
1.5.5. Alternative treatment with acidifying agents	37
1.5.6. Treatment of abnormal vaginal microflora in pregnancy	39
2. NOVELTY OF THE STUDY	42
3. MATERIALS AND METHODS	43
3.1. Study population, time frame and settings	43
3.1.1. Summary of inclusion criteria	43
3.1.2. Summary of exclusion criteria	44
3.2. Study design	46
3.3. Ethical issues	46
3.4. Methods	46
3.5. Outcome assessment	49
3.6. Statistical analysis	50
4. RESULTS	52
4.1. Factors associated with abnormal vaginal microflora	52
4.2. Symptoms and microscopical findings related to increased vaginal pH.....	58
4.3. Vaginal bacteriological study	61
4.4. Vaginal ascorbic acid (vitamin C) study	65
4.4.1. Results in pregnant women subgroup	65
4.4.2. Results in total study population and non-pregnant subgroup	67
4.4.3. Side effects of vaginal vitamin C	71
4.5. Results if the vaginal clindamycin group	72
4.6. Pregnancy outcome	72

5. DISCUSSION	79
5.1. Risk factors of abnormal vaginal microflora in the first trimester of pregnancy	79
5.2. Bed-side diagnostic methods of abnormal vaginal microflora	84
5.2.1. Value of vaginal pH test in abnormal vaginal microflora diagnostic in the first trimester of pregnancy	86
5.2.2. Correlations of bed-side diagnostic tests with vaginal bacteriological findings	89
5.3. Impact of vaginal ascorbic acid on abnormal vaginal microflora	92
5.4. Pregnancy outcome	95
5.5. Limitations of the study	98
5.6. Summary	98
6. CONCLUSIONS	100
7. CLINICAL IMPLICATION AND FUTURE ASPECTS	101
8. REFERENCES	102
9. PUBLICATIONS AND CONFERENCE THESIS	125
10. APPENDICES	128
Appendix No. 1. Rīga Stradiņš University Ethical Committee Study approval	128
Appendix No. 2. Written participant information sheet for consent (in Latvian)	129
Appendix No. 3. Written participant information sheet for consent (in English)	133
Appendix No. 4. Questionnaire	138
Appendix No. 5. Vitamin C study diary.....	149
11. ACKNOWLEDGMENTS	150

TEXT ABBREVIATIONS

AV	aerobic vaginitis
AVF	abnormal vaginal flora
<i>A. vaginae</i>	<i>Atopobium vaginae</i>
BMJ	British Medical Journal
BV	bacterial vaginosis
CI	confidence intervals
CFU	colony forming unit
CN	coagulase negative
CP	coagulase positive
DNA	deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
FIRS	fetal inflammatory response syndrome
GBS	group B streptococci
<i>G. vaginalis</i>	<i>Gardnerella vaginalis</i>
HIV	Human Immunodeficiency Virus
H ₂ O ₂	hydrogen peroxide
hpf	high power field
IL	interleukin
ITT	intention to treat
KOH	potassium hydroxide
LBG	lactobacillary grade
<i>L. crispatus</i>	<i>Lactobacillus crispatus</i>
Mixed AV-BV	mixed aerobic vaginitis and bacterial vaginosis
<i>M. hominis</i>	<i>Mycoplasma hominis</i>
PASW	Predictive Analytics Software
PCR	polymerase chain reaction
PP	per protocol
rRNS	ribosomal ribonucleic acid
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>Str. agalactiae</i>	<i>Streptococcus agalactiae</i>
spp	species

SPSS	Statistical Package for the Social Sciences
STI	sexually transmitted infections
TNF	tumour necrosis factor
<i>U. urealyticum</i>	<i>Ureaplasma urealyticum</i>
vs.	<i>versus</i>

INTRODUCTION

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% per 100 000 live births 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 them 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 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 microorganisms 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.

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.

STUDY OBJECTIVES

1. Primary objective – to investigate the impact of vaginal ascorbic acid (vitamin C) in a treatment and maintenance regimen on abnormal vaginal environment, which is characterized by increased vaginal pH and abnormal microflora on wet mount in pregnant and non-pregnant women.
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).

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. Non-treated abnormal vaginal microflora is associated with adverse pregnancy outcome, which is improved by application of vaginal vitamin C.

1. REVIEW OF THE LITERATURE

1.1. Vaginal microflora types

1.1.1. Normal vaginal microflora

Normal vaginal environment has long been recognized as an important women's health factor and is maintained by high numbers of different *Lactobacillus* species [Redondo-Lopez, et al., 1990]. Although the data are still not fully convincing, estrogen seems to be a determining factor for colonization of lactobacilli by enhancing vaginal epithelial-cell production of glycogen, which then breaks down into glucose and acts as a substrate for the bacteria [Linda, et al., 2006; Gustaffson, et al., 2011]. Studies show, that *Lactobacillus* flora are more prevalent in the vagina of fertile women, than in postmenopausal [Gustaffson, et al., 2011]. Co-aggregation, production of acids, hydrogen peroxide (H₂O₂), bacteriocins and bacteriocin-like substances, and competition for nutrients, ecological niches, blocking pathogen attachment to vaginal epithelium, are factors believed to influence the relation between lactobacilli and potentially pathogenic bacteria [Redondo-Lopez, et al., 1990; Osset, et al., 2011].

In 1892, Professor Albert Döderlein published a monograph in which he said that cultured organisms were a source of lactic acid that could inhibit the growth of pathogens *in vitro* and *in vivo* [Döderlein, 1892]. In 1928, Stanley Thomas identified Döderlein's bacillus as *Lactobacillus acidophilus*, adding, prophetically, that this was either a characteristic of a group of related species, or a species that underwent a remarkable transformation [Thomas, 1928]. In 1980, a group of organisms previously known as *Lactobacillus acidophilus* was shown to be highly heterogeneous [Lauer, et al., 1980]. Today, high numbers of different *Lactobacillus* species are found in the woman's vagina. Verhelst in her study combined cultures with molecular identification and detected 17 *Lactobacillus* types from 197 unselected pregnant women vaginal swabs, and the most common species recovered were: *Lactobacillus crispatus* (*L. crispatus*), *L. jensenii*, *L. gasseri*, *L. iners*, *L. vaginalis* [Verhelst, et al., 2005]. *L. crispatus* are known as strong H₂O₂-producing species, *L. iners* – weak H₂O₂ producers [Antonio, et al., 1999; Rabe, et al., 2003], but Wilks found that *L. jensenii* and *L. vaginalis* produces the highest levels of H₂O₂ and reduces the incidence of ascending infection of the uterus and preterm birth the most [Wilks, et al., 2003].

Culture-independent diagnostic techniques demonstrated that some healthy women (7–33%) lack high numbers of *Lactobacillus* species in the vagina, and that these may be replaced by other lactic acid producing bacteria like *Atopobium vaginae* (*A. vaginae*), *Megasphaera* and *Leptotrichia* species [Zhou, et al., 2004; Anukam, et al., 2006]. Consequently, the presence of these organisms and the absence of lactobacilli do not necessarily constitute an abnormal state [Hymen, et al., 2005]. Ravel et al. data suggest that approximately one-fourth of asymptomatic reproductive age women don't have a *Lactobacillus*-dominated microbiota and almost half have pH higher than 4.5 [Ravel, et al., 2011]. There are many factors recognized to influence prevalence of *Lactobacillus* species. The vaginal microbial communities undergo shifts in their representation, abundance and virulence, and are affected by many factors, including hormonal fluctuations, menstruations, sex practices, vaginal douches, vaginal lubricant use, feminine hygiene products, and other factors [Srinivasan, et al., 2010, Zhou, et al., 2010]. Racial variations and geographic area are just as important [Pavlova, et al., 2002]. Different racial groups within the same geographical region have significant differences in what is the dominant vaginal organism [Anukam, et al., 2006]. These differences can be explained by genetic, environmental factors and diet, which might influence the *Lactobacillus* species resident in the gastrointestinal tract, and hence the vagina, as the lactobacilli of the gut varies between women of different ethnicities [Ahrne, et al., 1998].

Abnormal vaginal flora (AVF) may occur because of sexually transmitted infections, e.g. trichomoniasis, colonization by organisms that are not part of the normal vaginal community, like *Streptococcus pneumoniae*, *Listeria monocytogenes*, or by overgrowth or increased virulence of organisms that are constituents of normal vaginal flora, like *Escherichia coli* (*E. coli*) [Lamont (a), et al., 2011]. The most common vaginal flora disorder is bacterial vaginosis (BV), aerobic vaginitis (AV) is another one. Alterations of vaginal flora do not necessarily imply disease or result in symptoms, as disease results from the interplay between microbial virulence, numerical dominance, and innate and adaptive immune response of the host [Lamont (a), et al., 2011].

1.1.2. Bacterial vaginosis

Since Gardner and Dukes discovered a new genus of bacteria present in vaginal smears devoid of *Lactobacillus*, the condition called bacterial vaginosis has been known [Gardner&Dukes, 1955]. No single organism causes BV. BV is caused by an overgrowth of endogenous vaginal flora representatives like *Gardnerella vaginalis* (*G.vaginalis*), anaerobes, *Mycoplasma hominis* (*M.hominis*), *Ureaplasma urealyticum* (*U.urealyticum*) [Hill, 1993] and clinically diagnosed by Amsel criteria [Amsel, 1983].

G.vaginalis is one of the most common BV-associated microorganisms. Svidsinski et al., using a broad range of fluorescent bacterial group-specific ribosomal ribonucleic acid (rRNS) targeted oligonucleotide probes, identified *G. vaginalis* biofilms, adherent to epithelial cells in vaginal biopsies from women with BV [Svidsinski, et al., 2005]. In this study, the presence of *G. vaginalis* was further referred to as “dispersed” *G. vaginalis*, and consisted of loosely dispersed bacteria cells, and as “cohesive” *G. vaginalis*, consisting of *Gardnerella* cells clustered to the epithelium. Although the bacteria became less metabolically active, the biofilm persisted even one week after treatment.

M.hominis may act symbiotically with other BV bacteria or as a major pathogen [Taylor-Robinson, et al., 2011]. *M. hominis* can be found in large numbers in the vagina of most women with BV, but is found less often in healthy women [Taylor-Robinson, et al., 2011]. Although *Ureaplasma* species may not be independently associated with BV, the prevalence of vaginal colonization by ureaplasmas may be increased by about twofold [Grevett, et al., 1986].

Using various molecular-based techniques and usually the Amsel clinical criteria or Nugent score (explained on the page 28) to classify normal or abnormal flora, a number of studies have demonstrated a considerable diversity of other organisms in women with BV as compared to women with normal flora. *A.vaginae* has been frequently detected in the vagina and is found much more commonly in women with BV than in those with normal flora [Zhou, et al., 2004; Ferris, et al., 2004; Menard, et al., 2008]. *A.vaginae* is strictly anaerobic and is very sensitive to clindamycin, but highly resistant to metronidazole [Ferris, et al., 2004] – a medication often used in BV treatment. Not only *A. vaginae*, but other anaerobic bacteria have been identified using molecular techniques in women with clinical BV, like *Prevotella*, *Megasphaera*, *Mobiluncus*, *Finegoldia*, *Sneathia* [Zhou, et al., 2010].

The only *Lactobacillus* species detected in BV-positive women is *L. iners* [Fredricks, et al., 2005; Wertz, et al., 2008]. *L. iners* – a low H₂O₂ producing organism – is found in 99% of women with BV and 92% of women with normal flora, making it the most ubiquitous *Lactobacillus* in the vagina of women worldwide [Fredricks, et al., 2007]. This may be because *L. iners* adapts better to the conditions associated with BV [Wertz, et al., 2008]. The presence of *L. iners* and *L. gasseri* in the vagina is likely to vary over the time and may lead to a strong predisposition to overgrowth of abnormal bacteria [Verstraelen (a), et al., 2009].

1.1.3. Aerobic vaginitis

Besides anaerobic BV, another type of vaginal flora disturbance is often encountered. It is called aerobic vaginitis (AV) and its most severe form is similar, or equal, to desquamative inflammatory vaginitis [Gardner, 1968; Sobel, 1994; Donders, et al., 2002]. Donders analysed abnormal vaginal microflora, which were neither normal, nor could be defined as BV [Donders, et al., 2002]. AV in his study was diagnosed if smears were deficient in lactobacilli, positive for cocci or coarse bacilli, parabasal epithelial cells, and increased vaginal leucocytes. AV was associated with growth of group B streptococci (GBS), *E. coli*, *Staphylococcus aureus* (*S.aureus*), and had different immunological inflammation reaction and clinical signs. The clinical signs of severe AV include presence of red, inflamed vaginal mucosa, yellowish sticky discharge, high vaginal pH, and “not fish-like” bad odour [Donders, et al., 2002].

1.2. Role of the abnormal vaginal microflora in pregnancy and other non-obstetrical complications

1.2.1. Adverse pregnancy outcome

BV is present in 15–42% of pregnant women [Subramaniam, et al., 2012]. Having a decreased number of *Lactobacillus* and abnormal vaginal flora types – BV and AV, are 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 is a major obstetric problem and is still a challenging question in perinatology. It is a leading cause of neonatal mortality worldwide [Hack, et al., 2000]. In Latvia, the rate of preterm deliveries increased from 4.8% per 100 000 live births in 2008 to 5.8% in 2011 [Statistical Yearbook of Health Care in Latvia, 2012], and is similar than in neighbouring countries like Finland (5.5%), Estonia (5.7%) and Lithuania (5.7%), but lower than in Russia (7%) and Germany (9.2%) [Blencowe, et al., 2012]. Infection related morbidity in the perinatal period in Latvia is 39.2 per 1000 live births in 2011, compared to 28.8 in 2010 [Statistical Yearbook of Health Care in Latvia, 2012].

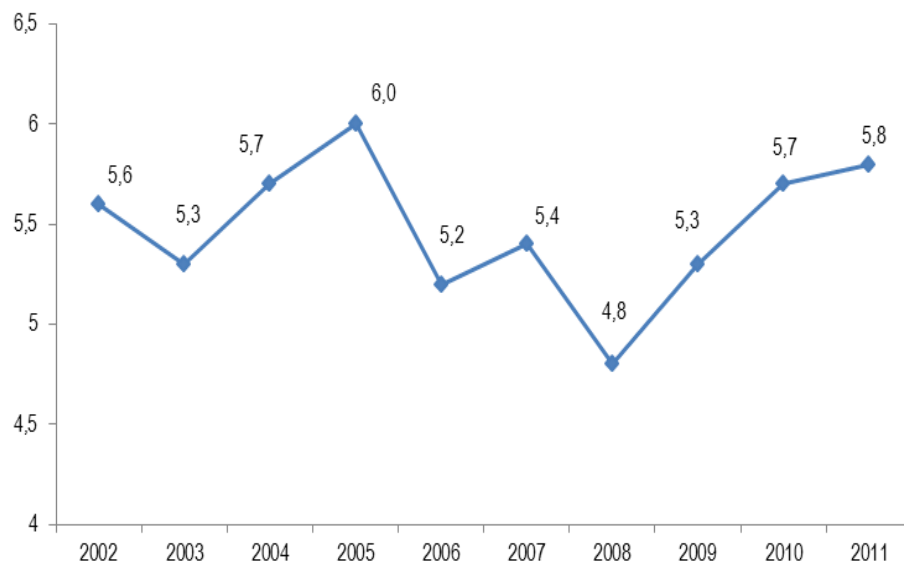


Figure 1.1. Preterm deliveries in Latvia, %
[Statistical Yearbook of Health Care in Latvia, 2012]

Mechanisms of causation of preterm labor include infection/inflammation, vascular disease, uterine over-distention, abnormal allograft reaction, an allergic-like phenomenon, progesterone deficiency, and cervical disorders [Lamont (b), et al., 2011].

Intrauterine infection is an important mechanism that might account for 25–40% of preterm births [Goldenberg, et al., 2000]. The mechanisms responsible for this process have been identified and involve pattern recognition receptors (like Toll like receptors) [Abrahams, et al., 2005], chemokines [Hamill, et al., 2008] or inflammatory

cytokines [Romero, et al., 1992]. Organisms most commonly gain access to the uterus and fetus by ascending from the vagina and cervix [Goldenberg, et al., 2000]. Intrauterine infection begins in the decidua, extends to the space between the amnion and chorion, and finally reaches the amniotic cavity and fetus [Goldenberg, et al., 2000]. Intrauterine infection is usually chronic and asymptomatic until labor begins or the membrane ruptures [Goldenberg, et al., 2008]. Histological placental inflammation is associated with both BV and aerobic bacteria, while funisitis is more often related more to the vaginal colonization of aerobic bacteria and AV in the early pregnancy [Rezeberga, et al., 2008]. For intrauterine infection to cause preterm birth, microorganisms must enter the amniotic cavity, must be recognized as foreign by the host immune system and bacterial numbers must reach a threshold to trigger an intra-amniotic inflammatory response, which in turn induces preterm labor [Romero, et al., 2000]. Intra-amniotic infection and inflammatory response is associated with fetal inflammatory response syndrome (FIRS) and fetal injury [Bashiri, et al., 2006]. FIRS is considered to be the fetal counterpart of systemic inflammatory response syndrome and is defined by an elevated fetal plasma interleukin (IL) 6 concentration and/or funisitis [Kim, et al., 2001]. It can progress to multiple organ dysfunctions, including the hemopoietic system, the adrenals, heart, kidneys, thymus, and skin and is associated with infection-related neonatal complications, bronchopulmonary dysplasia, impaired neurological outcomes, including cerebral palsy [Mittendorf, et al., 2003].

Whether a sole microorganism or combination of different rather commensal microorganism can induce spontaneous preterm pregnancy termination, is not clearly known. Studies show that high concentrations of bacteria like *U. urealyticum* correlate strongly with chorioamnionitis, preterm delivery, and lower birth weight [Kasper, et al., 2010]. Also, *M. hominis* in high concentration (more than 10^5 colony forming units) were more likely to be detected in patients with preterm birth, but not in the control patients [Lamont, et al., 1987; Rosenstein, et al., 1996; Kacerovsky, et al., 2012]. *E. coli* and GBS, thought to originate from the natural flora of pregnant women, are the major causes of early-onset neonatal infectious complications [Watt, et al., 2003]. Numerous studies have identified obstetric risk factors for early onset GBS disease, but others have recognized the increasing role of *E. coli* in the development of early neonatal disease and sepsis, especially in preterm babies [Lin, et al., 2011; Shane, et al., 2013]. *E. coli* vaginal colonization is observed in 3 to 20%, and GBS in 6.5 to 36% of pregnant women [Watt, et al., 2003; Barcaite, et al., 2008]. Neonatal pathogenicity due to *E. coli*

correlates with the presence of various virulence factors [Watt, et al., 2003]. There are some data that both *E. coli* and *Klebsiella* are associated with preterm birth [Carey, et al., 2005], and *Streptococcus viridians* (*S. viridans*) was more often found in culture associated with intra-amniotic infections [Veleminsky, et al., 2008], though these correlations have not been found in other studies. Prevention of perinatal transmission of GBS through the use of intrapartum antibiotics has generated concerns of antibacterial resistance and perinatal infections caused by antibacterial resistant organisms [Lamont (c), et al., 2011].

Also, there is a strong body of evidence that abnormal vaginal flora is associated with a range of adverse pregnancy outcomes, evidence compiled in the Cochrane Reviews does not support the concept of widespread screening for BV and treatment to prevent premature delivery [Brocklehurst, et al., 2013]. The Centre for the Disease Control of United States has recommended that only patients at high risk for preterm delivery – specifically only those with a history of a spontaneous preterm birth – should be treated with antibiotics, if they are found to have bacterial vaginosis [Romero, et al., 2004], even though recent Cochrane meta-analysis did not show reduction of preterm birth after treatment in this group of women [Brocklehurst, et al., 2013]. Although the natural history of abnormal vaginal flora in pregnancy is poorly understood, there are reports of spontaneous resolution in up to 31% in late pregnancy. This may be explained in part by an increase in the concentration of protective *Lactobacillus* species in the vagina and a decrease in the population of potentially pathogenic anaerobic bacteria [Ugwumadu, et al., 2004].

Since some women with BV have high concentrations of pro-inflammatory cytokines and others do not, Lamont recently has proposed that BV is a syndrome and not a single condition. Subsequently, outcomes of pregnancy in this subset of patients may vary [Lamont (a), et al., 2011]. In case of BV, increased risk for preterm delivery might be also affected by gene-environment interactions. For example, patients with symptomatic BV who carry a polymorphism for the tumour necrosis factor (TNF) α receptor gene have a significantly increased risk of preterm delivery [Gomez, et al., 2010].

AV is also independently associated with adverse pregnancy outcomes [Donders, et al., 2008; Donders, et al., 2009] and should be further investigated.

1.2.2. Non-obstetrical complications

In non-pregnant women, the presence of BV is associated with an increased risk of upper genital tract and sexually transmitted infections (Herpes simplex virus, *Chlamydia trachomatis*, *Trichomonas vaginalis*), with acquisition of Human immunodeficiency virus (HIV) and postoperative gynecological infections [Larsson, et al., 2002; Coleman, et al., 2007; Brotman, et al., 2010].

1.3. Risk factors of abnormal vaginal microflora

1.3.1. Sexual risk factors

Of all risk factors explored thus far, sexual behaviour-related characteristics have been most consistently associated with BV. New or multiple male partners, any female partner, ≥ 3 sex partners during the past year, oral sex, and no condom use have been found in many studies to be related to abnormal flora [Mbizvo, et al., 2001; Schwebke, et al., 2005; Beigi, et al., 2005; Fethers, et al., 2008]. On the other hand, BV is observed among sexually inexperienced girls, partner treatment studies have failed to document any benefit, and condom use was protective against incident BV, but not recurrent [Vogel (a), et al., 2006; Yotebieng, et al., 2009]. Studies in the population of pregnant women have not found any correlation between sexual practices and abnormal vaginal microflora [Vogel (a), et al., 2006]. Several lines of evidence corroborate that the BV incidence is increased by sexual activity, but also contradict exclusive heterosexual transmission. Data suggest that unprotected intercourse alters the vaginal environment, as alkaline prostatic ejaculate suppresses lactobacillary colonization, induces an imbalance at the level of the vaginal growth conditions and epithelial binding sites in favour of BV-associated microorganisms [Leppäluoto, 2008]. A second mechanism effecting the vaginal environment is vaginal penetration – it promotes the transfer of perianal, perineal, perivulvar bacteria to the vagina, thereby possibly inducing abnormal vaginal flora by introduction of enteric gram-negative bacteria [Verstraelen, et al., 2010]. These mechanisms therefore suggest that BV may behave as a sexually enhanced disease rather than an exclusively sexually transmitted infection [Verstraelen, et al., 2010].

1.3.2. Socioeconomic risk factors

Since education promotes increasing awareness, responsibility and knowledge of self-care, healthy lifestyle and behaviour [Koch, *et al.*, 2007], low social class (recipients of public benefits) and low level of education are also recognized as abnormal vaginal flora risk factors [Mbizvo, *et al.*, 2001; Vogel (a), *et al.*, 2006; Desseauve, *et al.*, 2012].

Also low income level is related to reproductive tract infections [Goto, *et al.*, 2005; Ashraf-Ganjoei, 2005].

1.3.3. Smoking

Tobacco use is recognized as a common AVF risk factor in many studies [Rezeberga, *et al.*, 2002; Vogel (a), *et al.*, 2006; Larsson, *et al.*, 2007; Desseauve, *et al.*, 2012]. The possible pathogenic mechanisms are not clear. One possible explanation is linked to the fact that cigarette smoke contains various chemical constituents, like nicotine, cintinine and benzopyrene diol epoxide. These chemicals have been found in the cervical mucus of smokers and may directly alter the vaginal microflora or may act by depleting Langerhans cells in cervical epithelium leading to local immunosuppression [Schwebke, *et al.*, 1999]. In pregnancy the effect of smoking on acquisition of BV could be due to a reduction in the placenta`s ability to produce estrogens; a factor which could result in decreased growth of *Lactobacillus* species [Barnea, 1994]. However, smoking is also known to be a social class indicator [Vogel (a), *et al.*, 2006].

1.3.4. Other risk factors

Factors such as vaginal douching, youth, and Africo-American ethnicity increase the risk for having an absence of protective lactobacillar flora [Beigi, *et al.*, 2005; Larsson, *et al.*, 2007; Klatt, *et al.*, 2010]. Vaginal douching can disrupt the normal vaginal environment, and, conversely, youth might be associated with sexual behaviour

risk factors. Different ethnicities have different vaginal microflora patterns [Anukam, *et al.*, 2006].

There are many reports about BV risk factors, but with a lesser extent about intermediate or AV flora.

1.4. Abnormal vaginal microflora diagnostic methods

1.4.1. Signs and symptoms

The signs and symptoms of AVF depend on the particular abnormal vaginal microflora type. Bacterial vaginosis is asymptomatic in at least half of the cases [Klebanoff, *et al.*, 2004]. Symptomatic BV is typically accompanied by foul-smell, profuse vaginal discharge in the absence of any appreciable signs of inflammation [Amsel, 1983; Donders, *et al.*, 2002]. Thin homogenous discharge however is poor predictor of BV, with sensitivity 48.8% and specificity 93.6% [Darwish, *et al.*, 2005]. Not all women complaining of and/or having abnormal vaginal discharge have BV, and abnormal vaginal flora is not always BV [Donders (a), 2007]. AV patients have different clinical signs with immunological inflammation reaction – such as red, inflamed vaginal mucosa, yellowish sticky discharge, high vaginal pH, and a “not fish-like” odour [Donders, *et al.*, 2002].

1.4.2. Clinical diagnosis

In 1983, Amsel *et al.*, introduced clinical diagnostic criteria for BV, which have proved particularly useful in clinical practice, and, hence, still in use today [Amsel, *et al.*, 1983] and accepted also in Latvia [Rezeberga, 2009]. The clinical diagnosis of BV is made if three of the four following signs are present – thin, greyish homogenous discharge; vaginal pH higher than 4.5; clue cells (vaginal epithelial cells with a heavy coating of bacteria that the peripheral borders are obscured) on saline wet mount, positive whiff test (detection/enhancement of fishy odour with or without addition of potassium hydroxide to the vaginal specimen). Amsel's criteria however have been criticized because two of them – appearance of the discharge and the appraisal of the

odour, are subjective and may lead to misdiagnosis. By contrast, having a pH higher than 4.5 are considered the most sensitive criterion, but the presence of clue cells is the single most specific predictor of BV [Verstraelen (b), et al., 2009].

1.4.3. Vaginal pH test

Vaginal pH is a quick, inexpensive, “bed-side” test. It correlates with the amount of lactobacilli in the vagina – mean vaginal pH is significantly lower in women with large numbers of lactobacilli [Rönnqvist, et al., 2006]. Therefore, pH reflects the vaginal environment. Studies have shown that an acidic vaginal pH significantly increases the binding capacity of lactobacilli to the vaginal epithelium and reduces the activity of several pathogenic bacterial enzymes such as sialidase [Hanna, et al., 1985].

