Bacterial imbalance of the vaginal flora

a cytological and biomolecular concept of *Gardnerella vaginalis*

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Colophon

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Bacteriële imbalans van de vaginal flora

een cytologisch and biomoleculair concept van *Gardnerella vaginalis*

(met een samenvatting in het Nederlands)

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Voor mijn ouders

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Introduction and aims of the thesis

Introduction

Diseases of the female genital tract are extremely common in clinical and pathological practice. Vaginal inflammation poses one of the most common problems in gynecology. The most frequent causes of infection are yeasts, bacteria, protozoa, viruses and parasites.

As bacterial imbalance of the vaginal flora is a common cause of infective vaginal discharge in women of reproductive age,[1] vaginal complaints constitute one of the main motives that lead women attending gynecological clinics.[2]

In 1955 the bacterium *Gardnerella vaginalis*, at the time known as *Haemophilus vaginalis*, was introduced as one of the main causative organisms of these symptoms.[3] In this context bacterial imbalance represents a complex, microbiological change of the vaginal flora characterized by an increase in the concentration of *Gardnerella vaginalis* at the expense of the usually dominant hydrogen-peroxide producing lactobacilli. Although the etiology remains poorly understood, bacterial imbalance is associated with an increased risk of gynecological and obstetrical complications.[4-6]

Since vaginal inflammation has been suggested as a possible co-factor in cervical carcinogenesis, research has drawn more attention to this topic.[7,8]

The vaginal flora

The human epithelium is colonized by many types of bacteria. They establish a presence where they reproduce and become a feature of the epithelial surface. The presence and complexity of these populations, usually unknown to their host, are of interest to physicians for counseling their patients. Considering their numbers, they are only occasionally pathogenic and have found niches favourable for their survival that provide them with warmth, nutrition and protection. While the gut is better known for microbial habitation,[9] the vagina and cervix also harbour microbial populations whose dynamics are instructive, complex and have clinical significance.

The normal vaginal flora is best described as a broad spectrum of facultative organisms including *Gardnerella vaginalis, Mobiluncus species, Bacteroides fragilis, Prevotella species, Mycoplasma species, Ureaplasma urealyticum, Fusobacterium nucleatum* and *Peptostreptococcus species*.[10,11] However, firstly identified in 1984 by the German physician A. Doderlein[12], *Lactobacillus* has been shown to be the predominant bacterium in the normal vaginal microbial flora found in women of reproductive age.[13]

Lactobacilli are facultative anaerobes that colonize the moist surface of the vaginal epithelium, but also the intestinal tract and oral cavity.[13,14] In women of reproductive age glycogen is deposited under estrogenic control onto the mature vaginal epithelium, where it is broken down to glucose by vaginal epithelial cells and bacterial enzymes.[13] The lactobacilli metabolize glucose to a final end product of lactic acid, which contributes to the maintenance of a low vaginal pH (4.0-4.5).[14,15] In addition to acid production, many isolates of vaginal lactobacilli produce various bacteriocines and H_2O_2, a compound having broad antimicrobial activity.[13,16-21] Hydrogen peroxide, possessing potent toxic properties, plays a crucial role in protecting against the overgrowth of pathogens in the vagina and is

capable to interact with hialide and peroxidase present in vaginal secretions.[16] The product of this reaction is a potent oxidant that is toxic to many bacteria.[17-23] H_2O_2-producing lactobacilli are capable to kill human immunodeficiency virus (HIV) in vitro as well as *Gardnerella vaginalis*, anaerobes and *Neisseria Gonorrhoeae*.[16,21-27] Furthermore, women with H_2O_2-producing lactobacilli are less likely to have bacterial vaginosis,[17,18] *Chlamydia trachomatis*[18] and *Trichomonas vaginalis*.[17,18] In addition, H_2O_2-producing lactobacilli seem to be more capable in sustaining long-term vaginal colonization than are lactobacilli that do not produce H_2O_2.[28]

Bacteria and imbalance

Bacteria occupy the mid-range in size of micro-organisms in the vagina and they are fundamental to the vaginal flora were they fulfill a useful function. However, they may also be pathogenic either in a restricted sense (that is, under certain circumstances) or without restrictions (i.e. there mere presence is sufficient).[29]

The term pathogenic means 'causing disease'. Restricted pathogenic bacteria are those that may induce sickness only under certain circumstances. Nutritional elements must be present in the environment and physiochemical factors (temperature, osmolarity, pH) will largely determine what bacteria will grow and function and in what order. In addition, superabundance of micro-organisms, lowered resistance of the host, change in the environment favourable to the micro-organism, destruction of the native flora and changes in or damage to the protective epithelium will alter the variety of bacteria.[29]

Bacteria have been classified according to their morphological characteristics, such as rod-shaped (bacilli), spherical (cocci), spiral-shaped (spirochetes), as well as according to their biochemical and physiological characteristics, such as an affinity for a certain stain (Gram-negative and Gram-positive), growth capacity and growth pattern in a specific medium, dependence on oxygen (aerobe, facultative aerobe and anaerobe) and resistance to specific antibiotics.[29]

A large variety of organisms can infect the female genital tract and, in total, account for considerable suffering and morbidity. Infections with *Gardnerella vaginalis*, are extremely common and may cause significant discomfort but can even remain asymptomatic. *Gardnerella vaginalis* is a small rod-shaped bacterium, and probably is the most inflicting agent in the vagina.[30]

The term 'bacterial imbalance' is not commonly used in literature, but covers the whole spectrum of cases in which a microbial disturbance is *cytologically* diagnosed. Although a significant proportion of cases of bacterial imbalance is diagnosed following symptoms reported by the woman, many women remain *asymptomatic* in the presence of a disturbed vaginal flora.[4,5] In this context bacterial imbalance seems to be closely related to the well-known diagnosis 'bacterial vaginosis'. Unfortunately, in literature the latter term is often confusing, whereas this syndrome reflects bacterial imbalance, but in practice is diagnosed on the basis of clinical findings.[31] This implies that bacterial imbalance is a microscopical diagnosis *independent* of clinical criteria, while the diagnosis bacterial vaginosis is *dependent* on clinical criteria.[32]

Factors of influence

Disruption of the vaginal ecosystem, resulting in bacterial imbalance is known to be influenced by age, phase of the menstrual cycle, sexual activity, contraceptive choice, pregnancy, presence of necrotic tissue or foreign bodies, and use of hygienic products or antibiotics. In this context, acquisition of bacterial imbalance has been associated with exposure to a new sex partner, use of intra-uterine devices, cigarette smoking and absence of H_2O_2 producing lactobacilli.[28,33-36] Moreover, Schwebke et al. showed that transient alteration in vaginal flora was correlated with behaviours such as a number of sex partners, frequent vaginal intercourse and frequent episodes of receptive oral sex.[37] Although sexual activity is a risk factor for acquisition of bacterial imbalance, this microbial change can also occur in women who have never had vaginal intercourse.[38] According to Vallor et al.[28] use of antibiotics increases the risk of losing colonization by H_2O_2 resulting in bacterial imbalance.

Consequences of bacterial imbalance

Bacterial imbalance diagnosed on the basis of clinical criteria has shown to be associated with adverse pregnancy outcomes, pelvic inflammatory disease, and increased post-operative infections. Women with bacterial vaginosis had an increased prevalence of HIV seropositivity, and, in a longitudinal study of pregnant women, women with bacterial vaginosis had an increased incidence of HIV infection.[28] More specific, infection with *Gardnerella vaginalis* was found to be a risk factor for the acquisition of HIV where stimulation of HIV-expression by *Gardnerella vaginalis* was demonstrated.[39,40] In addition, studies have demonstrated that post-abortion infection and pelvic inflammatory disease were correlated with symptomatic bacterial imbalance.[41,42]

Furthermore, symptomatic bacterial imbalance has been associated with preterm delivery, premature rupture of membranes, infection of the chorion and amnion, histological chorioamnionitis, and infection of the amniotic fluid.[43-45]

Diagnosis

Bacterial imbalance by *Gardnerella vaginalis* is diagnosed microscopically, representing a shift towards *Gardnerella vaginalis* with a sharp decrease in the number of lactobacilli, consisting of two different cytological categories, namely dysbacteriosis and *Gardnerella* infection. In cervical samples *Gardnerella vaginalis* is seen mainly on squamous epithelial cells, which as a result, stain blue.

Dysbacteriosis displays a shift in vaginal flora, in which the majority of lactobacilli is replaced by a mixture of anaerobic bacteria, mainly *Gardnerella vaginalis,* resulting in the presence of clue cells (CCs).[46,47] Cervical samples with *Gardnerella* infection show an *extreme* shift towards *Gardnerella vaginalis* having a lack of lactobacilli, resulting in the presence of blue mountain cells (BMCs).[48,49]

In contrast to bacterial imbalance, the syndrome bacterial vaginosis reflecting the clinical diagnosis of bacterial imbalance of the vaginal flora, can be identified by the presence of at least three of the four Amsel criteria, which is seen as the gold standard in diagnosis. The Amsel test comprises the following criteria: elevated vaginal pH (>4.5), increased vaginal discharge, presence of clue cells and amino odor after addition of potassium hydroxide.[32]

Another method used to detect bacterial vaginosis is Gram staining of vaginal smears which scores genital tract flora from 0-10 depending on the numbers of three bacterial morphotypes observed on the slide.[50] A commercially available test for *Gardnerella vaginalis* has also been shown to have utility in detecting patients with bacterial vaginosis.[51]
There has been expanding interest in identifying bacteria in a mixture of organisms on an epithelial surface with molecular methods.[52,53] The difficulty has been in obtaining molecular probes specific to the genotypes of all the bacteria.[54] This obstacle has been overcome using molecular methods to identify the genotypes of bacteria in patients with bacterial vaginosis.[52-55] These technologies are based on the sequence of 16S ribosomal regions of the bacterial genome. While each bacterium has a 16S region, there are specific portions in the 16S region that are coding for a genotype of a bacterium. These hallmark studies have not only identified the species specific 16S regions, but have produced probes that may be used with in situ hybridization to identify and enumerate the relative abundance of individual bacteria species in a smear. The technology for this research may take several years to become available to office practice or rapid diagnosis but it will allow a rapid quantitative determination of the bacterial type and disease potential.

Treatment
Bacterial imbalance is not treated, unless symptoms occur. The same accounts for asymptomatic bacterial vaginosis, which is only treated in case of pregnancy. For women with clinical symptoms, diagnosed with bacterial vaginosis according to the Amsel test, medicamentation is used in order to restore the normal vaginal microflora. Treatment is indicated for relief of symptoms and to prevent postoperative infection in those with asymptomatic infection prior to abortion or hysterectomy.
Metronidazole or clindamycin administered either orally or intravaginally will result in high rates of clinical cure (70-80 percent at four weeks follow up).[56-58] Oral medication is more convenient, but associated with a higher rate of systemic side effects than vaginal administration. Follow-up is unnecessary if symptoms revolve.

Aims of the thesis

Given the above concerns, it is unsurprising that for many years research has drawn more attention to the implications of disturbances in the vaginal ecosystem as inflammatory patterns of the vaginal flora could possibly be a co-factor in cervical carcinogenesis.
As bacterial vaginosis is mainly diagnosed in symptomatic women on the basis of clinical criteria, population-based cervical screening reveals the interesting fact that a remarkable part of asymptomatic women shows a cytological bacterial imbalance of the vaginal flora with a shift towards *Gardnerella vaginalis*. At screening of cervical samples not only squamous cells are visible, but also the composition of the vaginal flora becomes clear. Results of cervical smears reporting the presence of cervical *Gardnerella* infection are regularly encountered by pathologists, yet the significance of this finding is not well studied.

In the Netherlands each year about 900,000 cervical samples are taken for cytology screening, as cervical cancer is the second most common malignancy in women worldwide and contributes to 9.8% of all female cancers.[59] In 1988, the Netherlands introduced nationwide cytological screening for cervical cancer and its precursor lesions as a method to reduce cervical cancer morbidity and mortality.[60] The majority of all cervical smears is taken as part of the Dutch national screening program, in which all women between 30 and 60 years of age are invited for a smear by a personal letter of the city council on a 5-yearly basis. Sampling is performed by the general practitioner. The remaining smears are those taken for other reasons, a combination of opportunistic reasons and medical indications.[61]

In 1996, the Dutch national coding system for cervical cytology, KOPAC, was introduced to uniformly describe cytomorphological findings in order to increase the efficacy of the screening program and to decrease equivocal results.[62] This system interprets cervical smears by using a rating system, which includes information on specimen composition, inflammatory characteristics and adequacy of the smear. KOPAC is an acronym for this coding system in which K (kompositie = composition), O (ontstekingsverschijnselen = inflammation signs), P (plaveiselepitheel = squamous epithelium), A (andere endometrium afwijkingen = other endometrial abnormalities) and C (cylinderepitheel endocervix = endocervical cylindrical epithelium) are used to indicate the composition and morphology of the smears. Squamous, columnar and other cells are graded for the presence of dyskariosis (dysplasia). These values determine the interpretation of the smear.[62] Besides determining the cervical abnormalities, the inflammatory status of the vaginal microflora is also coded. This is established by the O-category comprising nine different subgroups: koilocytosis (O1, see Fig. 1), *Trichomonas vaginalis* (O2, see Fig. 2), dysbacteriosis (O3, see Fig. 3), *Candida* (O4, see Fig. 4), *Gardnerella vaginalis* (O5, see Fig. 5), normal flora (O6, see Fig. 6), *Actinomyces* (O7, see Fig. 7), *Chlamydia trachomatis* (O8, see Fig. 8), non-specific changes (O9, see Fig. 9). All are ThinPrep slides.

The large cytological databases in the Netherlands can be exploited to study a variety of relationships. The aim of this thesis is to study prevalence, diagnosis and implications of bacterial imbalance caused by *Gardnerella vaginalis* among asymptomatic women as established in population-based cervical screening.

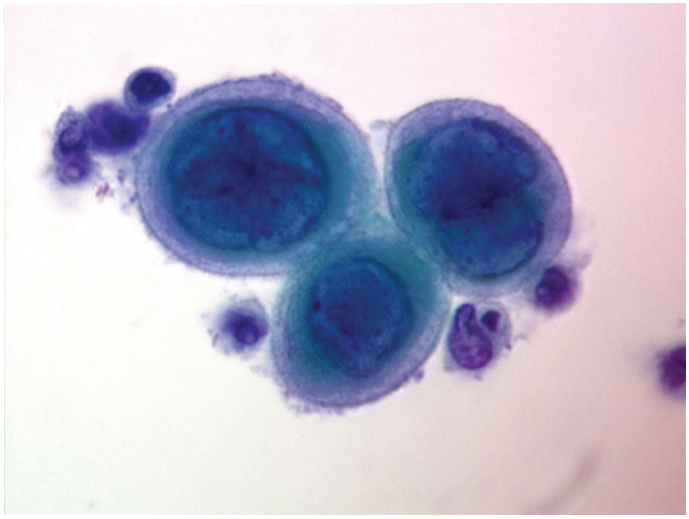

Figure 1 Koilocytosis (magnification x 1000)

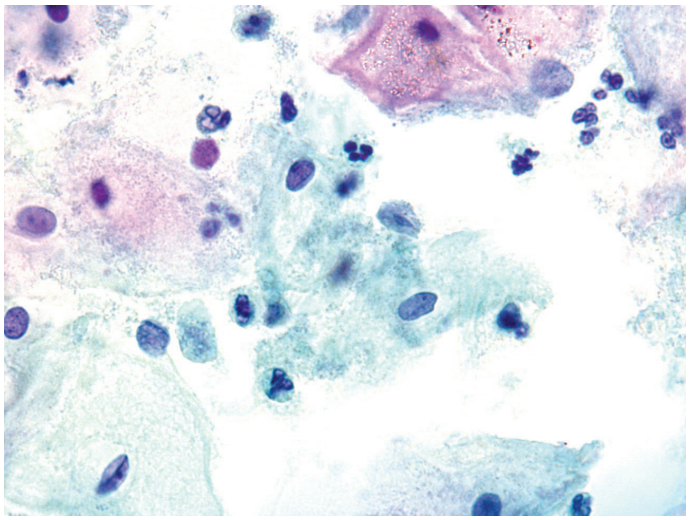

Figure 2 *Trichomonas vaginalis* (magnification x 1000)

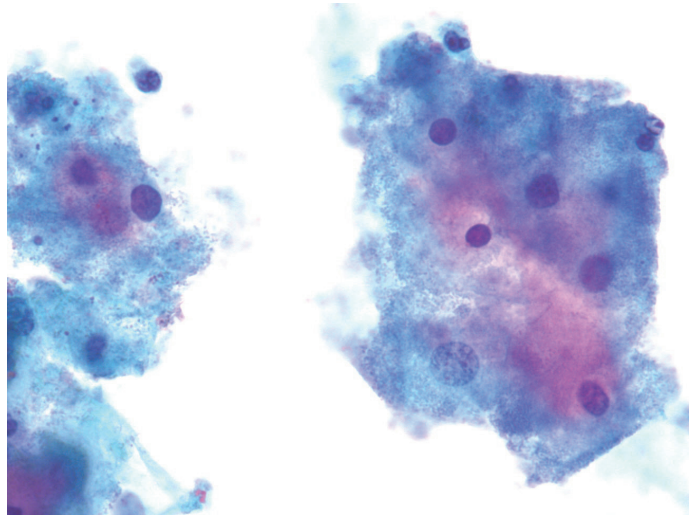

Figure 3 Dysbacteriosis (magnification x 1000)

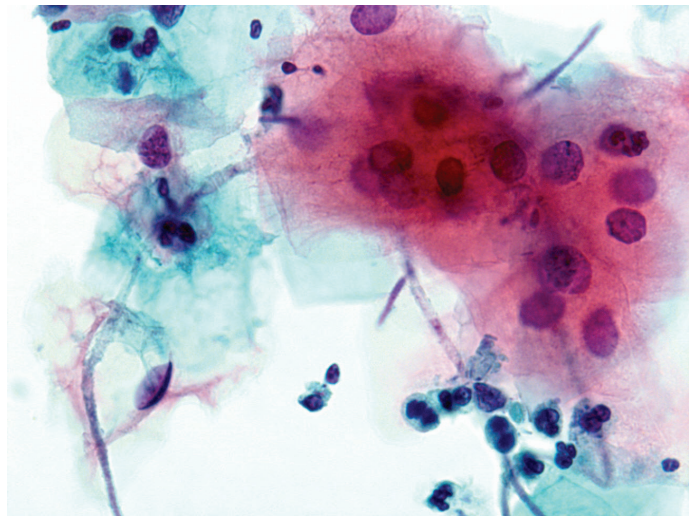

Figure 4 *Candida albicans* (magnification x 1000)

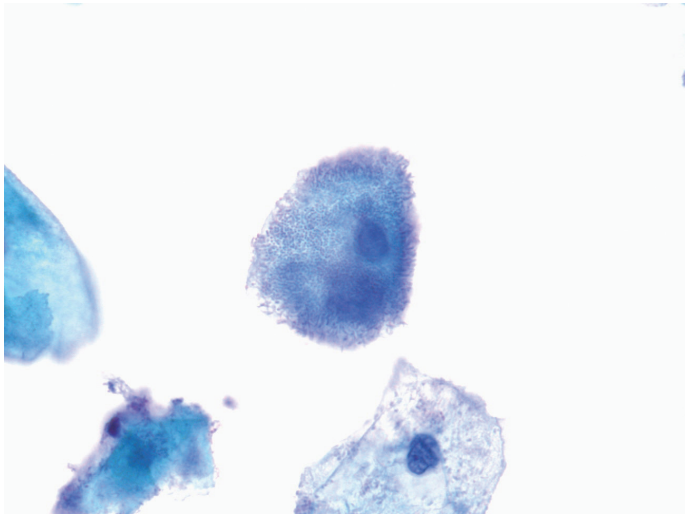

Figure 5 *Gardnerella vaginalis* (magnification x 1000)

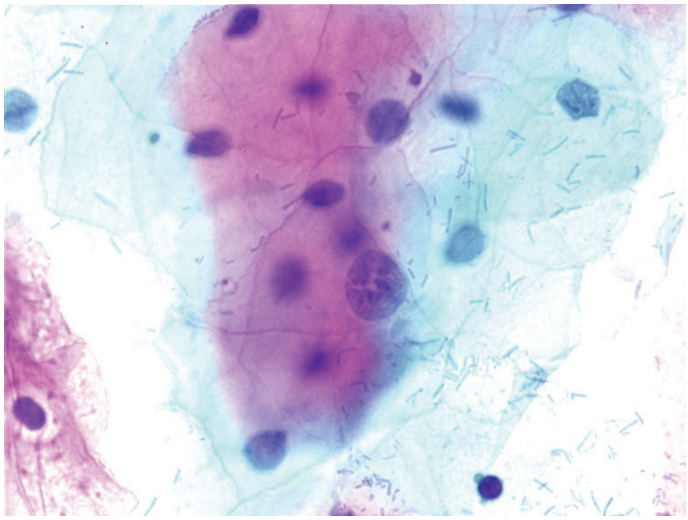

Figure 6 Normal flora (magnification x 1000)

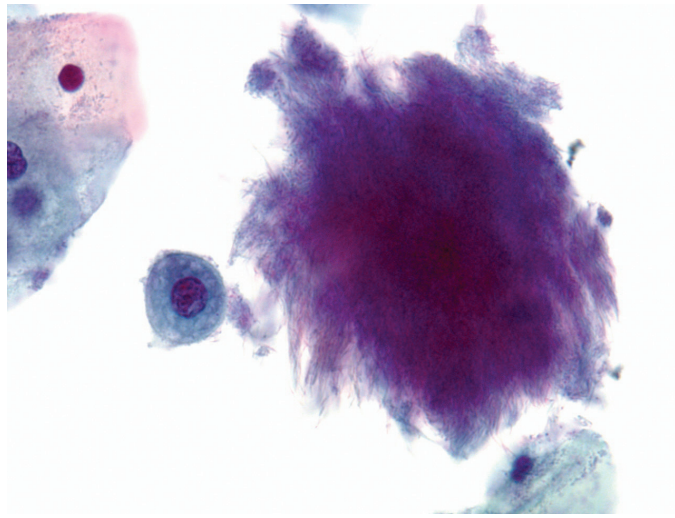

Figure 7 *Actinomyces* (magnification x 1000)

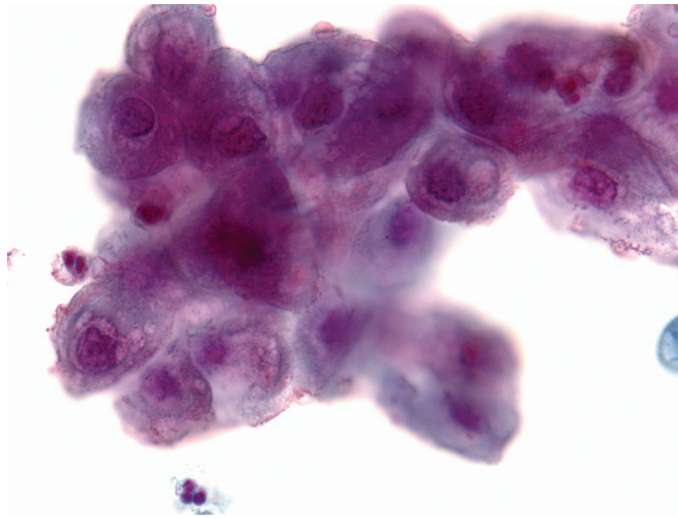

Figure 8 *Chlamydia trachomatis* (magnification x 1000)

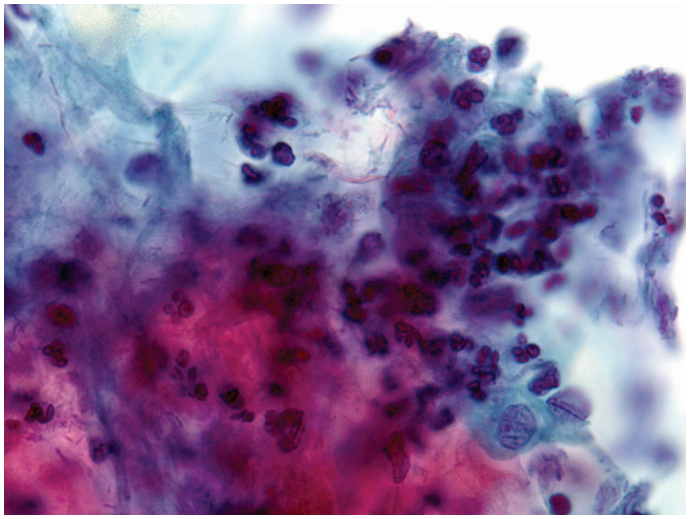

Figure 9 Non-specific inflammatory changes (magnification x 1000)

In the present thesis we attempted to answer the following questions concerning bacterial imbalance by *Gardnerella vaginalis*:

1) Is there a change in prevalence of inflammatory states of the vaginal flora (as coded in the Dutch KOPAC system) among women participating in the national screening program over the last decade?
2) Is there an association between dysbacteriotic smears containing *Gardnerella vaginalis* and a shortage of lactobacilli and cervical (pre)neoplasia?
3) Is there an association between *Gardnerella* infection and the presence of cervical (pre)neoplasia?
4) Is it possible to distinguish *Gardnerella* infection and dysbacteriosis based on cytological criteria?
5) Is it possible to identify and quantify the DNA of adherent bacteria in liquid-based cytology samples of healthy and disturbed vaginal flora?
6) How is the colonization pattern of *Gardnerella vaginalis* and *Lactobacillus crispatus* in the general population of asymptomatic women?
7) Do DNA-PCR concentrations of *Gardnerella vaginalis* and *Lactobacillus crispatus* and cytology express the same phenomenon?
8) Do high-risk human papillomavirus genotypes prefer bacterial imbalance?

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Trends in inflammatory status of the vaginal flora as established in the Dutch national screening program for cervical cancer over the last decade

Abstract

Objective: This study describes recent trends in prevalence of inflammatory patterns of the vaginal flora (koilocytosis, *Trichomonas vaginalis*, dysbacteriosis, *Candida*, *Gardnerella vaginalis, Actinomyces, Chlamydia trachomatis* and non-specific inflammatory signs) over the last decade.

Methods: From 1996-2005 more than 500,000 cervical smears were screened in the western region of the Netherlands in the context of the Dutch national screening program. Data of the first screening period (1996-2000) were compared with that of the second screening period (2001-2005).

Results: Bacterial imbalance shows a decline in all age groups with dysbacteriosis decreasing from 34.8 to 27.4 per 1000 smears and *Gardnerella* infection from 3.0 to 1.2. Cases of HPV-related koilocytosis have significantly increased among young women (age cohort 30 and 35 years) from around 2.0 to 6.7 per 1000 smears.

Conclusion: Bacterial imbalance of the vaginal flora has significantly decreased during the past decade in all age cohorts. Consciousness of vaginal hygiene might have contributed to this amazing effect although further research needs to elucidate the exact underlying mechanism. We ought to be concerned about the increase of HPV-related koilocytosis.

Introduction

Since cervical inflammation has been proposed as etiological cofactor in the development of cervical cancer,[1] much research has been done to unmask the influence of several vaginal infections on cervical carcinogenesis. It is now well established that infection with putative oncogenic human papillomaviruses plays a central role in cervical carcinogenesis.[2-5] In addition, studies have noted that cervical cytological abnormalities occur more often in women with bacterial imbalance of the vaginal flora than in those without this condition.[6,7] In this context, Verbruggen et al. showed a higher prevalence of squamous abnormalities in women with dysbacteriosis.[8] In contrast, according to Engberts et al., presence of *Candida* is not associated with cervical (pre)neoplasia.[9]

To our knowledge little is known about the prevalence of the different vaginal inflammations. Moreover, we do not know either what trends developed in the recent years.

In 1996, the current version of the Dutch national coding system for pathology findings in cervical cytology, KOPAC, was introduced.[10] Accordingly, all Dutch smears are coded for cytological status of the vaginal flora, comprising nine different subgroups (Table 1). According to this system, the Dutch screening program can provide epidemiological data on the presence of these conditions in the general population. In fact, the situation is ideal because the purpose of the program, detection of cancer, is not aimed at the detection of cervical infections and the women do not have a smear taken because of complaints but because they received a personal invitation sent by the health authorities.

We present the analysis of more than 500,000 cervical smears and describe the cytological prevalences of koilocytosis, *Trichomonas vaginalis*, dysbacteriosis, *Candida*, *Gardnerella vaginalis*, *Actinomyces*, *Chlamydia trachomatis* and non-specific inflammatory changes over the last decade enabling us to compare data from 1996-2000 (first screening) with data from 2001-2005 (second screening). This could possibly contribute in identifying trends and age-profiles resulting in detection of women that perhaps warrant more intensive surveillance concerning life-style factors and treatment. We have studied this relation by analysing large datasets of systematically coded, Papanicolaou-stained smears.[11]

Material and methods

Cervical screening program

Between January, 1996, and December, 2005, 514,129 smears taken in the western region (population around 2 million) of the Netherlands were used in this study. All women between the age of 30 and 60 years are invited once every 5 year for cervical sampling in connection with the national prevention program on cervical carcinoma. As they reach the age of 30, 35, 40, 45, 50, 55 and 60, they receive a letter of invitation by the health authorities to have a smear taken. This results in seven age cohorts, which are screened at 5 year intervals.[12] Over the study period, response was almost 70%. For all women, personal data and screening results were entered into a database of the screening organization, the SBBW (Stichting Bevolkingsonderzoek Baarmoederhalskanker regio

West). Throughout the study period there occurred no major changes in the population, except from forgotten invitations, moving houses and death, resulting in an overlap of 70%.

O codes of the KOPAC system	Cytological diagnosis
O1	Koilocytosis
O2	*Trichomonas vaginalis*
O3	Dysbacteriosis
O4	*Candida*
O5	*Gardnerella vaginalis*
O6	Normal flora
O7	*Actinomyces*
O8	*Chlamydia trachomatis*
O9	Non-specific inflammatory pattern

Table 1 O codes and their cytological diagnosis within the KOPAC system.

Cervical smear coding

All smears, screened by six pathology laboratories, were coded according to KOPAC in which the letter 'O' stands for 'Ontsteking' (inflammation) consisting of nine different categories (KOPAC O1-O9, Table 1).[10] Dutch screeners are taught how to recognize evidence of every inflammatory pattern.

'O1' is diagnosed in the presence of koilocytotic cells; cells with a cavitation of the cytoplasm due to active HPV infection.[13,14] In case of 'O2', smears show trichomonads. A cervical specimen is coded 'O3' (dysbacteriosis) when, by microscope, a remarkable shift is seen in the microflora with a sharp decrease in the number of lactobacilli and overgrowth with coccoid bacteria, mainly *Gardnerella vaginalis*.[15,16] *Candida* (O4) is coded when the smear shows hyphes and spores of *Candida*.[15] The code 'O5' stands for *Gardnerella* infection and is only given when the cervical smear shows an abundance of the so-called blue mountain cells (BMCs), that is, cells staining dark blue in the Papanicolaou stain because they are covered by a mountain of blue staining round bacteria.[10,15] In addition, few lactobacilli are present. 'O6' was given when a cervical smear showed no evidence of inflammation thus with a normal pattern. A cervical smear with *Actinomyces* (O7) is diagnosed in the presence of a microscopically large, tight dark blue colouring mass of this bacterium. *Chlamydia trachomatis* (O8) is seen when so-called 'indicator cells' are present. These are metaplastic cells with an altered staining due to the small cytoplasmic inclusions giving the cytoplasm a freckled, mottled appearance.[17] O9 was diagnosed in case of presence of non-specific inflammatory signs. When a smear showed two or more inflammatory patterns, a choice was made whereas only one O-code could be given. As a result, each smear receives an O-code (O1-O9) allowing for a study on prevalence of cytologically diagnosed vaginal inflammation. Finally, prevalence per 1,000 smears was calculated for every O-code. Data were stratified for age.

Results

In the first screening, 239,880 women participated, and in the second 274,365. The largest age cohort was the 40 year cohort in the second screening (49,251 women) and the smallest the 60 year cohort in the first screening with 14,909 women (Table 2).

Age (years)	First screening (1996-2000)		Second screening (2001-2005)	
	Year of birth	Number	Year of birth	Number
30	1966-1970	40,464	1971-1975	36,706
35	1961-1965	45,177	1966-1970	45,752
40	1956-1960	43,050	1961-1965	49,251
45	1951-1955	36,888	1956-1960	45,086
50	1946-1950	36,308	1951-1955	38,727
55	1941-1945	23,084	1946-1950	36,750
60	1936-1940	14,909	1941-1945	22,093
Total women		**239,880**		**274,365**

Table 2 Numbers of women participating in the Dutch national screening program for cervical cancer in different age cohort during their first and second screening. Stratification by age.

In Table 3A the prevalence of different inflammatory states of the vagina in the first screening (1996-2000) and in Table 3B the results of the second screening (2001-2005) are presented. The highest prevalence was for dysbacteriosis with a value of 34.8 per thousand smears in the first screening and the lowest for *Chlamydia trachomatis*, 0.2‰.

	O1 Koilocytosis		O2 Trichomonas		O3 Dysbacteriosis		O4 Candida		O5 Gardnerella		O6 Normal flora		O7 Actinomyces		O8 Chlamydia		O9 Non-specific inflammatory pattern	
Age	n	‰	n	‰	n	‰	n	‰	n	‰	n	‰	n	‰	n	‰	n	‰
30	81	2.0	61	1.5	1,254	31.0	688	17.0	105	2.6	32,982	815.1	202	5.0	12	0.3	5,082	125.6
35	81	1.8	86	1.9	1,351	29.9	718	15.9	140	3.1	36,688	812.1	357	7.9	9	0.2	5,747	127.2
40	60	1.4	116	2.7	1,468	34.1	702	16.3	116	2.7	34,681	805.6	418	9.7	9	0.2	5,480	127.3
45	37	1.0	114	3.1	1,413	38.3	472	12.8	136	3.7	29,905	810.7	343	9.3	7	0.2	4,452	120.7
50	40	1.1	182	5.0	1,532	42.2	378	10.4	145	4.0	29,685	817.6	283	7.8	7	0.2	4,012	110.5
55	14	0.6	99	4.3	912	39.5	164	7.1	58	2.5	19,437	842.0	67	2.9	7	0.3	2,325	100.7
60	6	0.4	37	2.5	417	28.0	78	5.2	28	1.9	12,761	855.9	7	0.5	7	0.5	1,565	105.0
Total	319	1.3	695	2.9	8,347	34.8	3,200	13.3	728	3.0	196,139	817	1,677	7.0	58	0.2	28,663	119.5

Table 3A Prevalence per 1000 women of different inflammatory states of the vagina in the first screening stratified by age cohort.