Usually, elevated vaginal pH is discussed as a BV screening tool, but it can be increased by other vaginal abnormal flora types, infections and non-infectious conditions. In the Pastore study 22% of the cohort with BV had normal vaginal pH and 57% of women with a high pH did not have BV [Pastore, et al., 2002]. The sensitivity of elevated vaginal pH for the diagnosis of BV is 88.3 %, but specificity is less: 58.6% [Sodhani, et al., 2005]. *Trichomonas vaginalis* infection is strongly associated with vaginal pH ≥ 4.5 [Bell, et al., 2007]. An elevated vaginal pH level has been shown to be 100% sensitive and 92% specific in screening non-pregnant premenopausal women for aerobic bacterial pathogens [Caillouette, et al., 1997]. A large increase in pH correlates with severe AV [Donders, et al., 2002]. Rönnqvist showed that the prevalence of aerobic bacteria GBS in subjects with high numbers of lactobacilli is lower than in women with low or average numbers [Rönnqvist, et al., 2006]. Also *Chlamydia trachomatis* infection in women can be associated with increased vaginal pH [Das, et al., 2005].

There have been several studies of vaginal pH as a screening tool during pregnancy. Hauth in his study concluded, that women with vaginal pH of 5.0 or higher or a vaginal pH 4.5 or higher and a Gram stain score of 9–10 by Nugent had significantly increased rates of preterm birth and low birth weight [Hauth, et al., 2003]. Hoyme was able to lower preterm risk in the province of Tübingen by sending patients with increased self-measured vaginal pH for treatment, offering a simple method for self- detection high risk for preterm birth. This self-detection was to be seen as an alarm

sign to go and see a doctor for diagnosis and treatment [Hoyme, et al., 2004]. In Latvia vaginal pH measurement strips were not available until 2007, when the author and group of co-authors started the first studies about the use of this simple method in the evaluation of vaginal microflora and even presently it is not applied in most gynecology clinics in Latvia.

Still some limitations of pH based screening strategy exist: vaginal pH can increase after intercourse and vaginal douching, the presence of blood, and/or abundant cervical mucus [Hillier (a), 1993; Sagawa, et al., 1995].

Hence, use of routine vaginal pH in screening of abnormal vaginal microflora in pregnancy is somewhat controversial. Especially in Latvia, where this test is not widely used, it is important to analyse and to verify the validity of the vaginal pH test for use in the detection of abnormal vaginal microflora and in assessment for the risk of an adverse pregnancy outcome.

1.4.4. Microscopic examinations

In addition to the clinical presentation, laboratory diagnosis is critical in detection of abnormal vaginal microflora. Wet preparations and Gram stains are the two most useful microscopic procedures.

Wet preps are a simple, but efficient and reliable diagnostic method to determine the source of vaginal discharge [Franko, 2007]. It can be done as a bed-side test by a gynaecologist or other health care provider. A wet mount preparation is obtained by diluting the vaginal discharge with one drop of 0.9 percent normal saline solution and is examined using high power field (hpf) microscope [Egan, et al., 2000]. The presence of bacteria, unusual numbers of cells (red and white blood cells) and microorganisms are tested for. Wet mount preparations can have a sensitivity of 60% and a specificity of 98% for the detection of BV and a sensitivity of 60% and a specificity of 99% for the detection of trichomonas [Egan, et al., 2000]. Detection of lactobacillary grades and clue cells are more accurate when phase contrast is used in addition to simple light transmission microscopy [Donders, et al., 2009(b)]. Wet mounts can be classified according to Schröders's original classification, as further refined by Donders [Schröders, 1921; Donders, 1999]. This classification includes lactobacillary grades (LBG), the morphology of lactobacilli (normal, leptosomic or short morphotypes),

number of leucocytes (less than 10 per hpf, score zero, more than 10 per hpf, but less than 10 per epithelial cell, score one, and 10 or more per epithelial cell, score two), the presence of red blood cells and sperm cells, type of epithelial cells (cytolytic, superficial, intermediate, parabasal) and a screen for potential pathogens.

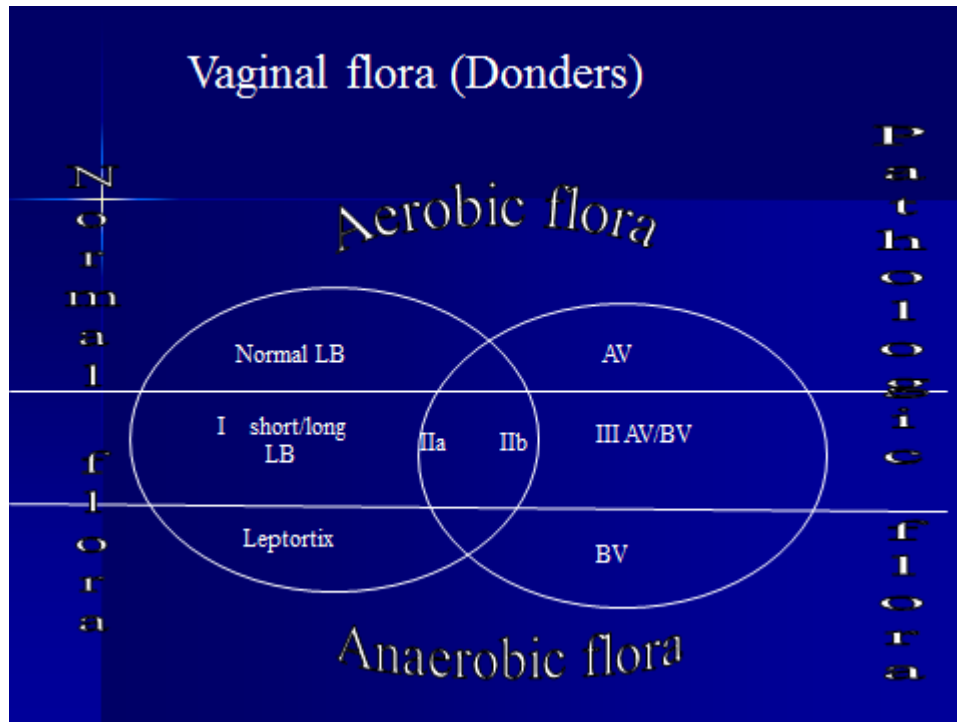


Figure 1.2. General overview of types of flora [Donders (a), 2007]

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

- LBG I consists of the predominant presence of *Lactobacillus* morphotypes, with very few coccoid bacteria presented,
- LBG IIa (intermediate) lactobacilli outnumbering other microorganisms,
- LBG IIb (intermediate) microorganisms outnumbering lactobacillary morphotypes,
- LBG III (completely disturbed flora) has no lactobacilli present. LBG III is further divided into three subgroups: BV, aerobic vaginitis, 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) are defined as full blown BV, while mixed areas with streaks of BV-like flora or sporadic clue cells

combined with other types of microflora are classified as partial BV [Donders (a) et al, 2007, Donders et al, 2009].

AV is diagnosed if short bacilli or cocci, leucocytes and/or parabasal cells are found [Donders, et al., 2002]. The severity of aerobic vaginitis is represented by a composite AV score: LBG is the basis to which any of the four following variables are added – leucocytes (scoring see above), presence of toxic leucocytes (granular appearance: score zero, if there are no such leucocytes, score one, if less than 50% were toxic and score two, if more than 50% of leucocytes have a toxic appearance), presence of parabasal cells (no parabasal cells, score zero, parabasal cells representing <10% of the epithelial cells, score one, parabasal cells representing >10% of the epithelial cells, score two), background flora (score zero, if the background flora is unremarkable, score one, if lactobacillary morphotypes are very coarse or resemble small bacilli and score two, if cocci are dominant) [Donders, et al., 2002], Table 1.1.

Table 1.1.

Aerobic vaginitis score

AV score	LBG	Number of leucocytes	Proportion of toxic leucocytes	Background flora	Proportion of parabasal epitheliocytes
0	I and IIa	≤ 10/hpf	None or sporadic	Unremarkable or cytolytic	None or < 1%
1	II b	> 10/hpf and ≤ 10/epithelial cell	≤ 50% of leucocytes	Small coliform bacilli	≤ 10%
2	III	> 10/epithelial cell	> 50% of leucocytes	Cocci or chains	> 10%

A composite score of one to two represents normality; score three to four corresponds to slight AV, five to six to moderate AV and a score above six means severe AV.

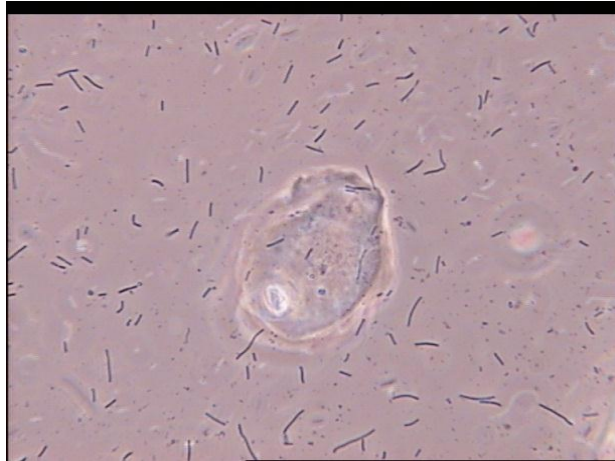


Figure 1.3. LBG I (pictures courtesy Professor G. Donders)

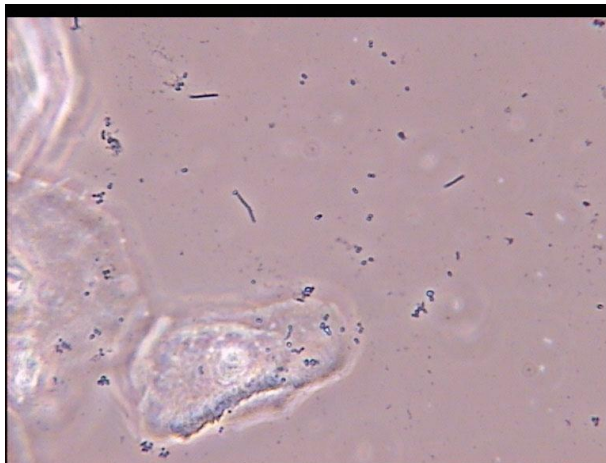


Figure 1.4. LBG IIb



Figure 1.5. LBG III BV

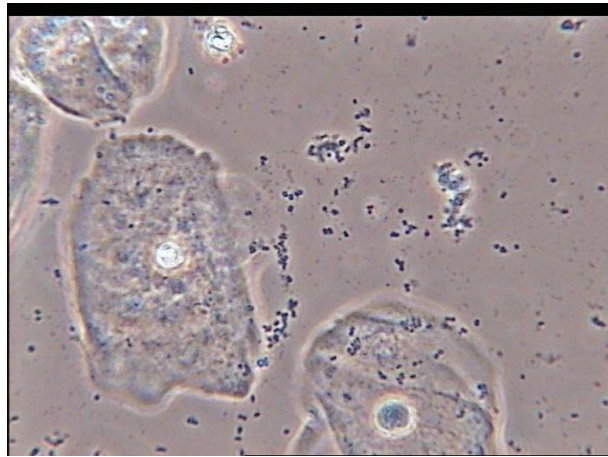


Figure 1.6. LBG III AV

Native microscopy allows better validation of lactobacillary flora grades, probably because of lactobacilli loss during Gram fixation and the staining process [Donders (a), et al., 2000]. Even dried-rehydrated samples are reliable [Donders, et al., 1996].

Among the laboratory methods, Gram-stained vaginal smears are the least expensive, require the least time to perform and are most widely available. However, this is the most interpretive of the laboratory methods [Nugent, et al., 1991]. To perform a Gram stain, vaginal fluid is collected on a glass slide, allowed to air-dry, stained in the laboratory and examined under an oil immersion microscope for the presence of specific bacteria [Verstraelen (b), et al., 2009]. The most widely performed Gram-stain scoring system was developed by Nugent et al. [Nugent, et al., 1991], Table 1.2.

Table 1.2.

Nugent scoring system

Score	<i>Lactobacillus</i> morphotypes	<i>Gardnerella</i> and <i>Bacteroides</i> spp. morphotypes	<i>Mobiluncus</i>
0	4+ (> 30 per hpf)	0	0
1	3+ (5-30 per hpf)	1+ (< 1 per hpf)	1-2+
2	2+ (1-5 per hpf)	2+ (1-5 per hpf)	3-4+
3	1+ (< 1 per hpf)	3+ (5-30 per hpf)	
4	0	4+ (> 30 per hpf)	

The *Nugent* score for the diagnosis of BV is ≥ 7 , intermediate flora 4–6 and for normal flora ≤ 3 . Overall, the *Nugent* scoring system for Gram-stained vaginal smears has shown high intracenter and intercenter reliability and reproducibility. However, practitioners are not usually familiar with performing an in-office Gram-stain-based diagnosis and *Nugent*'s criteria are widely applied in the absence of standardized pre-analytical and analytical conditions and interpretation, especially of the so called intermediate flora, is also a matter of concern [Donders (a), 2007; Verstraelen (a), et al., 2009].

To improve interpretation of Gram-stain smears, Claeys' modified *Ison&Hay* criteria were introduced – based on comparison between Gram-stain preps and deoxyribonucleic acid (DNA) – based techniques [Verhelst, et al., 2005]. Separate categories were recognized:

- Grade Ia – *L. crispatus* cell types were present (plump, mostly short rods)
- Grade Ib – other *Lactobacillus* cell types – smaller or more elongated were present
- Grade Iab – all previous types present
- Grade I – like – Gram positive rods, quite small, short, irregular
- Grade II (intermediate) – both *Lactobacillus* or *Bacteroides-Prevotella* cell types were present
- Grade III (BV) – *Lactobacillus* absent and only *Gardnerella*, *Bacteroides-Prevotella* and *Mobiluncus* cell types were present
- Grade IV – Gram positive cocci present.

1.4.5. Cultures based examinations

Since Albert Döderlein studies 100 years ago, lactobacilli have become recognized as the dominant members of normal vaginal microflora [Döderlein, 1892]. Further studies relying on the cultivation of organisms have shown that a diverse array of bacteria such as *Staphylococcus*, *Ureaplasmas*, *Corynebacterium*, *Streptococcus*, *Peptostreptococcus*, *Gardnerella*, *Bacteroides*, *Mycoplasma*, *Enterococcus*, *Escherichia*, *Veilonella*, *Candida* can be normally present, but typically in much lower

numbers [Larsen, et al., 2001]. However, when clinical diagnosis or microscopy findings are doubtful, cultures of BV-associated or aerobic bacteria (*E. coli*, streptococci, staphylococci) might help [Donders (a), 2007].

Positive culture results for genital mycoplasmas – *M. hominis*, *U. urealyticum* and *U. parvum*, are associated with pregnancy complications, such as late miscarriage, preterm birth, low birth weight and neonatal respiratory diseases [Taylor-Robinson, et al., 2007; Donders, et al., 2009] and may help to delineate the pathogenicity of certain types of abnormal vaginal flora [Donders (a), 2007]. On the other hand, genital colonization with ureaplasmas is common and there is not sufficient evidence from antibiotic treatment trials that ureaplasmas should be cultured and treated in all cases [Rayness-Greenow, et al., 2011]. Vaginal carriage of ureaplasmas is not reliably predictive of preterm labor, but there is an association when they are present in amniotic fluid or placenta [Carey, et al., 1991]. The mere presence of small numbers of *M. hominis* and ureaplasmas in the lower genital tract would not justify treatment [Taylor-Robinson, et al., 2011], since only high loads of *M. hominis* and ureaplasmas are associated with adverse pregnancy outcomes [Lamont, et al., 1987; Rosenstein, et al., 1996; Kasper, et al., 2010], but overgrowth of mycoplasmas is mostly detected when BV is diagnosed. This may not be the case for *M. genitalium*, which is potentially of greater pathogenicity, although it probably behaves more independently of BV than *M. hominis* [Taylor-Robinson, et al., 2011]. Adherence of mycoplasmas to host cells is a prerequisite for pathogenicity, and mycoplasmal membrane adhesion protein components are important. The 140-kDa adhesion of *M. genitalium* is probably the most extensively characterized [Taylor-Robinson, et al., 2011].

Although the cultivation of microorganisms is essential to fully understanding the physiology and phenotypic properties of organisms [Zhou, et al., 2010], cultivation of microbes as a means to characterize microbial communities in a natural ecosystem has major shortcomings. It is recognized that many microbes, like *M. genitalium*, *A. vaginae*, *Megasphaera* and *Leptotrichia* species [Zhou, et al., 2004; Anukam, et al., 2006], in different ecosystems, cannot be cultivated using standard culture techniques [Verstraelen (b), et al., 2009] and their identification requires the use of other cultivation-independent diagnostic methods.

1.4.6. Molecular diagnostics

Development and introduction of molecular-based techniques have provided new information about the composition of normal and abnormal vaginal microflora [Lamont (a), et al., 2011].

Bacterial DNA is extracted from samples, and is amplified using the polymerase chain reaction (PCR) using either universal or specific primers. As it is present in all bacteria, the most common target for molecular identification of bacteria is the small ribosomal subunit of the 16S ribosomal ribonucleic acid (rRNS) gene. Once the 16S rRNS gene has been sequenced, the variable regions can be used for species-specific PCR in a qualitative or quantitative manner. The sequences are aligned and compared with a large database [Oakley, et al., 2008].

Hillier has concluded, that: “Cultivation-independent techniques may show greater diversity by overcoming cultivation problems and the identification of fastidious organisms, but are limited by their tendency to sample only the most prevalent bacteria in a community, such as low-abundance species are likely to be missed” [Hillier, 2005]. Hence cultivation studies remain an important part of vaginal microbiology, and will need to be used in combination with cultivation-independent techniques [Margulies, et al., 2005]. Still cultivation and, even more, molecular diagnostic methods are expensive and not accessible in all settings.

1.4.7. Definition of abnormal vaginal microflora

There are different approaches to define abnormal vaginal microflora. Historically A. Döderlein using saline on microscopy showed a *Lactobacillus* deficient flora in women with postpartum endometritis [Döderlein, 1892]. Schröder started to implement this information in the clinical use and introduced lactobacillary grades, where LBG I corresponded to a healthy flora (predominant lactobacillar morphotypes), LBG II – intermediate flora (partial replacement of the lactobacilli by other bacteria) and LBG III – abnormal flora (lactobacilli completely replaced by other bacterial morphotypes) [Schröder, 1921]. In 1983 Amsel introduced clinical and later Nugent microbiological BV diagnostic criteria [Amsel, 1983; Nugent, 1991]. The Nugent scoring system accounts for three bacterial cell morphotypes quantitated from 0

to 4+ in the high power field– *Lactobacillus* (large Gram positive rods), *Gardnerella* and *Bacteroides* (small Gram variable or Gram negative rods) and *Mobiluncus* (curved Gram variable rods), and score for BV is ≥ 7 .

However AVF is not only BV, but also AV, mixed abnormal and/or intermediate flora vaginitis [*Gardner, 1968; Hay, et al., 1994; Sobel, 1994; Donders, et al., 2002; Ugwumadu, et al., 2004*], but Nugent score combine women with different vaginal microflora in a single category and does not include assessment of the potentially pathogenic aerobic flora. There are some studies showing that the absence of *Lactobacillus* is a more powerful predictor of preterm birth than solely presence of BV [*Hay, et al., 1994*]. Donders has further refined *Schröder* classification and subdivided LBG II into a less severe LBG IIa and LBG IIb, and also introduced scoring system for aerobic vaginal microflora [*Donders (a), 2007*]. According to Donders modified *Schröder* classification abnormal vaginal microflora on wet mounts is defined in case of LBG III and LBG IIb [*Donders (a), 2007*].

1.5. Treatment of abnormal vaginal microflora

Treatment of AVF is primarily targeted at managing symptoms, though, because of possible complications, it also involves treatment of asymptomatic cases. While over the past six decades antibiotics have served as the mainstay of AVF treatment, they are currently poorly armed to ensure normal vaginal environment in the long run [*Verstraelen (b), et al., 2009*], in larger meta-analysis are not effective in reducing preterm delivery risk [*Brocklehurst, et al., 2013*], can cause microbial resistance [*Austin, et al., 2005*], cause a shift in pathogens causing neonatal sepsis [*Baltimore, 2007*] and are not excepted by all women, especially in pregnancy.

Several other treatment modalities have been investigated. They can be categorized as treatment with antiseptics and disinfectants, with vaginal acidification or buffering agents, probiotics as a mono-therapy or in conjunction with antibiotics [*Verstraelen (b), et al., 2009*].

1.5.1. Standard treatment with antibiotics

Oral 500 mg metronidazole twice daily for seven days was the first BV treatment regimen studied [Pfeifer, et al., 1978]. Ten years later, an oral regimen of 300 mg clindamycin twice daily was proven to be as effective as the metronidazole regimen [Greaves, et al., 1988]. Since then, metronidazole and clindamycin have been the drugs of choice in the treatment of BV.

Metronidazole and other nitroimidazoles target anaerobic bacteria, including *Bacteroides fragilis* and protozoans. Conversely, clindamycin, an antibiotic of the lincosamide group, has a larger scale antibacterial activity – against anaerobic gram-negative, aerobic gram-positive bacteria, *Mobiluncus species (spp.)* and *M. hominis* [Mylonas, 2011; Taylor-Robinson, et al., 2011]. Although *in vitro* studies have demonstrated that metronidazole and other nitroimidazoles are largely inactive against *G. vaginalis*, *M. hominis*, *U.urealyticum*, *A. vaginae*, *S. aureus* and streptococci, metronidazole administration to women with BV is associated with a treatment success rate similar to clindamycin. One potential explanation is that the efficacy of metronidazole has been attributed to the hydroxyl metabolite of the drug *in vivo* which is effective against the organisms involved in BV, and it changes microbial flora by eradication of bacteria susceptible to it and this favours cure of BV [Bradshaw(a), et al., 2006; Workowski, et al., 2007].

There have been many trials on treatment of BV with antibiotics, but they have never been subjected to a meta-analysis, due to methodological heterogeneity across studies [Verstraelen (b), et al., 2009]. The systematic review of the British Medical Journal (BMJ) Clinical Evidence series concluded that oral/vaginal metronidazole/clindamycin regimens have short-term benefits, with no obvious differences between the different recommended regimens [Joeseof, et al., 2005]. Therefore, several alternative antibiotics and regimens have recently been evaluated for their clinical efficacy.

5-nitroimidazole derivatives, other than metronidazole, such as ornidazole, tinidazole, are at least as effective as metronidazole itself, because of their pharmacokinetics [Thulkar, et al., 2012]. There was no extra therapeutic effect at all associated with the administration of azithromycin in combination with metronidazole [Schwebke, et al., 2007].

Rifaximin – a semisynthetic rifamycin derivative with a broad antimicrobial spectrum [Scarpignato, et al., 2005; Rivkin, et al., 2011], is another new antibiotic with a good safety profile because of its negligible grade of systemic absorption. Since its antibacterial activity covers *G. vaginalis* and other pathogens responsible for urogenital infections [Hoover, et al., 1993], rifaximin could be a suitable alternative for the local treatment of BV. There are some studies showing improvement of vaginal microflora after rifaximin treatment [Cruciani, et al., 2012] and its efficiency similar to clindamycin vaginal cream [Donders, et al., 2013].

AV treatment has not been as extensively studied as BV, albeit because of larger scale antibacterial activity, clindamycin vaginal gel, intermittent use of vaginal estrogens, vaginal pH lowering substances and intra-vaginal corticosteroids, can be successfully used. Despite the current success of clindamycin vaginal therapy, more needs to be known about this condition, so that better directed treatments can be devised [Ledger, et al., 2010].

There have been some reports of antimicrobial resistance of BV-associated anaerobes. Resistance to metronidazole following therapy is rare, except in cases of *A. vaginae*-associated BV [Verstraelen (b), et al., 2009]. By contrast, antibiotic resistance can rapidly develop to clindamycin [Kurkinen-Raty, et al., 2000].

1.5.2. Recurrence of bacterial vaginosis

Recurrence of BV even after a proper antibacterial treatment course is high, with some 30–50% of women experiencing a BV relapse within 2–3 months. It remains unclear at present whether BV recurrence reflects resistance, recurrence and /or reinfection [Verstraelen (b), et al., 2009], while change of partner was strongly associated with relapse of BV in some studies [Schwebke, et al., 2011; Larsson, et al., 2011]. Presence of *A. vaginae* is also an indicator of BV recurrence [Bradshaw (a), et al., 2006]. Persistence of the microbial biofilm after treatment might be associated with relapse of BV [Svidsinski, et al., 2008]. In Sobel's study, long term antibacterial suppression treatment did benefit in decreasing BV relapse rates, and, furthermore, it caused an increased occurrence of vaginal candidosis [Sobel, et al., 2006]. Larsson showed better results, although this study was not placebo controlled, non-conformistic and suprathreshold doses of medication were used [Larsson, et al., 2011].

In the Brandt's clinical trial the intravaginal application was as effective as the oral administration of metronidazole in treating BV. However, significantly more adverse events (nausea, abdominal pain, metallic taste) were reported after oral application of 2 grams metronidazole and probably led to a lower patient compliance [Brandt, et al., 2008].

1.5.3. Alternative treatment with probiotics

Probiotics are defined as live micro-organisms which, when administered in an adequate amount, confer a health benefit to the host [Andreu, 2004]. Probiotics have been shown to displace and kill pathogens and modulate the immune response [Reid, et al., 2003].

Various in-vitro studies have shown that specific strains of lactobacilli inhibit the growth of bacteria causing BV by producing H₂O₂, lactic acid, bacteriocins and inhibit the adherence of *G. vaginalis* to the vaginal epithelium [Mastromarino, et al., 2002]. Most relevant clinical trials have suggested that intravaginal administration of *L. acidophilus* or oral administration of *L. acidophilus* or *L. rhamnosus* GR-1 and *L. fermentum* RC-14 is able to increase the numbers of vaginal lactobacilli, restore the vaginal microbiota to normal and cure women with BV [Neri, et al., 1993; Shalev, et al., 1996; Reid (a), et al., 2003]. Although, several other trials have found that intravaginal instillation of lactobacilli had no significant effect on the treatment of acute BV [Reid, et al., 2004; Ozkinay, et al., 2005; Eriksson, et al., 2005].

Adjuvant probiotics, as a supplement together with or following antibacterial treatment of BV has been studied. Larsson, in a randomized double blind placebo controlled study using vaginal *L. rhamnosus* and *L. gasseri* followed after 2% clindamycin vaginal cream, could not show improvement of BV therapy during the first month, but adjunct treatment with lactobacilli significantly increased the time to relapse [Larsson, et al., 2008]. Some data indicate that more aggressive BV treatment with antibiotics (2% clindamycin vaginal cream together with oral clindamycin 600 mg per day 7 days, followed with vaginal metronidazole gel 5 days) combined with specific *Lactobacillus* strain and partner treatment can provide long lasting cure in some cases [Larsson, et al., 2011].

However, further randomized, controlled trials with larger samples of women with BV, in which lactobacilli are compared either with placebo or antibiotics, need to be conducted to draw definitive conclusions about whether probiotics represent an effective and safe method for treating women with BV [Falagas, et al., 2007].

1.5.4. Alternative treatment with antiseptics

Antiseptics can be considered from the same perspective as antibiotics. They have antibacterial actions against a wide range of aerobic and anaerobic bacteria, and they non-specifically disrupting bacterial cells membranes [Emilsson, 1977]. There are only sporadic reports of antimicrobial resistance against antiseptics agents, and these are safe for mucosal applications in appropriate concentrations and without systemic exposure [Verstraelen (b), et al., 2009].

Antiseptics have been administered to women with BV as vaginal suppositories, bioadhesive gel formulations and occasionally loaded on pessaries include benzydamine, chlorhexidine, dequalinium chloride, polyhexamethylene biguanide, povidone iodine, and hydrogen peroxide [Verstraelen (b), et al., 2009]. Stray-Pedersen has shown that vaginal douching with 0.2% chlorhexidine during labour can reduce both maternal and early neonatal infectious morbidity, but the main target for this treatment was to reduce the transmission of *E. coli*, GBS and *S. aureus* to prevent early-onset neonatal sepsis. [Stray-Pedersen, et al., 1999]. While there are some studies with cure rates at least as high as antibiotics, there are only a small number of such studies and, therefore no firm conclusions on these antimicrobial agents can be drawn from existing trials [Verstraelen (b), et al., 2009]. In a recent trial Weissenbacher et al tested dequalinium chloride vaginal tablet versus (vs.) clindamycin in a single blind, randomized trial [Weissenbacher, et al, 2011] found no difference in clinical cure rates of BV, and a non-significant reduction of candida infection in the dequalinium group.

1.5.5. Alternative treatment with acidifying agents

Verstraelen in his article has summarized, that “Alkalinisation of the vaginal milieu leads to decreased epithelial adherence of the lactobacilli and gives free rein to

the overgrowth of typical BV-associated microorganisms. Acidifying the vagina with naturally occurring acids like lactate or buffering the vagina against alkali exposure may enhance lactobacillary colonization and prevent anaerobic overgrowth” [Verstraelen (b), et al., 2009].

However, in recent trials the acidifying approach was not clearly proven to be effective: 0.92% acetic acid-based gel applied twice daily for seven days was not superior to a placebo [Holley, et al., 2004], lactic acid suppositories similarly placebo reduced BV in 49% compared to 83% treated with metronidazole [Boeke, et al., 1993]. Conversely polycarbophil-carbopol-based vaginal gel during a five week treatment course, however, was found to be effective – 93% were clinically cured, in comparison with 6% in the placebo group [Fiorilli, et al., 2005]. Probably the impact of different acidic substances depends on the rate of absorption, metabolism, adhesiveness to vaginal mucosa and clearance by vaginal discharge [Holley, et al., 2004].

One of the potentially beneficial, new acidifying therapeutic approaches is to use ascorbic acid per vaginam [Verstraelen (b), et al., 2009]. Ascorbic acid is available as silicon-coated tablets containing 250 mg vitamin C at a rate that ensure long-lasting reduction in vaginal pH and does not produce irritation [Polatti, et al., 2006]. Bacteria like lactobacilli, capable of reproduction even at low pH are favoured in growth, but undesirable anaerobes are severely inhibited by vitamin C-induced vaginal acidification [Petersen, et al., 2004].

There have been only 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 days mono-therapy regimen in the management of BV, but there are no data about long term use of vaginal ascorbic acid and its influence on different abnormal microflora types, let alone in pregnant women.

There are some promising results with vaginal acidifiers for the long-term treatment of recurrent BV [Andersch, et al., 1986]. Nonetheless, better designed trials are needed to evaluate the potential role of vaginal acidifying and buffering agents in the treatment and prevention of recurrent BV [Verstraelen (b), et al., 2009].

1.5.6. Treatment of abnormal vaginal flora in pregnancy

There are many studies showing ascending vaginal infections to be closely related to early/late miscarriage, premature rupture of membranes, preterm birth and newborn infections [Mylonas, 2011]. As a result, considerable efforts have been dedicated to evaluate antimicrobial therapy as an intervention to prevent preterm deliveries. Although meta-analysis showed reductions in maternal infections, no statistical difference was found in frequency of preterm birth and neonatal outcomes [King, et al., 2002; Brocklehurst, et al., 2013].

Several studies have subsequently evaluated the role of antibiotics in preventing preterm birth in BV cases. Early studies with metronidazole and clindamycin concluded that it might be associated with a reduction in preterm deliveries [Hauth, et al., 1995; Mc Gregor et al., 1995]. But, Carey's large prospective, randomized, double blind, controlled trial failed to show any effect on preterm birth reduction [Carey, et al., 2000]. Furthermore, in some studies the incidence of preterm delivery was actually higher in women treated with metronidazole than with placebo [Klebanoff, et al., 2001; Odendaal, et al., 2002; Sheennan, et al., 2005].

Limitations of the experimental design of these randomized clinical trials were called in to explain these unexpected failures According to Lamont , for antibiotics to be effective in reducing the rate of preterm delivery, several criteria must be met [Lamont (b), et al., 2011].:

- 1) antimicrobials must be effective against the target organism or the clinical condition under study (like BV);
- 2) antimicrobials should be used only in women who can benefit because they are at substantial risk for infections and infection-related condition;
- 3) antimicrobials must be used early enough so that eradication of the microorganisms would be followed by resolution of any inflammatory response and its unintended consequences (damage of the chorioamniotic membranes, microbial invasion of the amniotic cavity, fetal microbial invasion and fetal inflammation)".

Others have found that BV rarely develops as pregnancy progresses, but rather persists from the first or second trimester in those women who deliver preterm [Hay, et al., 1994], therefore clindamycin administered early in the second trimester to women who were positive for BV seemed to be more effective [Ugwumadu, et al., 2004;

Lamont, 2005]. A meta-analysis of randomized trials of clindamycin in pregnant women with BV before gestational 22 weeks has demonstrated that:

- 1) the mean gestational age at delivery was significantly higher in women treated with clindamycin;
- 2) the rate of preterm birth before 37 weeks of gestation is significantly lower than in the control group only for oral, but not vaginal clindamycin;
- 3) the rate of late miscarriage was lower in the clindamycin group;
- 4) there were no statistically significant differences in the risk of preterm birth < 33 weeks of gestation, low birth weight, admission to neonatal intensive care unit, stillbirth, peripartum infections [Lamont (b), et al., 2011].

Although there is some suggestion that treatment before 20–22 weeks' gestation may reduce the risk of preterm birth, this needs to be further verified.

Donders has recognized that absence of lactobacilli, partial BV and *M. hominis*, but not full BV are associated with an increased risk for preterm delivery after 24 weeks of gestation [Donders, et al., 2009]. Which might explain why metronidazole – single anti-anaerobic agent, is not as effective as clindamycin – broad spectrum antibiotic, active against anaerobic, aerobic organism like streptococci, *S. aureus*, *Mobiluncus spp.* and *M. hominis* [Donders, et al., 2009; Taylor-Robinson, et al., 2011] and has anti-inflammatory properties [Hand, et al., 1990]. Indeed meta-analyses such as that done by Cochrane provide little evidence, that screening and treating all low risk pregnant women with asymptomatic BV will prevent preterm birth and its consequences, however when screening criteria includes women with AVF (not only BV), there are 47% reduction of preterm birth [Brocklehurst, et al., 2013].

A review of eight randomized trials found that antibiotics reduce the risk of prelabour rupture of the membranes and the risk of preterm birth in a subgroup of pregnant women with BV who had experienced a previous preterm birth [Thinkhamrop, et al., 2009]. As a consequence, the guidelines of the Association of Latvian Gynecologists and Obstetricians recommend screening and treating BV only in high risk pregnant women with a history of late miscarriage/preterm birth and symptomatic BV [Rezeberga, 2009]. Nevertheless later updated Cochrane review pointed, that antibacterial treatment of BV did not affect the risk of subsequent preterm birth in women with an adverse history, but it increased the risk of side-effects sufficient to stop or change treatment [Brocklehurst, et al., 2013].

Not only lack of evidence about the efficiency of antibiotics to prevent infections related preterm deliveries, also even administration of antibiotics during pregnancy leads to concerns among doctors and patients about safety issues and antibacterial resistance. It is from this point of view that has led to the current research into non-antibacterial treatment options. Especially during pregnancy a treatment that restores normal vaginal microflora and acidity without systemic effects could be preferable to any other treatment [Othman, et al., 2012]. Probiotics appear to treat infections in pregnancy, but there are currently insufficient data from trials to assess an impact on pregnancy outcomes [Kraus-Silva, et al., 2011; Othman, et al., 2012]. Meta-analysis did not find any evidence to support the use of vaginal chlorhexidine during labour in preventing maternal and neonatal infections [Lumbiganon, et al., 2011].

Although there is some evidence that acidifying agents, like vaginal ascorbic acid, improve abnormal vaginal flora [Petersen, et al., 2011], there are no studies focused on the population of pregnant women and the prevention of infections related preterm births.

Vitamin C or ascorbic acid is essential to the human body for many systemic functions – it is an antioxidant, and important in collagen and synthesis of other substances [Hathcock, 2004]. Antioxidants have been proposed as potential inhibitors of premature fetal membrane remodelling and preterm rupture of fetal membranes, therefore, the use of ascorbic acid can have additional benefits in pregnancy – to prevent fetal membrane weakening and preterm rupture of membranes, and preventing preterm birth [Mercer, et al., 2010]. Although a low intake of vitamin C may be associated with complications in pregnancy such as pre-eclampsia, anaemia and intrauterine growth restriction, there are too little data to say that oral vitamin C supplementation either alone or in combination with other supplements is beneficial during pregnancy [Rumbold, et al., 2004; Conde-Agudelo, et al., 2011].

Odendaal in a randomized clinical trial among high preterm birth risk pregnant women with BV demonstrated that women receiving metronidazole had preterm deliveries more often than a control group receiving vitamin C orally as a placebo [Odendaal, et al., 2002]. However, further randomized controlled study of oral vitamin C and placebo did not show reduction of preterm birth rate in the treatment group [Schoeman, et al., 2005].

Although oral vitamin C was not proven to be effective in reduction of preterm delivery, research of vaginal vitamin C treatment as a modality to improve abnormal vaginal microflora in pregnancy and pregnancy outcome would be very interesting.

2. NOVELTY OF THE STUDY

- 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 in pregnant and non-pregnant women is evaluated.
- Abnormal vaginal microflora is defined in cases with both vaginal pH ≥ 4.5 and decreased numbers or absent *Lactobacillus* morphotypes on wet mounts.
- Different types, including BV, AV, intermediate and mixed flora were assessed.
- The study focuses on the application of simple and inexpensive bed-side AVF diagnostic tests.
- Correlations between vaginal pH, wet mounts, cultures and different types of abnormal vaginal microflora are analysed.

3. MATERIALS AND METHODS

3.1. Study population, time frame and settings

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 ≥ 4.5 were enrolled in a prospective intervention trial. Following enrolment of a woman with pH ≥ 4.5 , the next two pregnant seen in 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 of 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 ≥ 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 ascorbinic 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.

3.1.1. Summary of inclusion criteria

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

3.1.2. 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 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.

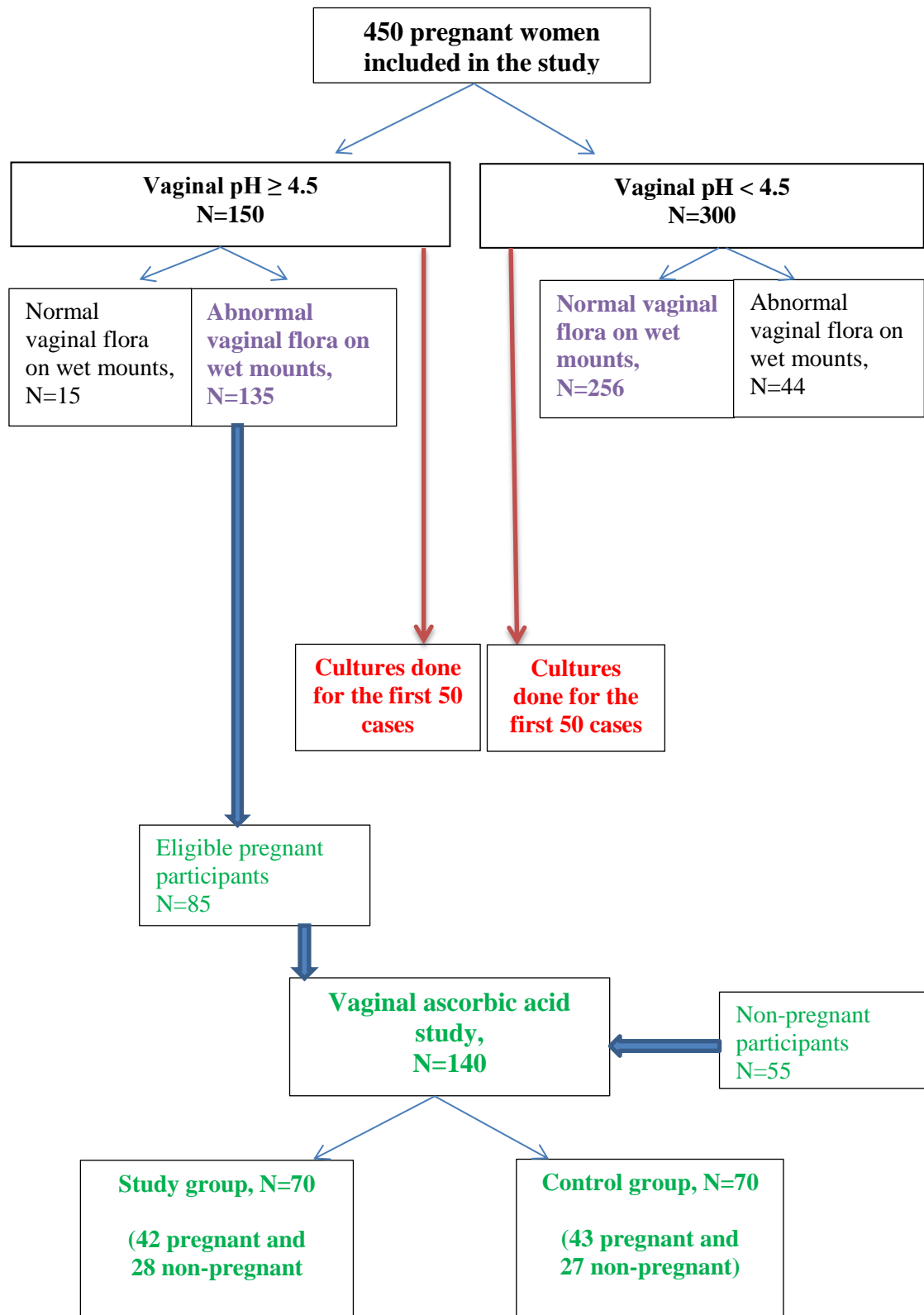


Figure 3.1. Flowchart of participant inclusion

3.2. Study design

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 outcome was assessed in the prospective cohort.

3.3. Ethical issues

The study was approved by the Ethics Committee of Rīga Stradiņš University (Appendix No. 1). All participants were informed, asked to sign an informed consent (Appendices No. 2, 3) 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 of Latvia No. 611 “Organization of delivery services”. The studies were done according to principles deriving from the Helsinki declaration.

3.4. Methods

There were three principle visits in the study: inclusion, follow-up and post-delivery. There was completed a questionnaire (Appendix No. 4), performed physical examination and collected specimen 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 (b), et al., 2007*].

Vaginal pH ≥ 4.5 was considered abnormal (elevated) [Amsel, et al., 1983]. Then one droplet of 10% KOH was added to evaluate “fishy” order of discharge [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. Patterns with considerably decreased or absent *Lactobacillus* morphotypes (LBG IIb and LBG III) were considered as AVF [Donders, 1999].

From the study population, vaginal cultures were done in the first 50 pregnant women with vaginal pH ≥ 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 *Str. pyogenes*, *Str. agalactiae* (or GBS), *Viridans* group streptococci, enterococci, *S. aureus*, *Candida 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 haemolysis. If there was β – haemolysis, 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 – haemolysis 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 *U. urealyticum* and *M. hominis* [Mahon, 2000]. More than 10⁵CFU/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 \geq 4.5 and abnormal vaginal flora 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, Table 3.1.

Table 3.1.

Vaginal vitamin C study overview assessments

	Visit 1: inclusion	Visit 1.1.: randomization	Visit 2: follow-up visit 4 months after randomization or 2–3 weeks after the last Vitamin C tablet	Visit 3: pregnancy outcome (for pregnant participants)
Informed consent	X			
Inclusion data	X			
Vaginal pH	X		X	
Vaginal smear microscopy	X		X	
Randomization in to the study group (receive medication) or control group		X		
Check diary			X	X
Check adverse events			X	X
Perinatal data				X

Intervention group participants made records in the diaries (Appendix No. 5) 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 late 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 (Appendix No. 4).

Visit 3 was 6–8 weeks after delivery. Questionnaires (Appendix No. 4) about pregnancy outcome were completed during that visit.

3.5. Outcome assessment

- 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.
- Efficacy 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 groups;
- intervention AVF and non-intervention AVF groups;
- vitamin C and control groups;
- vitamin C and all non-intervention AVF groups.

3.6. Statistical analysis

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 formula:

$$\text{Sensitivity} = a / (a + c)$$

(the proportion of those with increased vaginal pH, according to the standard tests - wet mounts, that are labelled positive by the vaginal pH test).

$$\text{Specificity} = d / (b + d)$$

(the proportion of those with normal vaginal pH, according to the standard test – wet mounts, that are labelled negative by the vaginal pH test).

a – number of individuals with abnormal vaginal flora and elevated vaginal pH: true positive.

b – number of individuals with normal vaginal flora and elevated vaginal pH: false positive.

c – number of individuals with abnormal vaginal flora and normal vaginal pH: false negative.

d – number of individuals with normal vaginal flora and normal vaginal pH: true negative.

a + c – total number of individuals with abnormal vaginal flora.

b + d – total number of individuals with normal vaginal flora [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.

4. RESULTS

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

4.1. Factors associated with abnormal vaginal microflora

Pregnant women with AVF compared to those with normal vaginal microflora were more often younger than 25 years, less educated, unmarried and single or not living with a partner, smoked more often before and during pregnancy, and had more often BV, but rarely *U. urealyticum*, candidas at least once during the year before pregnancy ($p < 0.01$), they also had a trend to be unemployed/housewife, 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, new partner, Table 4.1.

Table 4.1.

Comparison of abnormal vaginal microflora risk factors

Characteristics	Total (n=391) N (%)	Vaginal pH <4.5 and LBG I-IIa (n=256) N (%)	Vaginal pH ≥ 4.5 and LBG IIb-III (n=135) N (%)	P value
Age		28.5 \pm 4.6	27.8 \pm 5.2	0.153
Age groups:				0.008
< 25 years old	89 (23)	47 (18)	42 (31)	
26–29 years old	166 (42)	120 (47)	46 (34)	
≥ 30 years old	136 (35)	89 (35)	47 (35)	
Education:				< 0.001
primary	20 (5)	7 (3)	13 (10)	
secondary	145 (37)	74 (29)	71 (52)	
higher	226 (58)	175 (68)	51 (38)	
Employment:				0.060
employed	314 (80)	214 (84)	100 (74)	
housewife	45 (12)	23 (9)	22 (16)	
unemployed	32 (8)	19 (7)	13 (10)	

Continuation of Table 4.1.

Characteristics	Total (n=391) N (%)	Vaginal pH <4.5 and LBG I-IIa (n=256) N (%)	Vaginal pH ≥4.5 and LBG IIb-III (n=135) N (%)	P value
Marital status:				< 0.001
married	194 (50)	144 (56)	50 (37)	
living with partner	191 (49)	111 (43)	80 (59)	
not living with partner	6 (1)	1 (0.4)	5 (4)	
Smoking before pregnancy	121 (31)	57 (22)	64 (47)	< 0.001
Smoking during pregnancy	36 (9)	12 (5)	24 (18)	< 0.001
Nulliparas	197 (50)	129 (50)	68 (50)	0.997
Miscarriage in past history	75 (19)	49 (19)	26 (19)	0.997
Preterm birth in past history	11 (3)	5 (2)	6 (4)	0.199
Concomitant diseases	60 (15)	43 (17)	17 (13)	0.273
Use of medications	21 (5)	16 (6)	9 (7)	0.873
≥ 6 lifetime sexual partners	69 (18)	45 (18)	24 (18)	0.946
≥ 2 sexual partners during last year	25 (6)	12 (4)	13 (10)	0.058
Frequency of intercourses ≥ 10 times during last month	62 (16)	35 (14)	27 (20)	0.188
Sexual relationships with the last partner ≤ 6 months	91 (23)	53 (21)	38 (28)	0.208
Intercourse previous 48 hours	113 (30)	65 (27)	48 (36)	0.066
Genital infections in the year before pregnancy:				
<i>C. trachomatis</i>	5 (1)	4 (2)	1 (0.7)	0.663
<i>T. vaginalis</i>	2 (0.5)	1 (0.4)	1 (0.7)	1.000
herpes genitalis	9 (2)	5 (2)	4 (3)	0.504
bacterial vaginosis	34 (9)	16 (6)	18 (13)	0.019
candidosis	96 (25)	70 (28)	26 (19)	0.074
<i>U. urealyticum</i>	24 (6)	22 (9)	2 (2)	0.004
<i>M. hominis</i>	4 (1)	4 (2)	0	0.303
Antibiotics 2 weeks before sampling	6 (2)	4 (2)	2 (2)	1.000
Topical treatment of vaginal infections 2 weeks before sampling	13 (3)	9 (4)	4 (3)	1.000

Analysis of single risk factors associated with abnormal vaginal microflora is presented in Table 4.2.

Table 4.2.

Univariate analysis of significant abnormal vaginal microflora risk factors

Characteristic	pH<4.5&LBG I-IIa (n=256) N (%)	pH≥4.5&LBG IIb-III (n=135) N (%)	Odds ratio (OR) (95% confidence interval-CI)	P value
Age < 25 years	47 (18)	42 (31)	1.7 (0.9–2.9)	0.059
Primary (≤ 9 classes) education	7 (3)	13 (10)	6.4 ((2.4–16.8)	< 0.001
Secondary (12 classes) education	74 (29)	71 (52)	3.3 (2.1–5.0)	< 0.001
Not married, living with partner	111 (43)	80 (59)	2.1 (1.3–3.2)	0.001
Single/not living with partner	1 (0.4)	5 (4)	14.4 (1.6–126.2)	0.016
Housewife	23 (9)	22 (16)	2.1 (1.1–3.9)	0.026
≥ 2 sexual partners during last year	12 (4)	13 (10)	2.2 (0.1–4.9)	0.063
Intercourse previous 48 hours	65 (27)	48 (36)	2.1 (0.4–4.1)	0.067
Smoking before pregnancy	57 (22)	64 (47)	3.14 (2.0–4.9)	< 0.001
Smoking during pregnancy	12 (5)	24 (18)	4.4 (2.1–9.1)	< 0.001
BV one year before pregnancy	16 (6)	18 (13)	2.3 (1.1–4.7)	0.021
<i>U. urealyticum</i> one year before pregnancy	22 (9)	2 (2)	0.2 (0.4–0.7)	0.014
<i>Candida</i> one year before pregnancy	70 (28)	26 (19)	0.6 (0.4–1.0)	0.075

Women with primary education more often were smoking before pregnancy, as well those with primary or secondary education were more unmarried, Table 4.3.

Table 4.3.

Associations between educational level and other social factors

Characteristics	Higher education N (%)	Secondary education N (%)	Primary education N (%)	P value
Smoking before pregnancy:				< 0.001
yes	39 (17)	66 (46)	16 (80)	
no	187 (83)	79 (54)	4 (20)	
Smoking during pregnancy:				< 0.001
yes	4 (2)	24 (17)	8 (40)	
no	222 (98)	121 (83)	12 (60)	
Marital status:				< 0.001
married	142 (63)	46 (32)	6 (30)	
living with partner	82 (36)	96 (66)	13 (65)	
not living with partner	2 (1)	3 (2)	1 (5)	

Most of participants in all age groups had a high education (Table 4.4), but women with only primary education were more often younger (43%) than the age of 25 years ($p=0.05$).

Table 4.4.

Associations between age and educational level

Educational level	≥ 30 years N (%)	26–29 years N (%)	≤ 25 years N (%)	P value
Higher education	65 (73)	86 (72)	24 (51)	0.05
Secondary education	22 (25)	32 (26)	20 (43)	
Primary education	2 (2)	2 (2)	3 (6)	

Multivariate logistic regression analysis showed that the highest risk of abnormal vaginal flora is associated independently with low level of education, smoking before pregnancy and history of BV in a year before pregnancy, Table 4.5.

Table 4.5.

Multivariate analysis of significant abnormal vaginal microflora risk factors

Characteristic	OR (95% CI)	P value
Age < 25 years (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
<i>U. urealyticum</i> 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, but *U. urealyticum* was more frequently found in normal women and not or rarely in BV or AV women, Table 4.6.

Table 4.6.

Comparison of BV and AV microflora risk factors

Characteristics	Vaginal pH < 4.5 and LBG I-IIa (n=256) N (%)	Vaginal pH ≥4.5 and LBG III AV (n=21) N (%)	P value	Vaginal pH ≥4.5 and LBG III BV (n=75) N (%)	P value
Age	28.5±4.6	27±4.9	0.103	27.4±5.5	0.153
Education:			0.049		< 0.001
primary	7 (3)	3 (14)		7 (9)	
secondary	74 (29)	6 (29)		47 (63)	
higher	175 (68)	12 (57)		21 (11)	
Employment:			0.135		0.538
employed	214 (84)	15 (71)		59 (79)	
housewife	23 (9)	2 (10)		10 (13)	
unemployed	19 (7)	4 (19)		6 (8)	
Marital status:			0.313		< 0.001
married	144 (56)	9 (43)		22 (29)	
living with partner	111 (43)	12 (57)		49 (65)	
not living with partner	1 (0.4)	0		4 (5)	
Smoking before pregnancy	57 (22)	9 (43)	0.036	35 (47)	< 0.001
Smoking during pregnancy	12 (5)	4 (19)	0.025	14 (19)	< 0.001
Nulliparas	129 (50)	11 (52)	0.875	42 (56)	0.427
Miscarriage history	49 (19)	2 (10)	0.385	13 (17)	0.703
Preterm birth history	5 (2)	0		3 (4)	0.199
Concomitant diseases	43 (17)	4 (19)	0.766	6 (8)	0.056
Use of medicaments	16 (6)	1 (5)	1.000	2 (3)	0.540
≥ 6 life time sexual partners	45 (18)	4 (19)	0.092	12 (16)	0.858
≥ 2sexual partners during last year	12 (4)	1 (5)	1.000	7 (9)	0.240
Frequency of intercourses ≥ 10 times during preceding month	35 (14)	7 (33)	0.038	19 (26)	0.032
Sexual relationships with the last partner ≤ 6 months	53 (21)	7 (33)	0.394	24 (32)	0.130
Intercourse during the preceding 48 hours	65 (27)	11 (52)	0.002	28 (38)	0.068

Continuation of Table 4.6.