	O1 Koilocytosis		O2 Trichomonas		O3 Dysbacteriosis		O4 Candida		O5 Gardnerella		O6 Normal flora		O7 Actinomyces		O8 Chlamydia		O9 Non-specific inflammatory pattern	
Age	n	‰	n	‰	n	‰	n	‰	n	‰	n	‰	n	‰	n	‰	n	‰
30	250	6.8	22	0.6	1,009	27.5	679	18.5	44	1.2	29,702	809.2	195	5.3	11	0.3	4,794	130.6
35	206	4.5	46	1.0	1,171	25.6	705	15.4	69	1.5	36,753	803.3	384	8.4	9	0.2	6,405	140.0
40	158	3.2	54	1.1	1,335	27.1	729	14.8	64	1.3	39,750	807.1	399	8.1	10	0.2	6,752	137.1
45	131	2.9	95	2.1	1,371	30.4	600	13.3	72	1.6	36,952	819.6	352	7.8	9	0.2	5,505	122.1
50	58	1.5	85	2.2	1,185	30.6	352	9.1	43	1.1	32,693	844.2	287	7.4	8	0.2	4,016	103.7
55	15	0.4	66	1.8	992	27.0	165	4.5	15	0.4	31,712	862.9	118	3.2	11	0.3	3,657	99.5
60	7	0.3	29	1.3	453	20.5	75	3.4	9	0.4	19,654	889.6	13	0.6	9	0.4	1,845	83.5
Total	825	3.0	397	1.4	7,516	27.4	3,305	12.0	316	1.2	227,216	828.2	1,748	6.4	67	0.2	32,974	120.2

Table 3B Prevalence per 1000 women of different inflammatory states of the vagina in the second screening stratified by age cohort.

Figures 1A-1I demonstrate that for each inflammatory pattern there is an age profile, varying from declining (with increasing age), stable, to hill-shaped.

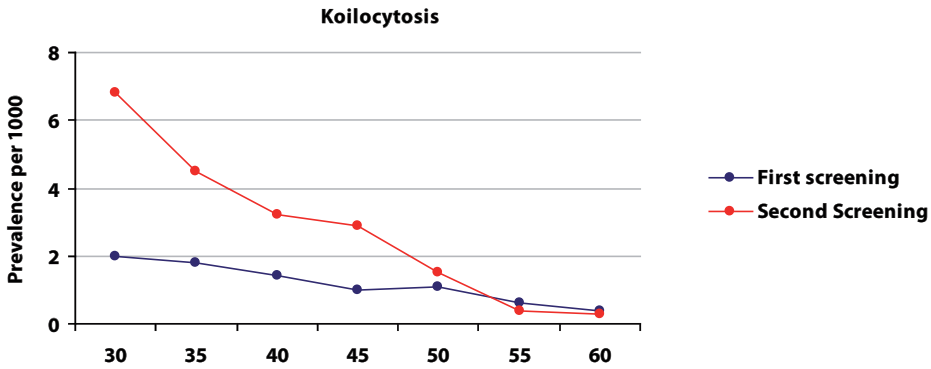

Figure 1A Prevalence of koilocytosis among the different age groups in the first and second screening: prevalence per 1000, declining age profile.

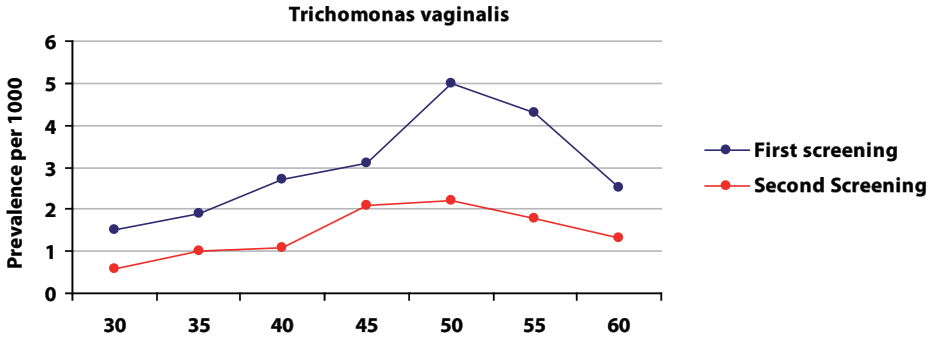

Figure 1B Prevalence of *Trichomonas vaginalis* among the different age groups in the first and second screening: prevalence per 1000, hill-shaped age-profile.

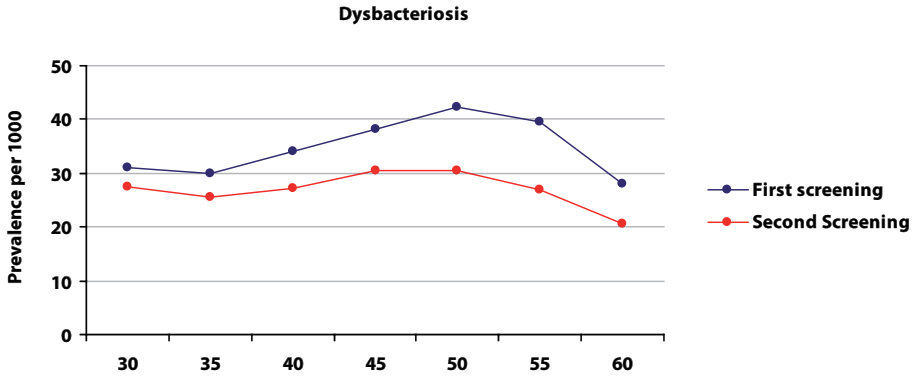

Figure 1C Prevalence of dysbacteriosis among the different age groups in the first and second screening: prevalence per 1000, hill-shaped age profile.

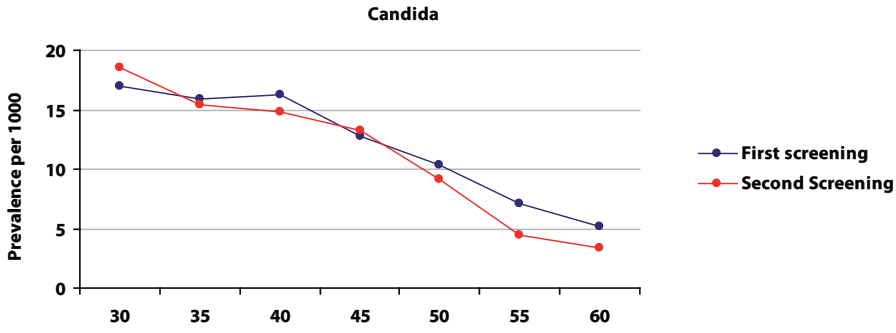

Figure 1D Prevalence of *Candida* among the different age groups in the first and second screening: prevalence per 1000, declining age profile.

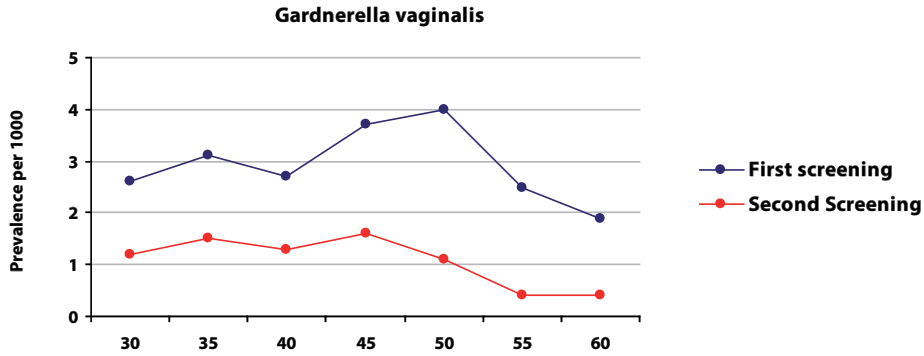

Figure 1E Prevalence of *Gardnerella vaginalis* infection among the different age groups in the first and second screening: prevalence per 1000, hill-shaped age profile.

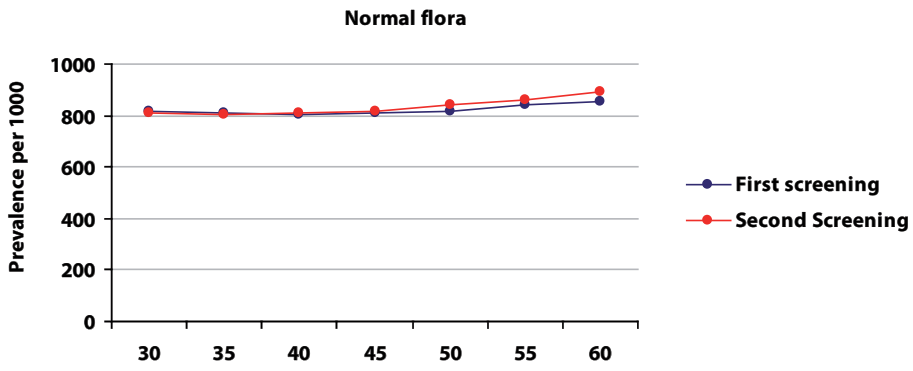

Figure 1F Prevalence of cytological normal flora among the different age groups in the first and second screening: prevalence per 1000, stable age profile.

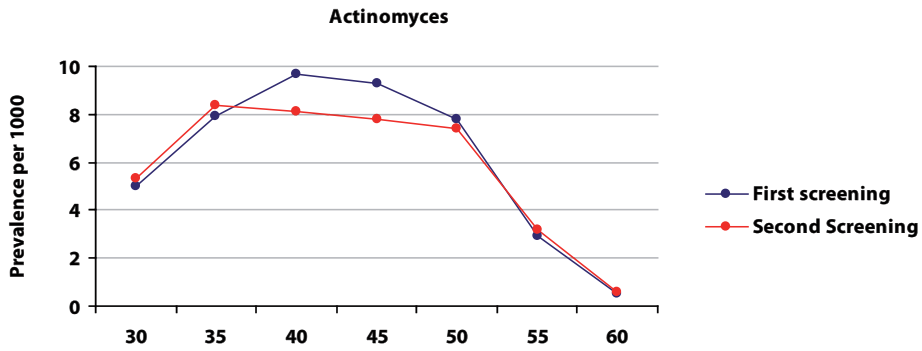

Figure 1G Prevalence of *Actinomyces* among the different age groups in the first and second screening: prevalence per 1000, hill-shaped age profile.

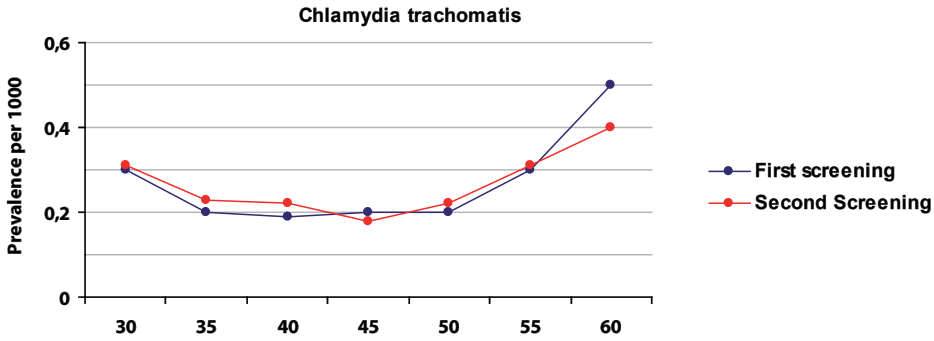

Figure 1H Prevalence of *Chlamydia trachomatis* among the different age groups in the first and second screening: prevalence per 1000, stable age profile.

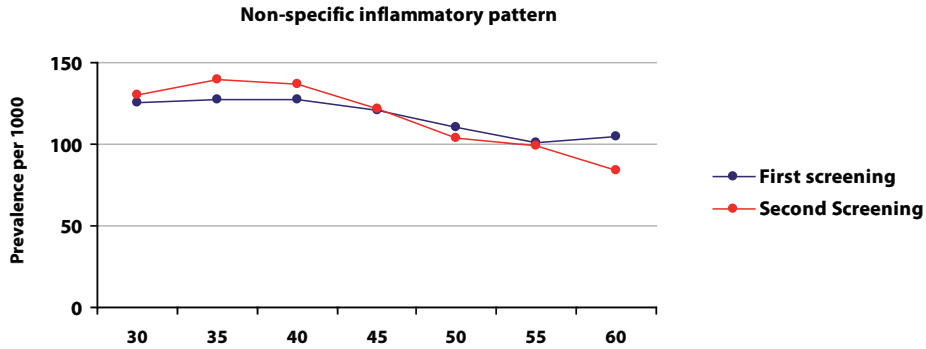

Figure 1I Prevalence of inflammatory pattern among the different age groups in the first and second screening: prevalence per 1000, stable age profile.

The prevalence of koilocytosis, shows an age-declining profile in both the first and second screening. Among young women between 30 en 35 years the prevalence of HPV-related koilocytosis increased significantly over the last decade (Figure 1A).
Trichomonas vaginalis (Figure 1B), dysbacteriosis (Figure 1C) and *Gardnerella vaginalis* (Figure 1E) all display higher prevalences among perimenopausal women, but showing a decline in all age groups. The peak prevalence for *Trichomonas* is at 50 years old, in contrast to koilocytosis were the peak age is 30 years. In *Trichomonas* there is an increase up to women 50 years and older, with a three times higher prevalence among 50 year old women compared to women in the 30 year age-group. While the prevalence declines in all age-groups, this trend is most steady among older women (above 50 years). The prevalences of *Candida*, normal flora, *Actinomyces*, *Chlamydia trachomatis* and non-specific inflammatory pattern did not change over the last ten years.

Discussion

This study establishes the prevalence of all cytologically diagnosed vaginal inflammations as stored in the KOPAC system over the 10 past years among women who participated in the Dutch national screening program for cervical cancer.

Firstly, our study shows a remarkable shift in the prevalence of dysbacteriosis and *Gardnerella* infection (both indicating bacterial imbalance of the vaginal flora) among all age cohorts, being strongly decreased in the second screening. Although prospective studies should elucidate the underlying cause, an increased awareness of the importance of a healthy vaginal flora might possibly contribute to this decrease. Women might have been more conscious of the importance of a lactobacilli flora and perhaps have achieved such by taking suitable measures. As a result of a lactobacilli-abundant vaginal flora, the chance on overgrowth with coccoid bacteria diminishes. The prevalence of *Gardnerella* infection as established in the cervical smear is actually very low, 1.2-3 per 1,000 smears, a tenth of that of dysbacteriosis, but with a similar hill-type age profile. It is of interest to remark that *Trichomonas* also shows a decline in the second screening, and that, similar to dysbacteriosis and *Gardnerella*, *Trichomonas* samples lack a lactobacilli flora.[18]

Secondly, our study reveals another change. Over the past decennium, there has been a strong increase of HPV-related koilocytosis among young women (30-35 year) which can be a sign of an HPV epidemic possibly caused by a change in sexual behaviour and/or unsafe sex.[19] From literature we know that the presence of koilocytic cells function as an indicator for active HPV-infection, whereas such an infection leads to excavitation of the cytoplasms of the cell, resulting in pathognomic abnormality of squaumous cells.[13,20,21] Not all samples from women with an active HPV-infection show koilocytosis, which means that the actual prevalence of HPV higher. As a matter of fact, the presence of HPV needs to be verified by PCR to validate trends concerning koilocytosis observed in cervical smears. Nowadays, it is well known that sexual transmission of human papilloma virus with oncogenic genotypes is a leading risk factor for cervical cancer.[2-5,22]

In this context a limitation of this study should be discussed. When a smear showed two or more inflammatory patterns a choice was made whereas only one O-code could be given. As a result, each smear receives an O-code which inevitably leads to deviations of real prevalences. For this reason prospective research should be done to study whether the significant increase of koilocytosis is possibly by caused by the decrease of bacterial imbalance.

Furthermore, we observed that a *Candida* infection is prevalent among young women with a lactobilli-abundant flora but can also develop when starting with estrogen suppletion at menopause. The age-declining profile of *Candida*, with high prevalences among young women and low prevalences among older women, resembles that of koilocytosis (Figure 1A). However, the prevalence did not change in the first and second screening.

The majority of women (over 800 per 1,000) had a healthy flora. Figure 1F shows that perimenopausal women more often have healthy vaginal flora which slightly increases in the second screening.

Actinomyces is often seen among women with an intra-uterine conceptive device (IUD),

diminishing when a new IUD is placed. Figure 1G reveals a hill-shaped age profile in both the first and second screening. However, the prevalence of *Actinomyces* slightly decreased among women from 35-50 years old. This effect could be contributed by a changed policy on placing IUDs by general practitioners.

Microscopy has a low sensitivity in diagnosis of *Chlamydia trachomatis*. Different types of 'diagnostic' inclusion bodies have been observed.[23,24] Studies on cytological diagnosis of *Chlamydia* infection based on the detection of various inclusion bodies display an average sensitivity of 27% and an average specificity of 79%, with large numbers of false-positive and false negative results.[14,22] Nowadays, the conclusive diagnosis of *Chlamydia trachomatis* requires confirmation by PCR. Therefore, the results displayed in Figure 1H do not reveal the real prevalence of *Chlamydia*, which actually is a serious health problem in the Netherlands.

Finally, the amount of cases in which a non-specific inflammatory pattern was seen, did not significantly change over the last decade and showed a stable age profile.

Although the calculated scores in this study can be considered as baseline prevalences in Dutch asymptomatic women, the results in this study do have limitations. In fact, the true prevalences as established by culture would be higher because cytological examination has limitations in detection of infective agents.

To our knowledge there has been no research on trends in vaginal inflammation over the last decade based on the spin-off of cervical screening. We do need to be informed about prevalences as vaginal inflammations are supposed to play an important role in cervical carcinogenesis. That is, although HPV infection is widely prevalent, only a few infected women will go on to develop cervical cancer, suggesting that also other factors are involved in malignant progression. Cervical inflammation has been proposed as one of the cofactors in cervical carcinogenesis, since disturbance of the vaginal flora is known to increase the risk of acquisition of HPV infection.[1,25-27] This is consistent with the general hypothesis that the local cervicovaginal milieu plays a role in susceptibility to HPV infection. In this context we ought to remark that it is not possible in the KOPAC system to code simultaneously for koilocytosis and dysbacteriosis or *Gardnerella*. Papanicolaou[28] and Mead et al.[29] already confirmed that women with cervical carcinoma often had a dysbacteriotic flora, lacking the normally protective lactobacilli. We recently found that cervical *Gardnerella* infection strongly covaries with the presence of (pre)neoplasia of the cervix.[30]

In conclusion, as disturbance of the vaginal flora seems to be related to the presence of cervical (pre)neoplasia, we can see the improvement of the vaginal balance of women in the western region of the Netherlands as a beneficial change. Nevertheless, the strong increase of HPV-related koilocytosis among young women is an ominous development, but should be further analysed by PCR. Further prospective research, concerning differences especially focussed on sexual behaviour and vaginal hygiene, is needed to acquire more insight in the reason of these differences.

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J.M. Klomp, M.E. Boon, M. van Haaften, A.P.M. Heintz

An increased prevalence of cervical (pre)neoplasia in dysbacteriotic smears containing *Gardnerella vaginalis* and shortage of lactobacilli

Abstract

Objective: Dysbacteriosis is defined as an unfavorable shift in the vaginal flora with a shortage of lactobacilli in the presence of clue cells (indicating a shift to *Gardnerella vaginalis*) as observed in the cervical smear and coded in the Dutch KOPAC system. As cervical inflammation has been proposed as a cofactor in the development of cervical cancer, the role of dysbacteriosis in cervical carcinogenesis is of great interest. This study focuses on the prevalence of cervical (pre)neoplasia in dysbacteriotic smears.
Methods: Data were collected from 800,498 Dutch asymptomatic women participating in the national screening program. Prevalences of (pre)neoplasia were calculated for dysbacteriotic smears using a healthy flora as reference. Data were weighted by age distribution.
Results: The prevalence of dysbacteriosis was 38.2 per thousand smears. The Odds ratio for HSIL/carcinoma was significantly higher in smears with dysbacteriosis compared to smears of women with a healthy vaginal flora (OR 2.0; CI 1.8-2.3), increasing from 1.5 for the 30 year category to 3.3 for the 60 year category.
Conclusion: Dysbacteriotic smears show a significant higher prevalence of cervical (pre) neoplasia. Cytotechnologists screening cervical smears should be aware of dysbacteriosis, in order to identify women harboring *Gardnerella vaginalis* and having a shortage of lactobacilli.

Introduction

In 1955 Gardner and Dukes introduced *Gardnerella vaginalis (Haemophilus vaginalis*) as one of the causative organisms of infection previously classified as non-specific vaginitis, and detected in cases of bacterial vaginosis.[1] Bacterial vaginosis is the most common cause of vaginitis in most clinical settings, and is characterized by the overgrowth of commensal anaerobic flora (mainly *Gardnerella vaginalis*) flora at the expense of H_2O_2-producing *Lactobacillus* species that predominate in the healthy vagina.[2-4]

Clinicians diagnose bacterial vaginosis by the characteristic fishy odor of the vaginal swab, a homogenous, grayish-white vaginal discharge, a vaginal pH > 4.5, and the presence of so called 'clue cells' in the unstained smear, the latter indicating a shift to *Gardnerella vaginalis*. [5-7] Apart from complaints of vaginal discharge caused by *Gardnerella* vaginitis, clinical studies have demonstrated a relationship between *Gardnerella vaginalis* and preterm delivery.[8,9] In another study *Gardnerella vaginalis* was found to be a risk factor for the acquisition of HIV where stimulation of HIV-expression by *Gardnerella vaginalis* was demonstrated. [10,11]

Although bacterial vaginosis is diagnosed by clinical criteria, many women remain *asymptomatic* in the presence of vaginitis or cervicitis.[12,13] Consequently, only symptomatic women will receive the diagnosis bacterial vaginosis. However, a larger group of women will actually have a vaginal flora with a shift to *Gardnerella vaginalis*, but still remain asymptomatic. This cytological shift is called dysbacteriosis. Although clinically diagnosed bacterial vaginosis (by Nugent criteria) has not been shown to be associated with cervical intra-epithelial neoplasia (CIN)[14], no published studies, however, have looked at the prevalence of cervical (pre)neoplasia among smears of *asymptomatic* women with a dysbacteriotic smear. Because of this large asymptomatic group of women, it is critical to indeed study the prevalence of cervical (pre)neoplastic cells in cytologically diagnosed dysbacteriosis.

The Bethesda System for coding screening results of cervical smears identifies five categories in cervical cytology concerning the vaginal ecosystem[15,16]: (1) *Trichomonas vaginalis*, (2) fungal organisms morphologically consistent with *Candida spp*, (3) shift in flora suggestive of bacterial vaginosis, (4) bacteria morphologically consistent with *Actinomyces spp* and (5) cellular changes consistent with herpes simplex virus. According to Meisels and Morin[17], dysbacteriosis appears to be a cytological subdivision of Bethesda's third category: 'shift in flora suggestive of bacterial vaginosis'. Dysbacteriosis therefore represents the microscopic diagnosis of a shift in vaginal flora, in which the majority of lactobacilli is replaced by a mixture of anaerobic bacteria, mainly *Gardnerella vaginalis*.[18,19]

In 1988, the Dutch national coding system for pathology findings in cervical cytology, KOPAC was introduced as a result of discussion between pathologists and gynecologists. Since then all smears screened in the Netherlands are also coded for inflammatory status of the vaginal flora.[20,21] Dutch cytotechnologists are taught how to recognize dysbacteriosis by the presence of clue cells and a shortage of lactobacilli in the stained smear.

The KOPAC system primary enables coding of the squamous epithelium: ranging from

KOPAC P1 (WNL or normal), via KOPAC P2-3 (ASCUS or borderline) to KOPAC P9 (macro invasive squamous cell carcinoma), accordingly the presence of cervical (pre)neoplasia and dysbacteriosis is recorded simultaneously. The study presented herewith focuses on the correlation between dysbacteriosis and the presence of (pre)neoplasia exploiting a large dataset of systematically coded, Papanicolaou-stained smears originating form the Dutch national screening program.[21,22]

Material and methods

Study set of 800,498 smears

Between January, 1991 and December, 2006, the Leiden Cytology and Pathology Laboratory (LCPL) received 800,498 cervical smears of women who participated in the Dutch national screening program. In the Netherlands all women between the age of 30 and 60 years are invited once every 5 year to have a smear taken in connection with prevention of cervical carcinoma. The invitation is based on a personal letter by the city council.

KOPAC coding

All smears, were coded according to KOPAC (the Dutch national coding system for cervical cytology) in which the letter 'O' stands for 'Ontsteking' (inflammation) consisting of the following nine different categories: koilocytosis (O1), *Trichomonas vaginalis* (O2), dysbacteriosis (O3), *Candida* (O4), *Gardnerella vaginalis* (O5), normal flora (O6), *Actinomyces* (O7), *Chlamydia trachomatis* (O8) and non-specific inflammatory changes (O9).

Furthermore, the letter 'P' within the Dutch coding system KOPAC is originating from the word 'Plaveiselepitheel' (squamous epithelium). Within this category, P1 stands for normal or benign, P2-3 for borderline changes, P4 for mild dysplasia, P5 for moderate dysplasia, P6 for severe dysplasia, P7 for carcinoma in situ, P8 for micro invasive carcinoma, and P9 for macro invasive squamous cell carcinoma. The relationship of the KOPAC P codes to the Bethesda system is shown in Table 1.

All diagnoses P5-P9 require referral to the gynecologist for colposcopic examination and, when necessary, a biopsy. For this reason, we grouped smears with P5 to P9 into a single category. Accordingly, statistical analyses are limited to this category.

Cytological criteria of O3 and O6

A cervical specimen is coded O3 (dysbacteriosis) when a remarkable shift is seen in the microflora with the presence of clue cells (CCs) that is, cells with adhering bacteria, mainly *Gardnerella vaginalis*. These CCs have an uneven blue color because of the haphazard bacterial adherence pattern (Figure 1). The number of lactobacilli is minimal. The dysbacteriotic smear, with coccoid bacteria in the smear background has a disorganized appearance (Figure 1).

A cervical smear is coded as O6 (normal flora) when there are lactobacilli and no, or only a few coccoid bacteria. In some of these O6 smears, very few bacteria are present. The O6 smear looks clean (Figure 2).

P-Codes of the KOPAC system	Description European systems	Bethesda system
P1	Normal	WNL
P2-P3	Borderline changes	ASCUS
P4	Mild dysplasia	LSIL
P5	Moderate dysplasia	HSIL
P6	Severe dysplasia	HSIL
P7	Carcinoma in situ	HSIL
P8	Micro invasive carcinoma	Carcinoma
P9	Squamous cell carcinoma	Carcinoma

WNL: within normal limits; ASCUS: atypical squamous cells of undetermined significance; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion.

Table 1 Relationship of the P-codes within the Dutch KOPAC system and other classification systems.

Statistical analysis

The prevalence per 1000 smears was calculated for dysbacteriosis (O3), and for cervical (pre)neoplasia (P2-3, P4 and P5-9). We also created a reference group consisting of non-inflammatory vaginal smears originating from women with a cytological normal flora. To make sure that this reference group was healthy, a special selection was made of women with at least three cervical smears classified as O6 and never had smears diagnosed other than O6.

The prevalence of cervical (pre)neoplasia among the two different groups, dysbacteriosis and normal flora, was calculated. Odds ratios (OR) with 95% confidence intervals (CI) were calculated for the dysbacteriosis group, by using SPSS 12.0. Age distribution differed between the two groups, with younger women (30-34) being overrepresented in the dysbacteriosis group. We therefore made a correction on age distribution in our calculations of the Odds ratios.

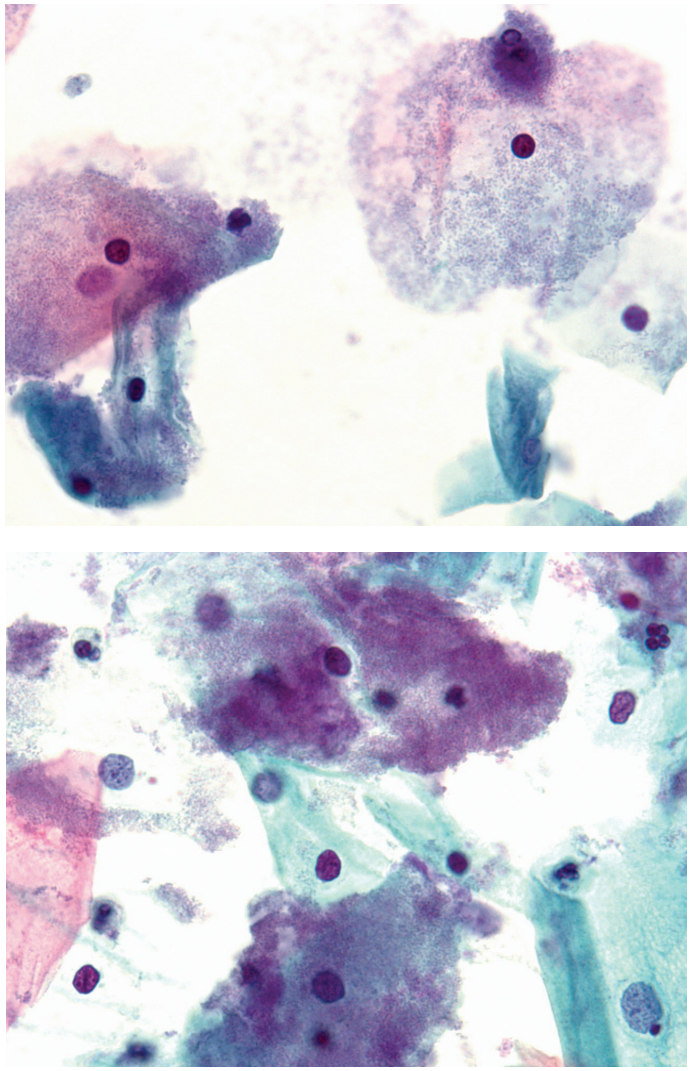

Figure 1 ThinPrep slides with a dysbacteriotic flora (O3). Note the shortage of lactobacilli. The coccoid bacteria are present in the background of the smears. Irregular distribution of the bacteria on the cells resulting in an uneven blue color. The image is 'disorderly' (Papanicolaou staining, magnification x 1000).

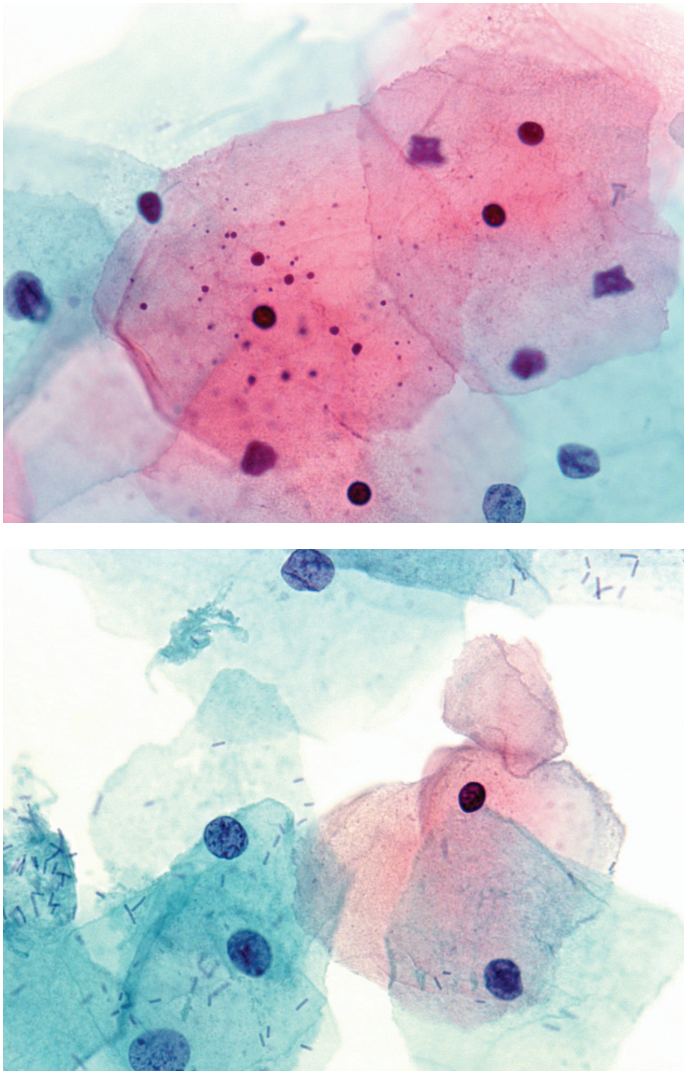

Figure 2 Smears with a healthy flora (O6). Note a few blue staining lactobacilli (A) and multiple blue staining lactobacilli (B). The smears have a 'clean' appearance (Papanicolaou staining, magnification x 1000).

Results

Table 2 displays the number of smears as well as the number of women from whom the smears originated. As shown, of all 800,498 cervical samples, 30,593 were coded O3. The prevalence of dysbacteriosis had a value of 38.2 per thousand smears. The reference group consisted of 227,580 smears. In addition, Table 2 also shows the prevalence of all other afore called vaginal inflammations grouped together, O1-O9.

	Smears	Women	Prevalence (smears)
Dysbacteriosis	30593	27186	38.2
Other inflammations	70580	62740	88.2
All	800498	442466	-

Table 2 Prevalences of dysbacteriosis and other vaginal inflammations per 1000 smears.

In Table 3, the presence of cervical (pre)neoplasia (P5-P9) in dysbacteriotic smears is presented. The same was done for the reference group. As shown, data were also stratified for age. The prevalence for dysbacteriosis decreased in the P4 (LSIL) series from 15.6 in the youngest age cohort to 2.6 in the oldest age group, and in the P5-P9 (HSIL/carcinoma) series from 14.7 to 5.7.