Characteristics	Vaginal pH < 4.5 and LBG I-IIa (n=256) N (%)	Vaginal pH ≥4.5 and LBG III AV (n=21) N (%)	P value	Vaginal pH ≥4.5 and LBG III BV (n=75) N (%)	P value
Genital infections in the year before pregnancy:					
<i>C. trachomatis</i>	4 (2)	0	1.000	1 (1)	1.000
<i>T. vaginalis</i>	1 (0.4)	0	1.000	0	1.000
herpes genitalis	5 (2)	0	1.000	3 (4)	0.390
bacterial vaginosis	16 (6)	3 (14)	0.170	12 (16)	0.016
candidosis	70 (28)	6 (29)	0.907	11 (15)	0.022
<i>U. urealyticum</i>	22 (9)	0	0.233	1 (1)	0.023
<i>M. hominis</i>	4 (2)	0	1.000	0	0.578
Antibiotic use in the 2 weeks before sampling	4 (2)	1 (5)	0.383	0	0.592
Topical treatment of vaginal infections in the 2 weeks before sampling	9 (4)	0	1.000	1 (1)	0.467

4.2. 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 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%) and 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 1 had BV), Table 4.7.

Table 4.7.

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
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)	

Continuation of Table 4.7.

Characteristic	Total (n=450) N (%)	pH < 4.5 (n=300) N (%)	pH ≥ 4.5 (n=150) N (%)	P value
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
Number of leucocytes:				0.001
<10 per 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%, Table 4.8.

Table 4.8.

pH test sensitivity and specificity

	Sensitivity (%)	Specificity (%)
Full BV	97	72
Partial BV	92	75
Total BV	95	83
Moderate AV	84	70
Severe AV	60	65
Total AV	78	70

All lactobacillary morphotypes were found more often in the pH ≤ 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% vs. 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% vs. 13%, $p = 0.001$.

4.3. 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, *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 Gram positive cocci group ($p = 0.015$). Correlations of abnormal vaginal microflora microscopic patterns with cultures were similar to those with elevated pH, Table 4.9.

Table 4.9.

Vaginal cultures results in the normal and elevated vaginal pH and different LBG groups

Cultured microbes	Total	Vaginal pH		P value	LBG				P value
		pH <4.5 n=50	pH ≥4.5 n=50		I N=35	IIa N=14	IIb N=17	III N=34	
<i>U. urealyticum</i>	34	10	24	0.017	7	5	2	20	0.002
<i>U. urealyticum</i> (high numbers)	15	3	12	0.023	2	2	0	11	0.006
<i>M. hominis</i>	15	1	14	<0.001	0	1	0	14	<0.001
<i>M. hominis</i> (high numbers)	9	0	9	0.017	0	0	0	9	0.001
<i>Str. agalactiae</i>	6	2	4	0.678	2	1	3	0	0.032

Continuation of Table 4.9.

Cultured microbes	Total	Vaginal pH		P value	LBG				P value
		pH <4.5 n=50	pH ≥4.5 n=50		I N=35	IIa N=14	IIb N=17	III N=34	
Coagulase positive (CP) <i>Staphylococcus</i>	4	1	3	0.618	1	0	1	2	0.896
CN <i>Staphylococcus</i>	56	27	29	0.618	18	10	8	20	0.172
<i>Str. viridians</i>	9	6	3	0.193	4	4	0	1	0.013
<i>Peptostreptococcus</i>	2	2	0	0.238	2	0	0	0	0.734
<i>Enterococcus faecalis</i>	5	2	3	1.000	2	0	1	2	1.000
<i>E. coli</i>	18	4	14	0.018	1	3	7	7	0.001
<i>Enterobacteriaceae</i>	2	1	1	1.000	0	0	1	1	0.476
<i>Acinetobacter spp</i>	4	0	4	0.121	1	0	1	2	0.896
Gram positive cocci and Gram negative bacilli:				0.015					0.000
Gram positive cocci (pure)	36	24	12		19	8	0	9	
Gram negative bacilli (pure)	7	3	4		4	0	0	3	
mixed Gram positive cocci and Gram negative bacilli	37	11	26		7	3	13	14	
<i>Candida spp</i>	16	8	8	1.000	7	0	4	5	0.265

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 abnormal vaginal flora 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 abnormal microflora 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* (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, Table 4.10.

Table 4.10.

Vaginal cultures results in the normal and abnormal vaginal microflora and acidity groups

Cultured microorganisms	Normal vaginal microflora, acidity group: pH < 4.5 and LBG I-IIa (n=43)	Abnormal vaginal microflora, acidity group: pH ≥ 4.5 and LBG IIb-III (n=44)	P values
<i>U. urealyticum</i>	9	21	0.019
<i>U. urealyticum</i> (high numbers)	3	11	0.043
<i>M. hominis</i>	1	14	<0.001
<i>M. hominis</i> (high numbers)	0	9	0.003
<i>Str. agalactiae</i>	2	3	1.000
CP <i>Staphylococcus</i>	0	2	0.496
CN <i>Staphylococcus</i>	25	26	0.600
<i>Str. viridians</i>	6	1	0.047
<i>Peptostreptococcus</i>	2	0	0.210
<i>Enterococcus faecalis</i>	2	3	1.000
<i>E. coli</i>	2	12	0.008

Continuation of Table 4.10.

Cultured microorganisms	Normal vaginal microflora, acidity group: pH < 4.5 and LBG I-IIa (n=43)	Abnormal vaginal microflora, acidity group: pH ≥ 4.5 and LBG IIb-III (n=44)	P values
<i>Enterobacteriaceae</i>	0	1	1.000
<i>Acinetobacter spp</i>	0	3	0.243
Gram positive cocci and Gram negative bacilli:			0.001
Gram positive cocci (pure)	24	9	
Gram negative bacilli (pure)	3	3	
mixed Gram positive cocci and Gram negative bacilli	7	23	
<i>Candida spp</i>	6	7	0.957

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 4.11.

Table 4.11.

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

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

4.4. Vaginal ascorbic acid (vitamin C) study

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

4.4.1. Results in pregnant women subgroup

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 4.4.2. The baseline characteristics of all cases of both groups were comparable, Table 4.12.

Table 4.12.

The baseline characteristics in the both groups in pregnant women population

Characteristics	Vitamin C group N=42	Control group N=43	P value
Age	28.8±5.1	27.1±5.3	0.498
Weight	68.2±13 kg	63.3±12 kg	0.109
Height	168.9±5.8 cm	167.5±6.5 cm	0.301
Higher education	18	15	0.174
Employed	35	30	0.369
Married	12	17	0.099
Smokers	9	9	1.000
Concomitant diseases	9	7	0.544
Use of medicaments	5	4	1.000
≥ 6 life time sexual partners	7	8	0.693
≥ 2sexual partners during last year	4	3	0.713
Frequency of intercourses ≥ 10 times during last month	9	6	0.479
Intercourse during the last 48 hours	22	20	0.723
Mean vaginal pH	5.05±0.37	5.02±0.30	0.764
LBG types on wet mounts			0.283
• LBG IIb	7	10	
• LBG III BV	9	11	
• LBG III AV	10	4	
• LBG III MF	16	18	

Continuation of Table 4.12.

Characteristics	Vitamin C group N=42	Control group N=43	P value
Number of leucocytes per hpf on wet mounts			0.948
• ≤10 per hpf	22	21	
• >10 per hpf, but < 10 per epithelial cell	9	10	
• ≥10 per epithelial cell	11	12	
Clue cells on wet mounts	24	28	0.451

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, Table 4.13.

Table 4.13.

Prevalence of normal vaginal pH and microflora types on follow-up visit

	Vitamin C group (n=42)	Control group (n=43)	P values
pH < 4.5 ITT population	85% (34)	36% (14)	<0.001
pH < 4.5 PP population	83% (29)	32% (12)	<0.001
LBG I-IIa ITT population	75% (30)	33% (13)	<0.001
LBG I-IIa PP population	74% (26)	30% (11)	<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 4.14.

Table 4.14.

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
Pure BV (20)	12	6	6	0.622
Pure AV (14)	7	6	1	0.315
Any BV (51)	26	16	10	0.012
Any AV (59)	28	21	7	0.001
Mixed AV-BV (34)	15	11	4	0.005

4.4.2. 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), Figure 4.1.

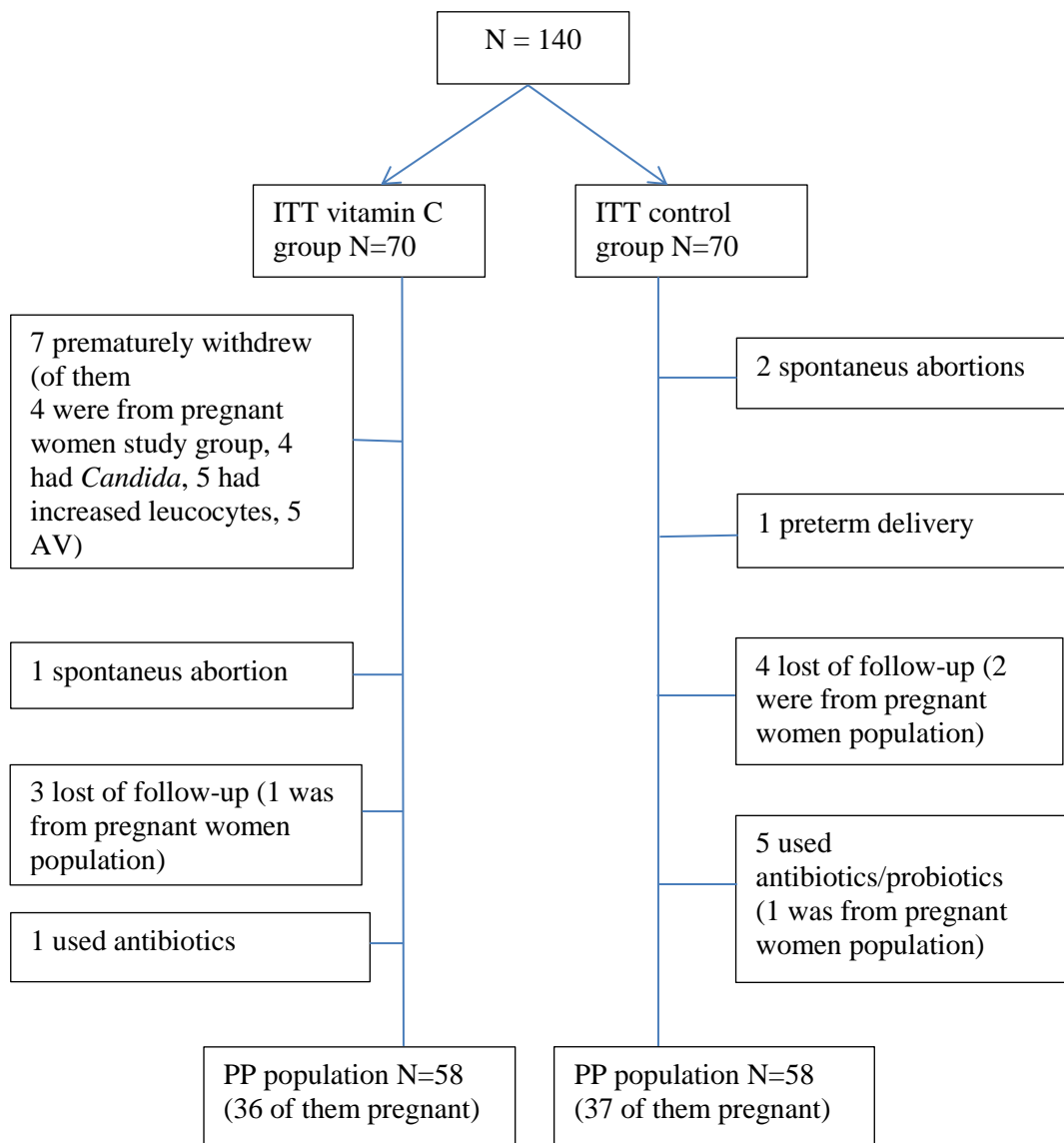


Figure 4.1. Overview of study population

The baseline characteristics of all cases of both groups were comparable, Table 4.15.

Table 4.15.

The baseline characteristics in the both groups

Characteristics	Vitamin C group N=70	Control group N=70	P value
Age	28.3±6.1	28.6±6.7	0.812
Weight	66.1±1.5 kg	63.3±1.3 kg	0.154
Height	168.6±5.8 cm	167.6±6.1 cm	0.320

Continuation of Table 4.15.

Characteristics	Vitamin C group N=70	Control group N=70	P value
Higher education	31	26	0.200
Employed	54	50	0.813
Married	22	25	0.749
Smokers	22	24	0.674
Pregnant women	42	43	0.801
Concomitant diseases	11	12	0.790
Use of medicaments	5	4	1.000
≥ 6 life time sexual partners	14	14	0.539
≥ 2sexual partners during last year	13	9	0.293
Frequency of intercourses ≥ 10 times during last month	14	13	0.782
Intercourse during the last 48 hours	26	23	0.723
Mean vaginal pH	5.09±0.37	5.06±0.33	0.616
LBG types on wet mounts			0.553
• LBG IIb	11	12	
• LBG III BV	16	20	
• LBG III AV	16	10	
• LBG III MF	27	28	
Number of leucocytes per hpf on wet mounts			0.674
• ≤10 per hpf	34	40	
• >10 per hpf, but < 10 per epithelial cell	19	16	
• ≥10 per epithelial cell	17	14	
Clue cells on wet mounts	43	48	0.376

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 not significant), Table 4.16.

Table 4.16.

Efficacy endpoint (normalization of the vaginal microflora and pH) rates in the vitamin C and control groups on follow-up visit

	Vitamin C group % (n)	Control group % (n)	Intergroup difference
Efficacy endpoint rates of ITT population (n=140)			
• Total	51.4% (36)	24.3% (17)	27.1% (95% CI 11.7–42.6, p<0.05)
Non-pregnant	58.3% (7)	41.7% (5)	6.5% (95% CI 15.3–28.2, p not significant)
Efficacy endpoint rates of PP population (n=116)			
• Total	53.5% (31)	22.4% (13)	31% (95% CI 14.3–47.8, p<0.05)
Non-pregnant	26.1% (6)	14.3% (3)	11.8% (95% CI 11.6–35.2, p not significant)

On follow-up visit prevalence of normal vaginal pH and microflora on wet mount was higher in ascorbic acid group, but not in the subgroup of non-pregnant participants, Table 4.17.

Table 4.17.

Prevalence of normal vaginal pH and microflora types on follow-up visit

	Vitamin C group (n=70)	Control group (n=70)	P values
pH < 4.5 ITT population			
• Total	67% (44)	30% (19)	<0.001
Non-pregnant	39% (10)	21% (5)	0.224
pH < 4.5 PP population			
• Total	64% (37)	26% (15)	<0.001
Non-pregnant	35% (8)	14% (3)	0.169
LBG I-IIa ITT population			
• Total	56% (39)	27% (19)	0.001
Non-pregnant	35% (9)	25% (6)	0.545
LBG I-IIa PP population			
• Total	57% (33)	26% (15)	0.001
Non-pregnant	30% (7)	19% (4)	0.494

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 vs. 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).

4.4.3. Side effects of vaginal vitamin C

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, Table 4.18.

Table 4.18.

Associations between side effects and LBG, number of leucocytes and presence of *Candida* on wet mounts

	Itching after treatment phase, N = 11	Itching after maintenance phase, N = 13	Irritation after treatment phase, N = 9	Irritation after maintenance phase, N = 6
LBG:	P = 0.940	P = 0.963	P = 0.959	P = 0.244
IIa	1	2	1	0
BV	2	3	2	1
AV	3	3	3	0
MF	5	5	3	5
Number of leucocytes:	P = 0.060	P = 1.000	P = 0.262	P = 1.000
< 10 per hpf	2	6	2	3
> 10 per hpf	9	7	7	3

Continuation of Table 4.18.

	Itching after treatment phase, N = 11	Itching after maintenance phase, N = 13	Irritation after treatment phase, N = 9	Irritation after maintenance phase, N = 6
Candida:	P = 0.287	P = 0.470	P = 0.249	P = 0.692
present	3	2	2	6
absent	8	11	7	0

4.5. Results of the vaginal clindamycin group

17 pregnant women received vaginal clindamycin (Dalacin) cream treatment. 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).

4.6. Pregnancy outcome

Pregnancy outcome data were available for 102 of 135 women with $\text{pH} \geq 4.5$ /LBG IIB-III and for 217 of 256 with normal and vaginal acidity and microflora.

In order to increase study compliance participants were phoned. 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.

Comparison of pregnancy outcome between normal and AVF groups (including cases with as well as without any intervention) did not show major differences. However, both first and fifth minute Apgar score levels were higher in newborns whose mothers` had both normal vaginal microflora and pH ($p = 0.027$ and $p = 0.023$). Furthermore we found a doubled rate of urinary tract infections in women with normal flora ($p = 0.05$) and a trend towards lower birth weight in women with abnormal flora, Table 4.19.

Table 4.19.

**Pregnancy complications and outcomes in normal and abnormal vaginal
microflora / pH groups**

Characteristic	Total (n=319) N (%)	Vaginal pH <4.5 and LBG I-IIa (n=217) N (%)	Vaginal pH ≥4.5 and LBG IIb-III (n=102) N (%)	P value
Bleeding	30 (9)	17 (8)	13 (14)	0.340
Hypertension	23 (8)	18 (9)	5 (5)	0.301
Rh isosensibilisation	1 (0.3)	0	1 (1)	0.314
Anaemia	100 (33)	73 (35)	27 (28)	0.251
Urinary tract infections	39 (13)	32 (15)	7 (7)	0.053
Respiratory tract infections	47 (15)	33 (16)	14 (15)	0.799
Fetal abnormalities	6 (2)	5 (2)	1 (1)	0.669
Other pathologies on ultrasound scan	19 (6)	13 (6)	6 (6)	0.984
Pregnancy outcomes:				0.121
early miscarriage	20 (6)	10 (5)	10 (8)	
late miscarriage	4 (1)	4 (2)	0	
preterm delivery at 22–26 weeks of gestation	0	0	0	
preterm delivery at 27–36 weeks of gestation	9 (3)	4 (2)	5 (4)	
term delivery	303 (90)	199 (92)	104 (87)	
Delivery type:				0.091
spontaneous	226 (88)	158 (90)	68 (83)	
induced	31 (12)	17 (10)	14 (17)	
Delivery mode:				0.835
vaginal	233 (79)	159 (78)	74 (80)	
planned caesarean section	37 (13)	27 (13)	10 (11)	
emergency caesarean section	27 (8)	19 (9)	8 (9)	
Newborn weight		3633±493	3527±494	0.072
Apgar score				
1. minute		7.7±0.1	7.4±1.3	0.027
5. minute		8.8±0.1	8.5±0.1	0.023
Newborn admission at intensive care unit	3 (1)	3 (1)	0	0.554
Newborn hospitalization at Children`s hospital (up to 28 days after birth)	5 (2)	4 (2)	1 (1)	0.665
Use of antibiotics (systemic or vaginal) during pregnancy	40 (12)	26 (12)	14 (12)	0.962

Comparison of pregnancy outcome between normal and non-intervention AVF group participants (58 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 4.20.

Table 4.20.

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, compared to non-intervention AVF group participants, Table 4.21.

Table 4.21.

Comparison of pregnancy outcomes in the AVF group

Characteristic	Total (n=118) N (%)	Non- intervention population (n=58) N (%)	Intervention population (n=60) N (%)	P value
Pregnancy outcomes:				0.107
early miscarriage	10 (9)	8 (14)	2 (3)	
late miscarriage	0	0	0	
preterm delivery at 22–26 weeks of gestation	0	0	0	
preterm delivery at 27–36 weeks of gestation	5 (4)	3 (5)	2 (3)	
term delivery	103 (87)	47 (81)	56 (93)	

Continuation of Table 4.21.

Characteristic	Total (n=118) N (%)	Non- intervention population (n=58) N (%)	Intervention population (n=60) N (%)	P value
Newborn weight		3515±553	3542±444	0.776
Apgar score				
1. minute		7.2±1.4	7.6±1.1	0.086
5. minute		8.3±1.7	8.8±0.4	0.033

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 4.22.

Table 4.22.

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 on newborns' weight in the study. As expected current smoking had the most negative effect on birth weight, Table 4.23.

Table 4.23.

Multivariate analysis of influence of different risk factors on newborns' lower birth weight

Characteristic	P value
Smoking during pregnancy	0.004
Abnormal vaginal microflora in the first trimester of pregnancy	0.349
Hypertension	0.869
Anaemia	0.632
Fetal abnormalities	0.313
Other abnormal ultrasound scan results	0.949

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, Table 4.24.

Table 4.24.

Pregnancy outcomes in vitamin C and control groups in ITT population

Characteristic	Vitamin C group (n=41) N (%)	Control group (n=38) N (%)	P values
Pregnancy outcomes:			0.113
early miscarriage	1 (2)	2 (6)	
late miscarriage	0	0	
preterm delivery at 22–26 weeks of gestation	0	0	
preterm delivery at 27–36 weeks of gestation	0	3 (8)	
term delivery	40 (98)	33 (86)	
Mean newborn weight	3528±422	3425±540	0.364
Apgar score			
1. minute	7.6±0.2	7.2±0.2	0.188
5. minute	8.8±0.1	8.1±0.3	0.031

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 4.25.

Table 4.25.

**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 non-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), Table 4.26.

Table 4.26.

Pregnancy outcomes in vitamin C and control groups in PP population

Characteristic	Vitamin C group (n=34) N (%)	Control group (n=33) N (%)	P values
Pregnancy outcomes:			0.239
early miscarriage	0	0	
late miscarriage	0	0	
preterm delivery at 22–26 weeks of gestation	0	0	
preterm delivery at 27–36 weeks of gestation	0	2(6)	
term delivery	34 (100)	31 (94)	
Mean newborn weight	3512±440	3457±517	0.641
Apgar score			
1. minute	7.8±0.1	7.1±0.3	0.009
5. minute	8.8±0.1	8.1±0.4	0.034

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, Tables 4.27., 4.28.

Table 4.27.

Comparison of pregnancy outcomes in Dalacin and control groups

Characteristic	Dalacin group (n=17) N (%)	Control group (n=38) N (%)	P values
Pregnancy outcomes:			0.187
early miscarriage	1 (8)	2 (6)	
late miscarriage	0	0	
preterm delivery at 22–26 weeks of gestation	0	0	
preterm delivery at 27–36 weeks of gestation	1(8)	3 (8)	
term delivery	15 (84)	33 (86)	
Mean newborn weight	3558±513	3425±540	0.458
Apgar score			
1. minute	7.6±0.5	7.2±0.2	0.248
5. minute	8.8±0.5	8.1±0.3	0.267

Table 4.28.

Comparison of pregnancy outcomes in Dalacin and vitamin C groups

Characteristic	Dalacin group (n=17) N (%)	Vitamin C group (n=41) N (%)	P values
Pregnancy outcomes:			0.187
early miscarriage	1 (8)	1 (3)	
late miscarriage	0	0	
preterm delivery at 22–26 weeks of gestation	0	0	
preterm delivery at 27–36 weeks of gestation	1(8)	0	
term delivery	15 (84)	40 (98)	
Mean newborn weight	3558±513	3528±422	0.837
Apgar score			
1. minute	7.6±0.5	7.6±0.2	0.844
5. minute	8.8±0.5	8.8±0.1	0.628

5. DISCUSSION

5.1. 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 many 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 ≥ 4.5 and LBG IIb/LBG III on wet mounts. We believe, that current approach could better select the patients with potentially AVF 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 AVF 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, Tables 4.1., 4.2., 4.5.

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. According to the data of Latvian Central Statistical Bureau the prevalence of higher education in Latvia is 23% and in Riga/surroundings 29-41% [Latvian Central Statistical Bureau, 2012]. Participants of the present study were even better educated (58% of total population, Table 4.1.), also in the age group ≤ 25 years (51% of younger women had higher education, Table 4.4.), but nonetheless those with lower education had significant trend to have more often AVF. In the light of the statistical power of the present study and the fact that proportion of lower education outside Riga is higher, the indirect impact of education on vaginal microflora and subsequently pregnancy outcomes might be more relevant in rural areas of Latvia.

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, Table 4.3.

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, Table 4.6.

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. In consideration of recent studies, the entire mucosal immune system should be considered as one organ and if environmental factors influence one part then it influences the entire mucosal system. The harmful influence of smoking increases the whole body's inflammatory response. During the damage process of epithelial cells, IL8 and TNF α are produced. These active substances stimulate macrophages to produce IL1 and IL6. IL1 and IL6 are inner body pyrogens and they influence the immunity of the whole body as a part of the innate inflammation mechanism. This means that they lower IL10 production levels (anti-inflammatory interleukin). If smoke inhalation is regular, the body's immune function depletes, and macrophages produce IL6 in lower amounts. It influences the full innate immunity cascade and also phagocytosis [Karavitis, et al., 2011]. 10% of pregnant women in Latvia smoke [The Newborn Register of Latvia, 2011], with a higher incidence in younger age groups [Statistical Yearbook of Health Care in Latvia, 2012]. Therefore, in young pregnant smokers, vaginal flora has to be evaluated more carefully in order to avoid pregnancy complications related to reproductive tract infections, Figure 5.1.

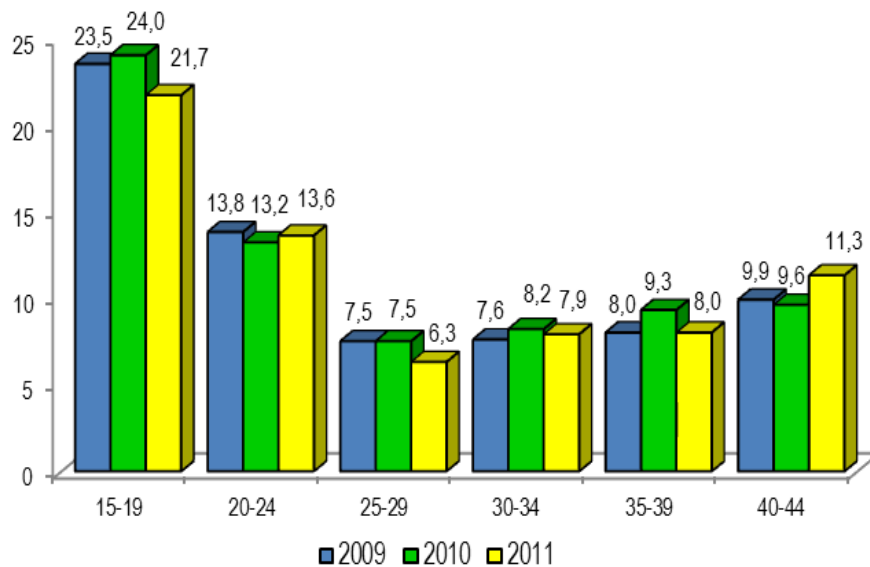


Figure 5.1. Fraction of Mothers who smoke of total mothers in respective age group, %
[Statistical Yearbook of Health Care in Latvia, 2012]

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, Table 4.1. 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 (odds ratio 2.4) [Larsson, *et al.*, 2007]. Although the exact mechanism of the effect of tobacco on the reproductive system is not clear, it is well known, that smoking is associated with preterm birth [Ahern, *et al.*, 2003], and fetal growth restriction [Vedmedovska, *et al.*, 2010]. Chang, from the 'Born Too Soon' preterm prevention analysis group in his recent report of "Preventing preterm births: analysis of trends and potential reductions with interventions in 39 countries with very high human development index," has identified smoking cessation as one of five interventions for reduction of preterm birth (besides decreasing multiple embryo transfers during assisted reproductive technologies, cervical cerclage, progesterone supplementation and reduction of non-medically indicated labour induction or caesarean delivery) [Chang., *et al.*, 2013]. At the same time smoking is also related to low socioeconomic status, as is low educational level. Both of these were associated with abnormal vaginal microflora, which indicates a high risk for adverse pregnancy outcome – which was further proved in the present study by increased

miscarriage/preterm birth rate in the group of AVF and lower newborn birth weight of pregnant women either smoking and presenting AVF. On the basis of the findings of the present and others studies and the fact, that pregnancy often is not planned, the smoking exposure time required to change vaginal microflora is not clear, it is important to educate women to generally avoid smoking.

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 (Table 4.6.). AV flora is usually associated with overgrowth of commensals, like GBS, *E.coli*, *S.aureus* [Donders, et al., 2002], also with sexually transmitted bacteria, like trichomonas [Donders. et al., 2002] and *Chlamydia trachomatis* [Marconi, et al. 2012].

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, Tables 4.1. 4.2. 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 intercours 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.

5.2. Bed-side 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.

Screening of genital infections is an important part of antenatal care. In Latvia pregnant women are screened for *gonorrhoea*, syphilis, HIV, hepatitis B, and *Chlamydia trachomatis* [Regulation of the Latvian Cabinet of Ministers Nr. 611], but since 2013 *Chlamydia trachomatis* is checked only in high risk groups and in women with cervicitis or urethritis. Independent from this, general screening for the presence of GBS in the lower genital tract is indicated at 35 to 37 weeks of gestation.

In Latvia, all pregnant women have upper vaginal smears taken for Gram staining in order to exclude *gonorrhoea* and evaluate vaginal microflora, and also upper vaginal and cervical smears for cytological examinations in the first antenatal visit, but the implications of these tests should be discussed.

For cervical cytological analysis, the *Leishman* methodology is applied in Latvia [Viberga, *et al.*, 2010], and the assessment of the so-called “degree of vaginal purity” is a part of this examination. There are 4 vaginal purity degrees, in which I and II degree represents normal vaginal microflora with predominant lactobacillar bacteria and acidic reaction, but III and IV degree represents abnormal vaginal flora with decreased or absent *Lactobacillus* morphotypes, overgrowth of other microorganisms and increased numbers of leucocytes [Bergmane, *et al.*, 1987]. In general, vaginal purity degrees correspond to LBG, but it takes time to get results from a laboratory, and also 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]. Therefore, cytology should not be used as a diagnostic method for vaginal microflora in pregnancy.

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. Since gonococci infect endocervical columnar epithelium, it is not accurate to take samples from the upper vagina to detect *gonorrhoea* and the sensitivity of Gram stain is not higher than 50% when compared to cultures and molecular diagnostic methods [Glass, *et al.*, 2005]. Compared to *Chlamydia* infection, the incidence of *gonorrhoea* is decreasing among women in Latvia [Survey of Reproductive Health of Inhabitants of Latvia, 2011]. *Chlamydia* is more common in the younger age group (15–24 years). As *Chlamydia trachomatis* is screened for only in the pregnant women risk group, apparently *gonorrhoea* also should be screened for the same population, but maybe with more sensitive tests.

There is an assumption that treatment of AVF before 20 week gestation may reduce genital infection related risk of preterm birth [Ugwumadu, *et al.*, 2004; Lamont, *et al.*, 2005], even more - so in patients with a history of adverse pregnancy outcomes [Thinkhamrop, *et al.*, 2009]. It might be crucial to identify pregnant women at high AVF risk who need early diagnostic and management of abnormal vaginal microflora. 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 *G.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.

5.2.1. 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 4.7.

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. In terms of morphology of the *Lactobacillus*, many authors have found that the normal appearing lactobacillary morphotypes mostly correspond to *L.gasseri* or *L.crispatus*, while the coarse types seem to correspond more often to *L.iners*, a ubiquitous lactobacillary morphotype, that is involved in different types of microflora, that switch more often from normal flora to LBG III and BV on follow up [Verhelst, et al., 2005; Verstraelen (a), et al., 2009]. In our study we could not demonstrate a similar association between vaginal acidity and lactobacillary morphology, Table 4.7.

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 (Table 4.8.). 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].

As expected, we observed a correlation between elevated pH and the presence of sperm in the smears (Table 4.7). Of interest, even when spermatozooids were unmistakably identified, two women reported that there had been no recent intercourse. As indicated by our study results, in pregnancy, self-reported data about sexual history and life style may not be fully reliable. Others have recognized an association between the presence of sperm or intercourse and increased vaginal pH [Amsel, *et al.*, 1983; Hillier (a), 1993]. There are different opinions about the influence of sperm on the vaginal pH. It can change vaginal acidity because of alkaline properties or enable disruption of vaginal microbiology [Hillier (a), 1993; Verhelst, *et al.*, 2005]. Though we also found mostly concurrent LBG III changes in cases with increased vaginal pH and spermatozooids, BV is more often associated with frequent intercourse, while AV was correlated to recent intercourse (Table 4.6.). This was previously not recognized, and is remarkable as in previous work severe AV was found to be associated with vaginal dyspareunia [Donders, *et al.*, 2002]. A diversity of antigenically distinct proteins is found in seminal plasma – proteases, protease inhibitors, signal transduction molecules, protein kinases and phosphatases, transporter proteins, structural molecules and immune response proteins [Fung, *et al.*, 2004]. Male reproductive proteins play a central role in embryo implantation and placentation by inducing inflammation. Sexual intercourse activates cytokines and recruitment of uterine and cervical macrophages, dendritic cells, lymphocytes and natural killer cell, and during the period from coitus to apposition these cells produce high levels of IL (IL- α , IL-1 β , IL-6); TNF and colony stimulating factor-1 [Chaouat, *et al.*, 2005]. Furthermore, postcoital cervical inflammation is eliminated by condom use, confirming an etiologic role in human reproduction failure of such proteins [Robertson, *et al.*, 2005]. In at term pregnancies, there is a link between

the frequency of vaginal intercourse and the timing of labour and delivery in some studies [Tan, et al., 2006], but it is not supported by others [Omar, et al., 2013]. Male reproductive proteins might also alter the composition of vaginal bacterial flora. They react with and deactivate H₂O₂ [O'Hanlon, et al., 2010], and thereby decrease the ability of H₂O₂-producing lactobacilli to inhibit growth of pathogens, like E. coli [Klebanoff, et al., 1984], which is consistent with our findings that recent intercourse is associated with AV, inflammatory type of abnormal vaginal flora. It was rather surprising that we could not correlate increased pH or abnormal vaginal flora with sexual risk behaviour (number of previous sexual partners, new partner during the previous six months) (Table 4.1.), except for frequent intercourse and pure BV or AV (Table 4.6.). This was true despite the fact that other studies have demonstrated a clear link between sexual activity and BV [Beigi, et al., 2005; Cherpes, et al., 2008]. The relation between frequency of sexual intercourse and preterm labor has been addressed by many authors and it is controversial [Petridou, et al., 2001; Berghella, et al., 2002;]. Male factors can contribute to preterm labor and other complications of pregnancy by both male reproductive protein inducing inflammation and by alteration of normal vaginal microflora. A Norwegian, population-based record linkage analysis showed that women with a new partner had a 1.8-2.5 fold increased risk of preterm birth, low birth weight and infant mortality [Vatten, et al., 2003]. Self-reported coitus during early pregnancy was not associated with an increased risk of recurrent preterm delivery and there was an association between the number of sexual partners in a woman's lifetime and the rate of recurrent preterm delivery in another study [Yost, et al., 2006]. Moreover, cigarette smoking, socioeconomic status or infection with STIs as a result of sexual partner change might confound the links between multiple or new partnership and preterm birth [Ness, et al., 2008].

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 4.7.

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]. The concentrations of vaginal IL-8 were significantly higher

in pregnant women with pathologic findings on vaginal wet mount, including elevated polymorphonuclear cell counts [Nenadic, et al., 2008] and associated with an increased risk of preterm birth [Brou, et al., 2012]. 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 4.7.). 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]. Hoyme “was able to lower the preterm birth risk in the province of Tübingen (Germany) by sending patients with increased self-measured vaginal pH to their doctor for further diagnosis and treatment” [Hoyme, et al., 2004].

5.2.2. 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* and also mixed Gram positive cocci/Gram negative bacilli, while solely Gram positive cocci (including *Str. agalactiae* and *Viridans* group streptococci) were more related to normal vaginal microflora (Table 4.9., 4.10.). Vaginal leucocytosis was significantly associated with *E. coli* colonization.

Although the association between *U. urealyticum*, *M. hominis* and pregnancy complications, such as late miscarriage, 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 (Tables 4.10., 4.11.). 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]. Surprisingly, some specific aerobic pathogenic bacteria, like *Str. viridians*, which can have a role in the pathogenesis of amniotic infections [Ariel, et al., 1991], were more often found in the normal vaginal microflora group in these series. Contrary to these findings, this group of bacteria has been associated with abnormal vaginal flora and reduced lactobacilli in other studies [Hillier,

1993]. Besides *Lactobacillus*, *Streptococcus* genera belong to order *Lactobacillales* - lactic acid bacteria, which ferment glucose to lactic acid and therefore might be associated with presence of an acidic environment. They are normal flora in humans in the oral cavity, the intestinal tract and the vagina, where they may even play a beneficial role [Todar, 2008]. Compared to some authors, we found a rather low prevalence of *Str. agalactiae* (6%), probably because the samples were taken from upper vagina not from the lower part of the vagina, perineum and rectum. The incidence is similar to earlier studies performed in Latvia with the same methodology [Rezeberga, et al., 2000].

Combination of different bacteria seems to be of importance, because cases with mixed Gram positive cocci/Gram negative bacilli in cultures were more often associated with both abnormal vaginal pH and AVF on wet mounts in the present study. 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 [Jacobsson, 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.

It is interesting that *Candida* species colonization were found with the same frequency in both the normal and abnormal vaginal flora groups and was not associated

with heavy leucocytosis. This demonstrates that *Candida* can exist in the different vaginal environment and can be as a part of co-infection. The predominance of *Candida* colonization in reproductive age women, and its infrequent occurrence in children and menopausal women, strongly suggests that colonization is hormone-dependent. Estrogen promotes glycogen production by vaginal epithelial cells, which is the primary nutrient source for *Candida*. Conditions associated with elevated hormone production (pregnancy, diabetes, oral contraception) are well established risk factors for increased growth of *Candida*. Conversion of an asymptomatic *Candida* colonization into a symptomatic infection following the ingestion of antibiotics, suggests that the vaginal flora, particularly *Lactobacillus* species, are important in the down regulation of the ability of *Candida* to proliferate [Ledger, et al., 2010]. *Candida* colonization not necessarily caused inflammation, it were more the presence of aerobic bacteria that was associated with heavy leucocytosis in our study.

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 [Thinkhamrop, 2009] 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. hominis* 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.

5.3. Impact of vaginal ascorbic acid on abnormal vaginal microflora

The lack 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. Clindamycin is the drug of choice for *M. hominis* infection, while quinolones

or tetracycline's are less effective. Against *U. urealyticum*, on the other hand, macrolides are more active, and for *E. coli*, an aerobic Gram negative rod, no macrolides, nor clindamycin, but β lactams, aminoglycosides, tetracycline's and quinolones are the antibiotics of choice [Taylor-Robinson, et al., 2011; Porter, et al., 2011]. Because of possible teratogenic effects, however, tetracycline's, quinolones and aminoglycosides are not recommended during pregnancy [Porter, et al., 2011]. Considerable efforts have been dedicated to evaluate antimicrobial therapy as an intervention to prevent preterm birth. Not only metronidazole and clindamycin have been studied [Hauth, et al., 1995; Lamont, 2005]. Randomized controlled studies using β lactams or azithromycin as mono-therapy failed to show any benefit, but the combination of β lactams and metronidazole, and of erythromycin and metronidazole given in the second trimester of pregnancy seemed to reduce preterm birth risk slightly. No large studies have been performed [Subramaniam, et al., 2011].

Resistance is another problem of antibiotics [Kurkinen-Raty, et al., 2000]. Hence, 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. In the previous study results 14 days after insertion of vaginal vitamin C had not been not as significant as after 6 days, showing that a six days therapy regimen may be too short and has 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, Tables 4.13., 4.14., 4.16., 4.17. 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 endpoint rates 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, Tables 4.16., 4.17. 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 reestablishing normal vaginal environment. This may also explain the spontaneous, partial improvement in vaginal pH in the control group, albeit less pronounced than 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 (Table 4.18.), 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, Table 4.14. 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.

Although not all acidifying approaches proved to be effective in clinical trials [Holley, *et al.*, 2004], in other studies using a polycarbophil-carbopol-based vaginal gel acidification seemed to be effective [Fiorilli, *et al.*, 2005]. Probably the impact of different acidic substances depends on several factors, such as the rate of absorption, metabolism, adhesiveness to vaginal mucosa and clearance by vaginal discharge [Holley, *et al.*, 2004].

5.4. Pregnancy outcomes

The observed preterm birth rate of 3% in our study was lower when compared to the general Latvian rate of preterm deliveries 5.8% in 2011 [Statistical Yearbook of Health Care in Latvia, 2012]. Probably this is associated with the exclusion of participants with STI, severe systemic diseases, multiple pregnancies – factors related to preterm birth.

We found that AVF 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. 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 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 (Tables 4.27., 4.28.), 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, Tables 4.21., 4.25. Same results were not achieved, analysing solely vitamin C and control group, probably because of few numbers, Tables 4.24., 4.26.

The higher Apgar scores in the intervention population with AVF was also observed, Tables 4.21., 4.24., 4.26. In 1952, Virginia Apgar proposed the Apgar score as a means of evaluating the physical condition of infants shortly after delivery [Apgar, 1953]. Each of five identifiable characteristics – heart rate, respiratory effort, muscle tone, reflex irritability and skin colour – is assessed and assigned a value of 0 to 2. The total score, evaluated at one and five minutes after delivery, is the sum of the five components, and a score of 7 or higher indicates that the baby's condition is good to excellent. Of the two scores, the five-minute score is regarded as the better predictor of disease-free survival in infancy [Lee, *et al.*, 2010]. The Apgar scoring system remains relevant for the prediction of neonatal survival today [Casey, *et al.*, 2001].

In the present study there was better Apgar score at 1 and 5 minutes after birth in the normal vaginal microflora/pH group, as compared to the AVF group, Table 4.19. In the non-intervention population, Table 4.20., the average Apgar score was lower than in those, who received any antibacterial/vitamin C treatment. Others have found that premature neonates, born between 28 and 35 weeks of gestation in BV positive mothers, more often had respiratory distress, required intermittent positive pressure ventilation, and had longer duration of stay in neonatal intensive care unit, but significant difference

in the mean birth weight, Apgar score, incidence of neonatal sepsis and perinatal mortality were not discovered [Laxmi, et al., 2012]. Low gestational age, small birth weight, low Apgar score as well as maternal chorioamnionitis significantly increase the risk of early-onset septicemia and early-onset pneumonia in newborns. These complications are frequently caused by Gram-positive cocci, *Str. agalactiae*, and Gram-negative bacilli [Wojkowska-Mach, et al., 2012]. 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 (Table 4.24., 4.25., 4.26.). However, small number of cases prevented us to find significant differences in neonatal infectious diseases.

Although smoking during pregnancy had the most negative effect on birth weight, AVF like AV and mixed AV-BV was also related to lower rates in the present study. 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 [Carrey, 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 4.22.). A major reason for this discrepancy could be that in others (and older) 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. viridans*, *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 FIRS, and most likely also impairment of fetal growth. This study did not find any adverse effect of first trimester vaginal colonization by *U. urealyticum*, *M. hominis* or aerobic microbes on pregnancy outcomes. This is probably because of the small number of participants in the bacteriological study, but the data showed that aerobes *E. coli*, *S. aureus*, *Klebsiella pneumonia* are significantly more prevalent in endocervical cultures from women in preterm than from those in term labour [Nadisauskiene, et al., 1995; Carey, et al., 2005]. By contrast, Holst found more

BV, not aerobic bacteria in cases of preterm labour [Holst, et al., 1994]. Although multivariate analysis in the present study showed smoking during pregnancy to be the most relevant low newborn weight risk factor, Table 4.23., the role of AVF, particularly associated with aerobic vaginitis, in the fetal growth deserves further investigation.

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.

5.5. 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.

5.6. Summary

The present study found low level of education, smoking before pregnancy and history of BV in the year before pregnancy to be the most important risk factors of

abnormal vaginal microflora in the first trimester of pregnancy, less significant associations were found with age <25 years, current smoking status, being unmarried, not employed, having ≥ 2 sexual partners in the year before pregnancy and intercourse 48 hours before sampling.

The study found that elevated vaginal pH in the first trimester of pregnancy is associated with complaints of increased, bad smell, also with thin, homogenous, yellow discharge on examination and abnormal vaginal flora, increased number of leucocytes (> 10 per epithelial cell), presence of sperm cells on wet mounts.

Pregnant women with elevated vaginal pH and abnormal vaginal flora on native microscopy were more likely to have *M. hominis*, *U. urealyticum*, *E. coli* and also mixed Gram positive cocci/Gram negative bacilli positive cultures, while cultures with solely Gram positive cocci (including *Str. agalactiae* and *Viridans* group streptococci) were more related to normal vaginal microflora. Also, vaginal leucocytosis was significantly associated with *E. coli* colonization.

According to data in the study, long term use of vaginal ascorbic acid creates a better abnormal vaginal microflora in pregnant women, but did not show a significant improvement on pure AV or BV microflora.

In the present study abnormal vaginal microflora/pH during the first trimester of pregnancy was associated with early miscarriage and preterm delivery, lower Apgar score. AV and mixed BV-AV microflora was related to lower birth weights of newborns.

Pregnant women, who received vitamin C, had less miscarriage and no preterm deliveries, compared to non-intervention AVF group, however same results were not achieved, analysing solely vitamin C and control group. The vaginal ascorbic acid improves infants Apgar score at five minutes of age in both ITT and PP populations.

6. CONCLUSIONS

1. Vaginal ascorbic acid (vitamin C) in a treatment/maintenance regimen improves abnormal vaginal microflora in pregnancy, but 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 flora (aerobic vaginitis, bacterial vaginosis and mixed flora) on wet mounts, 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.

7. 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 a 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.

8. REFERENCES

1. Abrahams V.M., Mor G. Toll-like receptors and their role in the trophoblast. *Placenta*, 2005; 26: 540–547.
2. Ahrne S., Nobaek S., Jepsson B., Adlerberth I., Wold A.E., Molin G. The normal *Lactobacillus* flora on healthy human rectal and oral mucosa. *J Appl Microbiol*, 1998; 85: 88–94.
3. Ahern J., Pickett K.E., Selvin S., Abrams B. Preterm birth among African American and white women: a multilevel analysis of socioeconomic characteristics and cigarette smoking. *J Epidemiol Community Health*, 2003; 57: 606–611.
4. Amsel R., Totten P.A., Spiegel C.A. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. *Am J Med*, 1983; 74: 14–22.
5. Andersch B., Forssman L., Lincoln K., Torstensson P. Treatment of bacterial vaginosis with an acid cream: a comparison between the effect of lactate-gel and metronidazole. *Gynecol Obstet Invest*, 1986; 21: 19–25.
6. Andreu A. *Lactobacillus* as probiotic for preventing urogenital infections. *Rev Med Microbiol*, 2004; 15: 1–6.
7. Antonio M.A., Hawes S.E., Hillier S.L. The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species. *J Infect Dis*, 1999; 180: 1950–1956.
8. Anukam K.C., Osazuwa E.O., Ahonkhai I., Reid G. *Lactobacillus* vaginal microbiota of women attending a reproductive health care service in Benin city, Nigeria. *Sex Transm Dis*, 2006; 33: 59–62.
9. Apgar V. Proposal for new method of evaluation of newborn infant. *Anesth Analg*, 1953; 32: 260–267.
10. Arbyn M., Antilla A., Jordan J., Ronco G., Schenck U., Segnan N., et al. European guidelines for quality assurance in cervical cancer screening; second edition. Belgium: European communities, 2008. Available from: <http://www.bookshop.europa.eu/guidelines.cervical-cancer-screening/>. Retrieved from the internet on 01.05.13.
11. Ariel I., Singer D.B. *Streptococcus viridans* infections in midgestation. *Pediatr Pathol*, 1991; 11 (1): 75–83.

12. Ashraf-Ganjoei T. Risk factors for bacterial vaginosis in women attending a hospital in Kerman, Islamic Republic of Iran. *East Mediterr Health J*, 2005; 11 (3): 410–415.
13. Austin M.N., Beigi R.H., Meyn L.A., Hiller S.L. Microbiological response to treatment of bacterial vaginosis with topical clindamycin or metronidazole. *J Clin Microbiol*, 2005; 43 (9): 4492–4497.
14. Baltimore R.S. Consequences of prophylaxis for group B streptococcal infections of the neonate. *Semin Perinatol*, 2007; 31 (1): 33–38.
15. Barnea E.R. Modulatory effect of maternal serum on xenobiotic metabolizing activity of placental explants: modification by cigarette smoking. *Human Reprod*, 1994; 9 (6): 1017–1021.
16. Barcaite E., Bartusevicius A., Tameliene R., Kliucinskas M., Maleckiene I., Nadisauskiene R. Prevalence of maternal group B streptococcal colonisation in European countries. *Acta Obstet Gynecol Scand*, 2008; 87 (3): 260–271.
17. Bashiri A., Burstein E., Mazor M. Cerebral palsy and fetal inflammatory response syndrome: a review. *J Perinat Med*, 2006; 34 (1): 5–12.
18. Beigi R.H., Wiesenfeld H.C., Hillier S.L., Straw T., Krohn M.A. Factors associated with absence of H₂O₂ – producing *Lactobacillus* among women with bacterial vaginosis. *J Infect Dis*, 2005; 191: 924–929.
19. Bell C., Houhg E., Smith A., Greene L. Targeted screening for *Trichomonas vaginalis* in women, a pH-based approach. *Intern J STD AIDS*, 2007; 18: 402–403.
20. Berghella V., Klebanoff M., Mc Pherson C., Carey J.C., Hauth J.C., Ernest J.M., et al. Sexual intercourse association with asymptomatic bacterial vaginosis and *Trichomona vaginalis* treatment in relationship to preterm birth. *Am J Obstet Gynecol*, 2002; 187 (5): 1277–1282.
21. Bergmane A., et al. Normālā dzemdniecība. Zvaigzne, Rīga: 1987; pages 32–35.
22. Blencowe H., Cousens S., Oestergaard M.Z., Chou D., Moller A.B., Narwal R., et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet*, 2012; 379 (9832): 2162–2172.
23. Boeke A.J., Dekker J.H., Van Ejik J.T., Kostenze P.J., Bezemer P.D. Effect of lactic acid suppositories compared with oral metronidazole and placebo in bacterial vaginosis: a randomised clinical trial. *Genitourin Med*, 1993; 69: 388–392.

24. Bradshaw C.S. (a), Tabrizi S.N., Fairley C.K., Morton A.N., Rudland E., Garland S.M. The association of *Atopobium vaginae* and *Gardnerella vaginalis* with bacterial vaginosis and recurrence after oral metronidazole therapy. *J Infect Dis*, 2006; 194: 828–836.
25. Bradshaw C.S. (b), Morton A.N., Hocking J., Garland S.M., Morris M.B., Horvath L.B., et al. High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. *J Infect Dis*, 2006; 1478–1486.
26. Brandt M., Abels C., May T., Lohmann K., Schmidts-Winkler I., Hoyme U.B. Intravaginally applied metronidazole is as effective as orally applied in the treatment of bacterial vaginosis, but exhibits significantly less side effects. *Eur J Obstet Gynecol Reprod Biol*, 2008; 141 (2): 158–162.
27. Brocklehurst P., Gordon A., Heatley E., Milan S.J. Antibiotics for treating bacterial vaginosis in pregnancy. *Cochrane Database of Systematic Reviews*, 2013; doi: 10.1002/14651858.
28. Brotman R.M., Klebanoff M.A., Nansel T.R., Yu K.F., Andrews W.W., Zhang J., et al. Bacterial Vaginosis Assessed by Gram Stain and Diminished Colonization Resistance to Incident Gonococcal, Chlamydial, and Trichomonal Genital Infection. *J Infect Dis*, 2010; 202 (12): 1907–1915.
29. Brou L., Almlı L.M., Pearce B.D., Bhat G., Drobek C.O., Fortunato S., et al. Dysregulated biomarkers induce distinct pathways in preterm birth. *BJOG*, 2012; 119 (4): 458–473.
30. Caillouette J.C., Sharp C.F., Zimmerman G.J., Roy S. Vaginal pH as a marker for bacterial pathogens and menopausal status. *Am J Obstet Gynecol*, 1997; 170: 1270–1277.
31. Carey J., Blackwelder W.C., Nugent R.P., Matteson M.A., Rao A.V., Eschenbach D.A., et al. Antepartum cultures of *Ureaplasma urealyticum* are not useful in predicting pregnancy outcome. *Am J Obstet Gyn*, 1991; 164: 728–733.
32. Carey J., Klebanoff M.A., Hauth J.C., Hillier S.L., Thom E.A., Ernest J.M., et al. Metronidazole to prevent preterm delivery in pregnant women with asymptomatic bacterial vaginosis. *N Engl J Med*, 2000; 342: 534–540.
33. Carey J., Christopher M.D., Klebanoff M.A. Is change in the vaginal flora associated with an increased risk of preterm birth? *Am J Obstet Gyn*, 2005; 192 (4): 1341–1347.