	Age	n	P1	P2-P3	P4	P5-P9
Dysbacteriosis	30-34	5243	933.4	36.2	15.6	14.7
	35-39	6104	945.5	32.0	12.3	10.3
	40-44	6882	950.0	28.3	12.9	8.7
	45-49	5950	960.2	28.1	5.9	5.9
	50-54	4493	965.1	24.5	5.3	5.1
	55-59	1921	963.6	28.1	2.6	5.7
	All	30593	951.3	29.8	10.1	8.8
Reference group	30-34	21519	935.7	43.5	11.5	9.3
	35-39	40648	957.1	29.5	7.1	6.3
	40-44	53613	966.3	24.6	5.1	4.0
	45-49	49536	968.2	25.2	4.3	2.3
	50-54	39074	974.3	20.3	3.1	2.3
	55-59	23190	986.7	10.0	1.6	1.7
	All	227580	965.6	25.2	5.2	4.0

P1: normal; P2-P3: ASCUS ; P4:LSIL; P5-P9: HSIL & carcinoma.

Table 3 Data stratified by inflammatory status of the cervical smear and age: prevalence of P1-P9 per 1000 smears for dysbacteriosis (A) and the reference group (B).

Table 4 presents Odds ratios per age category. Since the age cohorts 50-54 years and 55-59 years for dysbacteriosis were relatively small, with respectively 4,493 and 1,921 smears, we grouped these into an age cohort of 50-59 years. Finally, the Odds ratio for all women was weighted by age. The Odds ratio of the presence of cervical (pre)neoplasia (KOPAC P5-P9) was significantly higher in the dysbacteriosis group compared to reference group (OR 2.0; CI 1.8-3.2). Moreover, the Odds ratio increased from 1.5 for the youngest cohort to 3.3 for the oldest cohort.

Dysbacteriosis	
Age/years	**KOPAC P5-P9**
	OR (95% CI)
30-34	1.5 (1.1-2.1)
35-39	1.5 (1.2-2.1)
40-44	2.2 (1.7-2.9)
45-49	2.6 (1.8-3.7)
50-59*	3.3 (2.1-5.0)
All	2.0 (1.8-2.3)

Table 4 Odds ratios for P5-P9 (HSIL/carcinoma) in dysbacteriotic smears (weighted by age).

* Since the age cohorts 50-54 years and 55-59 years were relatively small, we grouped these into age cohort 50-59 years.

In Figure 3 prevalence of (pre)neoplasia (P5-P9) in dysbacteriotic smears is depicted (8.8‰) versus 4.0‰ for the reference group.

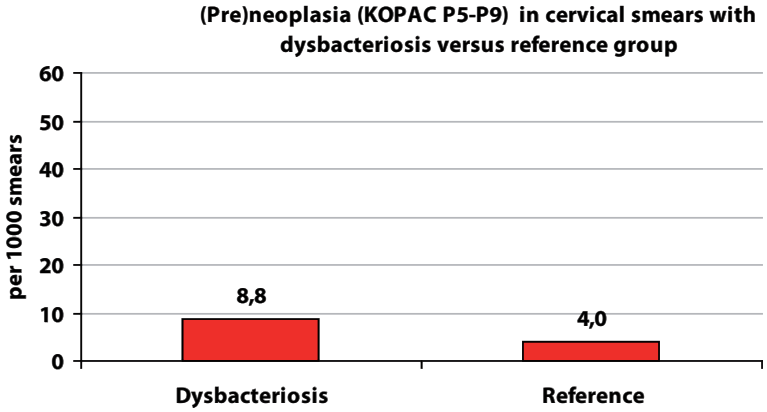

Figure 3 (Pre)neoplasia in cervical smears with dysbacteriosis versus reference smears (prevalence per 1000 smears).

Discussion

Although clinically diagnosed bacterial vaginosis (diagnosed by the Amsel test) was found to be unimportant in the etiology of cervical neoplasia in a relative small study by Peters et al.[14], the prevalence of cervical (pre)neoplasia in a large number of smears of *asymptomatic* women with cytologically diagnosed dysbacteriosis might be relevant to elucidate the correlation between dysbacteriosis and cervical neoplasia. With the exception of *Candida* infection, cervical inflammation has been proposed as etiological cofactor in the development of cervical cancer.[23-26] Moreover, pure *Gardnerella* patterns are associated with a significant higher proportion of HSIL/carcinoma.[27] In short, it is worthwhile to focus on smears with a shift in bacterial flora towards *Gardnerella vaginalis*.

To the best of our knowledge, our study is the first main report focusing on the covariation of dysbacteriosis and the presence of cervical (pre)neoplasia as established in asymptomatic women in population-based cervical screening. In a dataset of over 800,000 smears we found that dysbacteriosis is related to an increased prevalence of cervical (pre)neoplasia in the same smear.

It is a well known fact that infection with putative oncogenic human papillomaviruses contributes to the development of cervical cancer.[28-31] Although HPV infection is widely prevalent, only a few infected women will go on to develop cervical cancer, suggesting that also other factors such as a shift in bacterial flora might be involved in malignant progression.[31-33] In the early years of cytology, Papanicolaou[34] and Mead et al.[32] already observed that women with cervical carcinoma often had a disturbed flora, lacking the normally protective lactobacilli and with an overgrowth of coccoid bacteria. This seems to be consistent with the general hypothesis that the local cervicovaginal milieu plays a role in susceptibility to HPV infection.[28,29] A shortage of lactobacilli resulting in a shift toward a flora containing *Gardnerella vaginalis* increases the effects of HPV[23,31] and a disturbance of the vaginal flora enhances acquisition of HPV infection.[27,32,36]

According to the differences in the presence of (pre)malignant cells in dysbacteriosis and healthy smears, our findings might be relevant for cytotechnologists screening cervical smears. They should be taught how to accurately recognize bacterial dysbalance of the vaginal flora. During the process of screening, awareness of the possible consequences of a disturbed flora is essential. Secondly, women with dysbacteriosis should be treated efficiently, for instance with metronidazole, to irradicate *Gardnerella vaginalis*.[38-40] In addition, the dysbacteriotic pattern can also serve to identify women who perhaps warrant more intensive surveillance concerning life-style factors including post coital hygiene.[2]

In our study, we show that large databases from organized screening can be used to study cervical (pre)neoplasia and dysbacteriosis. We proved that there is an increased prevalence of cervical (pre)neoplasia in dysbacteriotic smears containing *Gardnerella vaginalis* and lacking lactobacilli. In the context of studying the influence of the vaginal flora on cervical carcinogenesis it might be of interest to establish the effects of interventions with lactobacilli supplements and metronidazole treatments of women with a disturbed vaginal flora. Finally, we remark that it would be of interest to embark on research focused on sexual behavior and vaginal hygiene using smears to establish shifts in the vaginal flora.

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American Journal of Obstetrics and Gynecology, in press

J.M. Klomp, M.E. Boon, M. van Haaften, A.P.M. Heintz

Cytologically diagnosed *Gardnerella* infection and cervical (pre)neoplasia as established in population-based cervical screening

Abstract

Objective: Cervical inflammation has been proposed as a cofactor in the development of cervical cancer. The purpose of this study was to document the prevalence of cervical (pre) neoplastic changes in asymptomatic women with a cytologically diagnosed *Gardnerella* infection.
Methods: Data were collected from 800,498 Dutch asymptomatic women, participating in the Dutch national screening program. Prevalences of (pre)neoplasia were calculated for *Gardnerella* smears using a healthy flora as reference.
Results: The prevalence of *Gardnerella* infection was 0.6 per thousand. The Odds ratio for (pre)neoplasia was significantly higher in smears with *Gardnerella* infection compared to smears of women with a healthy vaginal flora (OR 10.3; CI 6.6-16.1).
Conclusion: Cytologically diagnosed *Gardnerella* smears show a strong covariation with the presence of cervical (pre)neoplasia. Future research should therefore focus on the exact causal relation between cytological *Gardnerella* infection and the presence of (pre) neoplastic changes of the cervix.

Introduction

After breast cancer, cervical carcinoma is the second most common cancer among women worldwide.[1] Nowadays, it is well known that infection with putative oncogenic human papillomaviruses contributes to the development of cervical cancer.[2-5] Although HPV infection is widely prevalent, only a few infected women will go on to develop cervical cancer, suggesting that also other factors are involved in malignant progression. Cervical inflammation has been proposed as one of the cofactors in cervical carcinogenesis, since disturbance of the vaginal flora is known to increase the risk of acquisition of HPV infection.[6,7-9] This hypothesis resulted in many studies attempting to further evaluate the correlation between cervical inflammation and (pre)neoplasia. In this context, studies have noted that cervical cytological abnormalities occur more often in women with an imbalanced vaginal flora than in those without this condition.[10,11]

In 1955 Gardner and Dukes introduced *Gardnerella vaginalis (Haemophilus vaginalis*) as one of the causative organisms of infection previously classified as non-specific vaginitis, and detected in cases of bacterial vaginosis.[12] Worldwide, *Gardnerella vaginalis*-associated vaginitis is characterized by a malodorous, homogenous, grayish-white vaginal discharge, vaginal pH > 4.5 and a fishy odour.[13] But many women remain *asymptomatic* in the presence of vaginitis or cervicitis.[14] Apart from complaints of vaginal discharge caused by *Gardnerella* vaginitis, clinical studies have demonstrated a relationship between *Gardnerella vaginalis* and preterm delivery.[15,16] In another study, *Gardnerella vaginalis* was found to be a risk factor for the acquisition of HIV where stimulation of HIV-expression by *Gardnerella vaginalis* was demonstrated.[17,18]

Although clinically diagnosed bacterial vaginosis (by Nugent criteria) has not been shown to be associated with cervical intra-epithelial neoplasia (CIN)[19], no published studies, however, have looked at the prevalence of cervical (pre)neoplasia among smears of *asymptomatic* women with *Gardnerella* infection.

The Bethesda System identifies five categories of organisms in cervical cytology[20,21]: (1) *Trichomonas vaginalis*, (2) fungal organisms morphologically consistent with *Candida spp*, (3) shift in flora suggestive of bacterial vaginosis, (4) bacteria morphologically consistent with *Actinomyces spp* and (5) cellular changes consistent with herpes simplex virus.

According to Meisels and Morin, a cervical *Gardnerella* infection is a cytological subdivision of the category 'findings suggestive of bacterial vaginosis', that displays an extreme shift in flora, in which tiny rodlike organisms cling to the cellular membrane.[22] In the Papanicolaou stain this phenomenon has the appearance of a *blue mountain cell* (BMC), because the cell membrane is covered by a mountain of blue staining round bacteria.

In 1988, the Dutch national coding system for pathology findings in cervical cytology was introduced and especially designed to store the cytopathological findings of smears of asymptomatic women (30-60 years), including the presence of *Gardnerella* infection.[23] Dutch screeners are taught how to recognize evidence of *Gardnerella* infection by the presence of BMCs in the stained smear.

KOPAC is an acronym for this coding system in which K indicates comoposition [kompositie]; O, inflammation [ontstekingsverschijnselen]; P, squamous epithelium [plaveiselepitheel];

A, other abnormalities of the endometrium [andere afwijkingen van het endometrium]; C, endocervial columnar epithelium [cylinderepitheel endocervix].[23] Furthermore, the KOPAC system evaluates the squamous epithelium coded from P1 (WNL or normal), P2-3 (ASCUS or borderline) to P9 (macro invasive squamous cell carcinoma), allowing the recording of presence of cervical (pre)neoplasia in *Gardnerella* smears.
The objective of our study was to perform a population-based analysis to document the prevalence of cervical (pre)neoplasia in smears of asymptomatic women with cytologically diagnosed *Gardnerella* infection versus healthy vaginal flora. The Leiden database contains the cytological findings of 800,498 smears (originating from asymptomatic women) of which 498 smears with cytological evidence of a cervical *Gardnerella* infection. As a result, the prevalence of cervical (pre)neoplasia in *Gardnerella* infection can be calculated.[24]

Material and methods

Between January, 1991 and December, 2006, the Leiden Cytology and Pathology Laboratory (LCPL) received 800,498 cervical smears of asymptomatic women who participated in the Dutch national screening program. In the Netherlands all women between the age of 30 and 60 years are invited once every 5 year to have a smear taken in connection with prevention of cervical carcinoma. The invitation is based on a personal letter by the city council.
All smears, were coded according to KOPAC (the Dutch national coding system for cervical cytology) in which the letter 'O' stands for 'Ontsteking' (inflammation) consisting of the following nine different categories: koilocytosis (O1), *Trichomonas vaginalis* (O2), dysbacteriosis (O3), *Candida* (O4), *Gardnerella vaginalis* (O5), normal flora (O6), *Actinomyces* (O7), *Chlamydia trachomatis* (O8) and non-specific inflammatory changes (O9).
A *Gardnerella* infection (O5) is registered when, by microscope, the cervical smear shows so-called BMCs, thousands tiny rodlike organisms clinging to the cellular membrane, and a lack of lactobacilli. [22,23] In the Papanicolaou stain this phenomenon has the appearance of a *blue mountain cell* (BMC), because the cell membrane is covered by a mountain of blue staining round bacteria. A non-inflammatory cervical smear with a healthy flora is classified as O6.
Furthermore, the letter 'P' within the Dutch coding system KOPAC is originating from the word 'Plaveiselepitheel' (squamous epithelium). Within this category, P1 stands for normal or benign, P2-3 for borderline changes, P4 for mild dysplasia, P5 for moderate dysplasia, P6 for severe dysplasia, P7 for carcinoma in situ, P8 for micro invasive carcinoma, and P9 for macro invasive squamous cell carcinoma. The relationship of the P codes to The Bethesda System is shown in Table 1. All diagnoses P5-P9 require referral to the gynecologist for colposcopic examination and, when necessary, a biopsy. For this reason, we grouped smears with P5 to P9 into a single category. As a result, each smear receives both an O-code (O1-O9) and a P-code (P1-P9) allowing for a study of correlation between the prevalence of *Gardnerella* infection and (pre)neoplastic changes of the cervix.

P-Codes of the KOPAC system	Description European systems	Bethesda system
P1	Normal	WNL
P2-P3	Borderline changes	ASCUS
P4	Mild dysplasia	LSIL
P5	Moderate dysplasia	HSIL
P6	Severe dysplasia	HSIL
P7	Carcinoma in situ	HSIL
P8	Micro invasive carcinoma	Carcinoma
P9	Squamous cell carcinoma	Carcinoma

WNL: within normal limits; ASCUS: atypical squamous cells of undetermined significance; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion.

Table 1 Relationship of the P-codes within the Dutch KOPAC system and other classification systems.

Statistical analysis

The prevalence per 1000 smears was calculated for *Gardnerella* infection (O5) and cervical (pre)neoplasia (P2-3, P4 and P5-9). We also created a reference group consisting of smears displaying a normal flora. To make sure that this reference group was healthy, a special selection was made of women with at least three cervical smears classified as O6 (normal flora), who never had smears diagnosed other than O6.

The prevalence of cervical (pre)neoplasia among the two groups, *Gardnerella* and normal flora, was calculated. Odds ratios (OR) with 95% confidence intervals (CI) were calculated for both the *Gardnerella* group and the reference group, by using SPSS 12.0. Age distribution differed between both groups, with younger women (30-34 year) being overrepresented in the *Gardnerella* group. We therefore made a correction on age distribution in our calculations of the Odds ratios.

Results

Table 2 displays the number of smears, as well as the number of women from whom the smears originated. Of all 800,498 cervical samples, 498 were coded O5 composing the *Gardnerella* group. The prevalence of cytologically diagnosed *Gardnerella* infection was only 0.6 per thousand. Table 2 also shows the prevalence of all other afore called vaginal inflammations grouped together.

	Smears	Women	Prevalence (per 1000 smears)
Gardnerella infection	498	490	0.6
Other inflammations	100675	89436	125.8
All	800498	442466	-

Table 2 Number of smears: *Gardnerella* and other vaginal inflammations.

In Table 3, the presence of cervical (pre)neoplasia (P2-3, P4, P5-9) in *Gardnerella* smears is presented. The same was done for the reference group consisting of 227,580 smears. Data were also stratified by age.

	Age	n	P1	P2-P3	P4	P5-P9
Gardnerella	30-34	89	786.5	78.7	67.4	67.4
	35-39	106	811.3	75.5	84.9	28.3
	40-44	129	876.0	38.8	46.5	38.8
	45-49	95	873.7	63.2	0.0	63.2
	50-54	56	892.9	89.3	17.9	0.0
	55-59	23	869.6	43.5	43.5	43.5
	All	498	847.4	64.3	46.2	42.2
Reference group	30-34	21519	935.7	43.5	11.5	9.3
	35-39	40648	957.1	29.5	7.1	6.3
	40-44	53613	966.3	24.6	5.1	4.0
	45-49	49536	968.2	25.2	4.3	2.3
	50-54	39074	974.3	20.3	3.1	2.3
	55-59	23190	986.7	10.0	1.6	1.7
	All	227580	965.6	25.2	5.2	4.0

P1: normal. P2-P3: ASCUS. P4:LSIL. P5-P9: HSIL & carcinoma.

Table 3 Data stratified by age: prevalence per 1000 for *Gardnerella* and (pre)neoplasia (P2-P3, P4, P5-P9).

Table 4 presents Odds ratios for the different age cohorts. The results were weighted by age. The Odds ratio of the presence of cervical (pre)neoplasia (P5-P9) was significantly higher in the *Gardnerella* group compared to reference group (OR 10.3; CI 6.6-16.1).

Gardnerella		
Age/years	**KOPAC P5-P9**	
	OR	(95% CI)
30-34	7.6	(2.5-22.8)
35-39	6.1	(2.0-18.7)
40-44	11.2	(4.7-26.6)
45-49	28.9	(13.5-60.5)
50-59*	7.7	(1.9-30.8)
All	10.3	(6.6-16.1)

Table 4 Odds ratios for P5-P9 in *Gardnerella* smears (weighted by age)

*Since the age cohorts 50-54 years and 55-60 years were relatively small, we grouped these into age cohort 50-59 years.

In Figure 1 prevalences of (pre)neoplasia (P5-P9) in cytological *Gardnerella* infection are depicted, revealing a strong covariation.

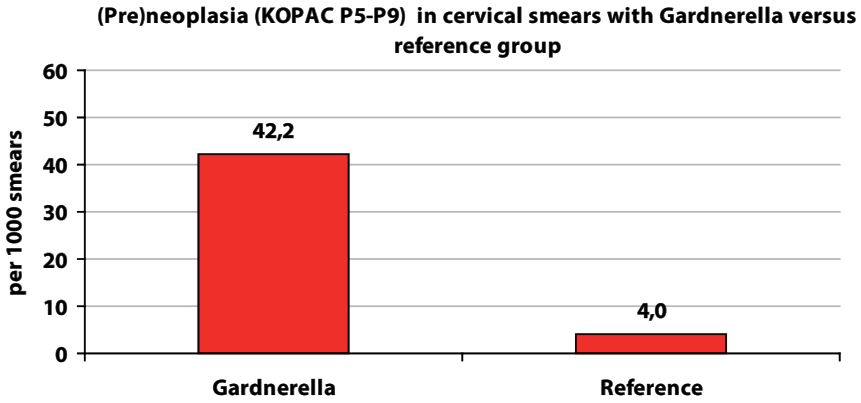

Figure 1 (Pre)neoplasia in cervical smears of asymptomatic women with cytologically diagnosed *Gardnerella* infection versus reference smears (prevalence per 1000 smears).

Discussion

In our study we established that the presence of cytologically diagnosed cervical *Gardnerella* infection in asymptomatic women strongly covaries with the presence of cervical (pre)neoplasia in the same smear.

Papanicolaou[9] and Mead et al.[25] already confirmed that women with cervical carcinoma often had a vaginal flora lacking the normally protective lactobacilli. In this context the relationship of bacterial inflammation and (pre)neoplasia has recently been investigated. Verbruggen et al. found that women with a cytologically diagnosed bacterial imbalance had a significantly higher presence of cervical (pre)neoplasia.[26] This finding is partially supported by other studies that investigated women with bacterial vaginosis and cervical (pre)neoplasia.[7,11,27] In contrast, several studies indicated that bacterial vaginosis diagnosed by the Nugent criteria showed not to be associated with CIN.[14,28] In addition, two studies showed similar frequencies of clinically diagnosed bacterial vaginosis among women with squamous intraepithelial lesions (SIL).[19,29] According to Engberts et al., presence of *Candida* is not associated with cervical (pre)neoplasia.[30]

We believe our study the first report on the prevalence of cervical (pre)neoplasia in cytologically diagnosed *Gardnerella* infection in asymptomatic women.

It should be noted that, in the Netherlands, the prevalence of cervical cancer is highest among young women around the age of 35 years. A peak in prevalence is also seen at the age of 55.[31] Our results show significant differences in age distribution between the *Gardnerella* group and the reference group. As this could be a possible confounder, our results were corrected for age. Because the prevalence of both a *Gardnerella* pattern and the presence of cervical (pre)neoplastic cells is low, the number of smears is small, but

nevertheless statistically significant.

As far as pre-cancer could possibly be causing the BMCs, we investigated on this by additional screening of 100 cervical smears, without evidence of cervical inflammation, but with the presence of HSIL/carcinoma (P5-P9). Ergo, in these 100 cases, (pre)neoplasia did not result in BMCs.

Although we did not investigate the nature of the link between *Gardnerella* infection and (pre)neoplasia, the general hypothesis is that the local cervicovaginal milieu plays a role in susceptibility to HPV infection. Since women with cervical *Gardnerella* infection are likely to possess an unhealthy *Lactobacillus*-poor vaginal flora, they should also be at risk for acquiring HPV infection. Whereas HPV infection is associated with an increased risk of developing squamous abnormalities[2-5] and a *Lactobacillus*-poor environment increases HPV effects,[6,32] our study suggests that cervical *Gardnerella* infection might be a cofactor in the development of cervical cancer.

We therefore firstly propose that further research should be done to answer the question whether there exists a causal relationship between our findings.

Secondly, we propose that cytologically diagnosed *Gardnerella* infection is coded separately according to the Dutch KOPAC system. As a result, screeners have to be taught how to accurately recognize this specific cytological subdivision of bacterial disbalance of the vaginal flora. During the process of screening, awareness of the possible consequences is crucial. Our findings could be taken into account in further research concerning accuracy of identifying BMCs during the screening process as well as other predisposing factors for cervical carcinogenesis.

Thirdly, the *Gardnerella* pattern might also serve to identify women who perhaps warrant more intensive surveillance concerning life-style factors. Further research, concerning differences especially focused on sexual behavior and vaginal hygiene, is needed to acquire more insight in the reason of the documented differences.

In our study, we show that large databases from organized screening can be used to study the prevalence of cervical (pre)neoplasia in *Gardnerella* infection among asymptomatic women.

Finally, our data, that show a significant proportion of cervical (pre)neoplasia in smears of asymptomatic women with cytological *Gardnerella* infection, should prompt effective treatment with metronidazole, despite of the absence of clinical symptoms.[33,34] According to our results, we recommend future prospective research on women with *Gardnerella* smears to study the exact causal relationship between *Gardnerella* infection and cervical (pre)neoplasia. Such research may possibly be able to determine the effects of treatment of cytologically diagnosed *Gardnerella* infection on the inhibition of the various steps of cervical carcinogenesis.

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Gardnerella infection can be distinguished from cervical dysbacteriosis: a cytological study

Abstract

Objective: Dysbacteriosis and *Gardnerella* infection are both cytological diagnoses indicating a shift in vaginal flora, the former the mild version of ecological disturbance with the presence of clue cells (CCs), the latter the extreme version with so called blue mountain cells (BMCs). The purpose of this study was to evaluate these cytological diagnoses and to obtain insight into the diagnostic problems of *Gardnerella*.
Methods: One hundred randomly selected samples of each of the three diagnostic series were rescreened by two pathologists resulting in two rescreening diagnoses and a consensus diagnosis. A smear was considered unequivocal when the original O code and the O code of the consensus diagnoses were equal. A contrasting diagnosis in rescreening was considered discordant.
Results: Unequivocal diagnoses were established in 65% of the dysbacteriotic smears, 80% of the *Gardnerella* smears and as much as 93% of the healthy smears. Discordance was highest in the dysbacteriotic series (20%) and lowest in the normal flora-group (4%). Misclassification of *Gardnerella* occurred in the presence of clusters of bacteria mixed with spermatozoa.
Conclusion: *Gardnerella* infection can be identified unequivocally in cervical smears. Classification can be problematic in the presence of blood and/or spermatozoa. Because of the clinical importance of treating *Gardnerella*, such advantageous spin-offs of cervical screening should be exploited.

Introduction

In the process of screening for cervical (pre)neoplasia, the cytotechnologist can distinguish smears with a healthy flora containing lactobacilli from those with a shift in the vaginal ecosystem and/or the colonization of parasites or fungi.
The Bethesda System identifies five categories of organisms in cervical cytology:[1,2] (1) *Trichomonas vaginalis*, (2) fungal organisms morphologically consistent with *Candida spp*, (3) shift in flora suggestive of bacterial vaginosis, (4) bacteria morphologically consistent with *Actinomyces spp* and (5) cellular changes consistent with herpes simplex virus. According to Meisels and Morin[3], dysbacteriosis and *Gardnerella* infection appear to be two cytological subdivisions of Bethesda's third category: shift in flora suggestive of bacterial vaginosis. Dysbacteriosis represents the microscopic diagnosis of a shift in vaginal flora, in which the majority of lactobacilli are replaced by a mixture of anaerobic bacteria, mainly *Gardnerella vaginalis*[4,5] whereas pure *Gardnerella* infection displays an *extreme* shift of the vaginal flora with a lack of lactobacilli, in which tiny rodlike organisms cling to the cellular membrane.[3]
In KOPAC,[6] the Dutch national coding system for pathology findings in cervical cytology, dysbacteriotic smears are coded O3. In these smears lactobacilli are almost absent and coccoid bacteria including a few *Gardnerella vaginalis* bacteria are present on the so-called clue cells (CCs) and in addition in the background of the smear. Furthermore, the KOPAC system provides a category for pure *Gardnerella* patterns, coded as O5. In the Papanicolaou stain these smears contain *blue mountain cells* (BMCs) because they are completely covered by a mountain of blue staining round bacteria. Dutch screeners are taught how to recognize evidence of *Gardnerella* infection by the presence of BMCs in the stained smear and dysbacteriosis based on CCs.
Because of the high prevalence of (pre)neoplastic cervical cells among *Gardnerella* smears[7], it would be interesting to examine whether both states of imbalance of the vaginal flora can be distinguished. The present study was undertaken to evaluate problems that can hamper efficient identification of these two entities.

Material and methods

KOPAC coding of the vaginal flora
In connection with the Dutch national screening program on cervical cancer, all smears were routinely coded according to KOPAC (the Dutch national coding system for cervical cytology) in which the letter 'O' stands for 'Ontsteking' (inflammation) consisting of the following nine categories: koilocytosis (O1), *Trichomonas vaginalis* (O2), dysbacteriosis (O3), *Candida* (O4), *Gardnerella vaginalis* (O5), normal flora (O6), *Actinomyces* (O7), *Chlamydia trachomatis* (O8) and non-specific inflammatory changes (O9).

Cytological criteria of O3, O5 and O6
A cervical smear is coded O6 (normal flora) when there is clear evidence of the presence of lactobacilli and no, or only a few coccoid bacteria. The O6 smear looks clean at screening

magnification. In some O6 smears the number of lactobacilli is impressive and in these cytolysis may occur, leading to a less 'clean' impression. The shape of the lactobacilli can be judged at higher magnification (Figure 1).

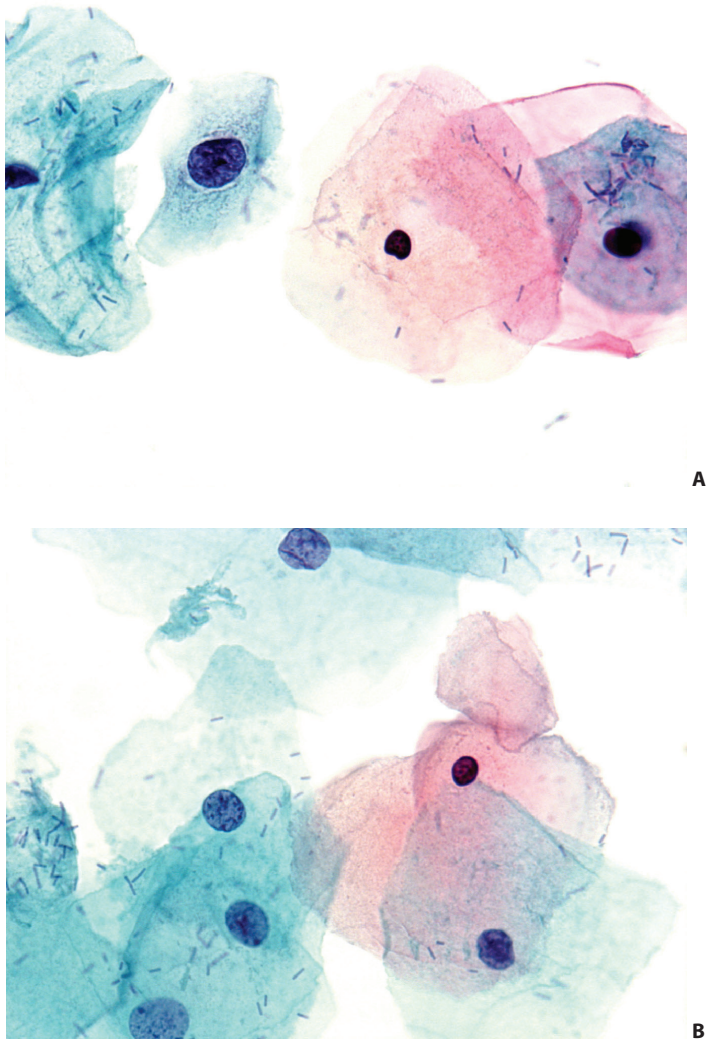


Figure 1 ThinPrep slides (A and B) with a normal flora (O6). Note multiple blue staining lactobacilli. The slides have a 'clean' appearance (Papanicolaou staining, magnification x 1000).

A cervical specimen is coded O3 (dysbacteriosis) when a shift in microflora is seen with evident shortage of lactobacilli and the presence of clue cells. These CCs prove to have an incomplete cover of coccoid bacteria displaying an uneven blue color because of the haphazard bacterial adherence pattern. The dysbacteriotic smear, with coccoid bacteria also in the background, has a disorganized appearance, as such clearly visible at screening

magnification. The coccoid shape of the bacteria clinging to the CCs should be judged at a higher magnification (Figure 2).

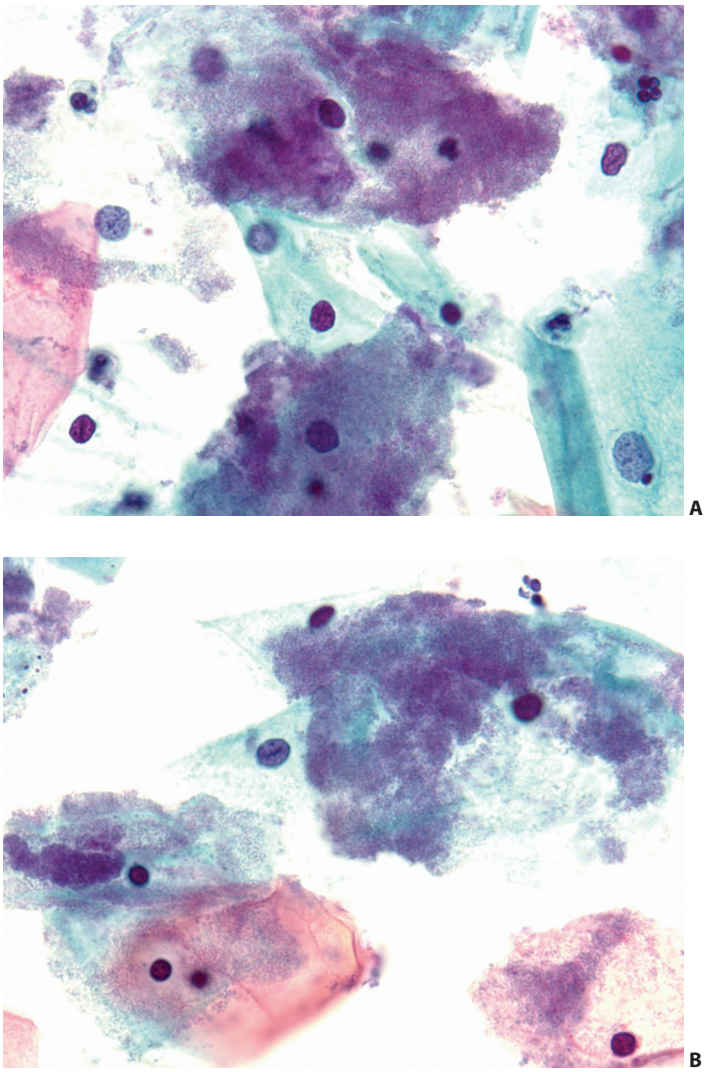


Figure 2 ThinPrep slides (A and B) with a dysbacteriotic flora (O3). Note the absence of lactobacilli. The coccoid bacteria are present in the background. Uneven distribution of the bacteria on the cells resulting in a blue color. The slides have a 'disorderly' appearance (Papanicolaou staining, magnification x 1000).