34. Casey B.M., McIntire D.D., Leveno K.J. The continuing value of the Apgar score for the assessment of newborn infants. *N Engl J Med*, 2001; 344 (7): 467–471.
35. Chang H.H., Larson J., Blencowe H., Spong C.Y., Howson C.P., Cairns-Smith S., et al. Born Too Soon preterm prevention analysis group. Preventing preterm births: analysis of trends and potential reductions with interventions in 39 countries with very high human development index. *Lancet*, 2013; 381 (9862): 223–234.
36. Chaouat G., Ledee-Bataille N., Chea K.B., Dubanchet S. Cytokines and implantation. *Chem Immunol Allergy*, 2005; 88: 34–63.
37. Chernes T.L., Hillier S.L., Meyn L.A., Busch J.L., Krohn M.A. A delicate balance: risk factors for acquisition of bacterial vaginosis include sexual activity, absence of hydrogen peroxide-producing lactobacilli, black race, and positive herpes simplex type 2 serology. *Sex Transm Dis*, 2008; 35 (1): 78–83.
38. Coleman J.S., Hitti J., Bukusi E.A., Mwachari C., Muliro A., Nguti R., et al. Infectious correlates of HIV-1 shedding in the female upper and lower genital tracts. *AIDS*, 2007; 21 (6): 755–759.
39. Conde-Agudelo A., Romero R., Kusanovic J.P., Hassan S. Supplementation with vitamins C and E during pregnancy for prevention of preeclampsia and other adverse maternal and perinatal outcomes: a systematic review and metaanalysis. *Am J Obstet Gynecol*, 2011; 204 (6):503. e1-12; doi: 10.1016/j.ajog. 2011. 02. 020.
40. Cruciani F., Brigidi P., Calanni F., Lauro V., Tacchi R., Donders G.G, et al. Efficacy of Rifaximin Vaginal Tablets in Treatment of Bacterial Vaginosis: a Molecular Characterization of the Vaginal Microbiota. *Antimicrob Agents Chemother*, 2012; 56 (8): 4062 – 4070; doi: 10.1128/AAC.00061-12.
41. Darwish A.M., Makarem M.H., Alnashar E.M., Hamadeh S.M. Screening for bacterial vaginosis in high risk pregnancy: the experience of a developing country. *Acta Obstet Gyn Scand*, 2005; 84: 483–485.
42. Das S., Sabin C., Allan S. Higher vaginal pH is associated with *Chlamydia trachomatis* infection in women: a prospective case-controlled study. *Intern J STD AIDS*, 2005; 16: 290–293.
43. Desseauve D., Chantrel J., Fruchart A., Khoshnood B., Brabant G., Ancel G., et al. Prevalence and risk factors of bacterial vaginosis during the first trimester of pregnancy in a large French population- based study. *Eur J Obstet Gynecol Reprod Biol*, 2012; 163 (1): 30–34.

44. Donders G.G., Vereecken A., Salembier G., van Bulk B., Spitz B. Assessment of lactobacillary flora in wet mount and fresh or delayed Grams stain. *Infect Dis Obstet Gynecol*, 1996; 4: 2–6.
45. Donders G.G. Microscopy of bacterial flora on fresh vaginal smears. *Infect Dis Obstet Gynecol*, 1999; 7: 126–127.
46. Donders G.G. (a), Vereecken A., Dekeersmaecker A., van Bulk B., Spitz B. Wet mount reflects functional vaginal lactobacillary flora better than Gram stain. *J Clin Pathol*, 2000; 53: 308–314.
47. Donders G.G. (b), Vereecken A., Bosmans E., Dekeersmaecker A., Van Bulck B., Spitz B. Pathogenesis of abnormal vaginal bacterial flora. *Am J Obstet Gynecol*, 2000; 182: 872–878.
48. Donders G.G. (c), Van B.B, Caudron J., Londers L., Vereecken A., Spitz B. Relationship of bacterial vaginosis and mycoplasmas to the risk of spontaneous abortion. *Am J Obstet Gynecol*, 2000; 183: 431–437.
49. Donders G.G., Vereecken A., Bosmans E., Dekeersmaecker A., Salembier G., Spitz B. Aerobic vaginitis: abnormal vaginal flora entity that is distinct from bacterial vaginosis. *BJOG*, 2002; 109:1–10.
50. Donders G.G. (a). Definition and classification of abnormal vaginal flora. *Best Pract Res Clin Obstet Gyn*, 2007; 21 (3): 355–373.
51. Donders G.G. (b), Caeyers T., Tydhof P., Riphagen I., Van den Bosch T., Bellen G. Comparison of two types of dipsticks to measure vaginal pH in clinical practice. *Eur J Obstet Gynecol Reprod Biol*, 2007; 134 (2): 220–224.
52. Donders G.G., Spitz B., Vereecken A., Van Bulck B. The ecology of the vaginal flora at first prenatal visit is associated with preterm delivery and low birth weight. *Open Inf Dis J*, 2008; 2: 45–51.
53. Donders G.G., Van Calsteren K., Reybrouck R., Van den Bosch T., Riphagen I., van Lierde S. Predictive value for preterm birth of abnormal vaginal flora, bacterial vaginosis and aerobic vaginitis during the first trimester of pregnancy. *BJOG*, 2009; 116 (10): 1315–1324.
54. Donders G.G., Larsson P.G., Platz-Christensen J.J., Hallén A., van der Meijden W., Wölner-Hanssen P. Variability in diagnosis of clue cells, lactobacillary grading and white blood cells in vaginal wet smears with conventional bright light and phase contrast microscopy *Eur J Obstet Gynecol Reprod Biol*, 2009 (b); 145: 109–112.

55. Donders G.G., Guaschino S., Peters K., Tacchi R., Lauro V. VARIANT 1 Study Group. A multicenter, double-blind, randomized, placebo-controlled study of rifaximin for the treatment of bacterial vaginosis. *Int J Gynaecol Obstet*, 2013; 120: 131–136.
56. Döderlein A. Das scheidensekret und seine bedeutung fur puerperalfieber. *Zentbl Bakteriol Hyg Abt*, 1892; 11: 699.
57. Eckert L.O. Acute vulvovaginitis. *N Engl J Med*, 2006; 355: 1244–1252.
58. Egan M.E., Lipsky M.S. Diagnosis of vaginitis. *Am F Phys*, 2000; 62 (5): 1095–1104.
59. Emilson C.G. Susceptibility of various microorganisms to chlorhexidine. *Scand J Dent Res* 1977; 85: 255–265.
60. Eriksson K., Carlsson B., Forsum U., Larsson P.G. A double-blind treatment study of bacterial vaginosis with normal vaginal lactobacilli after an open treatment with vaginal clindamycin ovules. *Acta Derm Venereol*, 2005; 85: 42–46.
61. European Commission, 2011. Demography report 2010 – Older, more numerous and diverse Europeans. Available at: <http://epp.eurostat.ec.europa.eu/portal/page/portal/population/documents/Tab/report.pdf>. Retrieved from internet on 12.01.13.
62. Falagas M.E., Betsi G.I., Athanasiou S. Probiotics for the treatment of women with bacterial vaginosis. *Clin Microbiol Infect*, 2007; 13: 657–664.
63. Ferris M.J., Masztal A., Aldridge K.E., Fortenberry J.D., Fidel P.L., Martin D.H. Association of *Atopobium vaginae*, a recently described metronidazole resistant anaerobe, with bacterial vaginosis. *BMC Infect Dis*, 2004; 4: 5; doi: 10.1186/1471-2334-4-5.
64. Fethers K.A., Fairley C.K., Hocking J.S., Gurrin L., Bradshaw C.S. Sexual risk factors and bacterial vaginosis: a systemic review and meta-analysis. *Clin Infect Dis*, 2008; 47 (11): 1426–1435.
65. Fiorilli A., Molteni B., Milani M. Successful treatment of bacterial vaginosis with a polycarbophil-carbopol acidic vaginal gel: results from a randomised double-blind, placebo-controlled trial. *Eur J Obstet Gynecol Reprod Biol*, 2005; 120 (2): 202–205.
66. Franko E.A. Microscopy for public health nurses. Georgia Department of Human Resources, 2007: 37. Available at: <http://health.state.ga.us/pdfs/epi/std/Microscope%20Manual%2006.pdf>. Retrieved from internet on 01.05.13.

67. Fredricks D.N., Fiedler T.L., Marrazzo J.M. Molecular identification of bacteria associated with bacterial vaginosis. *N Engl J Med*, 2005; 353: 1899–1911.
68. Fredricks D.N., Fiedler T.L., Thomas K.K., Oakley B.B., Marrazzo J.M. Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. *J Clin Microbiol*, 2007; 45: 3270–3276.
69. Fung K.Y., Glode L.M., Green S., Duncan M.W. A comprehensive characterization of the peptide and protein constituents of human seminal fluid. *Prostate*, 2004; 61: 171–181.
70. Gardner H.L., Dukes C.D. *Haemophilus vaginalis* vaginitis. A newly defined specific infection previously classified “Nonspecific” vaginitis. *Am J Obstet Gynecol*, 1955; 69: 962–976.
71. Gardner H.L. Desquamative inflammatory vaginitis: a newly defined entity. *Am J Obstet Gynecol*, 1968; 102: 1102–1105.
72. Glass N., Nelson H., Villemeyer K. Screening for Gonorrhea. Evidence Synthesis No. 40. (Prepared by the Oregon Evidence-based Practice Center under Contract No. 290-02-0024). Rockville, Md: Agency for Healthcare Research and Quality. May 2005. Available at: <http://www.ahrq.gov/clinic/serfiles.htm>. Retrieved from internet at 13.01.13.
73. Goldenberg R.L., Hauth J.C., Andrews W.W. Intrauterine infection and preterm delivery. *N Engl J Med*, 2000; 342 (20): 1500–1507.
74. Goldenberg R.L., Culhane J.F., Iams J.D., Romero R. Epidemiology and causes of preterm birth. *Lancet*, 2008; 371: 75–78.
75. Gomez L.M., Sammel M.D., Appleby D.H., Elovitz M.A., Baldwin D.A., Jeffcoat M.K. Evidence of a gene-environment interaction that predispose to spontaneous preterm birth: a role for asymptomatic bacterial vaginosis and DNA variants in genes that control the inflammatory response. *Am J Obstet Gynecol*, 2010; 202 (4): 386.e1 – 386.e6.
76. Goto A., Nguyen Q.V., Pham N.M., Kato K., Cao T.P., Le T.H., et al. Prevalence of and factors associated with reproductive tract infections among pregnant women in ten communes in Nghe An Province, Vietnam. *J Epidemiol*, 2005; 15 (5): 163–172.
77. Greaves W.L., Cungafung J., Morris B., Haile A., Townsend J.L. Clindamycin versus metronidazole in the treatment of bacterial vaginosis. *Obstet Gynecol*, 1988; 72 (5): 799–802.

78. Grevett M.G., Eschenbach D.A. Possible role of *Ureaplasma urealyticum* in preterm premature rupture of the fetal membranes. *Pediatr Infect Dis*, 1986; 5: 253–257.
79. Gustaffson R.J., Ahrne S., Jeppsson B., Benoni C., Olsson C., Stjernquist M., et al. The *Lactobacillus* flora in vagina and rectum of fertile and postmenopausal healthy Swedish women. *BMC Womens Health*, 2011; doi: 10.1186/1472-6874-11-17.
80. Hack M., Fanaroff A.A. Outcomes of children of extremely low birthweight and gestational age in the 1990s. *Semin Neonat*, 2000; 5 (2): 89–106.
81. Hamill N., Romero R., Gotsch F., Kusanovic J.P., Edwin S., Erez O., et al. Exodus-1 (CCL20): evidence for the participation of this chemokine in spontaneous labor at term, preterm labor, and intrauterine infection. *J Perinat Med*, 2008; 36: 217–227.
82. Hanna N.F., Taylor-Robinson D., Kalodiki-Karamanoli M. The relation between vaginal pH and the microbiological status in vaginitis. *BJOG*, 1985; 92: 1267–1271.
83. Hand W.L., Hand D.L., King-Thompson N.L. Antibiotic inhibition of the respiratory burst response in human polymorphonuclear leukocytes. *Antimicrob Agents Chemother*, 1990; 34: 863–870.
84. Hathcock J.N. Vitamin and mineral safety. Council for responsible nutrition. 2nd edition. 2004
Available at: <http://www.crnusa.org/safety.html>. Retrieved from internet on 01.05.13.
85. Hauth J.C., Goldenberg R.L., Andrews W.W., Dubard M.B., Cooper R.L. Reduced incidence of preterm delivery with metronidazole and erythromycin in women with bacterial vaginosis. *N Engl J Med*, 1995; 333 (26): 1732–1736.
86. Hauth J.C., McPerson C., Carey J.C., Klebanoff M.A., Hiller S.L., Ernest J.M., et al. Early pregnancy threshold vaginal pH and Gram stain scores predictive of subsequent preterm birth in asymptomatic women. *Am J Obstet Gynecol*, 2003; 188 (3): 831–835.
87. Hay P.E., Lamont R.F., Taylor-Robinson D., Morgan D.J., Ison C., Pearson J. Abnormal bacterial colonisation of the genital tract and subsequent preterm delivery and late miscarriage. *BMJ*, 1994; 308 (6924): 295–298.
88. Hill G.B. The microbiology of bacterial vaginosis. *Am J Obstet Gynecol*, 1993; 169: 450–454.

89. Hillier S.L. (a) Diagnostic microbiology of bacterial vaginosis. *Am J Obstet Gynecol*, 1993; 169: 455–459.
90. Hillier S.L. (b), Krohn M.A., Rabe L.K., Klebanoff S.J., Eschenbach D.A. The normal vaginal flora, H₂O₂-producing lactobacilli, and bacterial vaginosis in pregnant women. *Clin Infect Dis*, 1993; 16 (4): 273–281.
91. Hillier S.L. The complexity of microbial diversity in bacterial vaginosis. *N Engl J Med*, 2005; 353: 1886–1887.
92. Hobel C.J., Dunkel-Schetter C., Roesch S.C., Castro L.C., Arora C.P. Maternal plasma corticotropin-releasing hormone associated with stress at 20 weeks` gestation in pregnancies ending in preterm delivery. *Am J Obstet Gynecol*, 1999; 180: 257– 263.
93. Holley R.L., Richter H.E., Varner R.E., Pair L., Schwebke J.R. A randomized, double-blind clinical trial of vaginal acidification versus placebo for the treatment of symptomatic bacterial vaginosis. *Sex Transm Dis*, 2004; 31: 236–238.
94. Holst E., Goffeng A.R., Andersch B. Bacterial vaginosis and vaginal microorganisms in idiopathic premature labor and association with pregnancy outcome. *J Clin Microbiol*, 1994; 32: 176–186.
95. Hoover W.W., Gerlach E.H., Hoban D., Eliopoulos G.M., Pfaller M.A., Jones R.N. Antimicrobial activity and spectrum of rifaximin, a new topical rifamycin derivative. *Diagn. Microbiol. Infect. Dis*, 1993; 16: 111–118.
96. Hoyme U.B., Saling E. Efficient prematurity prevention is possible by pH-self measurement and immediate therapy of threatening ascending infection. *Eur J Obstet Gynecol Reprod Biol*, 2004; 115 (2): 148–153.
97. Huttunen R., Heikinen T., Sirjanen J. Smoking and the outcome of infection. *J Intern Med*, 2011; 269: 258–269.
98. Hymen R.W., Fukushima M., Diamond L., Kumm J., Giudice L.C., Davis R.W. Microbes on the human vaginal epithelium. *Proc Natl Acad Sci USA*, 2005; 102: 7952–7957.
99. Jacobsson B., Mattsby-Baltzer I., Andresch B. Microbial invasion and cytokine response in amniotic fluid in a Swedish population of women with preterm prelabor rupture of membranes. *Acta Obstet Gynecol Scand*, 2003; 82 (5): 423–431.
100. Joesoef M.R., Schmid G. Bacterial vaginosis. *BMJ Clinical Evidence*, 2005; 04:1601. Available at: <http://clinicalevidence.bmj.com/x/mce/file/2005-04-1601.pdf>. Retrieved from internet on 01.05.13.

101. Juárez Tomás M.S., Ocaña V.S., Wiese B., Nader-Macías M.E. Growth and lactic acid production by vaginal *Lactobacillus acidophilus* CRL 1259, and inhibition of uropathogenic *Escherichia coli*. *J Medl Microbiol*, 2003; 52: 1117–1124.
102. Kacerovsky M., Pliskova L., Bolehovska R., Skogstrand K., Hougaard D., et al. The impact of the microbial load of genital mycoplasmas and gestational age on the intensity of intraamniotic inflammation. *Am J Obstet Gynecol*, 2012; 206 (4): 342.e1 - 342349.
103. Karavitis J., Kovacs E.J. Macrophage phagocytosis: effects of environmental pollutants, alcohol, cigarette smoke and other external factors. *J Leukoc Biol*, 2011; 90: 1065–1078.
104. Kasper D.C., Mechtler T.P., Reischer G.H., Witt A., Langgartner M., Pollak A., et al. The bacterial load of *Ureaplasma parvum* in amniotic fluid is correlated with an increased intrauterine inflammatory response. *Diagn Microbiol Infect Dis*, 2010; 67 (2): 117–121.
105. Kim C.J., Yoon B.H., Romero R., Moon J.B., Kim M., Park S.S., et al. Umbilical arteritis and phlebitis mark different stages of the fetal inflammatory response. *Am J Obstet Gyn*, 2001; 185: 496–500.
106. King J.F., Flenady V. Prophylactic antibiotics for inhibiting preterm labour with intact membranes. *Cochrane Database of Systematic Reviews*, 2002; doi: 10.1002/14651858.CD000246.
107. Klebanoff S.J., Waltersdorff A.M., Rosen H. Antimicrobial activity of myeloperoxidase. *Methods Enzymol*, 1984; 105: 399–403.
108. Klebanoff M.A., Carey J.C., Hauth J.C., Hillier S.L., Nugent R.P., Thom E.A., et al. Failure of metronidazole to prevent preterm delivery among pregnant women with asymptomatic *Trichomonas vaginalis* infection. *N Engl J Med*, 2001; 345 (7): 487– 493.
109. Klebanoff M.A., Schwebke J.R., Zhang J., Nansel T.R., Yu K.F., Andrews W.W. Vulvovaginal symptoms in women with bacterial vaginosis. *Obstet Gynecol*, 2004; 104 (2): 267.
110. Klatt T.E., Cole D.C., Eastwood D.C., Barnabei V.M. Factors associated with recurrent bacterial vaginosis. *J Reprod Med*, 2010; 55: 55–61.
111. Koch E., Thorp J., Bravo M., Gatica S., Romero C.X., Aguilera H., et al. Women's Education Level, Maternal Health Facilities, Abortion Legislation and Maternal

- Deaths: A Natural Experiment in Chile from 1957 to 2007. *PLoS ONE*, 2012, 7 (5): e36613; doi:10.1371/journal.pone.0036613.
112. Kraus-Silva L., Moreira M.E., Alves M.B., Braga A., Camacho K.G., Batista M.R., et al. Randomised controlled trial of probiotics for the prevention of spontaneous preterm delivery associated with bacterial vaginosis: preliminary results. *Trials*, 2011; 12 (1): 239–241.
113. Kurkinen-Raty S., Vuopala M., Koskela A., Kekki M., Kurki T., Paarvonen J., et al. A randomised controlled trial of vaginal clindamycin for early pregnancy bacterial vaginosis. *BJOG* 2000; 107 (11): 1427–1432.
114. Lamont R.F., Taylor-Robinson D., Wigglesworth J.S., Furr P.M., Evans R.T., Elder M.G. The role of mycoplasmas, ureaplasmas and chlamydia in the genital tract of women presenting in spontaneous early preterm delivery. *J Med Microbiol*, 1987; 24: 253–257.
115. Lamont R.F., Duncan S.L., Mandal D., Bassett P. Intravaginal clindamycin to reduce preterm birth in women with abnormal genital tract flora. *Obstet Gynecol*, 2003; 101: 516–522.
116. Lamont R. F. Can antibiotics prevent preterm birth-the pro and con debate. *BJOG*, 2005; 112 (1): 67–73.
117. Lamont R.F. (a), Sobel J.D., Akins R.A., Hassan S.S., Chaiworapongsa T., Kusanovic J.P. The vaginal microbiome: new information about genital tract flora using molecular based techniques. *BJOG*, 2011; 118: 533–549.
118. Lamont R.F. (b), Nhan-Chang C.L., Sobel J.D., Workowski K., Conde-Agudelo A., Romero R. Treatment of abnormal vaginal flora in early pregnancy with clindamycin for the prevention of spontaneous preterm birth: a systematic review and metaanalysis. *Am J Obstet Gynecol*, 2011; 205 (3): 177–190.
119. Lamont R.F. (c), Sobel J.D., Kusanovic J.P., Vaisbuch E., Mazaki-Tovi S., Kim S.K., et al. Current debate on use of antibiotic prophylaxis for caesarean section. *BJOG*, 2011; 118: 193–201.
120. Larsen B., Monif G.R. Understanding the bacterial flora of the female genital tract. *Clin Infect Dis*, 2001; 4: e69 - e77.
121. Larsson P.G., Platz-Christensen J.J. Enumeration of clue cells in rehydrated air-dried vaginal wet smears for the diagnosis of bacterial vaginosis. *Obstet Gynecol*, 1990; 76: 727–730.

122. Larsson P.G., Carlsson B. Does pre- and postoperative metronidazole treatment lower vaginal cuff infection rate after abdominal hysterectomy among women with bacterial vaginosis? *Infect Dis Obstet Gynecol*, 2002; 10 (3): 133–140.
123. Larsson P.G., Fahraeus L., Carlsson B., Jakobsson T., Forsum U. Late miscarriage and preterm birth after treatment with clindamycin: a randomized consent design study according to Zelen. *BJOG*, 2006; 113: 629–637.
124. Larsson P.G., Fahraeus L., Carlsson B., Jakobsson T., Forsum U. Predisposing factors for bacterial vaginosis, treatment efficacy and pregnancy outcome among term deliveries; results from a preterm delivery study. *BMC Womens Health*, 2007; 7:20; doi: 10.1186/1472-6874-7-20.
125. Larsson P.G., Stray-Pedersen B., Ryttig K.R., Larsen S. Human lactobacilli as supplements of clindamycin to patients with bacterial vaginosis reduce the recurrence rate; a 6-month, double-blind, randomized, placebo-controlled study. *BMC Womens Health*, 2008; doi: 10.1186/1472-6874-8-3.
126. Larsson P.G., Brandsborg E., Forsum U., Pendharkar S., Andersen K.K., Nasic S. Extended antimicrobial treatment of bacterial vaginosis combined with human lactobacilli to find the best treatment and minimize the risk of relapses. *BMC Infect Dis*, 2011; doi: 10.1186/1471-2334-11-223.
127. Latvian Association of Gynecologists and Obstetricians.
Available at: <http://www.ginasoc.lv>. Retrieved from internet on 13.01.13.
128. Latvian Central Statistical Bureau. Available at: <http://www.csb.gov.lv/en/about-us>. Retrieved from internet on 13.01.13.
129. Lauer E., Helming C., Kandler O. Heterogeneity of the species *Lactobacillus acidophilus* (Moro) Hansen and Mosquit as revealed by biochemical characteristics and DNA–DNA hybridization. *Zentbl Bakteriol Microbiol Hyg Abt*, 1980; 1: 150–168.
130. Laxmi U., Agrawal S., Raghunandan C., Randhawa V.S., Saili A. Association of bacterial vaginosis with adverse fetomaternal outcome in women with spontaneous preterm labor: a prospective cohort study. *J Matern Fetal Neonatal Med*, 2012; 25 (1): 64–67.
131. Ledger W.J., Witkin S.S. *Vulvovaginal infections*. London, UK: Manson Publishing Ltd; 2010, page 52.

132. Lee H.C., Subeh M., Gould J.B. Low Apgar score and mortality in extremely preterm neonates born in the United States. *Acta Paediatr*, 2010; 99 (12): 1785–1789.
133. Lee J., Taneja V., Vassallo R. Cigarette smoking and inflammation: cellular and molecular mechanisms. *J Dent Res*, 2012; 91 (2): 142–149.
134. Leitich H., Kiss H. Asymptomatic bacterial vaginosis and intermediate flora as risk factors for adverse pregnancy outcome. *Best Pract Res Clin Obstet Gynaecol*, 2007; 21 (3): 375–339.
135. Leppäluoto P.A. Autopsy of bacterial vaginosis: a physiological entity rather than a contagious disease. *Acta Obstet Gynecol Scand*, 2008; 87: 578–579.
136. Lin C.Y., Hsu C.H., Huang F.Y., Chang J.K., Hung H.Y., Hao K.A., et al. The changing face of early-onset neonatal sepsis after the implementation of a maternal group B Streptococcus screening and intrapartum prophylaxis policy-a study in one medical center. *Pediatr Neonatol*, 2011; 52 (2): 78–84.
137. Linda O., Eckert M.D. Acute vulvovaginitis. *N Engl J Med*, 2006; 335: 1244–1255.
138. Llahy-Camp J.M., Rai R., Ison C., Regan L., Taylor-Robinson D. Association of bacterial vaginosis with the history of second trimester miscarriage. *Hum Reprod*, 1996; 11: 1575–1578.
139. Lumbiganon P., Thinkamrop J., Thinkamrop B., Tolosa JE. Vaginal chlorhexidine during labour for preventing maternal and neonatal infections (excluding Group B streptococcal and HIV). *Cocharene Database of Systematic Reviews*, 2011; doi: 10.1002/14651858.CD004070.pub2.
140. Mahon C.R., Manuselis G. *Textbook of diagnostic microbiology*. W.B. Saunders Company, Philadelphia, 2000; pages 339–408.
141. Marconi C., Donders G.G., Martin L.F., Ramos B.R., Duarte M.T., Parada C.M., Tristão A.R., Silva M.G. Chlamydial infection in a high risk population: association with vaginal flora patterns. *Arch Gynecol Obstet*, 2012; 285 (4): 1013–1018.
142. Margulies M., Egholm M., Altman W.E., Attiya S., Bader J.S., Bemben L.A., et al. Genome sequencing in microfabricated high-density picolitre reactors. *Nature*, 2005; 437: 376–380.
143. Mastromarino P., Brigidi P., Macchia S., Maggi L., Pirovano F., Trinchieri V., et al. Characterization and selection of vaginal *Lactobacillus* strains for the preparation of vaginal tablets. *J Appl Microbiol*, 2002; 93: 884–893.

144. Mbizvo E.M., Msuya S.E., Stray-Pedersen B., Sundby J., Chirenje Z.M., Hussain A. Determinants of reproductive tract infections among asymptomatic women in Harare, Zimbabwe. *Centr Afr J Med*, 2001; 47 (3): 57–64.
145. McCann M.F., Irwin D.E., Walton L.A., Hulka B.S., Morton J.L., Axelrad C.M. Nicotine and cotinine in the cervical mucus of smokers, passive smokers, and nonsmokers. *Cancer Epidemiol Biomarkers Prev.* 1992; 1 (2): 125–129.
146. McGregor J.A., French J.I., Parker R., Draper D., Patterson E., Jones W., et al. Prevention of premature birth by screening and treatment for common genital tract infections: results of a prospective controlled evaluation. *Am J Obstet Gynecol*, 1995 Jul; 173 (1):157–167.
147. Menard J.P., Fenollar F., Henry M., Bretelle F., Raoult D. Molecular quantification of *Gardnerella vaginalis* and *Atopobium vaginae* loads to predict bacterial vaginosis. *Clin Infect Dis*, 2008; 47: 33–43.
148. Mercer B.M., Abdelrahim A., Moore R.M., Novak J., Kumar D., Mansour J.M., et al. The impact of vitamin C supplementation in pregnancy and in-vitro upon fetal membrane strength and remodeling. *Reprod Sci*, 2010; 17(7): 685–695.
149. Mittendorf R., Montag A.G., ManMillan W., Janeczek S., Pryde P.G., Besinger R.E., et al. Components of the systemic fetal inflammatory response syndrome as predictors of impaired neurologic outcomes in children. *Am J Obstet Gynecol*, 2003; 188: 1438–1440.
150. Msuya S.E., Uriyo J., Stray-Pedersen B., Sam N.E., Mbizvo E.M. The effectiveness of a syndromic approach in managing vaginal infections among pregnant women in northern Tanzania. *East Afr J Public Health*, 2009; 6 (3): 263–267.
151. Mylonas I. Antibiotic chemotherapy during pregnancy and lactation period: aspects for consideration. *Arch Gynaecol Obstet*, 2011; 283: 7–18.
152. Nadisauskiene R., Bergström S., Stankeviciene I., Spukaite T. Endocervical pathogens in women with preterm and term labour. *Gynecol Obstet Invest*, 1995; 40(3): 179–182.
153. Nenadić D.B., Pavlović M.D. Cervical fluid cytokines in pregnant women: relation to vaginal wet mount findings and polymorphonuclear leukocyte counts. *Eur J Obstet Gynecol Reprod Biol.* 2008; 140 (2): 165–170.
154. Neri A., Sabah G., Samra Z. Bacterial vaginosis in pregnancy treated with yoghurt. *Acta Obstet Gynecol Scandin*, 1993; (72): 17–19.

155. Ness R.B., Grainger D.A. Male reproductive proteins and reproductive outcomes. *Am J Obstet Gynecol*, 2008; doi: 10.1016/j.ajog.2007.09.017.
156. Newborn register of Latvia. [Internet]. Latvia: Center of Disease Control of Latvia. Available from: www.spkc.gov.lv. Retrieved from internet on 13.01.13.
157. Nugent R.P., Krohn M.A., Hillier S.L. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol*, 1991; 29 (2): 297–301.
158. Oakley B.B., Fiedler T.L., Marazzo J.M., Fredricks D.N. Diversity of human vaginal bacterial communities and association with clinically defined bacterial vaginosis. *Appl Environ Microbiol*, 2008; 74: 4898–4909.
159. Odendaal H.J., Popov I., Schoeman J., Smith M., Grove D. Preterm labour – is bacterial vaginosis involved? *S Afr Med J*, 2002; 92 (3): 231–234.
160. O'Hanlon D. E., Lanier B. R., Moench T. R., Cone R. A. Cervicovaginal fluid and semen block the microbicidal activity of hydrogen peroxide produced by vaginal lactobacilli. *BMC Infect Dis*, 2010; doi: 10.1186/1471-2334-10-120.
161. Omar N.S., Tan P.C., Sabir N., Yusop E.S., Omar S.Z. Coitus to expedite the onset of labour: a randomised trial. *BJOG*, 2013; 120 (3): 338–345.
162. Othman M., Neilson J.P., Alfirevic Z. Probiotics for preventing preterm labour. *Cochrane Database of Systematic Reviews*, 2012; doi: 10.1002/14651858.
163. Osset J., Bartolome R.M., Garcia E., et al. Assessment of the capacity of *Lactobacillus* to inhibit the growth of uropathogens and block their adhesion to vaginal epithelial cells *J Infect Dis*, 2011; 183 (3): 485–491.
164. Ozkinay E., Terek M.C., Yayci M., Kaiser R., Grob P., Tuncay G. The effectiveness of live lactobacilli in combination with low dose oestriol (Gynoflor) to restore the vaginal flora after treatment of vaginal infections. *Int J Obstet Gynecol* 2005; 112: 234–240.
165. Parent D., Bossens M., Bayot D., Kirkpatrick C., Graft F., Wilkinson F.E., et al. Therapy of bacterial vaginosis using exogeneously-applied *Lactobacilli acidophili* and a low-dose of estriol: A placebo-controlled multicentric clinical trial. *Arzneimittelforschung*, 1996; 46: 68–73.
166. Pastore L.M., Thorp J.M., Royce R.A., Savitz D.A., Jackson T.P. Risk score for antenatal bacterial vaginosis: BV PIN points. *J Perinatol*, 2002; 22: 125–132.

167. Pavlova S.I., Kilic A.O., Kilic S.S., So J.S., Nader-Marcias M.E., Somoes J.A., et al. Genetic diversity of vaginal lactobacilli from women in different countries based on 16S rRNA gene sequences. *J Appl Microbiol*, 2002; 92: 451–459.
168. Petersen E.E., Magnani P. Efficacy and safety of Vitamin C vaginal tablets in the treatment of non-specific vaginitis. A randomized, double-blind, placebo-controlled study. *Obstet Gynecol*, 2004; 117: 70–75.
169. Petersen E.E., Genet M., Caserini M., Palmieri R. Efficacy of vitamin C vaginal tablets in the treatment of bacterial vaginosis: a randomized, double-blind, placebo-controlled clinical trial. *Arzneimittelforschung*, 2011; 61 (4): 260–265.
170. Petridou E., Salvanos H., Skalkidou A., Dessypris N., Moustaki M., Trichopoulos D. Are there common triggers of preterm deliveries? *BJOG*, 2001; 108 (6): 598–604.
171. Pfeifer T.A., Forsyth P.S., Durfee M.A., Pollock H.M., Holmes K.K. Nonspecific vaginitis: role of *Haemophilus vaginalis* and treatment with metronidazole. *N Engl J Med*, 1978; 298: 1429–1434.
172. Polatti F., Rampino M., Mahnani P., Mascarucci P. Vaginal pH-lowering effect of locally applied vitamin C in subjects with high vaginal pH. *Gynecol Endocrinol*, 2006; 22 (4): 230–234.
173. Porter R.S., Kaplan J.L. The Merck manual, 18th edition. United States of America: Merck Sharp&Dohme Corp, 2011, pages 1101–1120.
174. Rabe L.K., Hillier S.L. Optimization of media for detection of hydrogen peroxide by *Lactobacillus* species. *J Clin Microbiol*, 2003; 41: 3260–3264.
175. Ralph S.G., Rutherford A.J., Wilson J.D. Influence of bacterial vaginosis on conception and miscarriage in the first trimester: a cohort study. *BMJ*; 1999: 220–223.
176. Ravel J., Gajer P., Abdo Z., Schneider G.M., Koenig S.S., McCulle S.L., et al. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci U S A*, 2011; 108 (1): 4680–4687.
177. Rayness-Greenow C.H., Roberts C.L., Bell J.C., Peat B., Gilbert G.L., Parker S. Antibiotics for ureaplasma in the vagina in pregnancy. *The Cochrane Library* 2011; doi:10.1002/14651858.
178. Redondo-Lopez V., Cook R.L., Sobel J.D. Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. *Rev Infect Dis*, 1990; 164 (1): 94–100.

179. Regulation of the Latvian Cabinet of Ministers Nr. 611. Available from: <http://www.likumi.lv/doc.php?id=140695>. Retrieved from internet on 13.01.13.
180. Reid G., Charbonneau D., Erb J., Kochanowski B., Beuerman D., Poehner R., Bruce A.W. Oral use of *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. *FEMS Immunol Med Microbiol*, 2003(a); (35): 131–134.
181. Reid G., Bocking A. The potential for probiotics to prevent bacterial vaginosis and preterm labor. *Am J Obstet Gynecol*, 2003; 189 (4): 1202–1208.
182. Reid G., Burton J., Hammond J.A., Bruce A.W. Nucleic acid-based diagnosis of bacterial vaginosis and improved management using probiotic lactobacilli. *J Med Food*, 2004; 7: 223–228.
183. Rezeberga D., Lazdāne G., Kroiča J., Sokolova L. Seksuāli transmisīvo slimību sastopamība grūtniecēm Latvijā. *RSU Zinātniskie raksti*, 2000; 159–164.
184. Rezeberga D., Lazdāne G., Kroiča J., Sokolova L., Teibe U. Women's reproductive tract infections: influence on duration and outcome of pregnancy. *Proc Latv Ac Scien*, 2002; 56 (1/2): 42–47.
185. Rezeberga D., Lazdane G., Kroica J., Sokolova L., Donders G.G. Placental histological inflammation and reproductive tract infections in a low risk pregnant population in Latvia. *Acta Obstet Gynecol Scand*, 2008; 87 (3): 360–365.
186. Rezeberga D. Dzemdniecība. Medicīnas apgāds; Latvija, 2009; page 281.
187. Riegelman R.K. Studying a study and testing a test. Lippincot Williams&Wilkins; Philadelphia, 2000; pages 144–149.
188. Rivkin A., Gim S. Rifaximin: new therapeutic indication and future directions. *Clin. Ther.* 2011; 33: 812–827.
189. Robertson S.A. Seminal plasma and male factor signaling in the female reproductive tract. *Cell Tissue Res*, 2005; 322: 43–52.
190. Romero R., Mazor M., Sepulveda W., Avila C., Copeland D., Williams J. Tumor necrosis factor in preterm and term labor. *Am J Obstet Gynecol*, 1992; 166: 1576–1587.
191. Romero R., Espinoza J., Chaiworapongsa T., Kakache K. Infection and prematurity and the role of preventive strategies. *Semin Neonatol*, 2002; 7 (4): 259–274.
192. Romero R., Chaiworapongsa T., Kuivaniemi H., Tromp G. Bacterial vaginosis, the inflammatory response and the risk of preterm birth: a role for genetic

- epidemiology in the prevention of preterm birth. *Am J Obstet Gyn*, 2004; 190 (6): 1509–1519.
193. Romero R., Garite T.J. Twenty percent of very preterm neonates (23-32 weeks of gestation) are born with bacteremia caused by genital mycoplasmas. *Am J Obstet Gynecol*, 2008; 198: 1–3.
194. Rönnqvist P.D., Forsgren-Brusk U.B., Hakansson E.E. Lactobacilli in the female genital tract in relation to other genital microbes and vaginal pH. *Acta Obstet Gynecologica*, 2006; 85: 726–735.
195. Rosenstein I.J., Morgan D.J., Sheehan M., Lamont R.F., Taylor-Robinson D. Bacterial vaginosis in pregnancy: distribution of bacterial species in different Gram stain categories of the vaginal flora. *J Med Microbiol*, 1996; 45: 120–126.
196. Rumbold A., Crowther C.A. Vitamin C supplementation in pregnancy. *Cochrane database Syst Rev*, 2004; doi: 10.1002/14651858.
197. Sagawa T., Negishi H., Kishida T., Yamada H., Fujimoto S. Vaginal and cervical pH in bacterial vaginosis and cervicitis during pregnancy. *Hokk J Med Sc*, 1995; 70: 839–846.
198. Scarpignato C., Pelosini I. Rifaximin, a poorly absorbed antibiotic: pharmacology and clinical potential. *Chemotherapy*, 2005; 51: 36–66.
199. Schoeman J., Steyn P.S., Odendaal H.J., Grove D. Bacterial vaginosis diagnosed at the first antenatal visit better predicts preterm labour than later in pregnancy. *J Obstet Gynecol*, 2005; 25 (8): 751–753.
200. Schröder K. Zur pathogenese und Klinik des Vaginalbiocoenose auf sechs grundbilder. *Zentralblatt Gynekol*, 1921; 45: 1350–1361.
201. Scwebke J.R., Richey C.M., Weiss H.L. Correlation of behaviours with microbiological change in vaginal flora. *J Infect Dis*, 1999; 180: 1632–1636.
202. Schwebke J.R., Desmond R. Risk factors for bacterial vaginosis in women at high risk for sexually transmitted diseases. *Sex Transm Dis*, 2005; 32 (11): 654–658.
203. Schwebke J.R., Desmond R. A randomised trial of the duration of therapy with metronidazole plus or minus azithromycin for treatment of symptomatic bacterial vaginosis. *Clin Infect Dis*, 2007; 44: 213–219.
204. Schwebke J.R., Desmond R.A. Tinidazole versus Metronidazole for the Treatment of Bacterial Vaginosis. *Am J Obstet Gynecol*. *Am J Obstet Gynecol*, 2011; 204 (3): 211.e1–211.e6; doi: 10.1016/j.ajog.2010.10.898.

205. Schwiertz A., Taras D., Rusch K., Rusch V. Throwing the dice for the diagnosis of vaginal complaints? *Annals Clin Microbiol Antimicrob*, 2006; 5: 4; doi: 10.1186/1476-0711-5-4.
206. Shane A.L., Stoll B.J. Recent developments and current issues in the epidemiology, diagnosis, and management of bacterial and fungal neonatal sepsis. *Am J Perinatol*, 2013; 30 (2): 131–141.
207. Shalev E., Battino S., Weiner E., Colodner R., Keness Y. Ingestion of yogurt containing *Lactobacillus acidophilus* compared with pasteurized yogurt as prophylaxis for recurrent candidal vaginitis and bacterial vaginosis. *Archi Fam Medic*, 1996; (5): 593–596.
208. Shennan A., Crawshaw S., Briley A., Hawken J., Seed P., Jones G., Poston L. "General obstetrics: A randomised controlled trial of metronidazole for the prevention of preterm birth in women positive for cervicovaginal fetal fibronectin: The PREMETS Study". *BJOG*, 2005; 113 (1): 65–74.
209. Simhan H.N., Caritis S.N., Krohn M.A., Hillier S.L. Elevated vaginal pH and neutrophils are associated with early spontaneous preterm birth. *Am J Obstet Gynecol*, 2003; 189 (41): 1150–1154.
210. Simhan H.N., Caritis S.N., Krohn M.A., Hillier S.L. The vaginal inflammatory milieu and the risk of early premature preterm rupture of membranes. *Am J Obstet Gynecol*, 2005; 192 (1): 213–218.
211. Smart S., Singal A., Mindel A. Social and sexual risk factors for bacterial vaginosis. *Sex Transm Infect*, 2004; 80: 58–62.
212. Sobel J.D. Desquamative inflammatory vaginitis: a new subgroup of purulent vaginitis responsive to topical 2% clindamycin therapy. *Am J Obstet Gynecol*, 1994; 171: 1215–1220.
213. Sobel J.D., Ferris D., Schwebke J., Nyrjesy P., Wiesenfeld H.C., Peipert J., et al. Suppressive antibacterial therapy with 0.75% metronidazole vaginal gel to prevent recurrent bacterial vaginosis. *Am J Obstet Gynecol*, 2006; 194: 1283–1289.
214. Sodhani P., Garg S., Bhalla P. Prevalence of bacterial vaginosis in a community setting and role of the Pap smear in its detection. *Acta Cytol*, 2005; 49: 634–638.
215. Srinivasan S., Liu C., Mitchell C.M., Fiedler T.L., Thomas K.K., Agnew K.J., et al. Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. *PloS ONE*, 2010; 5: doi/10.1371/journal.pone.0010197.

216. Statistical Yearbook of Health Care in Latvia, 2012, [Internet]. Latvia: Center of Disease Control. Available from: www.spkc.gov.lv/veselibas-aprupes-statistika. Retrieved from internet on 13.01.13.
217. Stoll B.J., Hansen N.I., Sanchez P.J., Faix R.G., Poindexter B.B., Van Meurs K.P., et al. Early onset neonatal sepsis: the burden of group B streptococcal and *E. coli* continues. *Pediatrics*, 2011; 126 (5): 817–826.
218. Stray-Pedersen B., Bergan T., Hafstad A., Normann E., Grogaard J., Vangal M. Vaginal disinfection with chlorhexidine during childbirth. *Int J Antimicrob Agents*, 1999; 12 (3): 245–251.
219. Subramaniam A., Abramovici A., Andrews W.W., Tita A.T. Antimicrobials for preterm birth prevention: an overview. Doi: 10/2012/157159.
220. Survey of Reproductive Health of Inhabitants of Latvia, 2011.
221. Svare J.A., Schmidt H., Hansen B.B., Lose G. Bacterial vaginosis in a cohort of Danish pregnant women: prevalence and relationship with preterm delivery, low birthweight and perinatal infections. *BJOG*, 2006; 113 (12): 1419–1425.
222. Svidsinski A., Mending W., Loening-Baucke V., Ladhoff A., Svidsinski S., Hale L.P., Lochs H. Adherent biofilms in bacterial vaginosis. *Obstet Gynecol*, 2005; 106: 1013–1023.
223. Svidsinski A., Mending W., Loening-Baucke V., Svidsinski S., Dorffel J., Scholze J., et al. An adherent *Gardnerella vaginalis* biofilm persists on the vaginal epithelium after standart therapy with oral matronidazole. *Am J Obstet Gynecol*, 2008; 198: 97–106.
224. Tan P.C., Andi A., Azmi N., Noraihan M.N. Effect of coitus at term on length of gestation, induction of labor, and mode of delivery. *Obstet Gynecol*, 2006; 108 (1): 134–140.
225. Taylor-Robinson D. The role of mycoplasmas in pregnancy outcome. *Best Pract Res Clin Obstet Gynaecol*, 2007; 21: 425 – 438.
226. Taylor-Robinson D., Lamont R.F. Mycoplasmas in pregnancy. *BJOG*, 2011; 118: 164–174.
227. Thinkhamrop J., Hofmeyr G.J., Adetoro O., Lumbiganon P. Prophylactic antibiotic administration during second and third trimester in pregnancy for prevention infectiuos morbidity and mortality. *Cochrane Databasa of Systematic Reviews*, 2009; doi: 10.1002/14651858.

228. Thomas S. Döderlein`s bacillus: *Lactobacillus acidophilus*. J Infect Dis, 1928; 43: 218–227.
229. Thulkar J., Kriplani A., N. A comparative study of oral single dose of metronidazole, tinidazole, secnidazole and ornidazole in bacterial vaginosis. Indian J Pharmacol. 2012; 44 (2): 243 – 245; doi: 10.4103/0253-7613.93859.
230. Todar K. Todar`s online textbook of bacteriology. 2008-2012.
Available at: <http://textbookofbacteriology.net/lactics.html>. Retrieved from internet 13.01.13.
231. Ugwumadu A., Reid F., Hay P., Manyonda I. Natural history of bacterial vaginosis and intermediate flora in pregnancy and effect of oral clindamycin. Obstet Gynecol, 2004; 104: 114–119.
232. Vatten L.J., Skjaerven R. Effects on pregnancy outcome of changing partner between first two births: prospective population study. BMJ, 2003; 327: 1138; doi: 10.1136/bmj.327.7424.1138.
233. Vedmedovska N., Rezeberga D., Teibe U., Zodzika J., Donders G.G. Preventable maternal risk factors and association of genital infection with fetal growth restriction. Gynecol Obstet Invest, 2010; 70 (4): 291–298.
234. Veleminsky M., Tosner J. Relationship of vaginal microflora to PROM, pPROM and the risk of early-onset neonatal sepsis. Neuro Endocrin Lett, 2008; 29 (2): 205–221.
235. Verhelst R., Verstraelen H., Claeys G., Verchraegen G., Van Simaey L., De Ganck C, et al. Comparision between Gram stain and cultures for the characterization of vaginal microflora: definition og a distinct grade that resembles grade I microflora and revised categorization of grade I microflora. BMC Microbiol, 2005; 5: 61; doi:10.1186/1471-2180-5-61.
236. Verstraelen H. (a), Verhelst R., Claeys G., De Backer E., Temmerman M., Vanechoutte M. Longitudinal analysis of the vaginal microflora in pregnancy suggests that *L. crispatus* promotes the stability of the normal vaginal microflora and that *L. gasseri* and/or *L. iners* are more conducive to the occurance of abnormal vaginal microflora. BMC Microbiol, 2009; 9: 116; doi: 10.1186/1471-2180-9-116.
237. Verstraelen H. (b), Verhelst R. Bacterial vaginosis: an update on diagnosis and treatment. Expert Rev Anti Infect Ther, 2009; 7 (9): 1109–1124.

238. Verstraelen H., Verhelst R., Temmerman M., Vaneechoutte M. The epidemiology of bacterial vaginosis in relation to sexual behaviour. *BMC Infect Dis*, 2010; 10: 81; doi: 10.1186/1471-2334-10-81.
239. Viberga I., Engele L., Baili P. Past, present and future of cervical cancer screening in Latvia. *Tumori*, 2010; 96: 529–537.
240. Vogel I. (a), Thoresen P., Jeune B., Jacobsson B., Ebbeseb N., Arpi M., et al. Acquisition and elimination of bacterial vaginosis during pregnancy: a Danish population-based study. *Infect Dis Obstet Gyn*, 2006; doi 10.1155/IDOG/2006/94646.
241. Vogel I. (b), Thorsen P., Hogan V.K., Schieve L.A., Jacobsson B., Ferre C.D. The joint effect of vaginal *Ureaplasma urealyticum* and bacterial vaginosis on adverse pregnancy outcomes. *Acta Obstet Gynecol Scand*, 2006; 85 (7): 778–785.
242. Watt S., Lanotte P., Mereghetti L., Moulin-Schouleur M., Picard B., Quentin R. *Escherichia coli* strains from pregnant women and neonates: intraspecies genetic distribution and prevalence of virulence factors. *J Clin Microbiol*, 2003; 41 (5): 1929–1935.
243. Waits K.B., Katz B., Schelonka R.L. Mycoplasmas and Ureaplasmas as neonatal pathogens. *Clin Microbiol Rev*, 2005; 18 (4): 757–789.
244. Weissenbacher E.R., Donders G.G., Unzeitig V., Martinez de T.B., Gerber S., Halaska M. et al. A comparison of dequalinium chloride vaginal tablets (Fluomizin(R)) and clindamycin vaginal cream in the treatment of bacterial vaginosis: a single-blind, randomized clinical trial of efficacy and safety. *Gynecol Obstet Invest*, 2012; 73: 8–15.
245. Wertz J., Isaacs-Cosgrove N., Holzman C., Marsh T.L. Temporal shifts in microbial communities in nonpregnant African-American women with and without bacterial vaginosis. *Interdiscip Perspect Infect Dis*, 2008: 181253; doi: 10.1155/2008/181253.
246. Wilks M., Wiggins R., Whiley A., Hennessy E., Warwick S., Porter H., Corfield A., Millar M. Identification and H₂O₂ productions of vaginal Lactobacilli from pregnant women at high risk of preterm birth and relation with outcome. *J Clin Microbiol*, 2003; 42: 713–717.
247. Wójkowska-Mach J., Borszewska-Kornacka M., Domańska J., Gadzinowski J., Gulczyńska E., Helwich E., et al. Early-onset infections of very-low-birth-weight

- infants in Polish neonatal intensive care units. *Pediatr Infect Dis J*, 2012; 31 (7): 691–695.
248. Workowski K.A., Berman S.M. Centers for Disease Control and Prevention sexually transmitted diseases treatment guidelines. *Clin Infect Dis*, 2007; 3: S73–S76.
249. Yost N.P., Owen J., Berghella V., Thom E., Swain M., Dildy G.A. 3rd, et al. Effect of coitus on recurrent preterm birth. *Obstet Gynecol*, 2006; 107 (4): 793–797.
250. Yotebieng M., Turner A.N., Hoke T.H., Van Damme K., Rasolofomanana J.R., Behets F. Effect of consistent use on 6-month prevalence of bacterial vaginosis by baseline BV status. *Trop Med Int Health*, 2009; 14: 480–486.
251. Zhou X., Bent S.J., Schneider M.G., Davis C.C., Islam M.R., Forney L.J. Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. *Microbiol*, 2004; 150: 2565–2573.
252. Zhou X., Brotman R.M., Gajer P., Abdo Z., Schütte U., Ma S., et al. Recent advances in understanding the microbiology of the female reproductive tract and the causes of preterm birth. *Infect Dis Ob Gyn*, 2010; doi: 10.1155/2010/737425.

9. 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. *Archives in Gynecology and Obstetrics*, 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.
- Zodzika J., Rezeberga D., Vasina O., Bite R., Pundure I., Zile O., Pavlova Z., Vidnere I., Kampara I., Krumina S. Role of the native microscopy in the detection of abnormal vaginal flora in pregnancy. *Riga Stradins University Collection of Scientific Papers* 2011, 2012; 41–47.
- Zodzika J., Rezeberga D., Vasina O. Influence of socioeconomic factors and smoking on vaginal pH and microflora in the first trimester of pregnancy in Latvia. *Proceedings of 22nd European Congress of Obstetrics and Gynecology* 2012; 115–118
- Zodzika J., Rezeberga D., Vasina O., Jermakova I., Vedmedovska N., Donders G.G. Factors related to elevated vaginal pH in the first trimester of pregnancy. *Acta Obstetrica and Gynecologica Scandandinavica*, 2011; 90:41–46.
- Donders G.G., Zodzika J., Rezeberga D. Treatment of bacterial vaginosis: what we have and what we miss. *Expert Opinion on Pharmacotherapy*, 2014; doi: 10.1517/14656566.2014.881800.
- Vedmedovska N., Rezeberga D., Donders G.G., Teibe U., Zodzika J. Preventable maternal risk factors and association with genital infections with fetal growth restriction. *Gynecological and Obstetric Investigation*, 2010; 70: 219–226.

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., Vidnere I., Zile O., Pavlova Z., Kampara I., Krumina S. Influence of *Escherichia coli* on vaginal flora in pregnancy. 6th Latvian Congress in Obstetrics and Gynecology, 2011.

2.2. 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 Rīgas Stradiņa universitātes 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 Rīgas Stradiņa universitātes Zinātniskā konference.
- Zodzika J., Rezeberga D., Vasina O., Bite R., Pundure I., Vidnere I., Matule D., Zile O., Pavlova Z., Baranovska D., Dresmane A. Patoloģiska maksts pH iemesli grūtniecēm I trimestrī. 2010. gada Rīgas Stradiņa universitātes Zinātniskā konference.