The cytological diagnosis of *Gardnerella* infection (O5) is given when the smear contains cells completely covered with a thick layer of blue staining bacteria. These dark staining BMCs are already visible on screening magnification, clearly standing out in the clean smear background. These *Gardnerella* smears have an orderly appearance, in contrast to the dysbacteriotic smears (Figure 3).[3,6]

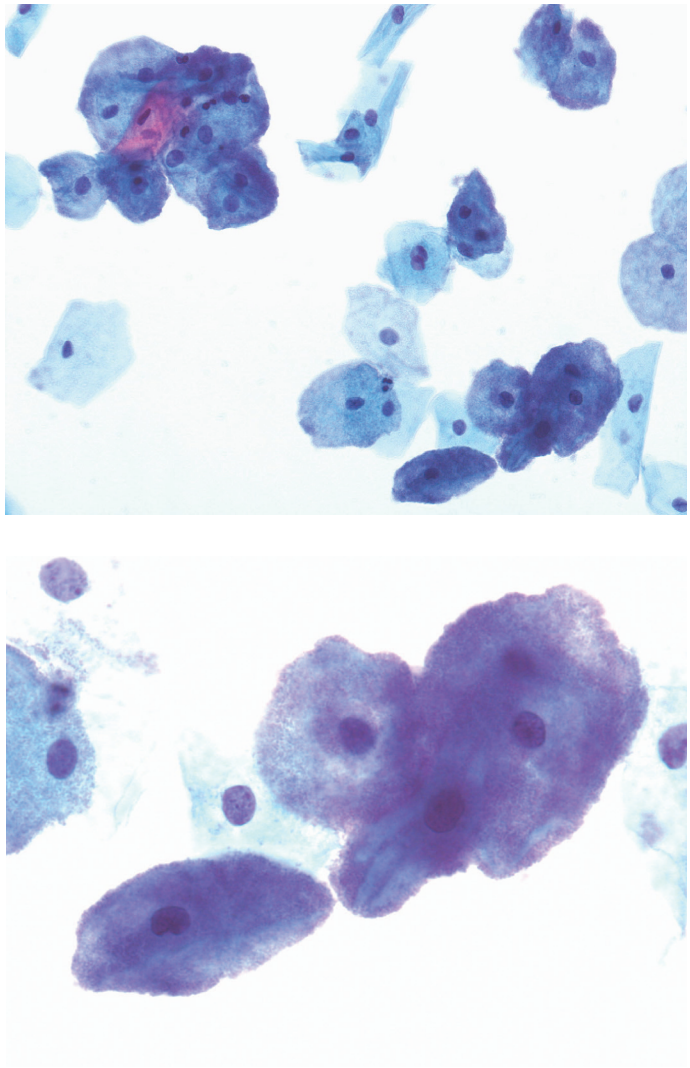


Figure 3 ThinPrep slides with a *Gardnerella* flora (O5). Note *blue mountain cells* (BMCs) completely covered by a thick layer of (blue staining) bacteria (A, Papanicolaou staining, magnification x 400). The slides have an 'orderly' appearance which is most evident at the screening magnification (B) (Papanicolaou staining, magnification x 1000).

The O3, O5 and O6 codes of eight cytotechnologists in 2007

The O3, O5 and O6 codes of 70,000 smears of eight cytotechnologists participating in the screening process were calculated. Ninety percent of the material consisted of ThinPrep slides, the remaining were conventional smears. There were no significant differences in O-codes between the ThinPrep and the conventional slides.

The rescreening process of the randomly selected 300 smears
One hundred cervical smears coded as O3 (dysbacteriosis), 100 coded as O5 (*Gardnerella* infection), and 100 coded as O6 (healthy flora) were randomly collected out of the 70,000 smears screened in the Leiden Cytology and Pathology Laboratory in 2007. Age distribution was equal in all three series.
These 300 smears were rescreened by two experienced pathologists independently, not knowing the original O code. Each rescreened smear received an O-code according to the KOPAC system and special cytological patterns as well as diagnostic problems encountered in the rescreening were documented.

Discordant, consensus and unequivocal diagnoses
During rescreening each smear received a 'consensus' diagnosis based on the diagnoses given by both pathologists. In case of contrasting diagnoses the smear was considered 'discordant'. However, these discordant smears were subsequently evaluated to obtain a consensus diagnosis.
A smear was classified 'unequivocal' when the original O code and the O code of the consensus diagnosis were exactly equal.
A stable diagnosis is presented by a high number of unequivocal diagnoses (same code in routine and re-screening by both pathologists) and a low discordance (contrasting diagnoses of the pathologists). For our study we compared these parameters for dysbacteriosis, *Gardnerella* infection and healthy flora in order to evaluate the cytological differentiation between both diagnoses.

Results

In Table 1, the screening results concerning O3, O5 and O6 codes of 70,000 smears of eight cytotechnologists participating in the screening process are shown. The O3 codes varied from 1.9 to 5.6%, the O5 codes from 0.02 to 0.09%, and the O6 codes from 77.4 to 88.4%.

Screener	O3		O5		O6	
	n	%	n	%	n	%
1	404	5.4	5	0.07	5,835	77.4
2	612	4.0	2	0.02	12,380	80.4
3	478	3.2	3	0.02	11,632	77.6
4	170	4.4	3	0.08	3,148	82.4
5	315	4.8	6	0.09	5,165	78.4
6	482	5.6	4	0.04	6,994	81.1
7	121	1.9	1	0.02	5,218	82.6
8	153	3.4	3	0.08	3,925	88.4

Table 1 O3, O5 and O6 diagnoses of eight cytotechnologists in 2007

Table 2 displays the diagnoses of the two pathologists in the dysbacteriotic series. Only 65/100 smears had a consensus diagnosis of dysbacteriosis (O3).

	Pathologist 1 Diagnosis	Pathologist 2 Diagnosis	Consensus diagnosis
Dysbacteriosis (O3)	67	58	65
Gardnerella (O5)	18	22	19
Normal flora (O6)	13	17	13
Inflammatory (O9)	2	1	2
Problematic identification	0	2	1
Total	100	100	100

Table 2 Diagnoses of the 100 rescreened smears originally coded as dysbacteriosis (O3).

In Table 3, the diagnoses of the two pathologists in the *Gardnerella* (O5) series are shown. The number of consensus diagnoses accounts for 80/100.

	Pathologist 1 Diagnosis	Pathologist 2 Diagnosis	Consensus diagnosis
Gardnerella (O5)	79	81	80
Dysbacteriosis (O3)	10	9	10
Normal flora (O6)	6	5	6
Inflammatory (O9)	3	1	2
Koilocytosis (O1)	1	1	1
Problematic identification	1	3	1
Total	100	100	100

Table 3 Diagnoses of the 100 rescreened smears originally coded as *Gardnerella* (O5).

Table 4 presents the diagnoses of the two pathologists concerning smears coded O6 (normal flora). As much as 93/100 smears received a consensus diagnosis. None of these smears were reclassified as *Gardnerella*.

	Pathologist 1 Diagnosis	Pathologist 2 Diagnosis	Consensus diagnosis
Normal flora (O6)	94	93	93
Dysbacteriosis (O3)	5	7	6
Gardnerella (O5)	0	0	0
Inflammatory (O9)	1	0	1
Total	100	100	100

Table 4 Diagnoses of the 100 rescreened smears originally coded as normal flora (O6).

In Table 5 the discordant and unequivocal diagnoses are brought together. A stable diagnosis is a diagnosis with low discordance and a high number of unequivocal diagnoses. *Gardnerella* evidently is a more stable diagnosis than dysbacteriosis. In dysbacteriosis as much as nine cases were discordant. In *Gardnerella* the number of consensus diagnoses was higher, whereas the amount of discordant diagnoses was less.

Original flora diagnosis	Discordant diagnosis	Unequivocal diagnosis
Dysbacteriosis (O3) (n=100)	20	65
Gardnerella (O5) (n=100)	8	80
Normal flora (O6) (n=100)	4	93

Table 5 Discordant and unequivocal diagnoses.

Diagnostic problems

A diagnostic problem in distinguishing O5 from O6 was caused by the presence of unattached clusters of bacteria, see Figure 4. In these clusters of spermatozoa can be identified. Such a smear should be classified as O6, but was coded O5 by the cytotechnologist in the screening process. Secondly, classification can be problematic in the presence of blood with red granular material covered with cells (Figure 5).

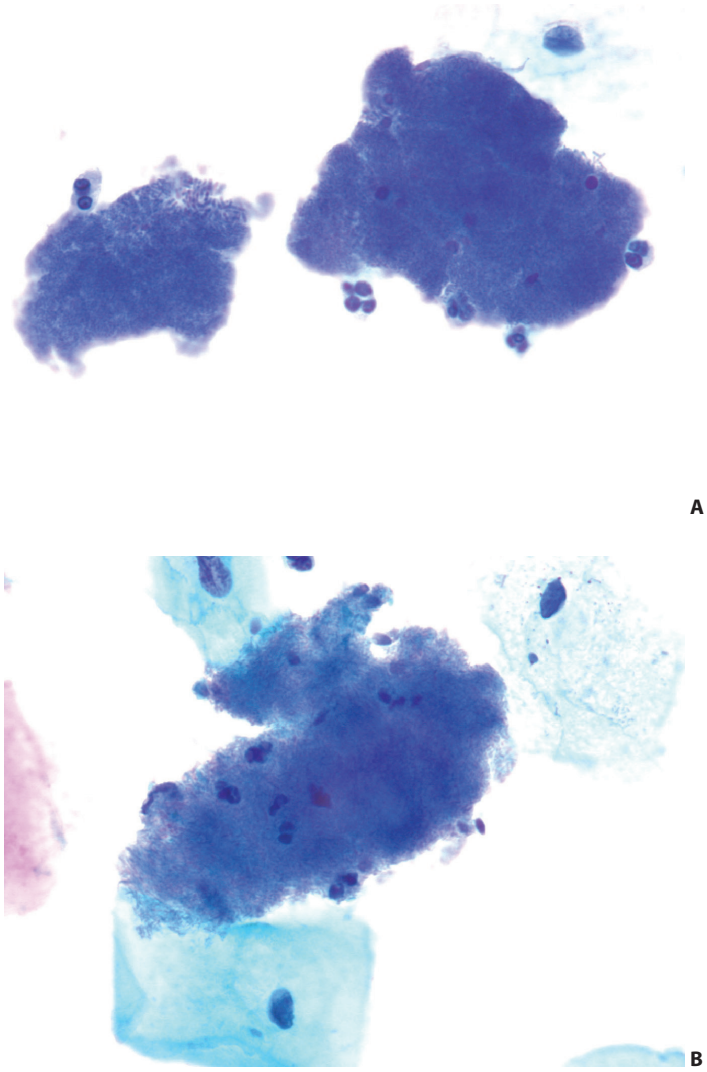
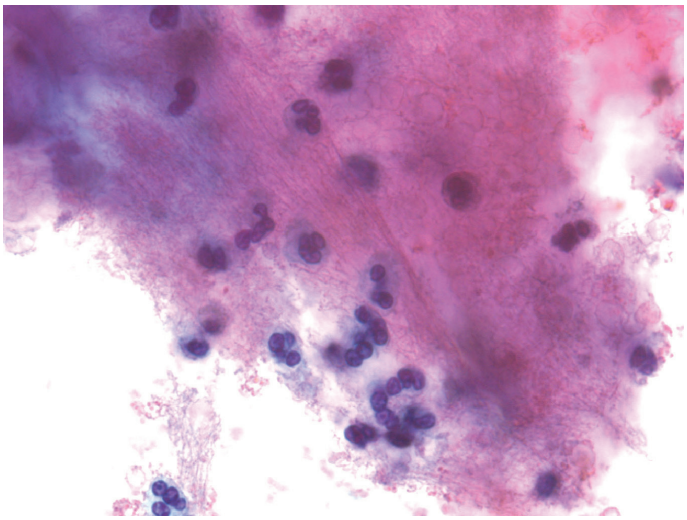
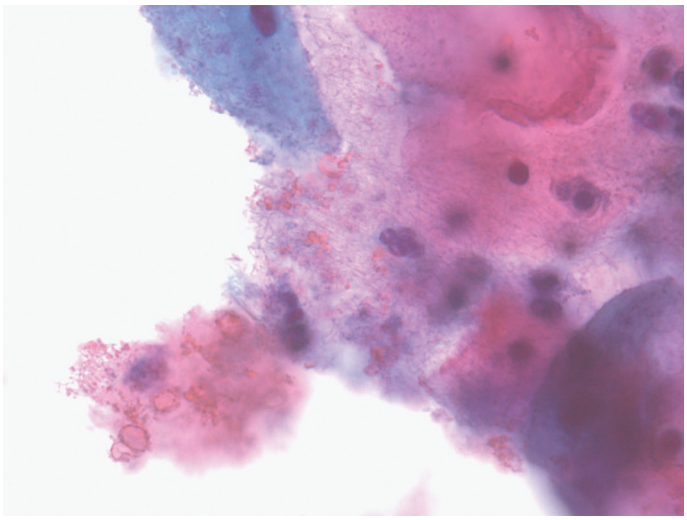


Figure 4 ThinPrep slides having dense clusters of bacteria (A). Spermatozoa in the cluster and sticking out of the cluster (B). These smears were classified as *Gardnerella* (O5) by the cytotechnologist in the original screening process (Papanicolaou staining, magnification x 1000).



A



B

Figure 5 Two ThinPrep slides (A and B) having flora difficult to classify due to reddish granular material in a bloody smear (Papanicolaou staining, magnification x 1000).

Discussion

Vaginal complaints due to a disturbed vaginal flora account for large numbers of visits to general practices in the Netherlands. Bacterial vaginosis (BV),[8] which is characterized by a malodorous, homogenous, grayish-white vaginal discharge, vaginal pH > 4.5 and a fishy odor due to the amines produced by *Gardnerella vaginalis* accounts for a large part of the cases of vaginal discomfort.[9-11] Approximately 500 to 1000 female patients per year visit their general practitioner for BV. Actually, the majority of women with a disturbed vaginal flora remain asymptomatic.[12] The present study focuses on asymptomatic women invited for cervical sampling in the national screening program with an interval of 5 years.

Studies have noted that cervical (pre)neoplastic changes occur more often in women with an imbalanced vaginal flora than in those without this condition.[13,14] The general hypothesis is that the local cervicovaginal milieu plays a role in susceptibility to HPV infection, whereas women that possess an unhealthy *Lactobacillus*-poor vaginal flora, should be more prone to acquiring HPV infection.[15-20] In this context, Verbruggen et al.[4] showed that asymptomatic women with dysbacteriotic smears had a significant higher presence of cervical (pre)neoplasia. We found a strong covariation between cytologically diagnosed *Gardnerella* infection and cervical (pre)neoplasia, being far more pronounced than in dysbacteriosis.[7]

In the present study we established that a cytological differentiation exists between *Gardnerella* infection, being the extreme form of bacterial dysbalance, and dysbacteriosis, the milder variant, with *Gardnerella vaginalis* as an important subdivision of the disturbed flora. This is a remarkable finding because it comprises the spin off of cervical screening which should be exploited for the benefit of the screened women. The lowest percentage of unequivocal diagnoses was obtained in the 100 dysbacteriotic smears (65%) whereas 93% of the smears with a healthy flora had an unequivocal diagnosis. Discordance was highest in the dysbacteriotic series (20%) and lowest in the healthy group (4%). The most interesting phenomenon leading to misclassification of *Gardnerella* infection was the presence of clusters of bacteria mixed with spermatozoa, both originating from the sperm of the sexual partner. These clusters differed from BMCs both in shape and size, as is visualized in Figure 4. We demonstrated that *Gardnerella* infection can be identified unequivocally in cervical smears and can be easily distinguished from smears with a healthy flora.

We showed that differentiation of dysbacteriosis and *Gardnerella* as subdivisions of Bethesda's category 'shift in vaginal flora suggestive of bacterial vaginosis' based on cytological criteria is not only possible but also worthwhile. Because of the clinical importance of treating pure *Gardnerella* infection[7] but also in case of a shift in flora towards *Gardnerella vaginalis* as in dysbacteriosis, it is important to teach cytotechnologists in identifying both cytological subdivisions. Such advantageous spin-offs of cervical screening should be exploited.

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Gardnerella vaginalis and *Lactobacillus sp* in liquid-based cervical samples in healthy and disturbed vaginal flora using cultivation-independent methods

Abstract

Objective: Our objective was to determine the morphotype of the adherent bacteria in liquid-based cytology (LBC) in smears with healthy and disturbed vaginal flora. And to use PCR technology on the same fixed cell sample to establish DNA patterns of the 16S RNA genes of the bacteria in the sample.

Methods: Thirty samples were randomly selected from a large group of cervical cell samples suspended in a commercial coagulant fixative. PCR was used to amplify DNA of five bacterial species: *Lactobacillus acidophilus*, *Lactobacillus crispatus, Lactobacillus jensenii, Gardnerella vaginalis* and *Mycoplasma hominis*. The LBC slides were analysed by light microscopy to estimate bacterial adhesion. DNA of lactobacilli was detected in all cell samples.

Results: Seventeen smears showed colonization with *Gardnerella vaginalis* (range $2.4x10^2$ - $5.6x10^5$ bacteria/µl). Two cases were identified as dysbacteriotic and had high DNA values for *Gardnerella vaginalis* and low values for *Lactobacillus crispatus*. The sample with the highest concentration for *Gardnerella vaginalis* showed an cytological *Gardnerella* infection.

Conclusion: This pilot-study indicates that the adherence pattern of a disturbed flora in liquid-based cervical samples can be identified unequivocally, and that these samples are suitable for quantitative PCR analysis. This cultivation independent method reveals a strong inverse relationship between *Gardnerella vaginalis* and *Lactobacillus crispatus* in dysbacteriosis and *Gardnerella* infection.

Introduction

The healthy vaginal flora is colonized by different bacteria. Lactobacilli usually predominate the bacterial flora of healthy premenopausal women.[1,2] Besides, coccoid bacteria are also inhabiting normal vaginal ecosystems given in small quantities. A disruption of this well-balanced microflora can occur increasing the total concentration of coccoid bacteria found up to 100 to 1000 fold.[1] In other words, the dominance of lactobacilli is replaced by an overgrowth of coccoid bacteria like *Gardnerella vaginalis, Prevotella, Mycoplasma hominis, Bacteriodes species, Ureaplasma species* and *Mobiluncus species*.[3,4]
In the Dutch national coding system for pathology findings in cervical cytology, KOPAC, the inflammatory status of the vaginal microflora is also registered.[5] In this system the letter 'O' stands for 'Ontsteking' (inflammation) consisting of nine different categories: Koilocytosis (O1), *Trichomonas vaginalis* (O2), dysbacteriosis (O3), *Candida* (O4), *Gardnerella vaginalis* (O5), normal flora (O6), *Actinomyces* (O7), *Chlamydia trachomatis* (O8) and non-specific inflammatory changes (O9).
In the KOPAC system bacterial imbalance is coded dysbacteriosis or *Gardnerella* infection. In this context, dysbacteriosis represents the microscopic diagnosis of a shift in vaginal flora, in which the majority of lactobacilli are replaced by a mixture of anaerobic bacteria, mainly *Gardnerella vaginalis*[4,5] whereas pure *Gardnerella* infection displays an *extreme* shift in vaginal flora towards *Gardnerella vaginalis* with a lack of lactobacilli, in which tiny rodlike organisms cling to the cellular membrane.[3] However, the prevalence of dysbacteriosis is substantially higher than *Gardnerella* infection. Both are diagnoses independent of clinical criteria.
Symptomatic bacterial imbalance (better known as the clinical syndrome bacterial vaginosis) frequently caused by *Gardnerella vaginalis* is one of the most common vaginal infections in fertile women. According to the CDC (Centers of Disease and Control Prevention), 16% of pregnant women in the United States of America have dysbacteriosis.[6] Unfortunately pathogenesis remains largely elusive.[7] Any woman could get the disease, but chances are increased by having multiple sex partners, vaginal douching or using intra-uterine devices.[6,8] There is a relationship between dysbacteriosis and an increased risk for HIV, preterm birth and low-birth-weight infants.[8]
Lactobacilli appear to be part of the defense mechanisms against pathogens in order to regulate the growth of other vaginal flora.[9] Diverse species and strains of *Lactobacillus* show properties which may protect the vaginal epithelial cells from colonization by pathogens.[10] First, some lactobacilli interfere with the pathogens by their ability to adhere to the vaginal epithelium and block the receptor sites to which other, potentially harmful, bacteria could attach, making a bacterial film.[3,11] Through adhesion to the epithelial cells, the lactobacilli form a defensive barrier that excludes the pathogens from the deeper vaginal epithelium. Boris et al. showed that the factors responsible for adherence to vaginal epithelial cells seemed to be glycoproteins and carbohydrates.[3] Secondly, hydrogen peroxide-producing strains may also provide an acidic environment through lactic acid production from

anaerobic fermentation of epithelial cell glycogen to lactic acid. The resulting low pH may be a defensive mechanism against invasion by other bacteria.[4]

The ability of rod-shaped lactobacilli to adhere to mucosal epithelial cells seems to be important in the establishment of an indigenous bacterial flora and perhaps in infectious disease.[12,13]

Dysbacteriotic smears show clue cells (CCs), epithelial cells which are entirely covered with tightly adherent, anaerobic bacteria, mainly *Gardnerella vaginalis*.[14-18] In such smears, many unattached bacteria can be found in the background. When such cell samples are brought into suspension, as is the case in liquid-based cytology (LBC),[19] the non-attached bacteria pass through the filter used for preparing the slides and the epithelial cells are seen in a clean background. Only the strongly adhering bacteria remain, they are still visible glued to the green staining squamous epithelial cells, lying on top or attached to the outer rim of the cells. Such epithelial cells, having a faint blue color thanks to the thousands of blue staining bacteria, should actually be called 'glue cells'.[19] In *Gardnerella* infection, those clue cells have developed into so-called blue mountain cells (BMCs), that is, cells staining dark blue in the Papanicolaou stain because they are completely covered by a mountain of blue staining round bacteria.

Mårdh et al. showed that lactobacilli have a lower adherence capacity per cell than *Gardnerella vaginalis.*[12,17] The mechanism of this is not yet understood. Sobel et al. and Peeters et al. both showed that adherence of *Gardnerella vaginalis* depends on pH of the vagina.[20-22] Zariffard et al. studied whether *Gardnerella vaginalis,* lactobacilli and *Mycoplasma hominis* could be detected in vaginal specimens, and also their relation to each other, using quantitative real-time PCR. They concluded that there is an inverse relationship between *Gardnerella vaginalis* and lactobacilli suggesting that these findings could be developed into a new method in detecting a disturbed vaginal microflora.[23]

For our LBC, we used a coagulant fixative[24] in which DNA is very well preserved thanks to fast change of the three-dimensional structure of the enzymes that degrade DNA[25,26] and can therefore be quantitated by real-time PCR. The aim of this study was to determine the morphotype of the adherent bacteria in the disturbed vaginal flora by liquid-based cytology (LBC) and to perform PCR on the same fixed cell sample to establish DNA patterns of the disturbed and healthy vaginal flora.

Material and methods

Patient material
Thirty cell samples were randomly collected from the Leiden database containing 80% smears origination from women who participated in the Dutch national screening program, western region. The remaining smears were indicated for individual or clinical reasons. In addition, the cellular samples were obtained with the CerviBrush (CellPath plc, Newton, Pawys, U.K.). Signed informed consent was obtained from participants and the protocol was approved by Institutional Ethical and Scientific Review Committee.

Sample preparation
After swabbing, the tip of the brush was broken off and transported in a cyto sample vial holding 15 mL of BoonFix® (Finetec, Tokyo, Japan) to the laboratory. BoonFix® is a formalin-free coagulant fixative containing ethyl alcohol and a low molecular weight PEG.[24] This suspension is preferred above formalin fixatives because it has as main preservative effect coagulation of proteins. DNA and RNA are not damaged allowing for successful PCR amplification of DNA.[24]

Primers and probes
Song et al. developed primers and probes for the detection of lactobacilli.[27] For the identification of H_2O_2-producing Lactobacilli, e.g. *Lactobacillus acidophilus, Lactobacillus crispatus* and *Lactobacillus jensenii,* the primer sets according to Song et al. were slightly adapted to become suitable for the LightCycler (Table 1). Another primer-probe set was designed for detecting a broad range of *Lactobacilli* species. The other two were for *Gardnerella vaginalis* and *Mycoplasma hominis*. None of the primers showed cross-reactivity to the other five bacteria.

Sensitivity and specificity
Sensitivity for *Lactobacillus crispatus* and *Gardnerella vaginalis* was tested and found equal at a concentration of $1.0x10^{-6}$ng/µl. Negative samples were documented as $1.0x10^{-8}$ng/µl. We did not test possible cross-reactivity with other bacterial species. All primers were synthesized and column purified by Tib MolBiol, Berlin, Germany.

Gardnerella	Gvag F	5′-ggCTAgAgTgCAgTAggg
Vaginalis	Gvag R	5′-gTTAgCTCCgACACAgAAC
	Gvag FL	5′-CgggAAgAACACCAATggC-FL
	Gvag LC	LC RED640-5′-AggCAggTCTCTgggCTgTT-3′-phos
Mycoplasma	MP_for	5′-ggAAgATATgTAACAAAAgAAggTgCTg
Hominis	MP_rev 1	5′-TTTATCTTCTggCgTAATgATATCTTCg
	MP_FL	5′-AgCAggTgCTAAAAAggTgTTTATTACTgCTCC-3′-FL
	MP_705	LC RED705-5′-gCTAAAAgCgAAggTgTTAAAACAgTTgTTTATTCAgTA-3′-phos
Lactobacillus	Laci-1	5′-TgCAAAgTggTAgCgTAAgC
Acidophilus	Laci-re	5′-ACCgggATTCCTCgTgTC
	Laci-FL	5′-gCgCgCTCgCAATTTCgCTTAC-FL
	Laci-LC	LC RED640-5′-gggCTCTCACCCTCTCTggCTTACCTTC-3′-phos
Lactobacillus	Ljen-3	5′-AAgAAggCACTgAgTACggA
Jensenii	Ljen-re	5′-ATCTACgggTCTTAgCTTACTTACA
	Ljen-FL	5′-gAACAgATTgTgAAAgCgAACCgAgAAg-FL
	Ljen-LC	LC RED640-5′-gAgATCTAggTAATAggTCAAgAAgAgAAgggCg-3′-phos
Lactobacillus	Lcri-FW	5′-CgAAgAAggACgTgACgAACTAC
Crispatus	Lcri-RE	5′-CAgATAATTCAACTATCTCTTACACTgCC
	Lcri-FL	5′-AgTgAATAgATAgCTAATCAAAggAAgACgCAgT-FL
	Lcri-LC	LC RED640-5′-AACTgAAACATCTAAgTAgCTgCAggAAgAgAA-3′-phos
Lactobacillus spp.	Lactobac F	5′-TggAAACAggTgCTAATACCg
	Lactobac R	5′-CCATTgTggAAgATTCCC
	Lactobac FL	5′-ggACTgAgACACggCCCAAAC-FL
	Lactobac LC	LC RED640-5′-CCTACgggAggCAgCAgTAgggA-3′-phos

Table 1 Primer and probe sequences (all primers and probes were synthesized by TIB MOLBIOL, Berlin, Germany).

PCR for bacteria

To detect the amounts of DNA from these bacteria in the patient specimens, the samples were randomly collected and analysed with real-time PCR (LightCycler 1.5, Roche, Mannheim, Germany), after automated purification (Qiagen M48 BioRobot, using MagAttract DNA mini M48 Kit. 953336, Qiagen, Hilden, Germany). A part of the 16S rRNA gene of the five bacteria was used for amplification primers. We used software (Primer-probe design, Roche, Mannheim, Germany) for designing primers and probes (Tib MolBiol, Berlin, Germany). See Table 1 for primer and probe sequences.[3,27,28]

PCR was performed with the LightCycler FastStart DNA Master PLUS Hybridization Probes by mixing 4µl of LightCycler DNA Master hyb probe, 7µl H2O and 1µl of each primer (final conc. 0,5µM) and probe (final conc. 0,2 µM) per bacteria. Five µl of purified DNA was added to the mix.

PCR was performed starting with a 95°C denaturation step for 10 minutes to activate taq polymerase. Amplification of the DNA was programmed for 45 cycles with denaturation of 5 s, 95°C, annealing of 10 s, 54°C and an elongation of 10 s, 72°C. All steps had a transition rate of 20°C/s. During annealing the concentration of the amplified DNA was measured. Quantification software (Roche, Mannheim, Germany) Fit points method (with an external standard curve using LightCycler Control Kit DNA, cat. No. 12158833001, Roche, Mannheim, Germany) was used for measuring the concentration of the bacterial DNA. All six primer sets resulted in amplification with DNA from the appropriate type.
The concentrations of the bacteria (ng/µl) were divided by the beta-globin gene concentration found using the LightCycler Control Kit DNA (cat. No. 12158833001 Roche, Mannheim, Germany). This quantitative control kit measures the concentration of the beta-globin gene present in the sample. This gene is a part of the human genome. We can therefore estimate the number of cervical cells, and concentrations of bacteria were calculated for 1 ng/µl beta-globin DNA.

Microscopic analysis
The 30 Papanicolaou-stained LBC slides were analysed by two observers, without knowing the original O-code from routine-screening. First, each smear was coded according to the Dutch KOPAC system. Two samples were coded as O3 (dysbacteriosis) by both observers, one sample was found O5 (*Gardnerella* infection). All 27 other cases showed a normal flora (O6).
In dysbacteriotic smears, so-called clue cells can be present. *Gardnerella* infection was diagnosed when a smear showed BMCs.
In each case the number of cells with bacterial adhesion was counted per 100 epithelial cells. The mean value of the adhesive cells per case was calculated. The morphotype of the adhering bacteria was coded either as coccoid, small (curved) rods or as large rod-shaped.

Results

The average age of the study population was 42 years (range 18-61 years), 10% of the women had reached menopause and 23% of the population used contraception, 3% using IUDs and 20% using oral contraceptives.

In Figure 1 the results of all 30 samples are depicted (concentrations bac/µl were corrected for the concentration of beta-globin DNA per sample). Lactobacilli were detected in all 30 samples, with a range of values from $1.31x10^{1}$ to $1.69x10^{7}$ bacteria/µl BoonFix® (not displayed). *Gardnerella vaginalis* was detected in 17 samples (range from $2.4x10^{2}$ to $5.6x10^{5}$ bacteria/µl BoonFix®, not displayed). *Mycoplasma hominis* was detected in 11 samples.

There were two dysbacteriotic samples (cases I and II, see Figure 2); both cases were coded as dysbacteriotic by both observers. In these two cases the mean values for cells with bacterial adherence were over 10/100, that is one with a mean value of

15.8/100 and the other with 38.9/100. In both cases many of these cells were almost completely covered by small bacteria, clearly visible against the clean background of the slide. Such cells were very easy to find because they were faint blue and thus differed from the other green or red staining squamous cells. Case III (see Figure 3), with bacterial adherence of 52.3/100, was coded *Gardnerella* infection. In contrast to both dysbacteriotic cases, the *Gardnerella* smear displayed blue mountain cells (BMCs).

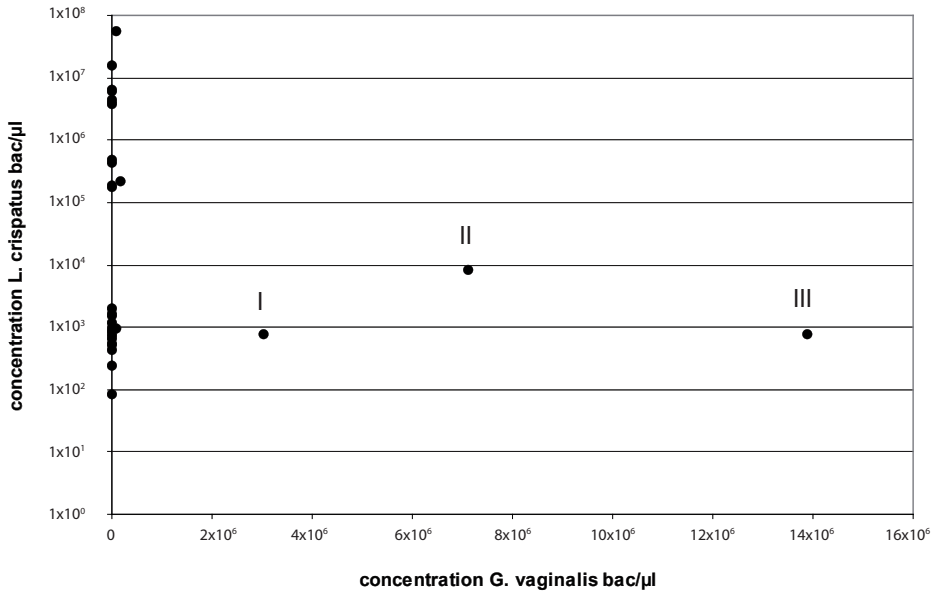

Figure 1 Data of 30 vaginal samples by DNA-PCR presented as a ratio of *Lactobacillus crispatus* and *Gardnerella vaginalis* relative to the entire beta-globin DNA load.
Case I and II: dysbacteriotic smears. Case III: smear with *Gardnerella* infection.
(*Lactobacillus crispatus* data were transformed to a $\log_{10}$ scale).

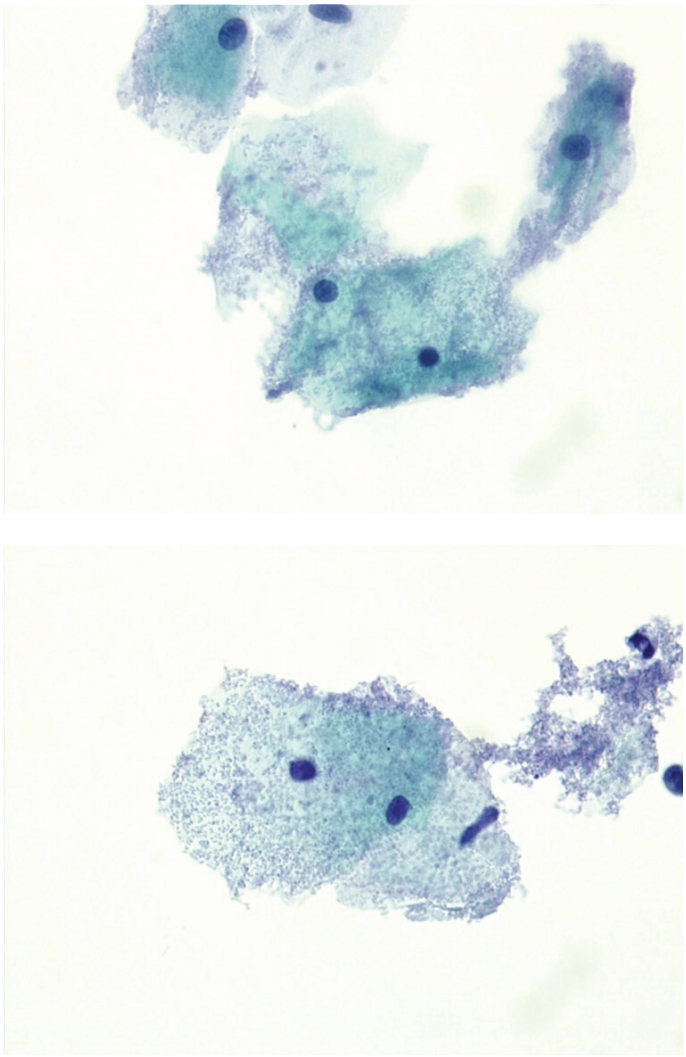

Figure 2 Case I and Case II: Dysbacteriosis
Thin Prep slides, note coccoid bacteria attached to squamous epithelial cells. (Papanicolaou-stained, magnification × 1000)

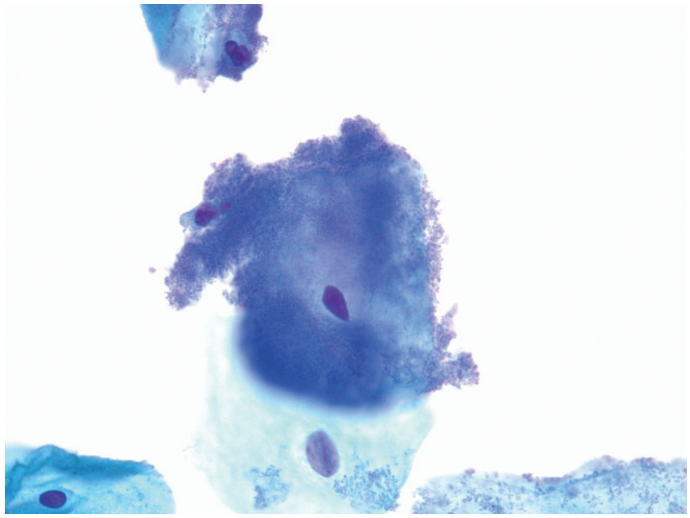

Figure 3 Case III: *Gardnerella* infection
ThinPrep slide, note presence of blue mountain cells. (Papanicolaou-stained, magnification x 1000)

All 27 remaining smears showed a predominantly lactobacillus flora attached to the epithelial cells and were all displaying a normal vaginal flora (coded O6). In all of those smears bacteria were large rod-shaped and less than 10% of the green staining cell surface was covered by bacteria: consequently these cells were not easy to find because the cytoplasm was green, just like the other squamous epithelial cells, the few blue staining rod-shaped adhering bacteria having very little effect on the colour of the cell. None of these cells had a tail of attached bacteria or showed CCs or BMCs as was the case in dysbacteriosis and *Gardnerella* infection. Furthermore, bacterial adhesion was not apparent.

To compare bacterial imbalance in smears displaying a normal vaginal flora, three cases (IV, V, VI, see Figure 4) coded O6, were selected from the remaining 27 smears that showed a normal vaginal flora (O6). Selection was made by matching age and use of contraceptives.

PCR findings

In the clue cell positive samples (Case I and II), as well as the BMC positive sample (Case III) the values for *Gardnerella vaginalis* were relatively high and the values for at least one of the lactobacilli species low (Table 2). In contrast, in the three cases with predominantly rod-shaped adhering bacteria (case IV, V and VI), the values for *Gardnerella vaginalis* were low while those for one of the lactobacillus species were high (Table 2). The ratio between *Gardnerella vaginalis* and *Lactobacillus crispatus* for each of the smears was resp. $8.5x10^{2}$ (Case I: dysbacteriosis); $3.9x10^{3}$ (Case II: dysbacteriosis); $1.8x10^{4}$ (Case III: unequivocal *Gardnerella* infection). The healthy smears had ratios $<1.0x10^{-2}$.

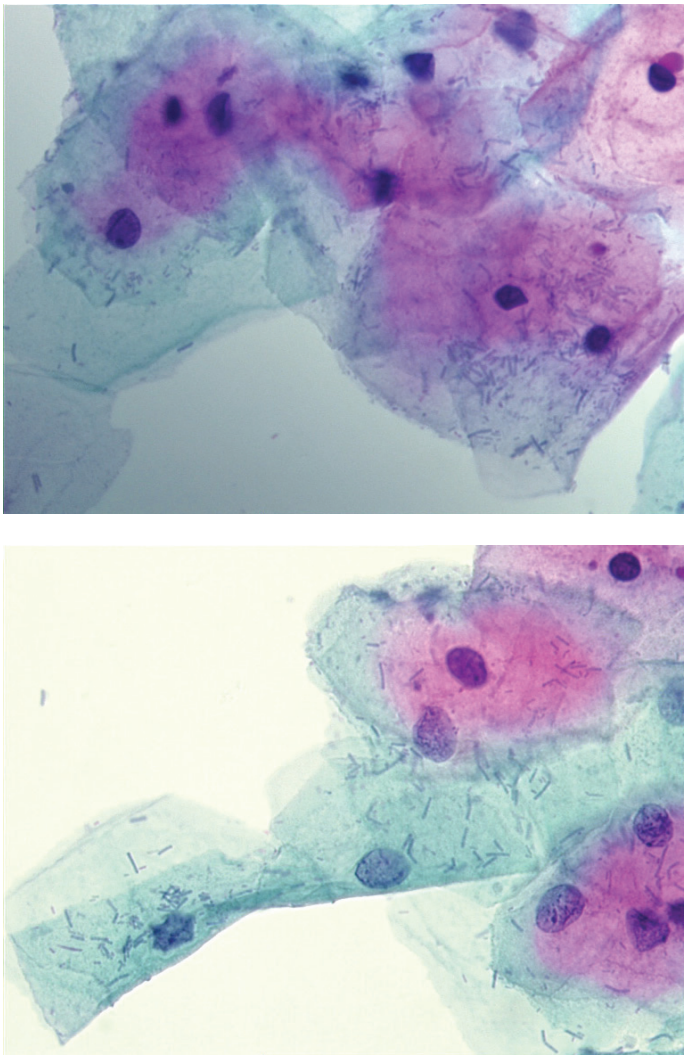

Figure 4 Case IV and V: Normal flora
Rod-shaped bacteria glued to epithelial cells. The density of these bacteria is less than in dysbacteriosis and *Gardnerella* infection. (Papanicolaou-stained, magnification × 1000).

In one of the dysbacteriotic cases *Mycoplasma hominis* was detected in very low numbers (2.07 bacteria/µl BoonFix®). There was one non-dysbacteriotic case which showed a relatively high value for *Mycoplasma hominis* that also had a large number of endocervical cells in the LBC sample.

	Morphotype of adhering bacteria					
	Coccoid/ small rods	Coccoid/ small rods	Coccoid/ small rods	Large rod-shaped	Large rod-shaped	Large rod-shaped
Bacteria	**Case I**	**Case II**	**Case III**	**Case IV**	**Case V**	**Case VI**
G.vaginalis	$3.76x10^5$	$3.00x10^5$	$5.60x10^5$	0	$1.43x10^3$	$2.40x10^2$
L.crispatus	$4.42x10^2$	$7.75x10^1$	$3.09x10^1$	$1.44x10^7$	$1.83x10^5$	$1.15x10^6$
L.jensenii	$9.73x10^1$	0	$0.11x10^1$	$6.99x10^3$	$1.08x10^4$	$1.43x10^4$
L.acidophilus	$7.01x10^1$	$2.96x10^1$	0	$3.04x10^2$	$1.25x10^2$	0
L.species	$4.65x10^5$	$8.70x10^3$	$1.05x10^5$	$6.02x10^6$	$3.41x10^6$	$8.44x10^5$

Table 2 Morphotype of the adhering bacteria and PCR findings. Amount of bacteria per microliter BoonFix® sample in two cases with a dysbacteriotic flora (I, II), one case with a *Gardnerella* infection (III) and three cases with a normal vaginal flora with predominant adherence of lactobacilli (IV, V, VI).

Discussion

Liquid-based cytology (LBC) is widely used for cancer screening because the detection of the (pre)malignant cells is enhanced by immediate fixation and lowering the background of the cell sample in the preparation process.[29-31] Hopwood et al. showed that it is feasible to use LBC samples (originally collected for cervical screening) for chlamydia screening.[32] In our LBC screening practice, we noticed that, in addition, the adhering properties of the vaginal flora are evident in the Papanicolaou-stained LBC slides thus facilitating the cytological diagnosis of a disturbed vaginal flora.

Our study indicates that the adhesion of a dysbacteriotic flora (case I, II) and a *Gardnerella* flora (Case III) is characterized by the number of adhering lactobacilli (case IV,V and VI), as shown in the photomicrographs (Figure 2-4). Samples collected for LBC may also be used for bacterial PCR screening.[28] According to literature, *Gardnerella vaginalis* seems to be the most prevalent bacteria present in the disturbed vaginal flora.[33] This pilot-study shows a marked inverse relationship between the concentration of *Gardnerella vaginalis* and *Lactobacillus crispatus*. The two dysbacteriotic cases had high values for *Gardnerella vaginalis* and low values for *Lactobacillus crispatus*, with ratios resp. $8.5x10^2$and $3.9x10^3$. However, for the *Gardnerella* smear, the ratio was $1.8x10^4$. In contrast, the smears showing a normal flora had ratios of $1.0x10^{-2}$ or less.

Large rod-shaped lactobacilli are numerous in healthy vaginas. In a recent study, Fredericks et al. detected considerably more bacterial diversity per sample among women with a disturbed flora than among women with a normal flora.[34] *Gardnerella vaginalis* is almost always present in high concentrations in women who have bacterial vaginosis or *Gardnerella* infection. Bacterial adhesion is prominent in microscopical samples of these women.[34] PCR studies of the many lactobacillus strains present in vaginal samples have recently become available. *Lactobacillus crispatus*, which was previously classified in the *Lactobacillus acidophilus* group, has also been identified as the most prevalent

lactobacillus in the normal vaginal flora.[1,33] Probably this is because *Lactobacillus crispatus* produces H_2O_2 and possesses strong aggregation and adhesive properties together with the ability to recognize and bind to the sugar chain of the A-antigen structure, which plays an important role in the immune system of the vagina.[35-37]

The status of *Mycoplasma hominis* as an indicator for a disturbed flora is doubtful. Some say that it is an indicator of dysbacteriosis,[38] whereas others state that there is no statistically significant difference between *Mycoplasma hominis* levels in the normal and a disturbed flora.[23] In our series there was only one case with a clear predominance of *Mycoplasma hominis*: it was interesting that this sample contained a large number of endocervical cells. This may indicate that *Mycoplasma hominis* thrives in the presence of endocervical cells and/or the mucus produced by these cells. The data of *Mycoplasma hominis* are too small to make an assumption concerning its role in the disturbed vaginal flora.

For our study we used the coagulant fixative Boonfix® because of the optimal fixation. Nevetheless, we suppose our methodology works also on the standard commercial American methods Surepath and Thinprep because these fixatives result even in adequately preserved cells allowing precise morphologic evaluation indicating that the direct fixation of the cells after sampling is the most important factor in the morphology of thin-layer slides. The cellular morphology in the boonfixed slides is closer to the SurePath slides than to the Thinprep ones. But we observed that the differences are small indeed.[19]

Simple microscopic observation of (disturbed) vaginal flora is a time-consuming and subjective activity. Therefore we suggest that diagnosis of a disturbed vaginal flora based on quantatative PCR could be useful in clinical practice. In addition, PCR-detection could be used in research to study the influence of specific organisms on either the development of dysbacteriosis and *Gardnerella* infection or their pathological outcomes.

In our study, the use of quantatative DNA-PCR amplified directly from vaginal swabs, enabled us to characterize the balance between *Gardnerella vaginalis* and *Lactobacillus crispatus* in the vaginal flora, and to compare the ratio of both bacteria in the normal vaginal flora with that in the disturbed vaginal flora in case of unequivocal *Gardnerella* infection and dysbacteriosis.

Our study has limitations because, firstly, our findings are based on a small number of cervical samples. We therefore recommend further research with large subsets. It is of interest to note that ongoing research with larger patient populations may give a better understanding of the complex vaginal bacterial populations and their patterns of adherence and succession.

Secondly, we did not determine cross-reactivity with other related species. Future research should investigate on specificity of the primers used in our study. Furthermore, according to our findings, it would be interesting to investigate the colonization pattern of *Gardnerella vaginalis* and *Lactobacillus crispatus* in a population of asymptomatic women. We could then possibly be able to answer the following question: is there an antagonistic effect between the concentrations of *Lactobacillus crispatus* and *Gardnerella vaginalis* in different inflammatory states of the vaginal flora?

This study indicates that the adherence pattern of bacteria in *Gardnerella* infection and dysbacteriotic flora in liquid-based cervical samples can be identified unequivocally. Secondly, the liquid-based samples are suitable for the novel approach of quantitative

PCR analysis. Further research, exploring the novel quantitative technologies with clinical samples, may give a better understanding of the complex vaginal bacterial populations and their patterns of adherence.

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Baseline-study of the vaginal flora of 1036 Dutch asymptomatic women: colonization of *Gardnerella vaginalis* and *Lactobacillus crispatus*

Abstract

Objective: *Lactobacillus crispatus* and *Gardnerella vaginalis* have shown to be important bacteria in maintaining a healthy vaginal flora. This study was designed to evaluate the colonization pattern of both bacteria in asymptomatic women of different ages and to determine whether *Lactobacillus crispatus* and *Gardnerella vaginalis* interfere with each other.

Methods: A total of 1036 samples was randomly collected from a large group of Dutch asymptomatic women, between 30-60 years, participating in the national screening program concerning cervical carcinoma. The cervical material was suspended in a commercial coagulant fixative (BoonFix®) and collected for PCR evaluation for *Lactobacillus crispatus* and *Gardnerella vaginalis* using primers based on a part of the 16S rRNA gene.

Results: *Gardnerella vaginalis* was present in 58.7% of the women, whereas 38.9% of the samples showed *Lactobacillus crispatus*. Colonization of *Lactobacillus crispatus* in postmenopausal women decreases from 40.5 to 19.5%. Colonization by *Gardnerella vaginalis* was more frequent in non-*Lactobacillus crispatus* flora (OR 1.3; CI 1.1-1.4), as *Lactobacillus crispatus* was oftentimes colonizing non-*Gardnerella* microflora (OR 1.4; CI 1.2-1.6).

Conclusion: Colonization of *Gardnerella vaginalis* is stable among all age groups. Cessation of production of female sex hormones significantly decreases the colonization pattern of *Lactobacillus crispatus*. *Gardnerella vaginalis* and *Lactobacillus crispatus* appear to interfere with each other. These results suggest that culture-independent methods can provide new insights into the key-role of a bacterium.

Introduction

The vaginal ecosystem is predominantly exhibited by *Lactobacillus* species but also by other bacteria including *Gardnerella vaginalis, Mobiluncus species, Bacteroides fragilis, Prevotella species, Mycoplasma species, Ureaplasma urealyticum, Fusobacterium nucleatum* and *Peptostreptococcus species*, all inhibitants of the commensal vaginal flora.[1,2]
First identified in 1894 by the German physician A. Doderlein[3], *Lactobacillus* has been shown to be the predominant bacterium in the normal vaginal microbial flora found in women of reproductive age[4], but it also colonizes the moist surface of the oral cavity and intestine tract.[5] Lactobacilli are one of the main defence mechanisms against pathogens and therefore play a significant role in maintaining the healthy balance of the vaginal flora.[4,6] Most lactobacilli function as endogenous microbicides through the production of lactic acid, which acidifies the vagina. In addition, some lactobacilli produce hydrogen peroxide (H_2O_2), which reacts with myeloperoxidase forming reactive molecules toxic to human immunodeficiency virus (HIV) and other pathogens.[7,8] In addition, women colonized by H_2O_2-producing lactobacilli have decreased acquisition of bacterial vaginosis.[9]
Several studies indicated that *Lactobacillus crispatus* is one of the most predominant H_2O_2-producing species found in the vaginal flora.[2,10,11] In addition, Vallor et al, demonstrated that this species is clearly associated with healthy microflora, and possibly better ensures stable healthy microflora than other lactobacilli.[12]
We recently found an inverse relation between *Gardnerella vaginalis* and *Lactobacillus crispatus* in dysbacteriosis and cytologically diagnosed *Gardnerella* infection.[13] Moreover, smears of asymptomatic women with *Gardnerella* infection showed a significant higher prevalence of HSIL/carcinoma.[14]
Because colonization of lactobacilli protects against acquisition of bacterial vaginosis and *Lactobacillus crispatus* has shown to fulfil a predominant role in the defence process, we sought to determine the colonization of *Gardnerella vaginalis* and *Lactobacillus crispatus*, as this has never been documented in a large asymptomatic population. In addition, based on those colonization patterns possible interference between *Gardnerella vaginalis* and *Lactobacillus crispatus* might be apparent.
Studies on many habitats have demonstrated the limitations of cultivation-dependent methods to assess microbial community composition. In most instances, this is due to the selectivity of growth media and conditions.[15] For that reason we turned to DNA-PCR in combination with formalin-free coagulant fixative to become a greater reliability of the results.

Material and methods

Patient material
Between January 1st, 2005 and December, 31st, 2005 the Leiden Cytological and Pathological Laboratory received 40.000 cervical smears of women in connection with the Dutch national screening program, western region. A group of 1036 cytology samples of women in the age of 30-60 years was randomly collected from the Leiden database in

order to perform DNA-PCR for the colonization of *Lactobacillus crispatus* and *Gardnerella vaginalis*. Signed informed consent was obtained from participants and the protocol was approved by the Institutional Ethical and Scientific Review Committee.

Sample preparation

After swabbing, accomplished by a general practitioner, the tip of the brush was broken off and transported in a cyto sample vial holding 15 mL of BoonFix® (Finetec, Tokyo, Japan) to the laboratory. BoonFix® is a formalin-free coagulant fixative containing ethyl alcohol and a low molecular weight PEG.[16] This suspension is preferred above formalin fixatives because it has as main preservative effect coagulation of proteins. DNA and RNA are not damaged allowing for successful PCR amplification of DNA.[16]

Primers and probes

Song et al. developed primers and probes for the detection of lactobacilli.[17] Part of the 16S rRNA gene of both bacteria was used for amplification primers. For the identification of H_20_2-producing *Lactobacillus crispatus,* the primer set according to Song et al. was slightly adapted to become suitable for the LightCycler. Software was used (Primer-probe design, Roche, Mannheim, Germany) for designing primers and probes (Tib MolBiol, Berlin, Germany). Table 1 lists all primers and probes used in this study.[17] All primers were synthesized and column purified by Tib MolBiol, Berlin, Germany.

G. vaginalis	Gvag F	5′-ggCTAgAgTgCAgTAggg
	Gvag R	5′-gTTAgCTCCgACACAgAAC
	Gvag FL	5′-CgggAAgAACACCAATggC-FL
	Gvag LC	LC RED640-5′-AggCAggTCTCTgggCTgTT-3′-phos
L. crispatus	Lcri-FW	5′-CgAAgAAggACgTgACgAACTAC
	Lcri-RE	5′-CAgATAATTCAACTATCTCTTACACTgCC
	Lcri-FL	5′-AgTgAATAgATAgCTAATCAAAggAAgACgCAgT-FL
	Lcri-LC	LCRED640-5′-AACTgAAACATCTAAgTAgCTgCAggAAgAgAA-3′-phos

Table 1 Primer and probe sequences (synthesized by TIB MOLBIOL, Berlin, Germany)

Sensitivity and specificity

Specificity of the primer sets was analysed by searching Gene Bank data base using the BLAST algorithm (National Center of Biotechnology Information, National Institutes of Health, Bethesda, MD, USA). Specificity of the primer sets was determined by testing the following strains: *Candida albicans, Mobiluncus mulieris, Mobiluncus curtisii, Raoultella planticola, Gardnerella vaginalis, Klebsiella sp., Lactobacillus johnsonii, Bifidobacterium scardovii, Staphylococcus epidermidi, Lactobacillus delbrueckii supsp. lactis, Lactobacillus helveticus, Lactobacillus acidophilus, Lactobacillus delbrueckii subsp. Bulgaricus, Bifidobacterium longum, Bifidobacterium minimum, Staphylococcus aureus subsp. Aureus, Streptococcus sp., Bifidobacterium bifidum, Peptoniphilus asaccharolyticus, Finegoldia magna, Enterococcus faecium (=Streptococcus faecium), Enterococcus faecalis (=Streptococcus faecalis),*

Prevotella bivia, Lactobacillus amylovorus, Anaerococcus prevotii, Lactobacillus jensenii, Lactobacillus crispatus, Streptococcus thermophilus, Bacteroides ureolyticus, Porphyromonas asaccharolytica, Escherichia coli, Pseudomonas aeruginosa.
No cross-reactivity was detected with DNA from any of the tested species. Sensitivity for *Lactobacillus crispatus* and *Gardnerella vaginalis* was tested and found equal at a concentration of $1.0x10^{-6}$ng/µl.

DNA extraction of bacteria and samples
To detect the amounts of DNA from these bacteria in the patient specimens, the samples were analysed with real-time PCR (LightCycler 1.5, Roche, Mannheim, Germany), after automated purification (Qiagen M48 BioRobot, using MagAttract DNA mini M48 Kit cat. no. 953336, Qiagen, Hilden, Germany). A part of the 16S rRNA gene of both bacteria was used for amplification primers.
PCR was performed with the LightCycler FastStart DNA Master PLUS Hybridization Probes (cat. no. 03515567001) by mixing 4 µl of LightCycler DNA Master hyb probe, 7 µl H2O and 1 µl of each primer (final conc. 0,5 µM) and probe (final conc. 0,2 µM) per bacteria. Five µl of purified DNA was added to the mix.
PCR was performed starting with a 95°C denaturation step for 10 minutes to activate taq-polymerase. Amplification of the DNA was programmed for 45 cycles with denaturation of 5 s, 95°C, annealing of 10 s, 54°C and an elongation of 10 s, 72°C. All steps had a transition rate of 20°C/s. During annealing the concentration of the amplified DNA was measured. Quantification software (Roche, Mannheim, Germany), fit points method (with an external standard curve using LightCycler Control Kit DNA, cat. No. 12158833001, Roche, Mannheim, Germany) was used for measuring the concentration of the bacterial DNA. Both primer sets resulted in amplification with DNA from the appropriate type. Finally, all samples were analysed by PCR for their DNA concentration of *Lactobacillus crispatus* and *Gardnerella vaginalis*. Negative samples were documented as $1.0x10^{-6}$ng/µl.

Statistical analysis
Statistical analysis was performed by using SPSS 12.0. A generalized X^2 test was performed to assess the significance of the colonization patterns of both *Gardnerella vaginalis* and *Lactobacillus crispatus* among pre- and postmenopausal women. Odds ratios (OR) with 95% confidence intervals (CI) were calculated for colonization of *Gardnerella vaginalis* in a LC-negative flora (without colonization of *Lactobacillus crispatus*) using a LC-positive flora as reference. The same was done for colonization of *Lactobacillus crispatus* in a GV-positive flora, whereas the reference group consisted of samples without colonization of *Gardnerella vaginalis*.

Results

Demographic features

The 1036 women whose samples were studied had a mean age of 39.9 years (30.2-60.7). Age distribution of the whole study-population is shown in Figure 1.

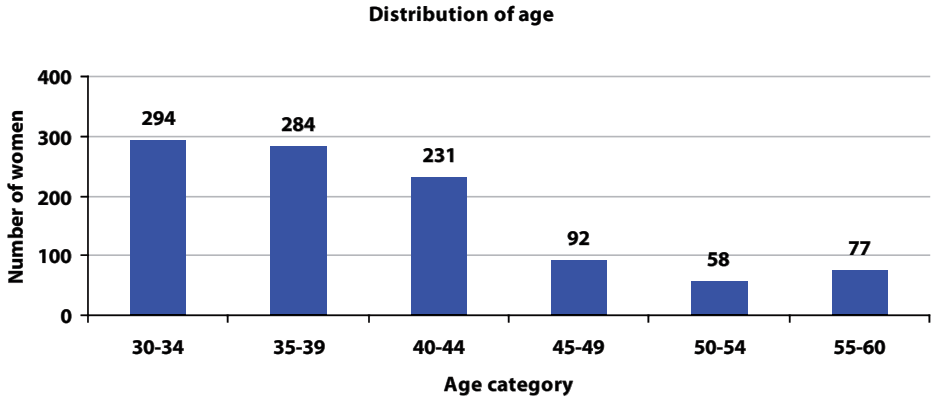

Figure 1 Age distribution depicted in 5-years age categories (N=1036)

PCR findings

As depicted in Figure 2, *Gardnerella vaginalis* was present in 608 of the 1036 women (58.7%). Only 38.9% of the smears (403) was colonized by *Lactobacillus crispatus*. According to Figure 3 there appeared to be four different clusters based on colonization of both microbial communities determined by DNA-PCR. Cluster A consisted of smears without *Lactobacillus crispatus* and *Gardnerella vaginalis*; cluster B with *Lactobacillus crispatus*, but without *Gardnerella vaginalis*; smears with both *Lactobacillus crispatus* and *Gardnerella vaginalis* (Cluster C) and those with only *Gardnerella vaginalis* (Cluster D).

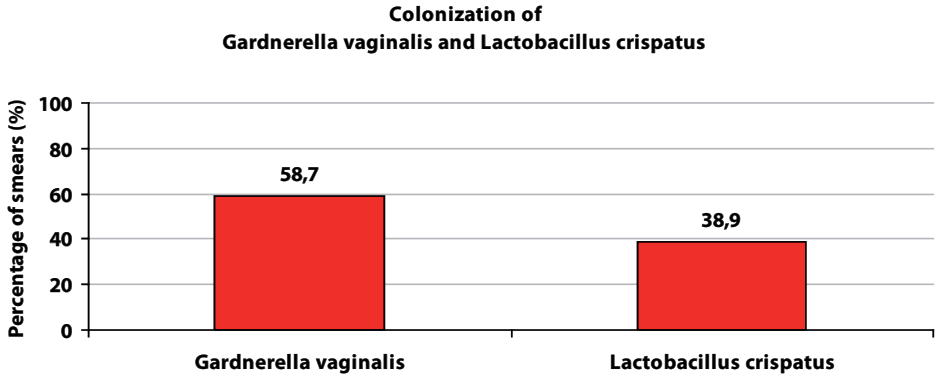

Figure 2 Proportion of the total study-population colonized by *Gardnerella vaginalis* and *Lactobacillus crispatus*.

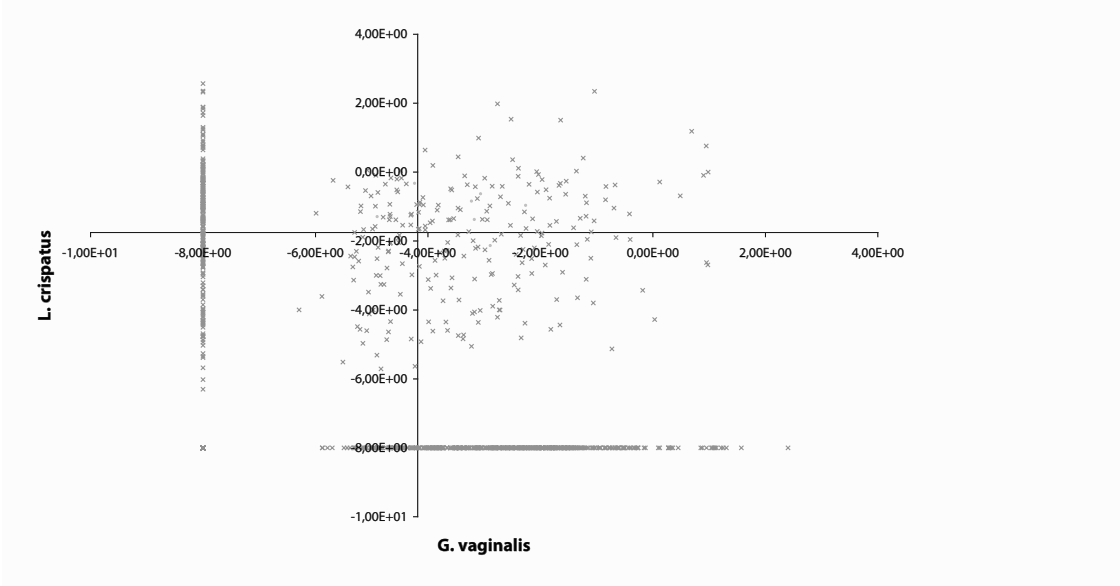

Figure 3 The DNA-concentrations of *Lactobacillus crispatus* and *Gardnerella vaginalis* per sample analysed by PCR (all data were transported to a log10-scale).

Stratified analysis

To evaluate the presence of both *Lactobacillus crispatus* and *Gardnerella vaginalis* in different lifetime periods we stratified for age in 5-years intervals. The distribution of age groups among the clusters A-D defined by colonization of *Lactobacillus crispatus* and/or *Gardnerella vaginalis* is shown in Table 2 revealing that almost half of the women aged 55-60 year were in Cluster D (only *Gardnerella vaginalis*). The second remarkable finding was that only 3.9% of the postmenopausal women were located in Cluster B. To demonstrate these findings clearly, Table 3 shows the distribution of premenopausal (<55) and postmenopausal (>55) women among the four different clusters.

	Cluster				
Age	**A** **No GV, No LC** % (N)	**B** **only LC** % (N)	**C** **GV and LC** % (N)	**D** **only GV** % (N)	**Total** % (N)
30 - 34	18.7 (55)	21.8 (64)	21.1 (62)	38.4 (113)	100 (294)
35 - 39	20.4 (58)	22.5 (64)	19.0 (54)	38.0 (108)	100 (284)
40 - 44	23.4 (54)	17.3 (40)	19.9 (46)	39.4 (91)	100 (231)
45 - 49	22.8 (21)	16.3 (15)	22.8 (21)	38.0 (35)	100 (92)
50 - 54	25.9 (15)	24.1 (14)	13.8 (8)	36.2 (21)	100 (58)
55 - 60	32.5 (25)	3.9 (3)	15.6 (12)	48.1 (37)	100 (77)

Table 2 Distribution of age groups among the clusters A-D defined by colonization of *Lactobacillus crispatus* and *Gardnerella vaginalis.*

	Cluster				
Age	**A** **No GV, No LC** % (N)	**B** **only LC** % (N)	**C** **GV and LC** % (N)	**D** **only GV** % (N)	**Total** % (N)
<55	21.2 (203)	20.5 (197)	19.9 (191)	38.4 (368)	100% (959)
55-60	32.5 (25)	3.9 (3)	15.6 (12)	48.1 (37)	100% (77)

Table 3 Distribution pre- (<55 year) and postmenopausal (55-60 year) women among the clusters A-D.

Figure 4 illustrates the independent colonization patterns of *Gardnerella vaginalis* and *Lactobacillus crispatus*, revealing a sharp decline in the colonization of *Lactobacillus crispatus* in the age group 55-60 years.

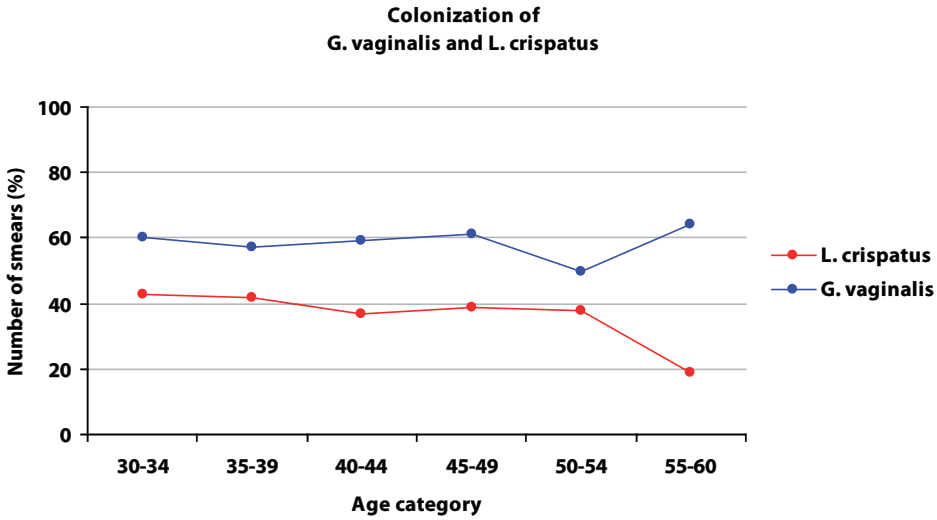

Figure 4 Number of colonized smears by respectively *Lactobacillus crispatus* and *Gardnerella vaginalis* in asymptomatic women.

The independent presence of absence of respectively *Lactobacillus crispatus* and *Gardnerella vaginalis* as distributed among pre- and postmenopausal women is presented in Table 4 and 5. The former table indicates that among the age group 55-60 year a significant decline is seen in the cases of colonization with *Lactobacillus crispatus,* respectively from 40% to 19% ($p<0.01$, χ^2 test). Table 5 displays that slightly more postmenopausal women are colonized by *Gardnerella vaginalis* although this is not significant.

Lactobacillus crispatus			
Age category	**Present** % (N)	**Absent** % (N)	**Total** % (N)
<55	40.5 (388)	59.5 (571)	100 (959)
55-60	19.5 (15)	80.5 (62)*	100 (77)

Table 4 Colonization of *Lactobacillus crispatus* among pre- (<55 year) and postmenopausal (55-60 year) women.

* generalized X^2 test statistics showed significance ($p<0.01$)

Gardnerella vaginalis			
Age category	**Present** % (N)	**Absent** % (N)	**Total** % (N)
<55	58.3 (559)	41.7 (400)	100 (959)
55-60	63.6 (49)	36.4 (28)*	100 (77)

Table 5 Colonization of *Gardnerella vaginalis* among women pre- (<55 year) and postmenopausal (55-60 year) women.

* generalized X^2 test statistics showed no significancy

Association of colonization

Finally, as shown in Table 6, we studied the association of colonization by *Gardnerella vaginalis* in a LC-negative flora (vaginal microflora without *Lactobacillus crispatus*) versus the reference group with a LC-positive flora (vaginal microflora with *Lactobacillus crispatus*) and also the colonization by *Lactobacillus crispatus* in GV-negative flora (vaginal microflora without *Gardnerella vaginalis*) versus the reference group with a GV-positive flora (vaginal microflora with *Gardnerella vaginalis*). Data were adjusted for age. Colonization by *Gardnerella vaginalis* was more frequent in LC-negative flora (OR 1.3; CI 1.1-1.4), as *Lactobacillus crispatus* was oftentimes colonizing GV-negative flora (OR 1.4; CI 1.2-1.6).