- Zodzika J., Rezeberga D., Vasina O., Jermakova I., Bite R., Pundure I., Strazdina L., Vidnere I., Baranovska D., Dresmane A., Donders G.G. Reliability of pH test for abnormal vaginal flora diagnose in the first trimester of pregnancy. The 13th World Congress on Controversies in Obstetrics and Gynecology& Infertility. Berlin, Germany, 2010.
- Zodzika J., Rezeberga D., Vasina O., Jermakova I., Bite R., Pundure I., Strazdina L., Vidnere I., Baranovska D., Dresmane A., Donders G.G. Association between increased vaginal pH and flora type in pregnant women. 21st European Congress of Obstetrics and Gynecology. Antwerpen, Belgium, 2010.
- Zodzika J., Rezeberga D., Kroica J., Strepmane I., Donders G.G. Comparison of different diagnostic methods in evaluation of bacterial vaginosis in pregnant women. VI Conference of European Society for Infectious diseases in Obstetrics and Gynecology. Leuven, Belgium, 2008.
- Zodzika J., Rezeberga D., Kroica J., Strepmane I., Donders G.G. Evaluation of vaginal flora in first trimester of pregnant women by native microscopy. VI Conference of European Society for Infectious diseases in Obstetrics and Gynecology. Leuven, Belgium, 2008.

APPENDICES

Appendix No. 1

Rīga Stradiņš University Ethical Committee Study approval

Veidlapa Nr E-9 (2)

RSU ĒTIKAS KOMITEJAS LĒMUMS

Rīga, Dzirciema iela 16, LV-1007
Tel.67409137

Komitejas sastāvs	Kvalifikācija	Nodarbošanās
1. Asoc. prof. Olafs Brūvers	Dr.miss.	teologs
2. Professore Vija Sīle	Dr.phil.	filozofs
3. Docente Santa Purviņa	Dr.med.	farmakologs
4. Asoc. prof. Voldemārs Arnis	Dr.biol.	rehabilitologs
5. Asoc. prof. Viesturs Liguts	Dr.med.	toksikologs
6. Professore Regīna Kleina	Dr.med.	patanatoms
7. Asoc. prof. Egils Korņevs	Dr.habil.med.	stomatologs
8. Asoc. prof. Guntars Pupelis	Dr.med.	ķirurgs

Pieteikuma iesniedzējs: Jana Žodžika
RSU, Doktorantūras nodaļa

Pētījuma nosaukums: Vaginālā vitamīna C ietekme uz grūtniecības norisi pacientēm ar izmainītu maksts vidi.

Iesniegšanas datums: 08.02.2010.

Pētījuma protokols:

- (X) Pētījuma veids: pētījuma instrumenti: anketa, pacientes dienasgrāmata, pētnieka brošūra
(X) Pētījuma populācija: MS,, ARS", SIA „ Rīgas Dzemdību nams", SIA „ Quartus", SIA „ Dzirciema poliklīnika" grūtnieces (n=500)
(X) Informācija par pētījumu:
(X) Piekrišana daļēbai pētījumā:

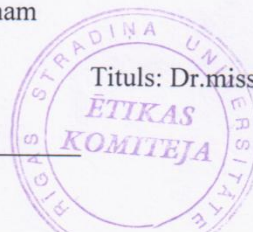
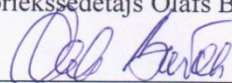
Citi dokumenti:

1. MS,, ARS" atļauja,
2. SIA „ Rīgas Dzemdību nams" atļauja,
3. SIA „ Quartus" atļauja,
4. SIA „ Dzirciema poliklīnika" atļauja

Lēmums: piekrist biomedicīniskajam pētījumam

Komitejas priekšsēdētājs Olafs Brūvers

Paraksts



Tituls: Dr.miss., asoc.prof.

Ētikas komitejas sēdes datums: 11.02.2010.

RAKSTISKS SKAIDROJUMS PACIENTEI PAR PĒTĪJUMU

(Written information sheet for consent – in Latvian)

Pētījuma nosaukums: IZMAINĪTA MAKSTS MIKROFLORA: TERAPIJAS
IESPĒJAS AR VAGINĀLO VITAMĪNU C.

Organizatori: Rīgas Stradiņa universitātes Dzemdniecības un
ginekoloģijas katedra

Pacientes identifikācijas numurs

Mēs vēlētos Jums piedāvāt piedalīties zinātniskajā pētījumā par makstī ievadāmā vitamīna C ietekmi uz izmainītu maksts mikrofloru grūtniecēm. Jūsu ārsts informēs par šo pētījumu. Pirms pievienojeties pētījumam, Jums ir jāzina, kāpēc šis pētījums tiek veikts un ko tas nozīmēs Jums.

IEVADS

Visu baktēriju kopumu makstī sauc par maksts mikrofloru. Normā maksts mikrofloru pamatā veido pienskābās baktērijas, kuras, izdalot dažādas vielas, nodrošina skābu maksts vidi. Šādā veidā maksts tiek pasargāta no infekcijām. Ja maksts vides pH palielinās, t.i., tā kļūst mazāk skāba (sārmaināka), tas nozīmē, ka ir savairojušās citas baktērijas – ir izmainīts maksts mikrofloras sastāvs. Šīs izmaiņas var noteikt ar maksts pH kontroles strēmelītēm, kā arī izmeklējot izdalījumus mikroskopiski vai veicot uzsmērjumus.

Pētījumos ir pierādīts, ka izmainīta maksts mikroflora ir saistīta ar grūtniecības neiznēsāšanas risku, tomēr joprojām nav atklāta efektīva taktika, kā iepriekš paredzēt un novērst šādus sarežģījumus. Pilnībā nav skaidrs, vai labāki rezultāti ir, nozīmējot kādu ārstēšanu vai arī vienkārši novērojot. Ir zināms, ka maksts infekciju gadījumā, tās var novērst ar antibakteriāliem līdzekļiem, taču nav pierādīts, vai tos jālieto visām

grūtniecēm un vai vienmēr šāda ārstēšana spēj novērst priekšlaicīgas dzemdības, antibiotiķu plaša lietošana ne visos gadījumos ir grūtniecēm pieņemama.

Pēdējā laikā arvien biežāk zinātnieki pēta citu ārstēšanas līdzekļu – „neantibakteriālu”, efektivitāti maksts mikrofloras uzlabošanā. Viens no šādiem medikamentiem ir makstī ievadāmās vitamīna C tabletes. Vitamīns C paskābina maksts vidi un veicina pienskābo baktēriju savairošanos. Vitamīna C makstī ievadāmās tabletes drīkst lietot grūtnieces, blaknes (nieze un dedzināšana makstī) ir reti sastopamas.

PĒTĪJUMA MĒRĶIS

Šajā pētījumā mēs vēlētos noskaidrot, vai izmainītas maksts mikrofloras gadījumā, lietojot makstī ievadāmo vitamīnu C, var uzlabot maksts vidi un novērst grūtniecības sarežģījumus.

PĒTĪJUMA IZMEKLĒJUMI

Lai pievienotos pētījumam, ir jābūt sekojošiem apstākļiem:

- Jums ir jābūt grūtniecībai mazāk par 14 nedēļām,
- ir vienaugļa grūtniecība,
- Jūs esiet vecāka par 18 gadiem,
- Jums nav hlamīdiju, gonokoku infekcijas vai sifiliss,
- Jums nav tādas slimības kā cukura diabēts, nieru mazspēja, arteriālā hipertensija,
- ja piedalīties C vitamīna izpētē – pēdējo 2 nedēļu laikā nav lietoti sistēmiski/lokāli antibiotiķi, pretsēņu preparāti un/vai pienskābo baktēriju preparāti; nav maksts infekciju simptomu, nav bijuši spontāni aborti pēc 14 grūtniecības nedēļām, kā arī priekšlaicīgas dzemdības; ja neesiet grūtniece – menopauze,
- Jums ir jāparaksta piekrišana dalībai pētījumā.

Jūsu ārsts izvērtēs, vai Jūs variet piedalīties pētījumā.

Ja Jūs pievienosieties pētījumam, Jūsu grūtniecības aprūpe neatšķirsies no parastās prakses, kas noteikta Latvijas grūtnieču aprūpes vadlīnijās. Kā papildus izmeklējumi pirmajā un 28–32 grūtniecības nedēļu vizītēs tiks veikti maksts vides pH mērījumi ar speciālām papīra testa strēmeliņiem, ar speciālu birstīti paņemtas analīzes no

maksts mikrobu izpētei, kā arī tiks paņemts uzsējums no maksts. Šie izmeklējumi nav sāpīgi vai traumatiski. Papildu izmeklējumi būs bez maksas.

Ja maksts pH būs palielināts un mikroskopijā konstatēs izmainītu maksts mikrofloru, tad Jūs iekļaus makstī ievadāmā vitamīna C izpētes grupā. Daļai no šīs grupas pacientēm tiks nozīmēta ārstēšana ar makstī ievadāmo vitamīnu C (preparāts *Feminella Vagi C*, ir atļauts izplatīt Latvijā). Šo pacientu izvēli noteiks datorizēti pēc nejaušības principa. Otra pacienšu daļa medikamentu nesaņems, bet tiks novērotas. Ja lietosiet C vitamīnu, tad Jums būs jāaizpilda dienasgrāmata par šī medikamenta lietošanu, sūdzībām, problēmām.

Gadījumos, ja Jums būs sūdzības, kas liecinās par maksts infekciju, tad ārsts Jums nozīmēs atbilstošu terapiju, kas rekomendēta Latvijas grūtnieču aprūpes vadlīnijās.

BRĪVPRĀTĪGA PIEDALĪŠANĀS

Piedalīšanās šajā pētījumā ir brīvprātīga. Jums ir tiesības pārtraukt dalību pētījumā jebkurā laikā jebkuru iemeslu dēļ. Jums ir tiesības piekrist tikai izmeklējumiem. Savāktie dati tiks izmantoti tikai pētījuma datu apkopošanai. Ja dalība pētījumā tiek pārtraukta, tas neradīs nekādu ietekmi uz turpmāku grūtniecības aprūpi.

KONFIDENCIALITĀTE UN PERSONAS DATU AIZSARDZĪBA

Konfidencialitāte šajā pētījumā ir ļoti svarīga. Visa informācija par Jums šajā pētījumā tiks identificēta pēc Jums piešķirtā numura. Jūsu vārds vai cita atpazīstama informācija netiks lietota nevienā dokumentā, rezultātu publikācijās. Pētījuma iniciatori uzglabās informāciju par pētījuma datiem, bet Jūsu vārdu zinās tikai Jūsu ārsts. Visi ar pētījumu saistītie dokumenti tiks uzglabāti drošās, citiem nepieejamās vietās.

Lai izvērtētu pētījuma rezultātus, informācija par Jums tiks analizēta elektroniski vai manuāli. Šī informācija tiks izmantota zinātniskos un medicīniskos nolūkos. Šādiem mērķiem dati tiks izmantoti tikai anonīmi!

Jums ir tiesības uzzināt no sava ārsta, kādas analīzes Jums tiek ņemtas un kādiem mērķiem. Jums ir tiesības neparakstīt piekrišanu pētījumam, kā arī pārtraukt dalību pētījumā jebkurā laikā.

PĒTĪJUMA DALĪBNIKĀ PIEKRIŠANAS LAPA

Es,

(pilns dalībnieces vārds, uzvārds)

apstiprinu, ka esmu izlasījusi un sapratusi visu informāciju par pētījumu „IZMAINĪTA MAKSTS MIKROFLORA: TERAPIJAS IESPĒJAS AR VAGINĀLO VITAMĪNU C”. Man ir izskaidrota informācija par pētījumu un plānotām aktivitātēm. Es apstiprinu, ka man bija iespēja uzdot jautājumus un esmu apmierināta ar saņemtām atbildēm. Man bija pietiekoši laiks, lai izlasītu informāciju pirms pieņemu lēmumu. Es brīvprātīgi dodu piekrišanu dalībai šajā pētījumā.

Esmu sapratusi, ka man ir tiesības atteikties no dalības šai pētījumā un ka varu pārtraukt dalību jebkurā laikā, kas neatstās iespaidu uz manu grūtniecības aprūpi.

Dalībnieces paraksts

Datums

Pētnieks

Vārds, uzvārds

Paraksts:

Datums

Written information sheet for consent
(translation in English)

STUDY TITLE: ABNORMAL VAGINAL MICROFLORA – IMPACT
OF VAGINAL VITAMIN C

ORGANIZED BY: DEPARTMENT OF OBSTETRICS AND
GYNECOLOGY OF RIGA STRADINS UNIVERSITY

Participant identification number

You were asked to join a study which aims to evaluate vaginal microbial alterations involved in the causation of preterm birth in women with increased vaginal pH and abnormal vaginal flora and to investigate the role of a prophylactic maintenance regimen using vaginal vitamin C to improve vaginal microflora and prevent adverse pregnancy outcome in women with abnormal vaginal flora. Before you can join the study, you should know why this study is performed and what it means for you.

INTRODUCTION

Healthy vaginal environment is acidic and is maintained by *Lactobacillus* bacteria. Increased vaginal pH reflects changes in normal microbial balance in the vagina. Vaginal infections can be treated by antibiotics, but treatment is not always effective in decreasing preterm births, it is not always acceptable to all women and might cause microbial resistance risks. Vaginal vitamin C cause acidic – healthy, vaginal environment, improve vaginal flora and interfere with pathological microbes. During this study your vaginal pH will be evaluated, several vaginal samples for infection analysis will be taken and in case of increased pH you will be offered to take treatment with vaginal vitamin C or alternatively only observed.

If you have any questions, or if you want more information, please don't hesitate to ask your doctor at any time.

When the study is clear to you, and you want to join it, you will be asked to sign this form. It is possible that there are some words in this form that you don't understand. Ask your study-doctor or study-nurse to explain them to you.

STUDY AIM

With the present work we would like to study different factors associated with abnormal vaginal flora early in pregnancy and investigate impact of vaginal vitamin C on abnormal vaginal microflora and pregnancy outcome.

STUDY PROCEDURES

You can be included in the study, if:

- your age is less than 18 years,
- your gestational age less than six and more than 14 weeks of gestation,
- no multiple pregnancy,
- you do not have systemic diseases, like diabetes, kidney failure, hypertension requiring medication,
- you will not have *Chlamydia*, gonorrhoea, syphilis and HIV infections,
- agree participate in the study and sign informed consent,
- additional criteria for vaginal vitamin C study:
 - currently/during the last 2 weeks you were not treated with systemic/local antibiotics, antimycotics and/or *Lactobacillus* preparations,
 - you have no symptoms of vaginal infections,
 - have no history of late miscarriage and preterm deliveries,
 - in addition for non-pregnant women – postmenopausal status.

Your study-doctor will evaluate if you can join the study.

If you will join the study, following procedures will be followed:

- You will have antenatal care according to national guidelines.
- No extra visits will be needed.

- Extra examinations during first and 28–32 week of gestation visits will be done and they will be free.
- The extra examination will be: vaginal pH, vaginal samples for native microscopy and for microbial cultures analysis.
- Randomization will be done by all study doctors: patients with increased vaginal pH will receive study vaginal tablets.
- You have to fill out a weekly dairy. In the dairy you will write the tablets usage, when you forget to introduce them, any periods of blood loss, sexual coitus, vulvo-vaginal symptoms such as itching, irritation, burning or pain, medication or treatments received for whatever reason and admission in hospitals during the last week.
- At all visits you will be asked about adverse effects, serious adverse events, signs and symptoms you might have.

VOLUNTARY PARTICIPATION

Participation in the study is voluntary. You have the right to withdrawn from the study at any time, for any reasons. You have the right to agree only for examinations. The data collected till that time will be used for analysis if relevant. Discontinuation has no disadvantages in the further follow-up of your pregnancy.

CONFIDENTIALITY AND PROTECTION OF YOUR PERSONAL ENVIRONMENT

Confidentiality is important in this study. All information about you will be identified by a number. Your name or any other kind of recognizable identification will not be mentioned at any place or document, nor on the reports or publications. The study initiator will store the study data, but only your study doctor will store the data with your name on the records. Your study doctor was asked to keep these documents in a safe place.

The information about you will be electronically or manual analyzed to determine the study results. Only the anonymous data will be used for this. Confidentiality will be respected.

You have the right to ask your study doctor what data about you are being collected and what the purpose is for that. You have the right to see your personal information in your medical file and have some corrections made by your study doctor.

Consent of the Informed participant

I, _____

(full name of the participant)

agree to participate in the study “Abnormal vaginal microflora – impact of vaginal vitamin C”.

Confirm that I read and understood all information. The study information and procedures have been explained to me. I confirm that I had the possibility to ask questions and that I am satisfied with the answers given. I was given sufficient time to read the information thorough before making a decision.

I voluntarily give my consent to participate in this study. I understand that I have the right to refuse to take part in this study and that I can withdraw at any time, without consequences for my further follow-up or treatment. I was informed about my rights to have access to my personnel records and have corrections made. I certify that I received a copy of this form for further reference.

Signature participant:

Date

(DD-MM-YY)

Investigator:

Name:

Signature:

Date:

(DD-MM-YY)

Questionnaire

Visit 1

1. Age
2. Education
 - 1) primary
 - 2) secondary
 - 3) higher
3. Employment
 - 1) employed
 - 2) housewife
 - 3) unemployed
4. Marital status
 - 1) married
 - 2) living with partner
 - 3) not living with partner
5. Smoking before pregnancy
6. Smoking during pregnancy
7. Gravidity
 1. 2. 3. 4. 5. and more
8. Outcomes of previous pregnancies
 - 1) term delivery
 - 2) preterm delivery
 - 3) early miscarriage (up to 14 weeks of pregnancy)
 - 4) late miscarriage (14–22 weeks)
 - 5) legal abortion
 - 6) ectopic pregnancy
9. Parity
 - 1) first
 - 2) second and more

10. History of miscarriage

- 1) no
- 2) yes, times atweeks of gestation

11. History of preterm deliveries

- 1) no
- 2) yes, times atweeks of gestation

12. Concomitant extra-genital diseases

- 1) none
- 2) urinary tract
- 3) cardiac
- 4) thyroid gland
- 5) respiratory
- 6) others

13. Use of medicaments

- 1) no
- 2) yes,

14. Number of sexual partners per lifetime

- 1) 1
- 2) 2
- 3) 3
- 4) 4
- 5) 5
- 6) 6 and more

15. Number of sexual partners during last year

- 1) 1
- 2) 2
- 3) 3 and more

16. New partner during last 6 months

- 1) yes
- 2) no

17. Number of sexual partners during last month

- 1) 0
- 2) 1
- 3) ≥ 2

18. Frequency of intercourses during last month

- 1) 0
- 2) 1 – 9
- 3) ≥ 10

19. Genital infections a year before pregnancy

No.	Type of infections	Yes	No
19.1	<i>C. trachomatis</i>		
19.2	<i>T. vaginalis</i>		
19.3	herpes genitalis		
19.4	bacterial vaginosis		
19.5	candidosis		
19.6	gonococcal infection		
19.7	<i>U. urealyticum</i>		
19.8	<i>M. hominis</i>		
19.9	<i>Luess</i>		
19.10	HIV		

20. Intercourse 2 days before sampling:

- 1) no
- 2) yes

21. Antibiotics 2 weeks before sampling

- 1) no
- 2) yes, name

22. Topical vaginal medications 2 weeks before sampling

- 1) no
- 2) yes, name

23. Complains

No.	Type of complains	Yes	No
23.1	increased discharge		
23.2	burning		
23.3	itching		
23.4	bad smell		
23.5	bloody discharge		
23.6	low abdominal pains		

24. Vaginal discharge

No.	Type of discharge	Yes	No
24.1	normal		
24.2	thin, homogeneous		

24.3	“cheese” like		
24.4	bloody		
24.5	yellow		

25. Vaginal pH

- 1) < 4.5
- 2) \geq 4.5

26. Vaginal pH

- 3.6
- 4.1
- 4.4
- 4.7
- 5.0
- 5.3
- 5.6
- 6.1

27. Amine test

- 1) negative
- 2) positive

28. Length _____ and weight _____

Visit 2

29. Number of sexual partners during last month

- 1) 0
- 2) 1
- 3) \geq 2

30. Frequency of intercourse during last month

- 1) 0
- 2) 1–9
- 3) \geq 10

31. Intercourse 2 days before sampling

- 1) no
- 2) yes

32. Antibiotics 2 weeks before sampling

- 1) no
- 2) yes, name

33. Topical vaginal medications 2 weeks before sampling

- 1) no
- 2) yes, name

34. Complains

No.	Type of complains	Yes	No
34.1	increased discharge		
34.2	burning		
34.3	itching		
34.4	bad smell		
34.5	bloody discharge		
34.6	low abdominal pains		

35. Vaginal discharge

No.	Type of discharge	Yes	No
35.1	normal		
35.2	thin, homogeneous		
35.3	“cheese” like		
35.4	bloody		
35.5	yellow		

36. Vaginal pH

- 1) < 4.5
- 2) ≥ 4.5

37. Vaginal pH

- 3.6
- 4.1
- 4.4
- 4.7
- 5.0
- 5.3
- 5.6
- 6.1

38. Amine test

- 1) negative
- 2) positive

Visit 3

39. Systemic antibiotics during pregnancy

- 1) no
- 2) yes, name

40. Topical vaginal medications during pregnancy

No.	Name	Yes	No
40.1	Study medication (vitamin C)		
40.2	Clindamycin		
40.3	Metronidasole		
40.4	Polygynax		
40.5	Antifungals		
40.6	Probiotics		
40.7	Progesterone		
40.8	Others, name.....		

41. History of genital infections during pregnancy

No.	Type of infections	Yes	No
41.1	<i>C. trachomatis</i>		
41.2	<i>T. vaginalis</i>		
41.3	herpes genitalis		
41.4	bacterial vaginosis		
41.5	candidosis		
41.6	gonococcal infection		
41.7	<i>U. urealyticum</i>		
41.8	<i>M. hominis</i>		
41.9	<i>Luess</i>		
41.10	HIV		

42. Pregnancy course, complications

No.	Pregnancy course, complications	Yes	No
42.1	No complication		
42.2	Uterine bleeding		
42.3	Hypertension		
42.4	Rh incompatibility		
42.5	Anemia		
42.6	Urinary infection		
42.7	Respiratory infection		

42.8	Other infections (type.....)		
42.9	Fetal anomalies		
42.10	Other		

43. Pregnancy outcome

- 1) early miscarriage (< 14 weeks of gestation)
- 2) late miscarriage (14–21 weeks 6 days)
- 3) preterm delivery at 22–26 weeks of gestation
- 4) preterm delivery at 27–36 weeks of gestation
- 5) term delivery

44. If miscarriage – weeks of gestation

45. Gestational age at birth

46. Place of birth

- 1) Riga Maternity house
- 2) Stradins University hospital
- 3) Jurmala hospital
- 4) other,

47. Delivery type

- 1) spontaneous
- 2) induced

48. Delivery mode

- 1) vaginal
- 2) planned cesarean section
- 3) emergency cesarean section

49. Birth weight

50. Apgar score

- 1) 1st minute
- 2) 5th minute

51. Newborn admission at Intensive care unit

- 1) no
- 2) yes

52. Newborn`s admission at Children`s hospital (up to 28 days after birth)

- 1) no
- 2) yes

Visit 1 microscopy results

53. Lactobacillary grade

No.	Grade	Yes	No
53.1	LBG I		
53.2	LBG IIa		
53.3	LBG IIb		
53.4	LBG III–BV		
53.5	LBG III–AV		
53.6	LBG III–MF		

54. Lactobacillary morphotypes

No.	Type	Yes	No
54.1	Normal		
54.2	Leptosomic		
54.3	Coarse, short		
54.4	Absent		

55. Background appearance

No.	Type	Yes	No
55.1	Clean		
55.2	Dirty, spawned with plasma		
55.3	Cytolplasmic debris		
55.4	Red blood cells		
55.5	Sperm cells		

56. Cytolysis

- 1) no
- 2) yes

57. Leucocytes

- 1) ≤ 10 per hpf
- 2) > 10 per hpf, but < 10 per epithelial cell
- 3) ≥ 10 per epithelial cell

58. Clue cells

- 1) no
- 2) yes

59. Bacterial vaginosis

- 1) no
- 2) partial
- 3) full

60. Aerobic vaginitis

- 1) < 3
- 2) 3–4
- 3) 5–6
- 4) 7–10

61. Candida

- 1) no
- 2) spores
- 3) hyphae
- 4) both

Visit II microscopy

62. Lactobacillary grade

No.	Grade	Yes	No
62.1	LBG I		
62.2	LBG IIa		
62.3	LBG IIb		
62.4	LBG III–BV		
62.5	LBG III–AV		
62.6	LBG III–MF		

63. Lactobacillary morphotypes

No.	Type	Yes	No
63.1	Normal		
63.2	Leptosomic		
63.3	Short coarse		
63.4	Absent		

64. Background appearance

No.	Type	Yes	No
64.1	Clean		
64.2	Dirty, spawned with plasma		
64.3	Cytoplasmic debris		
64.4	Red blood cells		
64.5	Sperm cells		

65. Cytolysis

- 1) no
- 2) yes

66. Leucocytes

- 1) ≤ 10 per hpf
- 2) > 10 per hpf, but < 10 per epithelial cell
- 3) ≥ 10 per epithelial cell

67. Clue cells

- 1) no
- 2) yes

68. Bacterial vaginosis

- 1) no
- 2) partial
- 3) full

69. Aerobic vaginitis

- 1) < 3
- 2) 3–4
- 3) 5–6
- 4) 7–10

70. Candida

- 1) no
- 2) spores
- 3) hyphae
- 4) both

Bacteriological results

71. Bacteriological findings

No.	Cultured microorganisms	Yes	No
72.0	Negative		
72.1	<i>U. urealyticum</i>		
72.2	<i>U. urealyticum</i> (high numbers)		
72.3	<i>M. hominis</i>		
72.4	<i>M. hominis</i> (high numbers)		
72.5	<i>Str. agalactiae</i>		

72.6	CP <i>Staphylococcus</i>		
72.7	CN <i>Staphylococcus</i>		
72.8	<i>Str. viridians</i>		
72.9	<i>Peptostreptococcus</i>		
72.10	<i>Enterococcus faecalis</i>		
72.11	<i>E. coli</i>		
72.12	<i>Enterobacteriaceae</i>		
72.13	<i>Acinetobacter spp</i>		
72.14	<i>Candida spp.</i>		

11. ACKNOWLEDGMENTS

I would like to express my sincere thanks to research supervisor Professor Dace Rezeberga and research scientific consultant Professor Gilbert Donders, University of Antwerp.

I am grateful to Irena Rogovska, PhD, from Rīga Stradiņš University, who helped me with statistical analysis.

My thanks to Olga Vasina, gynecology resident, for the questionnaire data input and participants randomization.

My appreciations to all my colleagues in the outpatient departments for enthusiastic support and to all women, who agreed to participate in the study.

I am in gratitude for possibility to study in Rīga Stradiņš University for obtaining the degree of a Doctor of Medicine and receiving grants from European Structural Funds and from Latvian Ministry of Education and Science.

I am also thankful to pharmaceutical companies for receiving study medicament from *Polichem*, Dalacin from *Pfizer* and grants for bacteriological study from *Wolff* and *Gedeon Richter*.

My warmest thanks I would like to express to my family members – for their love, patience and support.