Microflora	**Colonization pattern**			
	Gardnerella vaginalis		**Lactobacillus crispatus**	
	Prev	**OR (95% CI)**	**Prev**	**OR (95% CI)**
Non-Lactobacillus flora	64.0	1.3 (1.1-1.4)[a]		-
Non-Gardnerella flora		-	46.7	1.4 (1.2-1.6)[b]

Table 6 Association of colonization by *Gardnerella vaginalis* and *Lactobacillus crispatus* in the vaginal microflora (adjusted for age)

* prevalence per 100 smears

(a) the presence of Gardnerella vaginalis in women not colonized by Lactobacillus crispatus vs. women colonized by Lactobacillus crispatus.

(b) the presence of Lactobacillus crispatus in women not colonized by Gardnerella vaginalis vs. women colonized by Gardnerella vaginalis.

Discussion

The vaginal ecology plays an important role in the pathogenesis of vaginal inflammation in women. For this reason, the vaginal microflora has been extensively characterized in women of reproductive age, unmasking factors that might influence susceptibility for vaginal inflammation. Recently, we found an inverse relation between *Gardnerella vaginalis* and *Lactobacillus crispatus* in smears of asymptomatic women cytologically diagnosed as dysbacteriosis and *Gardnerella* infection.[13] However, little is known about possible differences in colonization status of these two bacteria among asymptomatic women. In our study, we examined colonization patterns of *Gardnerella vaginalis* and *Lactobacillus crispatus* in the vaginal flora of asymptomatic Dutch women participating in the national screening program on cervical cancer, using DNA-PCR, we detected interesting patterns.
Firstly, our data show that the prevalence of *Gardnerella vaginalis* colonization among asymptomatic women was 58.7% and in concordance with De Backer et al.[18] while the overall colonization of *Lactobacillus crispatus* was only 38.9%.
Secondly, we evaluated the colonization of both bacteria in postmenopausal women. According to the literature we used the general principle that women of 55 years and above are postmenopausal, since the overall median age at natural menopause is 51.3 (49-54 years).[19,20] The number of colonized smears by *Gardnerella vaginalis* was slightly higher among postmenopausal women (63.6%), versus 58.3% among premenopausal women, but this difference showed no significance. More interesting is the fact that only 19% of the vaginas of postmenopausal women was colonized by *Lactobacillus crispatus*. These results reveal a remarkable difference between the pre- and postmenopausal colonization of *Lactobacillus crispatus* among asymptomatic, women underlining that the menopause leads to a significant decline in *Lactobacillus crispatus*. The so-called menopausal transition implies a series of hormonal changes. As ovarian function decreases and fertility disappears, circulating estrogen levels are first increased and then decrease,[21] and there is a shift in estrogen production from the ovaries to extragonadal sites.[21,22] Loss of *Lactobacillus crispatus* could thus be caused by cessation of production of female sex hormones. However, with the appearance of climacteric symptoms, exogenous hormones are widely used; these hormones interact with a changing pre-existing hormonal status. In this context, Pabich et al.[23] demonstrated in their case-control study that 62% of the postmenopausal women were colonized by vaginal lactobacilli and were significantly more prevalent among women receiving hormone replacement therapy during the previous year. On that account, our data concerning the decline in colonization with *Lactobacillus crispatus* should be even more pronounced. It should be remarked that among premenopausal women colonization of both *Gardnerella vaginalis* and *Lactobacillus crispatus* showed not to be influenced by age.
Lastly, our study indicates that *Gardnerella vaginalis* and *Lactobacillus crispatus* might interfere with each other (Table 6). Whereas the former bacterium was significantly more frequent colonizing a non-*Lactobacillus crispatus* flora compared to a flora *with Lactobacillus crispatus*, the latter bacterium was significantly more frequent in a non-*Gardnerella vaginalis* flora compared to a flora *with* colonization of *Gardnerella vaginalis*.

These findings might support the hypothesis that *Gardnerella vaginalis* is inhibited by lactobacilli[24] and more specific by *Lactobacillus crispatus*,[25] which could be essential in developing probiotics for prophylactic use with appropriate ingredients. However, the exact inhibitory power of *Lactobacillus crispatus* has to be examined.

Strengths of the present study are: (1) the availability of a large number of data on asymptomatic women (2) objectivity of data, since the moment of sampling was not biased as the women were randomly invited for a cervical smear by the city council (3) objectivity of methods by avoiding culture dependent methods (4) use of formalin-free coagulant fixative (BoonFix®) in which DNA and RNA are not damaged.[16] A limitation of our study is that it was not possible to correct our data for the use of hormone replacement therapy, for the only reason that our data were collected from the national screening program in which such facts are not systematically registered.

In summary, this baseline study of asymptomatic women shows that the presence *Lactobacillus crispatus* depends on hormonal status. Moreover, our data suggest that diagnosis on the basis of PCR-techniques will probably provide more effective procedures in determining healthiness of vaginal flora. Future research is needed to evaluate the fascinating colonization patterns of *Gardnerella vaginalis* and *Lactobacillus crispatus* in vaginal flora of healthy women. It might become possible to predict the susceptibility to vaginal infections or cervical (pre)neoplasia using coagulate fixatives which allow for DNA-PCR on cervical samples.

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Concentration of *Gardnerella vaginalis* and *Lactobacillus crispatus* in women with a bacterial imbalanced vaginal flora

Abstract

Objective: Dysbacteriosis and cervical *Gardnerella* infection represent cytological subdivisions of a bacterial imbalanced vaginal flora. *Lactobacillus crispatus* and *Gardnerella vaginalis* are thought to be important bacteria in the balancing act of a healthy vaginal flora. The aim of this study was to examine the concentrations of *Lactobacillus crispatus* and *Gardnerella vaginalis* in bacterial imbalanced vaginal microflora versus normal flora.
Methods: From our database 29 cervical samples showing dysbacteriosis, 20 *Gardnerella* samples and 27 samples with a healthy vaginal flora were collected. All samples, suspended in a commercial coagulant fixative (BoonFix®) were used for PCR.
Results: There appeared to be four different quadrants (A-D) based on concentrations of both *Gardnerella vaginalis* and *Lactobacillus crispatus*. All samples with concentrations of *Gardnerella vaginalis* over $2.0x10^{-2}$ng/µl displayed bacterial imbalance, whereas *Gardnerella* samples, all with a concentration of *Lactobacillus crispatus* less than $3.8x10^{-4}$ng/µl, were located in quadrant D. Dysbacteriosis was found in all quadrants, while healthy flora was found only in quadrant A and B.
Conclusion: DNA-concentrations of *Gardnerella vaginalis* and *Lactobacillus crispatus* might be valuable markers of bacterial imbalance, however the differentiation between dysbacteriosis and *Gardnerella* infection could not be made. Our study reveals noteworthy data suggesting that culture-independent methods can provide new insights into diagnosis of bacterial imbalance of the vaginal flora.

Introduction

Vaginal complaints constitute one of the most common problems in clinical medicine, and it is one of the main motives that lead women to seek out their general practitioner or gynecologist.[1] Bacterial vaginosis is a common cause of vaginitis in most clinical settings, and it is characterized by the overgrowth of commensal anaerobic flora (mainly *Gardnerella vaginalis*) flora at the expense of H_2O_2-producing *Lactobacillus* species that predominate in the healthy vagina.[2-4]

Clinicians diagnose bacterial vaginosis by the characteristic fishy odor of the vaginal swab, a homogenous, grayish-white vaginal discharge, a vaginal pH > 4.5, and the presence of so called 'clue cells' in the unstained smear, the latter indicating a shift to *Gardnerella vaginalis*.[5-7] Although bacterial vaginosis is diagnosed by clinical criteria, 50% all women with bacterial vaginosis remain *asymptomatic* in the presence of vaginitis or cervicitis.[8,9] Consequently, only symptomatic women will receive the diagnosis bacterial vaginosis, however, a far more larger group of women will actually display a bacterial imbalanced vaginal microflora.

Gardnerella infection and dysbacteriosis are both cytological diagnoses of bacterial imbalance,[10] representing the cytological subdivisions of Bethesda's third category: 'shift in flora suggestive of bacterial vaginosis'.[11,12] Dysbacteriosis stands for the microscopic diagnosis of a shift in vaginal flora, in which the majority of lactobacilli is replaced by a mixture of anaerobic bacteria, mainly *Gardnerella vaginalis*.[13,14] A cervical *Gardnerella* infection is present when the cervical smear shows so-called BMCs (blue mountain cells) indicating an extreme shift towards *Gardnerella vaginalis* and a lack of lactobacilli.[10,15]

Recently, Verbruggen et al. demonstrated that dysbacteriotic smears had a significantly higher presence of cervical (pre)neoplasia.[13] We found that cytologically diagnosed *Gardnerella* infection shows a strong covariaton with (pre)neoplastic changes of the cervix.[16]

Unfortunately, since asymptomatic women will never visit their general practitioner or gynecologist because of vaginal complaints, asymptomatic bacterial imbalance will not be detected.

In the Netherlands, all cervical smears are coded for inflammatory status including bacterial imbalance, whereas the differentiation between dysbacteriosis and *Gardnerella* infection is also registered.[15]

Nowadays, in the process of screening the cytotechnologist can distinguish smears with a healthy flora containing lactobacilli from those with bacterial imbalance representing a shift towards *Gardnerella vaginalis* signifying an advantageous spin-off in traditional cervical cancer screening. However, the procedure remains largely laborious and subjective.

In our pilot-study, we found a marked inverse relationship between the DNA-concentration of *Lactobacillus crispatus* and *Gardnerella vaginalis* in smears with dysbacteriosis and *Gardnerella*.[17] As a consequence, this remarkable finding should be further examined in order to examine the possible diagnostic potential of this relation. We therefore questioned whether DNA-concentrations of *Gardnerella vaginalis* and *Lactobacillus crispatus* could be

useful in order to make diagnostic procedures less time-consuming and subjective.
This study was made with the objective of comparing the DNA-concentrations of *Lactobacillus crispatus* and *Gardnerella vaginalis* in the vaginal flora of Dutch asymptomatic women with cytological evidence for bacterial imbalance.

Material and methods

Patient material
Between January 1st, 2005 and December, 31st, 2005 the Leiden Cytological and Pathological Laboratory received 40.000 cervix cell samples of women (aged 30-60 years) in connection with the Dutch national screening program, western region. From our data base 29 dysbacteriotic samples, 20 samples with *Gardnerella* infection and 27 samples showing a healthy microflora were randomly selected in order to perform DNA-PCR for the colonization of *Lactobacillus crispatus* and *Gardnerella vaginalis*. Age distribution among the different groups was equal. Signed informed consent was obtained from participants and the protocol was approved by the Institutional Ethical and Scientific Review Committee.

Sample preparation
After swabbing, accomplished by a general practitioner, the tip of the brush was broken off and transported in a cyto sample vial holding 15 ml of BoonFix® (Finetec, Tokyo, Japan) to the laboratory. BoonFix® is a formalin-free coagulant fixative containing ethyl alcohol and a low molecular weight PEG.[18] This suspension is preferred above formalin fixatives because it has as main preservative effect coagulation of proteins. DNA and RNA are not damaged allowing for successful PCR amplification of DNA.[18]

Primers and probes
Song et al. developed primers and probes for the detection of lactobacilli.[19] A part of the 16S rRNA gene of both bacteria was used for amplification primers. For the identification of H_2O_2-producing *Lactobacillus crispatus,* the primer set according to Song et al. was slightly adapted to become suitable for the LightCycler (Table 1). Software was used (Primer-probe design, Roche, Mannheim, Germany) for designing primers and probes (Tib MolBiol, Berlin, Germany). Table 1 lists all primers and probes used in this study.[19] All primers were synthesized and column purified by Tib MolBiol, Berlin, Germany.

Sensitivity and specificity
Specificity of the primer sets was analysed by searching Gene Bank data base using the BLAST algorithm (National Center of Biotechnology Information, National Institutes of Health, Bethesda, MD, USA). Specificity of the primer sets was determined by testing the following strains: *Candida albicans, Mobiluncus mulieris, Mobiluncus curtisii, Raoultella planticola, Gardnerella vaginalis, Klebsiella sp., Lactobacillus johnsonii, Bifidobacterium scardovii, Staphylococcus epidermidi, Lactobacillus delbrueckii supsp. lactis, Lactobacillus helveticus, Lactobacillus acidophilus, Lactobacillus delbrueckii subsp. Bulgaricus, Bifidobacterium*

longum, Bifidobacterium minimum, Staphylococcus aureus subsp. Aureus, Streptococcus sp., Bifidobacterium bifidum, Peptoniphilus asaccharolyticus, Finegoldia magna, Enterococcus faecium (=Streptococcus faecium), Enterococcus faecalis (=Streptococcus faecalis), Prevotella bivia, Lactobacillus amylovorus, Anaerococcus prevotii, Lactobacillus jensenii, Lactobacillus crispatus, Streptococcus thermophilus, Bacteroides ureolyticus, Porphyromonas asaccharolytica, Escherichia coli, Pseudomonas aeruginosa.

No cross-reactivity was detected with DNA from any of the tested species. Sensitivity for *Lactobacillus crispatus* and *Gardnerella vaginalis* was tested and found equal at a concentration of $1.0x10^{-6}$ng/µl.

G. vaginalis	Gvag F	5'-ggCTAgAgTgCAgTAggg
	Gvag R	5'-gTTAgCTCCgACACAgAAC
	Gvag FL	5'-CgggAAgAACACCAATggC-FL
	Gvag LC	LC RED640-5'-AggCAggTCTCTgggCTgTT-3'-phos
L. crispatus	Lcri-FW	5'-CgAAgAAggACgTgACgAACTAC
	Lcri-RE	5'-CAgATAATTCAACTATCTCTTACACTgCC
	Lcri-FL	5'-AgTgAATAgATAgCTAATCAAAggAAgACgCAgT-FL
	Lcri-LC	LC RED640-5'-AACTgAAACATCTAAgTAgCTgCAggAAgAgAA-3'-phos

Table 1 Primer and probe sequences (synthesized by TIB MOLBIOL, Berlin, Germany)

DNA extraction of bacteria and samples

To detect the amounts of DNA from these bacteria in the patient specimens, the samples were randomly collected and analysed with real-time PCR (LightCycler 1.5, Roche, Mannheim, Germany), after automated purification (Qiagen M48 BioRobot, using MagAttract DNA mini M48 Kit cat. no. 953336, Qiagen, Hilden, Germany). A part of the 16S rRNA gene of both bacteria was used for amplification primers.

PCR was performed with the LightCycler FastStart DNA Master PLUS Hybridization Probes (cat. no. 03515567001) by mixing 4 µl of LightCycler DNA Master hyb probe, 7 µl H2O and 1 µl of each primer (final conc. 0,5 µM) and probe (final conc. 0,2 µM) per bacteria. Five µl of purified DNA was added to the mix.

PCR was performed starting with a 95°C denaturation step for 10 minutes to activate taq-polymerase. Amplification of the DNA was programmed for 45 cycles with denaturation of 5 s, 95°C, annealing of 10 s, 54°C and an elongation of 10 s, 72°C. All steps had a transition rate of 20°C/s. During annealing the concentration of the amplified DNA was measured. Quantification software (Roche, Mannheim, Germany), fit points method (with an external standard curve using LightCycler Control Kit DNA, cat. No. 12158833001, Roche, Mannheim, Germany) was used for measuring the concentration of the bacterial DNA. Both primer sets resulted in amplification with DNA from the appropriate type. Finally, all samples were analysed by PCR for their DNA concentration of *Lactobacillus crispatus* and *Gardnerella vaginalis*. PCR was negative if less than $1.0x10^{-6}$ng/µl was present in the vaginal swab, both for *Lactobacillus crispatus* and *Gardnerella vaginalis*. Negative samples were documented as $1.0x10^{-8}$ng/µl.

The concentrations of the bacteria (ng/µl) were divided by the beta-globin gene concentration found using the LightCycler Control Kit DNA (cat. No. 12158833001 Roche, Mannheim, Germany). This quantitative control kit measures the concentration of the beta-globin gene present in the sample. This gene is a part of the human genome. We can therefore estimate the number of cervical cells, and concentrations of bacteria were calculated for 1 ng/µl beta-globin DNA.

Microscopic analysis
Slides were reviewed as per routine procedure. Slides and results were classified according to the Dutch KOPAC system[20-23] in which abnormalities of all cell components and additional pathology present in the slide are recorded on a nationally used computerized database format.
A cervical specimen is coded O3 (dysbacteriosis) when a remarkable shift is seen in the microflora with the presence of clue cells (CC) that is, cells with adhering bacteria, mainly *Gardnerella vaginalis*. The number of lactobacilli is minimal.[10,14]
A *Gardnerella* infection (O5) is registered when, by microscope, the cervical smear shows so-called BMCs, thousands tiny rodlike organisms clinging to the cellular membrane, and a lack of lactobacilli.[10,16]
A non-inflammatory cervical smear representing a normal flora is classified as O6.

Results

Demographic features
The 76 women whose samples were studied had a mean age of 40.1 years (31.4-55.9). Age distribution among the three cytological groups was equal.

PCR findings
PCR was negative if less than $1.0x10^{-6}$ng/µl was present in the vaginal swab, both for *Lactobacillus crispatus* and *Gardnerella vaginalis*.
According to Figure 1 there appeared to be four different quadrants confined by concentrations of *Gardnerella vaginalis* and *Lactobacillus crispatus*, based on the cytological inflammatory status of the vaginal flora. The quadrant framework was established by the observation that, in general, all three inflammatory groups showed to have different concentrations of *Gardnerella vaginalis* and *Lactobacillus crispatus*.

Table 2 presents the distribution of inflammatory patterns among the quadrants A-D defined by the DNA-concentrations (ng/µl) of *Gardnerella vaginalis* and *Lactobacillus crispatus*. All samples with cytologically diagnosed *Gardnerella* infection showed concentrations of *Gardnerella vaginalis* > $2.0x10^{-2}$ ng/µl (-1.71E+00, log-scale), whereas 68.9% of the dysbacteriotic samples had a concentration of *Gardnerella vaginalis* > $2.0x10^{-2}$ ng/µl (-1.71E+00, log-scale). Moreover, all *Gardnerella* samples showed a concentration of < $3.8x10^{-4}$ ng/µl (-3.4E+00, log scale). All healthy samples were distributed among quadrant A and B indicating a concentration of *Gardnerella vaginalis* < $2.0x10^{-2}$ ng/µl (-1.71E+00, log-

scale). Besides, 31% of all dysbacteriotic samples was also distributed among quadrant A and B.

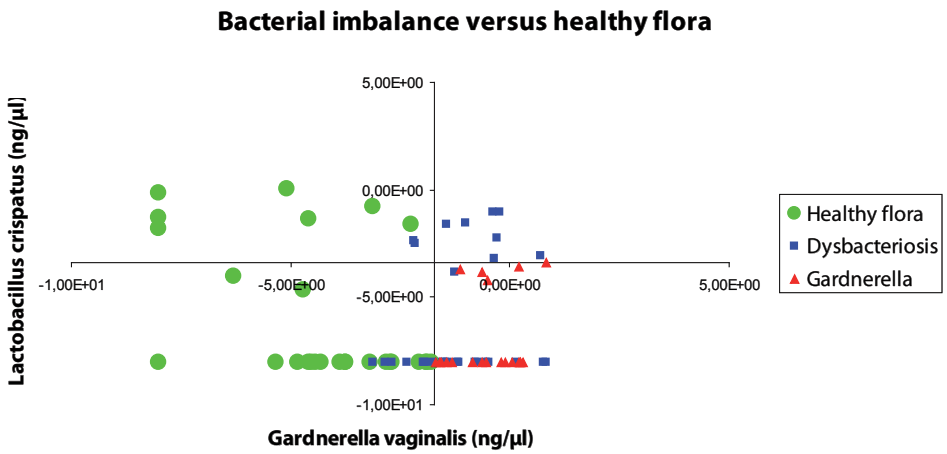

Figure 1 The DNA-concentrations of *Lactobacillus crispatus* and *Gardnerella vaginalis* per sample analysed by PCR (corrected for beta-globin). Age distribution among the three groups were equal (all data were transported to a log10-scale).

	Quadrant				
Inflammatory status	**A** % (N)	**B** % (N)	**C** % (N)	**D** % (N)	**Total** % (N)
Dysbacteriosis	24.1 (7)	6.9 (2)	24.1 (7)	44.8 (13)	100 (29)
Gardnerella	0.0 (0)	0.0 (0)	0.0 (0)	100.0 (20)	100 (20)
Healthy flora	74.1 (20)	25.9 (7)	0.0 (0)	0.0 (0)	100 (27)

Table 2 Distribution of dysbacteriosis, Gardnerella infection and healthy flora among quandrants A-D defined by the concentration (ng/µl) of *Lactobacillus crispatus* and *Gardnerella vaginalis*.

Quadrant A: G. vaginalis < -1.71E+00 and L. crispatus < -3.4E+00 (log scale)
Quadrant B: G. vaginalis < -1.71E+00 and L. crispatus > -3.4E+00 (log scale)
Quadrant C: G. vaginalis > -1.71E+00 and L. crispatus > -3.4E+00 (log scale)
Quadrant D: G. vaginalis > -1.71E+00 and L. crispatus < -3.4E+00 (log scale)

Discussion

We examined DNA concentrations of *Gardnerella vaginalis* and *Lactobacillus crispatus* in the vaginal flora of asymptomatic Dutch women by using PCR to determine the DNA-concentrations in all 76 cervical samples.
To our knowledge, we are presenting the first study on concentrations of both *Gardnerella vaginalis* and *Lactobacillus crispatus* established by DNA-PCR examining whether the

combination of *Lactobacillus crispatus* and *Gardnerella vaginalis* might function as an indicator for bacterial imbalance of the vaginal microflora.

Aroutcheva et al.[24] found that *Gardnerella vaginalis* appears to play a clinically significant role in the etiology of bacterial vaginosis. This is consistent with the findings of other studies in which recovery of *Gardnerella vaginalis* from samples from 98% of the patients with bacterial vaginosis supports the importance of *Gardnerella vaginalis* as a causal organism.[25,26]

Previous studies stated that lactobacilli are probably the most abundant organisms in healthy women.[4,27,28] Lactobacilli are one of the main defence mechanisms against pathogens and therefore play a significant role in maintaining homeostasis in the vaginal flora. Most lactobacilli produce hydrogen peroxide (H_2O_2) and provide an acidic environment through lactic acid production. This has been shown to be one of the most important mechanisms against pathogens.[29] *Lactobacillus crispatus* is one of the most predominant H_2O_2-producing species found in the vagina.[30]

Although there are diagnostic tools like the Amsel test, Nugent score, KOH-preparation, culture and microscopy that provide a helping hand in diagnosing an abnormal vaginal flora they all remain more or less subjective. Moreover, since smears with dysbacteriosis or *Gardnerella* infection show a higher prevalence of HSIL/carcinoma it is of great importance to create objective and simple diagnostic tools in detection of the bacterial imbalanced vaginal flora.

By means of PCR-techniques, diagnosis will become a far more efficient procedure. Several studies have used broad-range 16S rRNA gene PCR to characterize the community of vaginal bacteria and to assess the prevalence of each bacterial species in order to explore the potential of PCR for the microbiological diagnosis of bacterial vaginosis. Although studies have linked *Gardnerella vaginalis* as an indicator of bacterial vaginosis, Fredricks et al.[28] demonstrated that using the Amsel criteria as gold standard for bacterial vaginosis, *Gardnerella vaginalis* was detected by PCR in 96% of subjects with bacterial vaginosis, but was also detected in 70% of subjects without bacterial vaginosis, confirming the poor specificity of *Gardnerella vaginalis* on a qualitative basis of bacterial vaginosis. These findings made us hypothesize that a combination of concentration of *Gardnerella vaginalis* and *Lactobacillus crispatus* established by PCR would be a more reliable indicator of bacterial vaginosis.

Surprisingly, in the present study we found that bacterial imbalance might be characterized by the DNA concentrations of both *Gardnerella vaginalis* and *Lactobacillus crispatus* as established by DNA-PCR. Moreover, our data suggest that diagnosis on the basis of PCR-techniques will probably provide more effective procedures in determining healthiness of the vaginal flora. Unfortunately, this design appeared not to be capable of differentiating the two cytological subdivisions of bacterial imbalance, dysbacteriosis and *Gardnerella* infection.

There are several limitations to this study. First, although we randomly selected our study population, we examined a relatively small number of cervical samples. Second, although we carefully attempt to assay for every known vaginal bacterium that could interact with our primers, as shown in the material and methods section, additional bacterial species could be present in vaginal samples, which might compromise specificity.

Finally, based on our findings we propose that future research will discover whether there exist specific concentration ratios whereby the different inflammatory states of the vaginal flora can be diagnosed. In such studies concentrations of *Lactobacillus crispatus* and *Gardnerella vaginalis* should also be determined in other vaginal inflammations, e.g. *Candida* and *Trichomonas vaginalis*.

Second, cytological examination of cervical samples remains crucial in differentiating dysbacteriosis and *Gardnerella* infection.

In summary, our findings might be essential in developing a framework for diagnosing bacterial imbalance of the vaginal flora by concentrations of both *Gardnerella vaginalis* and *Lactobacillus crispatus* established by DNA-PCR. It might become possible to establish a new tool in diagnosing bacterial imbalance of the vaginal flora using formalin-free coagulant fixatives which allow for DNA-PCR on cervical samples.

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J.M. Klomp, H. Korporaal, M.E. Boon, M. van Haaften, A.P.M. Heintz

Bacterial imbalance of the vaginal flora containing *Gardnerella vaginalis* with a shortage of lactobacilli: preference of high-risk human papillomavirus

Abstract

Introduction: Cervical inflammation has been proposed as one of the cofactors in cervical carcinogenesis. Since disturbance of the vaginal flora is known to increase the risk of acquisition of HPV infection. It is of interest to investigate the statistical relation between bacterial imbalance by *Gardnerella vaginalis* with a shortage of lactobacilli and the presence of high-risk HPV infection. In addition, type-specific prevalence of high-risk HPV was assessed in bacterial imbalance versus normal vaginal flora.

Methods: A total of 19,793 cervical samples, suspended in BoonFix® (Finetec) was tested for 26 HPV-genotypes using the short fragment polymerase chain reaction (SPF) hybridization line probe assay (LiPA). Smears were analysed by light microscopy and coded for inflammatory status. We randomly collected a subset of 130 cervical samples with cytological evidence for bacterial imbalance of the vaginal flora. The reference group consisted of 3,293 smears with a cytological normal flora.

Results: Prevalence of hr-HPV infection was 44.6% among smears showing bacterial imbalance of the vaginal flora versus 25.8% in healthy flora (OR 1.6; CI 1.2-1.9). High-risk type specific prevalence of HPV genotypes appeared not significant.

Conclusions: Our findings indicate that high-risk HPV genotypes have a strong preference for a bacterial imbalanced flora showing *Gardnerella vaginalis* and a shortage of lactobacilli. Further research should investigate whether certain type specific HPV infections are more prominent in a bacterial imbalanced flora.

Introduction

Cervical inflammation has been proposed as one of the cofactors in cervical carcinogenesis, since disturbance of the vaginal flora is known to increase the risk of acquisition of human papilloma virus (HPV infection).[1,2,3,4]

Human papilloma viruses are prevalent worldwide and can cause cervical dysplasia, anogenital warts, and cervical carcinoma. Persistent infection with one or more types of high-risk human papilloma virus (hr-HPV) is the main risk factor for the development of cervical carcinoma.[5,6] The prevalence is highest in sexually active young women and decrease with age.[7-10] Although the lifetime risk of acquiring an HPV infection is about 80%[11], the incidence of cervical intraepithelial neoplasia and invasive carcinoma is relatively low.[8,12-14] This can be explained by the infection pattern of HPV which is mostly transient and asymptomatic.[7,15,16] In most cases the HPV infection disappears within 6 to 14 months.[7,17,18] So far, more than 100 different genotypes of HPV have been identified, about 40 genotypes have been shown to infect mucosal surfaces. Infections with HPV genotypes 16,18,26,31,33,35,39,45,51,52,53,58,59,66,68,73 of 82 have been frequently detected in patients with cervical cancer, whereas these genotypes are considered high-risk. Infections with HPV genotypes 6,11,40,43,44,54,61,70,72,81 are frequently detected in benign lesions such as condylomata acuminate and therefore termed low-risk HPV types.[19]

The general hypothesis is that the local cervicovaginal milieu plays a role in susceptibility to HPV infection. Since women with bacterial imbalance of the vaginal flora are likely to possess an unhealthy *Lactobacillus*-poor flora, they should be at risk for acquiring HPV infection.

Papanicolaou[20] and Mead et al[21] already confirmed that women with cervical carcinoma often had a dysbacteriotic flora having a shortage of the normally protective lactobacilli. In this context the relationship of bacterial inflammation and (pre)neoplasia has recently been investigated. Verbruggen et al. found that women with cytologically diagnosed dysbacteriotic smears had a significantly higher presence of cervical (pre)neoplasia.[22] This finding is partially supported by other studies that investigated women with dysbacteriosis and cervical (pre)neoplasia.[1,23-25] Klomp et al. recently found that cytologically diagnosed *Gardnerella* infection shows a strong covariaton with (pre)neoplastic changes of the cervix.[26]

The Bethesda System identifies one category of bacterial imbalance in cervical cytology[27,28]: 'shift in flora suggestive of bacterial vaginosis'. According to Meisels and Morin,[29] this category comprises two cytological subdivisions as there are (1) dysbacteriosis, representing the microscopic diagnosis of a shift in vaginal flora, in which the majority of lactobacilli is replaced by a mixture of anaerobic bacteria, mainly *Gardnerella vaginalis*[30,31] and (2) *Gardnerella* infection, which displays an *extreme* shift in flora towards *Gardnerella vaginalis*, lacking lactobacilli.[29]

In the Netherlands, these cytological subdivisions are scored in the KOPAC system that is primarily implemented for coding the squamous epithelium in cervical cytology.[32] KOPAC is an acronym in which K indicates comoposition [kompositie]; O, inflammation

[ontstekingsverschijnselen]; P, squamous epithelium [plaveiselepitheel]; A, other abnormalities of the endometrium [andere afwijkingen van het endometrium]; C, endocervial columnar epithelium [cylinderepitheel endocervix].[32]

The objective of our study was to document the prevalence of hr-HPV in women with bacterial imbalance of the vaginal flora versus women with a cytological normal flora. Therefore, 4,326 cervical samples of asymptomatic women, invited for a screening test in the national screening program, were collected to further our understanding of the prevalence of hr-HPV among bacterial imbalanced versus healthy vaginal flora. This knowledge will also help to clarify the role of HPV testing in primary screening to identity women at risk of developing cervical (pre)neoplasia.

Material and methods

Study population

From August 1993 to December 2007 almost 1 million conventional smears were received by the Leiden Cytology and Pathology Laboratory (Leiden, Netherlands), 80% originating from women who participated in the national screening program, the remaining part for reason of medical indication. In this period 19,793 HPV-PCR tests were performed as part of an ongoing quality control and assurance program. We randomly collected a subset of 130 samples with evidence of bacterial imbalance of the vaginal flora and 3,239 smears with a healthy flora. In all 4,326 specimens cervical intra-epithelial neoplasia was absent.

In the study population was assigned a unique study number to maintain anonymity to the non-clinical research staff. Only the pathologists responsible for the diagnosis had access to the full clinical data of the study population.

Cytological sample collection and classification

Cervical samples were collected using a standard brushing technique (Rovers Medical Devices B.V., Oss, Netherlands) provided free of charge to the general practitioners who take all smears in the Netherlands. Cell samples as well as brush heads were placed immediately into germ and DNA free 20 ml volumes of BoonFix® and referred by overnight mail to the testing laboratory (LCPL, Leiden, Netherlands).

A smear was prepared according to liquid based cytology methods using automated processing of cell suspensions (ThinPrep 3000 Processor). Cells were processed to and mounted on slides as per manufacturers' instruction. To visualize the disc and counterstain the cells, the cell disc was contrasted by adding one drop of haematoxylin and was subsequently stained according to Papanicolaou for conventional light microscopy as well as NNS scanner (Papnet®) supported assessment.

HPV testing

HPV PCR is established on a sub-sample of the original suspension fluid based on the proven and patented superior qualities with respect to DNA preservation over the storage period as existed in this study (4-8 days, inclusive of transport).

To obtain enough cervical cells we scraped the lower half of a PAP-smear by removing

the cover slip and the excess glue. Half of the smear, containing ecto- and endocervical cells, were scraped off with a sterile disposable blade under conditions which ensured a minimum risk of contamination.[33,34] The other half was returned to the archive. The scraped-off cells were suspended in 1000µl TRIS-HCL buffer (10mM, pH 8.3), and centrifuged for 5 minutes at 13.000rpm. This cell pellet was used for PCR as described in the paper of Van den Brule et al., 1990.[35]

For DNA extraction we used the automated Qiagen M48 Biorobot system (Qiagen, Venlo, Netherlands). Isolation was performed using the MagAttract DNA Mini M48 Kit (cat. no. 953336). The starting volume was set at 200µl cell suspension and final elution was in 100µl RNAse free water provided by the kit. Ten microliter of the eluted DNA was used for PCR using the Biometra T-gradient thermocycler (Biometra, Goettingen, Germany) with settings as followed. Initial denaturation of 9 minutes at 94˚C followed by the amplification which consisted of denaturation for 30 seconds at 94˚C, annealing for 45 seconds at 52˚C and an elongation for 45 seconds at 72˚C. After amplification a final elongation of 5 minutes at 72˚C took place. The mastermix contained a HPV primermix provided with the INNO-LiPA HPV genotyping v2 kit (Innogenetics, Gent, Belgium; cat. no. 80665). HPV-LiPA HPV is based on broad-spectrum amplification of HPV genotypes using SPF10 primers, which amplify only 65 bp at the L1 region. The other components of the mastermix were: 5µl PCR-buffer, 1.5 Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, USA; cat. no. N808-0241), 2.0mM MgCl2, 200µM of each dNTP (Amersham Biosciences, Freiburg, Germany; cat. no. 27-2035-01) and addition of H_2O up to 40µl. The line probe assay (LiPA) was performed according to the instructions of the AutoLiPA (Innogenetics, Gent, Belgium) provided by the manufacturer. The line probe assay is able to detect 26 types of gynecological HPV. The following types can be distinguished: 6,11,40,42,43,44,54,68,70 and 74 (low risk HPV) and 16,18,31,33,35,39,45,51,52,53,56,58,59,66,68 and 73 (high risk HPV). Five microliter of the PCR product was used for the hybridization assay. PCR products are seen and recorded as purple bands. Results were interpreted by direct observation.

Microscopic analysis

Slides were reviewed as per routine procedure. Slides and results were classified according to the Dutch KOPAC system[36-39] in which abnormalities of all cell components and additional pathology present in the slide are recorded on a nationally used computerized database format.

The bacterial imbalance group consisted of smears displaying vaginal flora with *Gardnerella* infection or dysbacteriosis. A cervical specimen is coded O3 (dysbacteriosis) when a remarkable shift is seen in the microflora with the presence of clue cells (CC) that is, cells with adhering bacteria, mainly *Gardnerella vaginalis*. The number of lactobacilli is minimal.[22,40] A *Gardnerella* infection (O5) is registered when, by microscope, the cervical smear shows so-called BMCs, thousands tiny rodlike organisms clinging to the cellular membrane, and a lack of lactobacilli.[29,32] A non-inflammatory cervical smear representing a normal flora is classified as O6. We created a reference group consisting of O6 smears, defined as non-inflammatory cervical smears originating from women with cytological normal vaginal flora. To make sure that this reference group was healthy, a special selection was made of women with at least three cervical smears classified as O6, who never had

smears diagnosed other than O6. This reference group consisted of 3,293 smears.

Statistical Analysis

The prevalence of hr-HPV among *Gardnerella* infection and normal flora was calculated. Odds ratios (OR) with 95% confidence intervals (CI) were calculated for bacterial imbalanced flora, by using SPSS 12.0. Age distribution differed between the two groups, with younger women (<20-29) being overrepresented in the bacterial imbalance group. We therefore made a correction on age distribution in our calculations of the Odds ratio.

Results

Mean age of the bacterial imbalance group was 37.1 years (range 18.2 to 59.0 years). The women who's smears were in the reference group had a mean age of 39.6 years (range 19.1 to 59.9 years). Age distributions of both groups are shown in Figure 1.

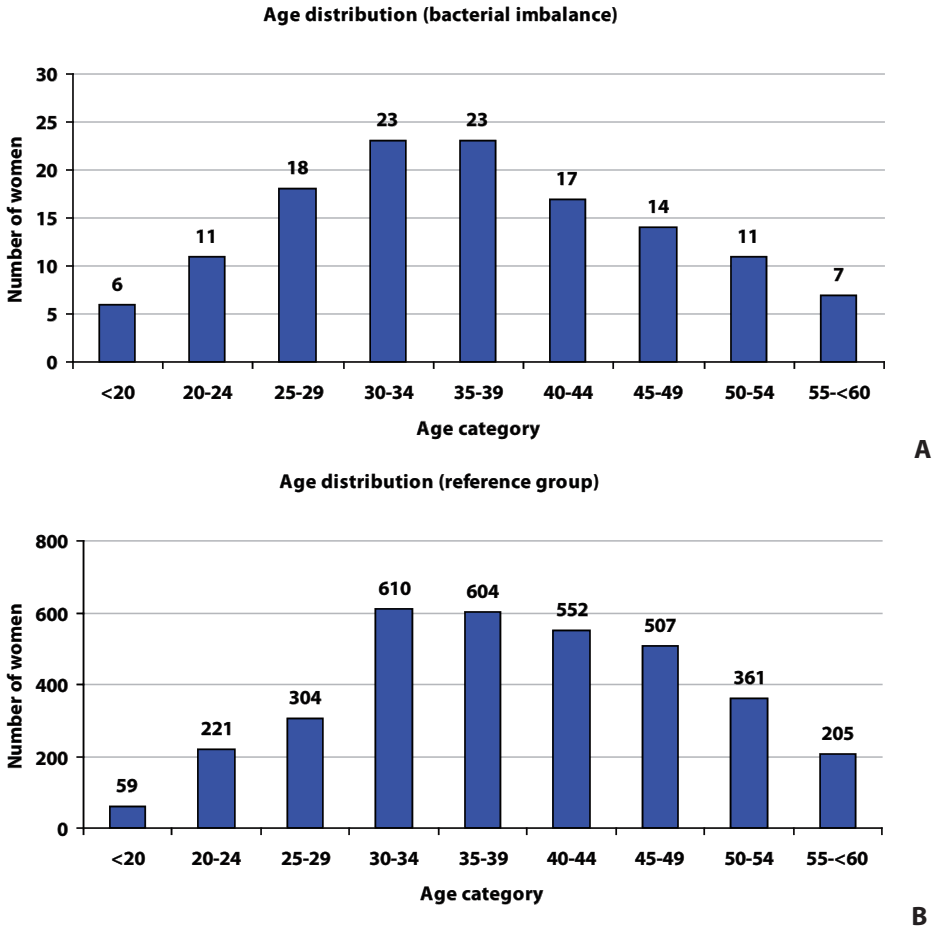

Figure 1 Age distribution of bacterial imbalance-group (A) and reference group (B)

The prevalence of high-risk HPV infection in bacterial imbalanced flora (44.8%) versus healthy flora (25.8%) is illustrated in Figure 2.

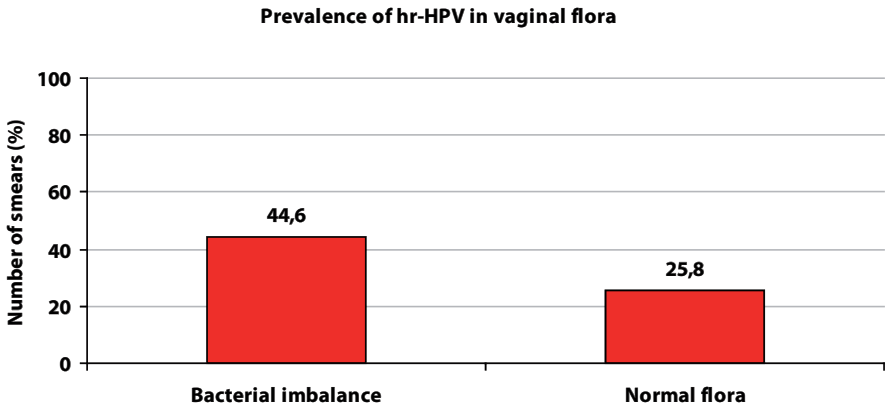

Figure 2 Prevalence of hr-HPV in bacterial imbalanced vaginal flora containing *Gardnerella vaginalis* versus normal vaginal flora.

Table 1 shows the prevalence of hr-HPV stratified by age. This table demonstrates that the prevalence of hr-HPV infection decreases with age.
Table 2 presents Odds ratios of presence of hr-HPV for the different age cohorts. The results were adjusted for age. The Odds ratio of the presence of hr-HPV in bacterial imbalanced flora was higher in the *Gardnerella* group compared to reference group (OR 1.6; CI 1.2-1.9).

	Age	n	Number of hr-HPV	Prevalence of hr-HPV
Bacterial imbalance	<20	6	5	83.3
	20-24	11	8	72.7
	25-29	18	10	55.6
	30-34	23	7	30.4
	35-39	23	17	73.9
	40-44	17	3	17.6
	45-49	14	3	21.4
	50-54	11	2	18.2
	55-<60	7	3	42.9
	All	130	58	44.6
Normal flora	<20	53	19	35.8
	20-24	210	95	45.2
	25-29	286	101	35.3
	30-34	587	191	32.5
	35-39	581	165	28.4
	40-44	535	107	20.0
	45-49	493	91	18.5
	50-54	350	59	16.9
	55-<60	198	22	11.1
	All	3293	850	25.8

Table 1 Data stratified by age: hr-HPV prevalence rates in bacterial imbalanced flora versus normal flora.

hr-HPV in bacterial imbalanced vaginal flora		
Age (years)	**OR**	**95% CI**
<20	2.8	1.9 – 4.0
20-29	1.6	1.1 – 2.3
30-39	1.7	1.3 – 2.3
40-49	1.0	0.5 – 1.9
50-<60	1.8	0.9 – 3.8
All	1.6*	1.2 – 1.9

Table 2 Prevalence of hr-HPV in bacterial imbalanced flora versus cytological normal flora.

* weighted by age

Table 3 displays the hr-HPV type-specific prevalence rates in bacterial imbalanced flora versus healthy flora. There were no significant differences. Owing to the low prevalence of cytologically diagnosed *Gardnerella* infection, the number of such slides was low (n=4). However, when focusing on the distribution of specific HPV genotypes, an interesting

pattern of distribution is apparent, that is, 75% (3) of the *Gardnerella* smears was infected by hr-HPV genotypes, whereas 25% (1) showed infection of an unknown type. This in contrast to smears showing a normal flora where 25.8% showed hr-HPV (data not displayed).

Type	O3+O5*	O6	O3+O5 versus O6**
	N=77	N=1137	
	n (%)	n (%)	OR (95% CI)
16	17 (22.1)	314 (27.6)	0.8 (0.5–1.2)
18	7 (9.1)	69 (6.1)	1.5 (0.7–3.1)
31	6 (7.8)	110 (9.7)	0.8 (0.4-1.8)
33	2 (2.6)	46 (4.0)	0.6 (0.2-2.6)
35	1 (1.3)	14 (1.2)	1.1 (0.1-7.9)
39	4 (5.2)	33 (2.9)	1.8 (0.7-4.9)
45	2 (2.6)	39 (3.4)	0.8 (0.2-3.1)
51	11 (14.3)	197 (17.3)	0.8 (0.5-1.4)
52	5 (6.5)	64 (5.6)	1.2 (0.5-2.8)
53	6 (7.8)	74 (6.5)	1.2 (0.5-2.7)
56	3 (3.9)	22 (1.9)	2.0 (0.6-6.6)
58	4 (5.2)	45 (4.0)	1.3 (0.5-3.6)
59	1 (1.3)	13 (1.1)	1.1 (0.2-8.6)
66	6 (7.8)	63 (5.5)	1.4 (0.6-3.1)
68	2 (2.6)	19 (1.7)	1.6 (0.4-6.6)
73	-	15 (1.3)	-

Table 3 High-risk HPV type-specific prevalence rates in bacterial imbalanced flora (O3+O5) versus normal flora (O6)

* adjusted for age

** generalized X^2 test statistics showed no significance

Discussion

Infection by certain types of HPV is recognised as causal and necessary factor for cervical cancer.[41-43] Cervical cancer represents the second most common malignancy in women around the world and contributes to 9.8% of all female cancers.[44] Clifford et al.[45] thoroughly evaluated the prevalence of HPV in 13 countries estimating that 6.6% of women in the age range 15-74 years with normal cytology are carriers of HPV DNA, with marked variation within and between world regions (range 1.4-25.6%). Unequivocally, HPV can be considered as the most common known sexually transmitted agent worldwide.

Nowadays the acquisition of HPV is generally believed to be the result of sexual behavior and frequency of sexual activity.[16] Our results append another aspect concerning the 'ambiance' that might provide a preferable milieu in which the hr-HPV thrives best.

In this study we investigated whether hr-HPV infection has a preference for a bacterial imbalanced vaginal flora, as determined in Papanicolaou stained cervical smears (OR 1.6; CI 1.2-1.9).

Our results might indicate that bacterial imbalance of the vaginal flora should be treated for the reason that hr-HPV infection better thrives in a milieu of bacterial imbalance.

Although numbers of *Gardnerella* infection in our study are very small (n=4), the observation that 75% of those smears shows prevalence of hr-HPV infection might challenge future research concerning the fact that *Gardnerella* infection represents an extreme shift of bacterial imbalance in which milieu the preference of hr-HPV could be more pronounced than in dysbacteriosis (a mild disturbance of the vaginal flora).

Furthermore we attempted to discover correlation patterns between type-specific hr-HPV infections and bacterial imbalance of the vaginal flora. According to our results a type-specific pattern could not be established since general statistics showed no significance. This may be due to the small number of samples coded as bacterial imbalance, which is inherent to the relative low prevalence of dysbacteriosis and *Gardnerella* infection. In the future times, we should therefore embark on research with larger numbers of smears showing bacterial imbalance of the vaginal flora. Profound study on this topic might unravel the possible pattern of preference of certain hr-HPV genotypes.

Finally, the prevalence of hr-HPV infection in this study decreases with increasing age. This is consistent with the findings of De Sanjosé et al.[46]. They found that in all world regions, HPV prevalence was highest among women younger than 35 years of age, decreasing in women of older age. The lower prevalence of hr-HPV infection observed in older women might be the result of a more stable sexual behavior, lower frequency of sexual activity or an acquired immunity to hr-HPV from previous exposure. Studies have shown that the clearance of HPV infection shows an interesting transition with age; the clearance of hr-HPV infection decreases with age.[16,47] Other possible mechanisms, which may influence hr-HPV clearance, include menopause-related changes of the cervicovaginal epithelium and age-related immunity.[48] We may conclude that although the prevalence of hr-HPV infection decreases with age there is never a complete absence of the risk.

The outcome of this study raises questions about the high prevalence of hr-HPV in bacterial imbalanced vaginal flora. Further research should answer the question about

the underlying cause: (1) is a bacterial imbalanced vaginal flora susceptible to acquiring hr-HPV (2) is clearance of hr-HPV compromised by a bacterial imbalanced flora?

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General discussion

General discussion

In 1996, the recent version of the Dutch national coding system for pathological findings in cervical cytology, KOPAC, was introduced.[1] Nowhere in the world such a specific coding system does exist. The internationally used standard on scoring vaginal microflora is the Bethesda System, which identifies five categories of organisms in cervical cytology[2,3]: (1) *Trichomonas vaginalis*, (2) fungal organisms morphologically consistent with *Candida spp*, (3) shift in flora suggestive of bacterial vaginosis, (4) bacteria morphologically consistent with *Actinomyces spp* and (5) cellular changes consistent with herpes simplex virus. Comparing both systems, it becomes clear that KOPAC is providing a more differentiated model of coding bacterial imbalance of the vaginal flora. In other words, dysbacteriosis and *Gardnerella* infection appear to be two cytological subdivisions of Bethesda's third category: 'shift in flora suggestive of bacterial vaginosis'.[4] In the Leiden Cytological and Pathological Laboratory (Leiden, Netherlands), both subdivisions were documented on a relative regular basis, but seemed to remain without clinical consequences in asymptomatic women.

As the Bethesda system only provides one category for bacterial imbalance and both inflammatory states, the question might raise whether both cytological states of bacterial imbalance can be differentiated unequivocally. For this reason we designed a cytological study. As demonstrated in chapter 3, in dysbacteriotic samples lactobacilli are few and replaced by a mixture of anaerobic bacteria, mainly *Gardnerella vaginalis,*[5,6] present on the so-called clue cells (CCs) and in addition in the background of the smear. Smears with pure *Gardnerella* patterns, reflecting an *extreme* shift in vaginal flora towards *Gardnerella vaginalis*, display *blue mountain cells* (BMCs) whereas they are completely covered by a mountain of blue staining round bacteria which cling to the cellular membrane (chapter 4).[4] Dutch screeners are taught how to recognize evidence of *Gardnerella* infection by the presence of BMCs in the stained smear and dysbacteriosis based on CCs.[5,6,7] Accordingly, a study was conducted to identify possible problems that can hamper clear identification of these two entities based on above mentioned cytological criteria (chapter 5). Therefore, 300 randomly selected samples (dysbacteriosis, *Gardnerella* infection and healthy flora) were rescreened by two pathologists. Rescreening resulted in 65% of unequivocal diagnoses in smears primarily diagnosed as dysbacteriotic. Eighty percent of the *Gardnerella* smears showed an unequivocal diagnosis and as much as 93% of the healthy smears was unequivocally diagnosed. Misclassification of *Gardnerella* infection occurred in the presence of clusters of bacteria mixed with spermatozoa (chapter 5). Based on these findings, this thesis clearly demonstrates that dysbacteriosis and *Gardnerella* infection can be identified unequivocally and can easily be distinguished from smears with a normal flora (chapter 5). This is an interesting finding since evaluation of the inflammatory status of the vaginal flora comprises the advantageous spin off of cervical screening which should be exploited for the benefit of the screened women. In fact, the situation is ideal because the purpose of the program, detection of (pre)cancer, is not aimed at the detection of cervical infections.

As the vaginal flora is influenced by different factors as sexual activity, contraceptive choice and personal hygiene, it is interesting to analyse possible trends concerning dysbacteriosis and *Gardnerella* infection over the last decade. This thesis, as described in chapter 2, illustrates a remarkable shift in the prevalence of dysbacteriosis (34.8‰) and *Gardnerella* infection (3.0‰), both indicating bacterial imbalance, among all age cohorts, being strongly decreased in the second screening (respectively 27.4 and 1.2‰). We suggest that this change might be caused by an increased awareness of the importance of a healthy vaginal flora. During the last ten years several women have become more concerned with a healthy vagina, as in such a flora the chance on overgrowth with *Gardnerella vaginalis* and other anaerobic bacteria diminishes.

As cervical inflammation has been proposed as cofactor in the development of cervical cancer,[8-11] this thesis documents the prevalences of cervical (pre)neoplastic disease in women with dysbacteriosis (chapter 3) or *Gardnerella* infection (chapter 4). Previous studies have investigated on this topic. Several studies indicated that bacterial vaginosis diagnosed by clinical criteria (e.g. Amsel test, Nugent score) showed not to be associated with CIN.[10,12-14] Other studies produced contrasting results demonstrating a strong association between the two conditions.[9,15,16] However, as bacterial vaginosis is diagnosed by clinical criteria[17,18] no published studies have looked at the prevalence of cervical (pre) neoplasia among smears of *asymptomatic* women with bacterial imbalance. Because of this large asymptomatic group of women, it is critical to indeed study the prevalence of cervical (pre)neoplastic cells in dysbacteriosis and *Gardnerella* infection. As the KOPAC system primarily enables coding of the squamous epithelium, ranging from KOPAC P1 (WNL or normal), via KOPAC P2-3 (ASCUS or borderline) to KOPAC P9 (macro invasive squamous cell carcinoma) prevalence of (pre)neoplastic abnormalities in dysbacteriosis and *Gardnerella* infection are presented in this thesis (chapter 3 and 4). Results demonstrate that *Gardnerella* infection strongly covaries with the presence of cervical (pre)neoplasia in the same smear. Concerning dysbacteriosis, the Odds ratio (weighted by age) of the presence of HSIL/carcinoma was significantly higher in the dysbacteriosis group compared to reference group (OR 2.0; CI 1.8-3.2). However, this relation is far more pronounced in smears with *Gardnerella* infection (OR 10.3; CI 6.6-16.1). This might be explained by the fact that *Gardnerella* comprises a pure pattern and an *extreme* shift towards *Gardnerella vaginalis*, reflecting a higher susceptibility to acquisition and persistence of HPV infection (as discussed further). This, at the same time, implicates a limitation on the study described in this thesis; as details concerning HPV status are not registered with smear taking in the national screening program, data could not be adjusted for HPV status.

Modern techniques in diagnosing bacterial imbalance

Although cytology has proven to be an accurate tool in diagnosing bacterial imbalance, modern technologies gain more and more terrain. This phenomenon challenges for further research on new diagnostic tools in determining bacterial imbalance.

Liquid-based cytology (LBC) is widely used for cancer screening because the detection of the (pre)malignant cells is enhanced by immediate fixation and lowering the background of the cell sample in the preparation process.[19-21] Hopwood et al. showed that it is

feasible to use LBC samples (originally collected for cervical screening) for chlamydia screening.[22] In our LBC screening practice, we noticed that, in addition, the adhering properties of the vaginal flora are evident in the Papanicolaou-stained LBC slides thus facilitating the cytological diagnosis of a disturbed vaginal flora. This thesis describes efforts to determine the morphotype of the adherent bacteria in LBC in smears with healthy and imbalanced vaginal flora and to use PCR technology on the same fixed cell sample to establish DNA patterns of the 16S RNA genes of the bacteria in the sample (chapter 6).

Studies on many habitats have demonstrated the limitations of cultivation-dependent methods to assess microbial community composition. In most instances, this is due to the selectivity of growth media and conditions.[23] For this reason we turned to DNA-PCR in combination with formalin-free coagulant fixative[24] in which DNA is very well preserved thanks to fast change of the three-dimensional structure of the enzymes that degrade DNA (chapter 6).[25,26] As a consequence reliability of the results presented in this thesis was increased.

According to previous studies diverse species and strains of *Lactobacillus* show properties which may protect the vaginal epithelial cells from colonization by pathogens.[27,28] The ability of rod-shaped lactobacilli to adhere to mucosal epithelial cells seems to be important in the establishment of an indigenous bacterial flora and perhaps in infectious disease.[29,30] PCR studies of the many lactobacillus strains present in vaginal samples have recently become available. *Lactobacillus crispatus*, which was previously classified in the *Lactobacillus acidophilus* group, has been identified as the most prevalent lactobacillus in the normal vaginal flora.[31,32] Probably this is because *Lactobacillus crispatus* produces H_2O_2 and possesses strong aggregation and adhesive properties together with the ability to recognize and bind to the sugar chain of the A-antigen structure, which plays an important role in the immune system of the vagina.[33-35] Mårdh et al. showed that lactobacilli have a lower adherence capacity per cell than *Gardnerella vaginalis*.[29] Sobel et al. and Peeters et al. both showed that adherence of *Gardnerella vaginalis* depends on pH of the vagina.[36-38] The mechanism of this is not yet understood. In addition, this thesis demonstrates that the adhesion pattern of bacteria in dysbacteriotic and *Gardnerella* smears appears to be characterized by the number of adhering lactobacilli (chapter 6).[39]

Zariffard et al. studied whether *Gardnerella vaginalis* and lactobacilli could be detected in vaginal specimens, and also their relation to each other, using quantitative real-time PCR. They concluded that there is an inverse relationship between *Gardnerella vaginalis* and lactobacilli suggesting that these findings could be developed into a new method in detecting a disturbed vaginal microflora.[40] In this context, this thesis also reveals a marked inverse relationship between the concentrations of *Gardnerella vaginalis* and *Lactobacillus crispatus* (chapter 6). That is, by means of quantitative PCR analysis, dysbacteriosis and *Gardnerella* infection are indicated by high values of *Gardnerella vaginalis* and low values of *Lactobacillus crispatus*, the phenomenon being more pronounced in smears displaying *Gardnerella* infection.

Based on literature, women colonized by H_2O_2-producing lactobacilli have decreased acquisition of bacterial vaginosis.[41] As afore mentioned and consistent with other studies,

the present thesis indicates that *Lactobacillus crispatus* is one of the most predominant H_2O_2-producing species found in the normal vaginal flora (chapter 6).[41-43] In addition, Vallor et al, demonstrated that this species is clearly associated with healthy microflora, and possibly better ensures a stable and healthy microflora than other lactobacilli.[44] Given these concerns including the fact that colonization of lactobacilli protects against acquisition of bacterial vaginosis and *Lactobacillus crispatus* fulfills a predominant role in the defence process, this thesis analysed the colonization of *Gardnerella vaginalis* and *Lactobacillus crispatus* among asymptomatic women as established in population-based screening (chapter 7). In literature, the colonization status of these two bacteria among asymptomatic women as well as the influence of age is unknown. Perhaps the difference in colonization patterns of vaginal smears could be helpful to improve the interpretation of clinical data in future studies, such as the understanding of response to treatment and recurrence of bacterial vaginosis in some women.[45]

The prevalence of colonization by *Gardnerella vaginalis* among asymptomatic women was stable at approximately 60% in concordance with De Backer et al.[46] while the overall colonization of *Lactobacillus crispatus* was only 38.9% (chapter 7). The number of colonized smears by *Gardnerella vaginalis* was slightly higher among postmenopausal women (63.6%), but this difference showed no significancy. Far more interesting was the fact that only 19% of the vaginas of postmenopausal women is colonized by *Lactobacillus crispatus* (chapter 7). These results reveal a remarkable difference between the pre- and postmenopausal colonization of *Lactobacillus crispatus* among asymptomatic, healthy women underlining that the menopause leads to a significant decline in *Lactobacillus crispatus*. The menopausal transition implies a series of hormonal changes. As ovarian function decreases and fertility disappears, circulating estrogen levels are first increased and then decrease,[47] and there is a shift in estrogen production from the ovaries to extragonadal sites.[47,48] Loss of *Lactobacillus crispatus* could thus be caused by cessation of production of female sex hormones. However, with the appearance of climacteric symptoms, exogenous hormones are widely used; these hormones interact with a changing preexisting hormonal status. In this context, Pabich et al.[49] demonstrated in their case-control study that 62% of the postmenopausal women was colonized by vaginal lactobacilli which were significantly more prevalent among women receiving hormone replacement therapy during the previous year. On that account, our data concerning the decline in colonization with *Lactobacillus crispatus* should be even more pronounced (chapter 7). Furthermore this thesis indicates that *Gardnerella vaginalis* and *Lactobacillus crispatus* might interfere with each other, although further research is needed (chapter 8). In this respect, Strus et al.[50] studied the in vitro effects of hydrogen peroxide on vaginal microbial communities, whereas the killing effect of hydrogen peroxide registered. *Lactobacillus crispatus* was found having high antimicriobial activity against *Gardnerella vaginalis*, suggesting inhibition.

As previously discussed in this section, a marked inverse relationship was found between the DNA-concentration of *Lactobacillus crispatus* and *Gardnerella vaginalis* in smears with dysbacteriosis and *Gardnerella*. As a consequence, this thesis presents further examination herewith in order to establish a potential tool in diagnosis of bacterial imbalance to make

diagnostic procedures less time-consuming and subjective (chapter 8). Moreover, since smears with dysbacteriosis or *Gardnerella* infection show a higher prevalence of HSIL/carcinoma it is of great importance to create objective and simple diagnostic tools in detection of the bacterial imbalanced vaginal flora.
Fredricks et al.[39] confirmed the poor specificity of *Gardnerella vaginalis* in bacterial vaginosis. Using the Amsel criteria as gold standard, *Gardnerella vaginalis* was detected by PCR in 96% of subjects with bacterial vaginosis, but was also in 70% of subjects without bacterial vaginosis. Studies in this thesis propose that a combination of concentration of *Gardnerella vaginalis* and *Lactobacillus crispatus* established by PCR would be a more reliable indicator of bacterial imbalance (chapter 6 and 8). Surprisingly, as discussed in the thesis, bacterial imbalance might indeed be characterized as hypothesized (chapter 8). Moreover, our data suggest that diagnosis on the basis of PCR-techniques will probably provide more effective procedures in determining healthiness of the vaginal flora. Unfortunately, this method appeared not to be capable of differentiating the two cytological subdivisions of bacterial imbalance. According to this finding it should be emphasized that cytology possesses the best diagnostic potential in differentiating the two states of bacterial imbalance. This advantageous feature is probably enclosed in the fact that cytology is focussing on the behaviour of bacteria instead of quantifying DNA-concentrations (chapter 5).

Bacterial imbalance and HPV infection
Reviewing literature it becomes clear that the presence of koilocytic cells function as an indicator for active HPV-infection, whereas such an infection leads to excavitation of the cytoplasms of the cell, resulting in pathognomic abnormality of squamous cells.[51-53] Researching the 10 year database of the western region of the Dutch national screening program this thesis reveals new data. Over the past decennium, there has been a strong increase of HPV-related koilocytosis among young women (30-35 year) which can be a sign of an HPV epidemic possibly caused by a changed sexual behaviour and/or unsafe sex (chapter 2).[54] Not all samples from women with an active HPV-infection show koilocytosis, which means that the actual prevalence of HPV higher. Nevertheless, determining the recent trend in prevalence of koilocytosis using the cytology in both screening periods provides a reliable image of the trend. In this context we ought to remark that the number of smears with bacterial imbalance significantly decreased over the last decade, which perhaps led to more frequent diagnosis of koilocytosis: when a smear showed two or more inflammation patterns, a choice was made whereas only one O-code could be given. In addition, as nowadays HPV infection is diagnosed by PCR, these cytological data should be seen as an illustration of the current trend in the acquisition of HPV.

Series of laboratory studies and epidemiological investigations have demonstrated HPV as a central cause of cervical cancer in women incapable clearing the virus.[55-60] However, since only a small fraction of infected women will go on to develop cancer, other factors should contribute to this process. The general hypothesis is that the local cervicovaginal milieu plays a role in susceptibility to HPV infection, whereas women that possess an unhealthy *Lactobacillus*-poor vaginal flora, should be more prone to acquiring HPV infection.[8,55-58] This is consistent with the findings reported in this thesis regarding the

high prevalence of HSIL/carcinoma in smears displaying bacterial imbalance. Persistent infection with one or more types of high-risk human papilloma virus (hr-HPV) is the main risk factor for the development of cervical carcinoma.[61,62] In accordance with the results in this thesis, the prevalence is highest in sexually active young women and decreases with age.[63-65] Although the lifetime risk of acquiring HPV infection is about 80%[66], the incidence of cervical intraepithelial neoplasia and invasive carcinoma is relatively low.[67-70] This can be explained by the infection pattern of HPV which is mostly transient and asymptomatic.[65,71,72] In most cases the HPV infection disappears within 6 to 14 months.[59,63,73] As described in chapter 9, a number of 19,793 cervical samples was tested for 26 HPV-genotypes using the short fragment polymerase chain reaction hybridization line probe assay (LiPA). We observed that hr-HPV is indeed preferring the bacterial imbalanced flora (OR 1.6; CI 1.2-1.9). According to the results of the concerning study, this thesis postulates that bacterial imbalanced states of the vaginal flora should be treated for reason that hr-HPV infection better thrives in a milieu of bacterial imbalance.

The lower prevalence of hr-HPV infection observed in older women might be the result of a more stable sexual behavior, lower frequency of sexual activity or an acquired immunity to hr-HPV from previous exposure. Studies have shown that the clearance of HPV infection shows an interesting transition with age; the clearance of hr-HPV infection decreases with age.[42,43] Other possible mechanisms, which may influence hr-HPV clearance, include menopause-related changes of the cervicovaginal epithelium and age-related immunity.[74] As the acquisition of HPV is generally believed to be the result of sexual behavior and frequency of sexual activity our results append another aspect concerning the 'ambiance' that might provide a preferable milieu in which hr-HPV thrives best. Unfortunately, the study was not able to establish a type-specific pattern since general statistics showed no significance. This may be due to the small number of samples coded as bacterial imbalanced, which is inherent to the prevalence of this inflammatory state in comparison to that of healthy smears.

Finally, a shortage of lactobacilli resulting in a shift towards a flora containing *Gardnerella vaginalis* increases the effects of HPV and a disturbance of the vaginal flora enhances acquisition of HPV infection.[67,75-77] This might explain the finding described in chapter 9; owing to the low prevalence of cytologically diagnosed *Gardnerella* infection, the number of such samples was low (4 samples). However, when focusing on the distribution of specific hr-HPV genotypes, an interesting pattern of distribution is apparent, that is, 75% (3 samples) of the *Gardnerella* smears was infected by hr-HPV genotypes, whereas 25% (1 sample) showed infection of an unknown type. This in contrast to smears showing a healthy flora where 25.8% showed hr-HPV and dysbacteriotic smears alone showed a percentage of high-risk infection of 42.6%. This finding shows that the extreme form of bacterial imbalance might be a very crucial co-factor along with HPV, although that further research with larger numbers of cervical samples on this topic is essential.

Conclusions

This thesis presents studies undertaken to investigate several aspects of cytologically diagnosed bacterial imbalance among asymptomatic women whose cervical samples displaying a shift towards *Gardnerella vaginalis*.

Based on the studies discussed in this thesis several conclusions can be made (numbers refer to the research questions as proposed in chapter 1).

1. As established in population-based screening the prevalence of dysbacteriosis (34.8‰) and *Gardnerella* infection (3.0‰), both representing bacterial imbalance has significantly decreased over the past decade, respectively 27.4‰ and 1.2‰.
2. Dysbacteriotic smears show a significant higher prevalence of cervical (pre) neoplasia.
3. Cytologically diagnosed *Gardnerella* smears show a strong covariation with the presence of cervical (pre)neoplasia being far more pronounced than in dysbacteriosis.
4. *Gardnerella* infection and dysbacteriosis can be identified unequivocally in cervical smears, whereas *Gardnerella* infection is a more stable diagnosis than dysbacteriosis.
5. The adherence pattern of a disturbed flora in liquid-based cervical samples can be identified unequivocally. Such samples also appeared to be suitable for quantitative PCR analysis, as an inverse relationship was seen between *Gardnerella vaginalis* and *Lactobacillus crispatus* in the presence of a vaginal flora characterized by bacterial imbalance.
6. As the prevalence of *Gardnerella vaginalis* shows no variation among asymptomatic women among different age groups (58.7%), colonization of *Lactobacillus crispatus* in postmenopausal women significantly decreases from 40.5 to 19.5%. Second, both bacteria might interfere as *Gardnerella vaginalis* was more frequent in non-*Lactobacillus crispatus* flora as *Lactobacillus crispatus* was oftentimes colonizing non-*Gardnerella* microflora.
7. DNA-concentrations of *Gardnerella vaginalis* and *Lactobacillus crispatus* might be valuable markers of bacterial imbalance, however the differentiation between dysbacteriosis and *Gardnerella* infection cannot not be made.
8. High-risk HPV genotypes have a strong preference for a bacterial imbalanced flora showing *Gardnerella vaginalis* and a shortage of lactobacilli.

Implications for clinical practice and future research

As disturbance of the vaginal flora seems to be related to the presence of cervical (pre) neoplasia, the improvement of bacterial balance among women in the western region of the Netherlands is considered a beneficial change. As the hypothesis is that women are more aware of the importance of a healthy vagina education concerning vaginal hygiene should be continued and intensified as should also be the instruction about sexual behaviour.

Nevertheless, the strong increase of HPV-related koilocytosis among young women is an ominous development. Further research, concerning differences especially focussed on sexual behaviour and vaginal hygiene, is needed to acquire more insight in the reason of these differences.

Most important, women with dysbacteriosis or *Gardnerella* infection should be treated efficiently, for instance with metronidazole, to irradicate *Gardnerella vaginalis*.[78-80] In

addition, bacterial imbalance of the vaginal flora can also serve to identify women who perhaps warrant more intensive surveillance concerning life-style factors including post coital hygiene. In this context it would be of interest to embark on research focused on sexual behavior and vaginal hygiene using smears to establish shifts in the vaginal flora.
Finally, the poor efficacy of antibiotics and their failure to eradicate *Gardnerella vaginalis* and restore a healthy lactobacilli population, has led to an increased interest in alternative approaches. This thesis postulates that *Lactobacillus crispatus* could perhaps be a valuable ingredient of probiotic vaginal suspensions. Recently, some probiotics have shown to reduce the risk of bacterial vaginosis and not only augment antibiotic cure through oral use, but also cure via direct intravaginal instillation.[81] In the context of studying the influence of the vaginal flora on cervical carcinogenesis it might be of interest to establish the effects of interventions with lactobacilli supplements and metronidazole treatments in women with a bacterial imbalanced vaginal flora.
As series of studies have investigated the impact of clinical syndromes on the risk of cervical carcinogenesis, data presented in this thesis underline that analysing large databases of asymptomatic women as provided by the Dutch national screening system, KOPAC, is of highly importance to detect whether asymptomatic features could also predispose for acquiring cervical (pre)neoplastic abnormalities. Therefore, coding of diverse and differentiated aspects of cervical samples in large databases of asymptomatic women will provide more insight in the complex pattern of co-factors related to cervical carcinogenesis.
Consequently, as this thesis shows that dysbacteriosis and cervical *Gardnerella* infection represent cytological subdivisions of a bacterial imbalanced vaginal flora showing different prevalences and impact concerning cervical (pre)neoplastic abnormalities, further research should be undertaken to discover whether Bethesda's category for coding vaginal flora is needed to be revised.

According to the differences in the prevalence of (pre)malignant cells in dysbacteriosis and cytological normal smears, our findings might be relevant for cytotechnologists screening cervical smears. They should be taught how to accurately recognize bacterial imbalance as awareness of the possible consequences of a disturbed flora is crucial during the process of screening.
Dysbacteriosis and cervical *Gardnerella* infection represent cytological subdivisions of a bacterial imbalanced vaginal flora. As this thesis demonstrated that *Lactobacillus crispatus* and *Gardnerella vaginalis* might provide a diagnostic tool in diagnosing bacterial imbalance, more research should be undertaken to come to a practical tool. Also, further research is needed to explore in what way the biomolecular differentiation can be made between dysbacteriosis and *Gardnerella* infection, using cultivation-independent methods as real-time PCR. Moreover, our data suggest that diagnosis on the basis of PCR-techniques will probably provide more effective procedures in determining healthiness of vaginal flora. Future research is needed to evaluate the colonization patterns of *Gardnerella vaginalis* and *Lactobacillus crispatus* in vaginal flora of healthy women. It might become possible to predict susceptibility to vaginal infections or cervical (pre)neoplasia using coagulant fixatives which allow for DNA-PCR on cervical samples.

Finally, based on our remarkable findings we propose that future research will discover whether there exist specific concentration ratios whereby the different inflammatory states of the vaginal flora can be diagnosed. In such studies concentrations of *Lactobacillus crispatus* and *Gardnerella vaginalis* should also be determined in other vaginal inflammations, e.g. *Candida* and *Trichomonas vaginalis*.
In short, our findings might be essential in developing a framework for diagnosing bacterial imbalance of the vaginal flora by concentrations of both *Gardnerella vaginalis* and *Lactobacillus crispatus* established by DNA-PCR. It might become possible to establish a new tool in diagnosing bacterial imbalance of the vaginal flora using formalin-free coagulant fixatives which allow for DNA-PCR on cervical samples.

The preference of high-risk HPV genotypes for a bacterial imbalanced milieu, as described by this thesis, insistently challenges future research as this thesis demonstrates that the grade of bacterial imbalance reflects the prevalence of hr-HPV. In the future, we should therefore embark on research with larger numbers of smears showing bacterial imbalance of the vaginal flora. Profound study on this topic will definitely elucidate the mechanism of preference of certain hr-HPV genotypes. Further research should answer questions about the underlying cause of the findings concerning HPV, as discussed in this thesis. Does the bacterial imbalanced vaginal flora by *Gardnerella vaginalis* provides a susceptibility to acquiring hr-HPV or is clearance of hr-HPV compromised in such a flora?

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11

Summary

Summary

Chapter 1
In the introduction the rationale for the studies presented in this thesis is explained. Since vaginal inflammation has been suggested as possible risk factor in cervical carcinogenesis, research has drawn attention to this topic. Bacterial imbalance is a microscopic diagnosis representing cytological disturbance of the vaginal microflora and therefore independent of clinical criteria.
In 1988, nationwide cytological screening for cervical cancer and its precursors has been introduced in the Netherlands. Since 1996 all women aged 30 to 60 years are invited for a cervical smear on a 5 year interval. KOPAC, the Dutch national coding system for cervical smears, introduced for determination of cervical abnormalities also evaluates the inflammatory status of the vaginal flora. In this system dysbacteriosis and *Gardnerella* infection comprise two different cytological entities reflecting bacterial imbalance, the former representing a mild disturbance, the latter an extreme shift towards *Gardnerella vaginalis*. This thesis addresses several aspects of bacterial imbalance caused by *Gardnerella vaginalis* among asymptomatic women as established in population-based cervical screening.

Chapter 2
This chapter presents an overview of recent trends in prevalence of inflammatory states of the vaginal flora over the last decade. Prevalence of koilocytosis, *Trichomonas vaginalis*, dysbacteriosis, *Candida, Gardnerella vaginalis*, *Actinomyces* and *Chlamydia trachomatis* and non-specific inflammatory changes, as coded according to KOPAC are calculated. From 1996-2005 the Leiden Cytology and Pathology Laboratory received more than 500,000 cervical smears in the context of the national screening program. Prevalences of the first screening period (1996-2000) were compared with that of the second screening period (2001-2005). Bacterial imbalance (dysbacteriosis and *Gardnerella* infection) has significantly decreased over the past decade in all age cohorts. From 2001-2005 prevalence of dysbacteriosis and *Gardnerella* infection accounted for respectively 27.4 and 1.2 per thousand smears, while prevalences in the first screening period were 34.8 and 3.0. Over the last decade women might have become more aware of the importance of a cytological normal vagina flora. The number of cases of HPV-related koilocytosis has dramatically increased among young women (age cohorts 30 and 35 years).

Chapter 3
This chapter documents the prevalence of cervical (pre)neoplasia in dysbacteriotic smears. Data were collected from 800,498 Dutch asymptomatic women participating in the national screening program. A total of 30,593 dysbacteriotic smears was compared with 227,580 smears displaying a healthy vaginal flora. Prevalence of (pre)neoplasia was calculated for dysbacteriotic smears using a healthy flora as reference. The Odds ratio for the presence of HSIL/carcinoma was significantly higher in smears with dysbacteriosis (OR 2.0; CI 1.8-2.3). Dysbacteriotic smears show a significant higher proportion of cervical (pre) neoplasia compared to smears displaying a healthy flora.

Chapter 4

This chapter outlines the relationship between *Gardnerella* infection and the presence of cervical (pre)neoplastic changes in asymptomatic women. The prevalence of HSIL/carcinoma was studied in 498 cervical smears displaying *Gardnerella* infection. Data were compared with a reference group consisting of 227,580 smears also originating from the cervical screening program. The Odds ratio for (pre)neoplasia was significantly higher in smears with *Gardnerella* infection compared to smears of women with a healthy vaginal flora (OR 10.3; CI 6.6-16.1). Cytologically diagnosed *Gardnerella* smears show a strong covariation with the presence of cervical (pre)neoplasia. Future research should therefore focus on the exact causal relation between cytological *Gardnerella* infection and the presence of (pre)neoplastic changes of the cervix.

Chapter 5

The study described in this chapter was conducted in order to prove that dysbacteriosis and *Gardnerella* infection can be clearly differentiated based on cytological criteria and to obtain insight into the diagnostic problems of *Gardnerella*. Dysbacteriosis and *Gardnerella* infection are both cytological diagnoses indicating a shift in vaginal flora, the former the mild version of ecological disturbance with the presence of clue cells (CCs), the latter the extreme version with so called blue mountain cells (BMCs). One hundred randomly selected samples of each of the three diagnostic series were rescreened by two pathologists resulting in a consensus diagnosis. A smear was considered unequivocal when the original code and the code of the consensus diagnoses were equal. Unequivocal diagnoses were established in 65% of the dysbacteriotic smears, 80% of the *Gardnerella* smears and as much as 93% of the healthy smears. Discordance (flora diagnoses of the two pathologists differed) was highest in the dysbacteriotic series (20%) and lowest in the healthy group (4%). Misclassification of *Gardnerella* occurred in the presence of blood and clusters of bacteria mixed with spermatozoa. This study demonstrates that *Gardnerella* infection can be identified unequivocally in cervical smears. Such advantageous spin-offs of cervical screening should be exploited.

Chapter 6

The purpose of this chapter to determine the morphotype of the adherent bacteria in liquid-based cytology (LBC) in smears with healthy and disturbed vaginal flora and to use PCR technology on the same fixed cell sample to establish DNA patterns of the 16S RNA genes of the bacteria in the sample. Thirty samples were randomly selected from a large group of cervical cell samples suspended in a formalin-free coagulant fixative. PCR was used to amplify DNA of five bacterial species: *Lactobacillus acidophilus*, *Lactobacillus crispatus, Lactobacillus jensenii, Gardnerella vaginalis* and *Mycoplasma hominis*. The LBC slides were then analysed by light microscopy to estimate bacterial adhesion. DNA of lactobacilli was detected in all cell samples. Seventeen smears showed colonization with *Gardnerella vaginalis* (range $2.4x10^2$ – $5.6x10^5$ bacteria/µl). Two cases were identified as dysbacteriotic with high DNA values for *Gardnerella vaginalis* and low values for *Lactobacillus crispatus*. The sample with the highest concentration for *Gardnerella vaginalis* showed to be a cytological *Gardnerella* infection. This study indicates that the adherence pattern of a

disturbed flora in liquid-based cervical samples can be identified unequivocally, and that these samples are suitable for quantitative PCR analysis. Finally, this study reveals a strong inverse relationship between *Gardnerella vaginalis* and *Lactobacillus crispatus* in dysbacteriosis and *Gardnerella* infection.

Chapter 7

As a previous study in this thesis indicated an inverse relationship between *Gardnerella vaginalis* and *Lactobacillus crispatus* (chapter 6), this chapter provides a baseline of colonization of these bacteria as this has never been established in asymptomatic women. A total of 1036 samples was randomly selected from a large group of Dutch asymptomatic women participating in the national screening program. The cervical material was suspended in a formalin-free coagulant fixative and collected for PCR evaluation for *Lactobacillus crispatus* and *Gardnerella vaginalis. Gardnerella vaginalis* was present in 58.7% of the women, whereas 38.9% of the samples showed *Lactobacillus crispatus*. Colonization of *Lactobacillus crispatus* in postmenopausal women decreases from 40.5 to 19.5%. Therefore, this study indicates that cessation of production of female sex hormones significantly decreases the colonization pattern of *Lactobacillus crispatus*. Furthermore, colonization by *Gardnerella vaginalis* was more frequent in non-*Lactobacillus crispatus* flora (OR 1.3; CI 1.1-1.4), as *Lactobacillus crispatus* was oftentimes colonizing non-*Gardnerella* microflora (OR 1.4; CI 1.2-1.6) suggesting a mechanism of interference.

Chapter 8

The purpose of this chapter was to examine the concentrations of *Lactobacillus crispatus* and *Gardnerella vaginalis* in bacterial imbalanced vaginal microflora as those bacteria are thought to be important in the balancing act of a healthy vaginal flora. From our large data base 29 cervical samples showing dysbacteriosis, 20 *Gardnerella* samples and 27 samples with a healthy vaginal flora were collected. All samples, suspended in a formalin-free coagulant fixative were used for PCR. Based on concentrations of *Gardnerella vaginalis* and *Lactobacillus crispatus* there appeared to be four different quadrants (A-D). All samples with concentrations of *Gardnerella vaginalis* over $2.0x10^{-2}$ng/µl displayed bacterial imbalance, whereas *Gardnerella* samples all having a concentration of *Lactobacillus crispatus* less than $3.8x10^{-4}$ng/µl were located in quadrant D. Dysbacteriosis was found in all quadrants, while healthy flora was found only in quadrant A and B. DNA-concentrations of *Gardnerella vaginalis* and *Lactobacillus crispatus* might be valuable markers of bacterial imbalance, however the differentiation between dysbacteriosis and *Gardnerella* infection could not be made. Our study reveals noteworthy data suggesting that culture-independent methods can provide new insights into diagnosis of bacterial imbalance of the vaginal flora.

Chapter 9

This chapter investigates the statistical relation between bacterial imbalance (with a shift towards *Gardnerella vaginalis* and a shortage of lactobacilli) and the presence of high-risk HPV infection. A total of 19,793 cervical samples (80% originating from routine cervical screening), suspended in a formalin-free coagulant fixative, was tested for 26 high and low-risk HPV-genotypes using the short fragment polymerase chain reaction

hybridization line probe assay (LiPA). Smears were analysed by light microscopy and coded for inflammatory status. Prevalence of hr-HPV infection was 44.6% (58/130) among smears showing bacterial imbalance of the vaginal flora versus 25.8% (850/3293) in healthy flora (OR 1.6; CI 1.2-1.9). High-risk type specific prevalence of HPV genotypes appeared not significant. Our findings indicate that high-risk HPV genotypes have a significant preference for a bacterial imbalanced flora showing *Gardnerella vaginalis* and a shortage of lactobacilli. Further research should investigate whether certain type specific HPV infections are more prominent in a bacterial imbalanced flora.

Chapter 10

This chapter finally discusses the conclusions which could be drawn from the studies presented in this thesis. Bacterial imbalance of the vaginal flora is associated with a significant higher prevalence of HSIL/carcinoma, the effect being far more pronounced in pure *Gardnerella* infections. On the basis of cytological criteria, both dysbacteriosis and *Gardnerella* infection can be clearly differentiated as established in smears originating of the Dutch national screening program.

Over the last decade, the prevalence of bacterial imbalance has significantly decreased, while HPV-related koilocytosis has dramatically increased during past decade. The former possibly caused by an increased awareness of vaginal hygiene, the latter as consequence of a changed sexual behaviour.

Second, although cytology is accurate in diagnosing bacterial imbalance, modern technologies gain more and more terrain. This thesis indicates that DNA-PCR might become a potential tool in diagnosis of bacterial imbalance.

Finally, this thesis demonstrates that high-risk HPV infection prefers the bacterial imbalanced milieu, leaving important questions as (1) does the bacterial imbalanced vaginal flora by *Gardnerella vaginalis* provides a susceptibility to acquiring hr-HPV or (2) is clearance of hr-HPV compromised by such a flora?

Samenvatting

Samenvatting

Hoofdstuk 1

Het eerste hoofdstuk vormt een inleiding op de studies die deel uitmaken van dit proefschrift, waarin een beschrijving wordt gegeven van diverse aspecten van de vaginale flora alsmede de definitie van bacteriële imbalans waarbij tevens diagnostiek en behandeling aan de orde komen. Tenslotte wordt het doel van dit proefschrift uiteengezet.

Sinds ontsteking van de vaginale flora mogelijk een rol zou kunnen spelen bij het ontstaan van baarmoederhalskanker, is wetenschappelijk onderzoek zich meer gaan richten op dit aspect. Wat betreft de rol van asymptomatische infectie met *Gardnerella vaginalis* is echter weinig tot niets bekend.

Bacteriële imbalans is een cytologische diagnose die een verstoring van het bacteriologisch evenwicht in de vaginale microflora weergeeft ongeacht de aanwezigheid van klinische symptomen. Beide diagnoses worden met enige regelmaat gesteld in de screening van uitstrijkjes in het kader van het bevolkingsonderzoek naar baarmoederhalskanker.

In 1988 werd in Nederland cytologische screening op cervixcarcinoom alsmede de voorstadia ervan geïntroduceerd. Sinds 1996 worden alle vrouwen tussen de 30 en 60 jaar iedere 5 jaar uitgenodigd om een uitstrijkje te laten maken bij hun huisarts. Alle uitstrijkjes worden volgens KOPAC, het Nederlandse coderingssysteem voor cervixcytologie, beoordeeld op de aanwezigheid van afwijkende cervixcellen, maar ook eventuele onstekingsverschijnselen van de vaginale flora worden gecodeerd aan de hand van de volgende negen categorieën, te weten: koilocytose, *Trichomonas vaginalis*, dysbacteriose, *Candida*, *Gardnerella vaginalis*, normale flora, *Actinomyces, Chlamydia trachomatis* en aspecifieke ontstekingsverschijnselen. Dysbacteriose en *Gardnerella* infectie zijn twee verschillende cytologische beelden die beide duiden op bacteriële imbalans ten gevolge van overgroei met *Gardnerella vaginalis*. In geval van dysbacteriose betreft het een milde verstoring van de vaginale flora met voornamelijk *Gardnerella vaginalis*, terwijl er bij een pure *Gardnerella* infectie sprake is van een extreme verandering in het bacteriële milieu van de vagina.

Dit proefschrift beschrijft de cytologische en biomoleculaire aspecten van bacteriële imbalans veroorzaakt door *Gardnerella vaginalis* onder asymptomatische vrouwen.

Hoofdstuk 2

Dit hoofdstuk beschrijft een retrospectieve studie waarin een overzicht wordt gegeven van recente trends in de prevalentie van de verschillende vormen van ontsteking van de vaginale flora, zoals gecodeerd binnen het KOPAC-systeem. In de periode tussen 1996 en 2005 werden door het Leids Cytologisch en Pathologisch Laboratorium te Leiden, meer dan 500.000 uitstrijkjes gescreend in het kader van het Nederlandse bevolkingsonderzoek. De gegevens van de eerste screeningsronde (1996-2000) werden vergeleken met de data van de tweede ronde (2001-2005). Op basis hiervan blijkt dat het aantal baarmoederhals uitstrijkjes met bacteriële imbalans (dysbacteriose en *Gardnerella* infectie) gedurende de afgelopen tien jaar aanzienlijk is afgenomen binnen alle leeftijdsgroepen. Van 2001 tot

2005 bedroegen de prevalenties van dysbacteriose en *Gardnerella* infectie respectievelijk 27.4 en 1.2 per 1000 uitstrijkjes, terwijl deze prevalenties in de eerste screeningsronde nog 34.8 en 3.0 bedroegen. Dit effect ligt mogelijk ten grondslag aan een toenemende bewustwording van de noodzaak van vaginale hygiëne. Het aantal gevallen van HPV-gerelateerde koilocytosis laat echter een zeer grote stijging zien onder vrouwen in de leeftijdsgroepen van 30 en 35 jaar.

Hoofdstuk 3
Dit hoofdstuk documenteert de prevalentie van cervicale (pre)neoplasie in uitstrijkjes met dysbacteriose. Een totaal van 800.498 uitstrijkjes van asymptomatische Nederlandse vrouwen uit het bevolkingsonderzoek werden verzameld. In 30.593 uitstrijkjes bleek er sprake te zijn van dysbacteriose, 227.580 uitstrijkjes toonden een gezonde flora. In beide cytologische groepen werd de prevalentie van cervicale (pre)neoplasie berekend. Uitstrijkjes met dysbacteriose tonen een significant hogere prevalentie van cervicale (pre) neoplasie in vergelijking met uitstrijkjes die een gezonde flora laten zien (OR 2.0; 1.8-2.3).

Hoofdstuk 4
De prevalentie van cervicale (pre)neoplasie in uitstrijkjes met *Gardnerella* infectie onder asymptomatische vrouwen wordt bestudeerd in hoofdstuk 4. Een groep van 498 uitstrijkjes met *Gardnerella* infectie werd vergeleken met een controlegroep bestaande uit uitstrijkjes met een gezonde flora (n=227.580), allen afkomstig uit het bevolkingsonderzoek. Uitstrijkjes met cytologisch gediagnosticeerde *Gardnerella* infectie laten een sterke samenhang zien met de aanwezigheid van cervicale (pre)neoplasie (OR 10.3; CI 6.6-16.1). Het verdient daarom aanbeveling om verder onderzoek te verrichten naar de exacte relatie tussen cytologische *Gardnerella* infectie en cervicale (pre)neoplasie alsook behandeling van asymptomatische *Gardnerella* infectie te overwegen.

Hoofdstuk 5
De studie die in dit hoofdstuk wordt beschreven werd ontworpen ten einde te bevestigen dat er een duidelijk onderscheid bestaat tussen dysbacteriose en *Gardnerella* infectie op basis van cytologische kenmerken. Ook is getracht eventuele problemen in de diagnostiek van *Gardnerella* infectie in kaart te brengen. Dysbacteriose wordt gekenmerkt door de aanwezigheid van 'clue cellen' (CCs). Bij een *Gardnerella* infectie zijn er cytologisch blue mountain cells (BMCs) waarneembaar.
Voor zowel dysbacteriose, *Gardnerella* infectie als normale flora werden honderd aselect verzamelde uitstrijkjes opnieuw gescreend door twee ervaren pathologen. De hieruit voortkomende zogenaamde consensus-diagnose werd vergeleken met de oorspronkelijke code afkomstig van routine screening. Wanneer de originele O-code en de consensus-diagnose identiek waren werd de diagnose betiteld als 'eenduidig'. Het percentage eenduidige diagnoses blijkt het hoogst in uitstrijken met een gezonde flora (93/100). Tachtig procent van de *Gardnerella* uitstrijken was eenduidig, terwijl dit voor 65% van de uitstrijken met dysbacteriose het geval was.
Wanneer het oordeel van beide pathologen niet eensluidend was werd onderling overlegd om alsnog tot een consensus-diagnose te komen. Echter, deze situatie werd betiteld

als 'incongruent'. Op grond van de bevindingen ten aanzien van eenduidigheid van de diagnoses en de mate van incongruentie blijkt dat cytologische *Gardnerella* infectie een stabiele diagnose is, welke goed is te differentiëren van dysbacteriose.

Hoofdstuk 6
Het doel van dit hoofdstuk is bepaling van het morphotype van bacteriën die aan de vaginale cellen kleven zoals wordt gezien in dunnelaag cytologie (liquid-based cytology). Op deze manier kan worden onderzocht of er verschil is in het soort aanklevende bacteriën binnen gezonde en verstoorde vaginale flora. Ook wordt beschreven op welke manier PCR (polymerase-chain-reaction) technologie gebruikt kan worden om in hetzelfde cervixmateriaal DNA patronen te detecteren van verschillende bacteriën middels 16S RNA genen van deze bacteriën. Hiertoe werden 30 aselect gekozen monsters met cervixmateriaal gesuspendeerd in een formaline-vrij fixatief. Middels PCR werd DNA vermenigvuldigd van de volgende vijf bacteriën: *Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus jensenii, Gardnerella vaginalis en Mycoplasma hominis*. De dunnelaag monsters werden vervolgens onderworpen aan microscopische analyse ten einde een schatting te maken van de mate van bacteriële adhesie.
In alle celmonsters werd DNA van lactobacilli gevonden. In 17 monsters werd *Gardnerella vaginalis* gemeten ($2.4x10^2$ – $5.6x10^5$ bacteriën/µl). Twee monsters toonden een dysbacteriotische flora en lieten eveneens hoge DNA-concentraties zien voor *Gardnerella vaginalis*, terwijl de concentratie *Lactobacillus crispatus* in beide monsters laag was. Het monster met de hoogste concentratie *Gardnerella vaginalis* bleek microscopisch een *Gardnerella* infectie.
Deze studie bewijst dat het kleefpatroon van bacteriën in een verstoorde flora duidelijk kan worden geïdentificeerd. Ook zijn dezelfde cervixmonsters geschikt voor kwantitatieve PCR analyse. Tenslotte toont deze studie een sterke tegengestelde relatie tussen de concentratie van *Gardnerella vaginalis* en *Lactobacillus crispatus* in dysbacteriose en *Gardnerella infectie*.

Hoofdstuk 7
Gezien de reeds eerder beschreven tegengestelde relatie tussen *Gardnerella vaginalis* en *Lactobacillus crispatus* in bacteriële imbalans van de vaginal microflora (hoofdstuk 6), wordt in dit hoofdstuk het kolonisatiepatroon van beide bacteriën in asymptomatische vrouwen beschreven. Hiertoe werden 1036 willekeurige uitstrijken geselecteerd merendeels afkomstig van vrouwen uit het Nederlandse bevolkingsonderzoek. Het cervixmateriaal werd gesuspendeerd in een formaline-vrij coagulerend fixatief en voorbereid voor PCR diagnostiek. *Gardnerella vaginalis* werd gemeten in 58.7% van de monsters, terwijl 38.9% kolonisatie met *Lactobacillus crispatus* liet zien. In post-menopauzale vrouwen daalde de totale kolonisatie met *Lactobacillus crispatus* aanzienlijk, van 40.5 (pre-menopauzaal) naar 19.5%. Dit hoofdstuk stelt dan ook dat het wegvallen van de productie van vrouwelijke geslachtshormonen een significante daling veroorzaakt in het kolonizatiepatroon van *Lactobacillus crispatus*. Ook blijkt dat kolonisatie met *Gardnerella vaginalis* freqeunter voorkomt in een flora zonder *Lactobacillus crispatus* (OR 1.3; 1.1-1.4), terwijl *Lactobacillus crispatus* vaker een flora zonder *Gardnerella vaginalis* koloniseert (OR 1.4; 1.2-1.6). Deze

bevinding zou mogelijk kunnen duiden op interferentie tussen beide bacteriën.

Hoofdstuk 8
Het doel van dit hoofdstuk is het bestuderen van de concentratie van zowel *Lactobacillus crispatus* als *Gardnerella vaginalis* in een vaginale flora met bacteriële imbalans. Zoals eerder beschreven worden beide bacteriën belangrijk geacht in het behouden van een gebalanceerde vaginale flora. Uit de database van het Leids Cytologisch en Pathologisch Laboratorium werden de volgende aantallen cervixmonsters aselect gekozen: dysbacteriose (29), *Gardnerella* infectie (20), normale flora (27). Alle monsters, gesuspendeerd in een formaline-vrij coagulerend fixatief werden gebruikt voor PCR-analyse. Op basis van de concentraties *Gardnerella vaginalis* en *Lactobacillus crispatus* bleek een viertal kwadranten te kunnen worden onderscheiden (A-D). In alle monsters met een concentratie van *Gardnerella vaginalis* $>2.0x10^{-2}$ng/µl bleek er cytologisch sprake te zijn van bacteriële imbalans. Daarbij toonden alle monsters met cytologische *Gardnerella infectie* (kwadrant D) een concentratie van *Lactobacillus crispatus* $<3.8x10^{-4}$ng/µl. Dysbacteriose werd binnen alle kwadranten gevonden, terwijl normale flora slechts verspreid was over kwadrant A en B. Dit hoofdstuk eindigt met de conclusie dat DNA-concentraties van *Gardnerella vaginalis* en *Lactobacillus crispatus* waardevolle markers zouden kunnen zijn bij het diagnosticeren van bacteriële imbalans van de vaginale flora. Echter, differentiatie tussen dysbacteriose en *Gardnerella* infectie lijkt op basis van deze methode niet mogelijk. Verder onderzoek naar deze methode moet ertoe leiden dat PCR in de toekomst een belangrijke en snelle methode wordt in de diagnostiek van de verschillende vormen van bacteriële imbalans van de vaginale flora.

Hoofdstuk 9
Dit hoofdstuk beschrijft de relatie tussen bacteriële imbalans en de aanwezigheid van hoog-risico HPV-infectie. Een totaal van 19,793 cervicale monsters, de meerderheid afkomstig uit het Nederlandse bevolkingsonderzoek, werd getest op 26 hoog en laag risico HPV-genotypes. De vaginale flora van cervixmateriaal werd geanalyseerd met behulp van lichtmicroscopie en gecodeerd volgens het KOPAC-systeem.
De prevalentie van hoog-risico HPV infectie was 44.6% (58/130) in uitstrijken met bacteriële imbalans versus 25.8% (850/3293) in gezonde flora (OR 1.6; CI 1.2-1.9). De prevalentie van specifieke hoog-risico genotypes bleek niet significant.
De bevindingen in dit hoofdstuk tonen aan dat hoog-risico HPV genotypes vaker voorkomen in een flora met bacteriële imbalans ten gevolge van *Gardnerella vaginalis* met een tekort aan lactobacillen. Verder onderzoek zal verricht moeten worden ten einde te ontdekken welke genotypes vaker voorkomen in een flora met bacteriële imbalans.

Hoofdstuk 10
Dit hoofdstuk bespreekt tenslotte de conclusies die in dit proefschrift naar voren zijn gebracht zoals beschreven in de verschillende voorafgaande hoofdstukken.
Dit proefschrift toont dat bacteriële imbalans van de vaginale flora geassocieerd is met een significant hogere prevalentie van cervicale (pre)kankercellen. Onder uitstrijkjes met pure *Gardnerella* infectie is deze relatie echter veel sterker dan in dysbacteriose.

Ook bevestigt dit proefschrift dat op basis van cytologische criteria zowel *Gardnerella* infectie als dysbacteriose duidelijk kunnen worden onderscheiden.
Gedurende het laatste decennium is de prevalentie van bacteriële imbalans van de vaginale flora aanzienlijk gedaald, terwijl het percentage uitstrijken met HPV-gerelateerde koilocytose een grote stijging laat zien over dezelfde periode. Het eerste fenomeen zou veroorzaakt kunnen zijn door een toegenomen bewustwording omtrent vaginale hygiëne. De toename van koilocytosis ligt mogelijk ten grondslag aan een verandering in seksueel gedrag.
Hoewel cytologie een accurate methode is in de diagnostiek van bacteriële imbalans winnen moderne technieken meer en meer terrein. Dit proefschrift suggereert dat DNA-PCR in de toekomst een belangrijke rol zou kunnen gaan spelen in de diagnostiek van bacteriële imbalans.
Tenslotte, dit proefschrift toont dat hoog-risico HPV infectie beter gedijt in een milieu gekenmerkt door imbalans van de bacteriële flora van de vagina. Dit gegeven laat belangrijke vragen achter met betrekking tot het hieraan ten grondslag liggende mechanisme. Leidt bacteriële imbalans van de vaginale flora tot een verhoogde gevoeligheid van hoog-risico HPV of is de klaring van het virus gecompromitteerd door een dergelijke verstoring?

Dankwoord

Dankwoord

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Annemarie,
April 2008

Curriculum vitae

Curriculum Vitae

Johanna Marieke (Annemarie) Klomp werd geboren op 10 oktober 1978 te Leeuwarden. In 1997 behaalde zij haar diploma voor het Voortgezet Wetenschappelijk Onderwijs op Scholengemeenschap 'Guido de Brès' te Amersfoort. Wegens uitloting voor de studie geneeskunde vertrok zij naar Cambridge, Engeland, alwaar zij aan de University of Cambridge enkele maanden engels studeerde. Na in 1998 wederom te zijn uitgeloot, begon zij met de studie psychologie aan de Universiteit Utrecht. Na het behalen van haar propedeuse in 1999 werd zij uiteindelijk ingeloot voor de studie geneeskunde aan de Universiteit Utrecht.

Tijdens de doctoraalfase van haar studie werkte zij als student-assistent bij de vakgroep anatomie. Ook was zij betrokken bij wetenschappelijk onderzoek binnen de divisies heelkunde en gynaecologie van het Universitair Medisch Centrum Utrecht.

Na afronding van haar wetenschappelijke stage, waarbij zij onderzoek deed naar het behoud van ovariumfunctie bij vrouwen met cervixcarcinoom na Wertheim-Meigs operatie en chirurgische ovariumtranspositie, ontving zij op 25 augustus 2005 haar artsendiploma, waarna zij ging werken als arts-assistent heelkunde in Ziekenhuis Rivierenland te Tiel. In januari 2006 begon zij haar promotie-onderzoek onder leiding van prof. dr. A.P.M. Heintz (divisie chirurgische gynaecologie en oncologie) in samenwerking met het Leids Cytologisch en Pathologisch Laboratorium te Leiden. De resultaten van dit onderzoek worden gepresenteerd in dit proefschrift. Naast haar promotie-onderzoek werkte zij vanaf april 2006 als arts-assistent gynaecologie in het Diakonessenhuis te Utrecht (toenmalig opleider Dr. M. van Haaften). Vanaf 1 juli 2008 zal zij gaan werken als arts-assistent heelkunde in Ziekenhuis Gelderse Vallei te Ede (opleider Dr. J.H.C. Kuijpers).

List of publications

List of publications

Klomp JM, Boon ME, Dorman MZ, van Haaften M, Heintz APM. Trends in inflammatory status of the vaginal flora as established in the first and second screening of the Dutch national screening program for cervical cancer.
Submitted for publication. (Chapter 2)

Klomp JM, Boon ME, van Haaften M, Heintz APM. An increased prevalence of cervical (pre)neoplasia in dysbacteriotic smears containing *Gardnerella vaginalis* and shortage of lactobacilli.
Submitted for publication. (Chapter 3)

Klomp JM, Boon ME, van Haaften M, Heintz APM. Cytologically diagnosed *Gardnerella* infection and cervical (pre)neoplasia as established in population-based cervical screening.
American Journal of Obstetrics and Gynecology. In press. (Chapter 4)

Klomp JM, Ouwerkerk-Noordam E, Boon ME, van Haaften M, Heintz APM. *Gardnerella* infection can be distinguished from dysbacteriosis: a cytological study.
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Klomp JM, Verbruggen BSM, Korporaal H, Boon ME, de Jong P, Kramer GC, van Haaften M, Heintz APM. *Gardnerella vaginalis* and *Lactobacillus* sp in liquid-based cervical samples in healthy and disturbed vaginal flora using cultivation-independent methods
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Klomp JM, Korporaal H, Boon ME, van Haaften M, Heintz APM. Concentration of *Gardnerella vaginalis* and *Lactobacillus crispatus* in women with a bacterial imbalanced vaginal flora.
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Cell oncology 2008;30(1):1-11

Notities