

Review

Infection through structured polymicrobial *Gardnerella* biofilms (StPM-GB)

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Summary. BACKGROUND: We analysed data on bacterial vaginosis (BV) contradicting the paradigm of mono-infection.

METHODOLOGY: Tissues and epithelial cells of vagina, uterus, fallopian tubes and perianal region were investigated using fluorescence in situ hybridization (FISH) in women with BV and controls.

RESULTS: Healthy vagina was free of biofilms. Proliferic structured polymicrobial (StPM) *Gardnerella*-dominated biofilm characterised BV. The intact StPM-*Gardnerella*-biofilm enveloped desquamated vaginal/prepuce epithelial cells and was secreted with urine and sperma. The disease involved both genders and occurred in pairs. Children born to women with BV were negative. Monotherapy with metronidazole, moxifloxacin or local antiseptics suppressed but often did not eradicate StPM-*Gardnerella*-biofilms. There was no BV without *Gardnerella*, but *Gardnerella* was not BV. Outside of StPM-biofilm, *Gardnerella* was also found in a subset of children and healthy adults, but was dispersed, temporal and did not transform into StPM-*Gardnerella*-biofilm.

CONCLUSIONS: StPM-*Gardnerella*-biofilm is an infectious subject. The assembly of single players to StPM-*Gardnerella*-biofilm is a not trivial every day process, but probably an evolutionary event with a long history of growth, propagation and selection for viability and ability to reshape the environment. The evolutionary

memory is cemented in the structural differentiation of StPM-*Gardnerella*-biofilms and imparts them to resist previous and emerging challenges.

Key words: Bacterial vaginosis, Structured polymicrobial *Gardnerella* dominated bacterial biofilm, Antibiotic resistance, FISH, Preterm birth, Pregnancy, Occurrence, dependence on age, Sex and pregnancy, Anatomic extension, Response to therapy, Evolution

Introduction

In 1683, Antoni van Leeuwenhoek sketched different morphotypes of “animalcules” in smears of dental film. Although he used other termini, rods, spirochetes, and cocci can be clearly recognized in his drawings. Leeuwenhoek’s discovery inspired research, which for the next 200 years is pointed to the appearance of microorganisms. Multiple “taxonomic” categories emerge, most of which were lost later and are unknown now. Ironically the name “bacterium” was introduced for non-relevant unclassifiable morphotypes. Despite a firework of microbial discoveries not a single pathogen was identified at that time. The idea of pathogens and likewise of antiseptics or asepsis simply did not fit into

Abbreviations: FISH, fluorescence in situ hybridization; Cy3, FITC, Cy5, DAPI, different fluorescent dyes corresponding to orange, green, dark red, and blue colors; BV, Bacterial vaginosis; Carnoy solution, a fixative with composition of 6/6/1 vol. ethanol/glacial acetic acid/chloroform; StPM, structured polymicrobial

the existing logical framework. Microscopy in the first days disclosed complex microbial communities nearly everywhere. The abundance of bacteria, their ubiquity, polymorphism and readiness to emerge in organic substance made a practical link to involvement in disease difficult to imagine. The mainstream opinion was that bacteria can archigenetically arise from nowhere (abiogenesis).

Pasteur brought the archigenetic hypothesis down. He demonstrated that all microorganisms are pre-existing, and need specific conditions for propagation, growth and transfection. The paradigm changed. Active isolation, transfection and culturing became standard procedures that identified the impact of microorganisms on disease, spoiling of food or fermentation. The idea of intangible complexity was reduced to a handsome agglomerate of microorganisms, which can be approached one by one. The success was overwhelming. Due to isolation, a vast number of infectious diseases have been characterized and effectively controlled within the last 150 years. The emerging difficulties seemed to be temporary and solvable with development of new tools. The expectations remained unfulfilled.

By the end of the twentieth century, it became increasingly apparent that the singularity is just one part of the story. As it is impossible to understand the human body by dissecting it into single cells, it is also impossible to deduce the functional potency of polymicrobial communities from the sum of involved bacteria. Bacteria exist mainly in the form of polymicrobial communities within the natural environment and thrive under conditions in which each of the community composing the microorganisms alone would perish. Microbiomes of the human body interfacing environment: skin, mouth, intestine, genitalia – are also polymicrobial. Infections such as bacterial vaginosis, appendicitis, parodontosis, caries, tonsillitis, small bowel overgrowth, gallstones, inflammatory bowel disease, remain poorly understood, despite 300 years of intensive research.

The vaginal microbiome is a typical example. Complexly composed, it shows remarkable constancy of its main players, which makes it unmistakably different from other microbial communities such as skin, mouth, and intestine. The sustainability of the vaginal microbiome is disturbed in conditions such as bacterial vaginosis, aerobic vaginitis, and fungal overgrowth. Bacterial vaginosis (BV) is the most common vaginal disorder. The disease was recognized in 1955 when Gardner and Dukes isolated *Haemophilus vaginalis*, now known as *Gardnerella vaginalis* from the vaginas of 92% of women with characteristic symptoms now known as bacterial vaginosis. In the same year, Gardner and Dukes were able to transmit BV using vaginal secretions of diseased women, but not when using a pure culture of *Gardnerella* (Gardner and Dukes, 1955). Criswell induced BV through inoculation with overnight cultures of *Gardnerella* (Criswell et al., 1969). Although these cultures could be impure, Koch's criteria seemed

to be in general fulfilled: 1. isolation of pathogen from diseased but not from healthy persons, 2. cultivation of pathogen, 3. induction of infection by culture. The further progress came to a hold and even seemed to roll backwards. Investigators began to report up to 30-40% occurrence of *G. vaginalis* among subgroups of apparently healthy women, which made it difficult to accept *G. vaginalis* as a pathogen. At the same time, bacteria like *Mobiluncus* were isolated in BV, which were absent in healthy women. Unfortunately, the prevalence and persistence of these bacteria in BV was too low to explain the pathogenesis of the disease. The introduction of gene sequencing amplified the number of involved participants and further undermined understanding. More than 400 different bacterial groups were allocated in vaginal smears of BV women. Since none of them fulfilled Koch's criteria, bacterial vaginosis is traditionally explained by bacterial imbalance and with lactobacilli overshadowed by diverse gram labile microbiota. Unfortunately, the imbalance is a statement and not an explanation. The widespread reasoning making a displacement of "good" lactobacilli responsible for increase of "bad" bacteria is even more ambiguous. Why do species, which are ubiquitously present in the environment, suddenly vanish and do not recolonize the vaginal habitat? Why children, who have no stable indigenes, do not develop the disease at any time, even when they are born to women with BV? Why is the incidence of BV not increased in the geriatric population or in newborns: groups that should be especially vulnerable to dysbiosis of any kind? Let us suppose the lack of lactobacilli is crucial. Could, for example, a lack of lactobacilli be restored (readmitted) by sex with a healthy partner? - Definitely no. Most studies show the sexual transmission of BV from diseased to healthy and not vice versa. Are changes of local pH, or substitution of lactobacilli curative? None of the previous studies could definitively demonstrate a sustained effect. Antibiotics, which in theory destabilize the vaginal microbiome, are the only substances with confirmed positive effects on BV.

The history of bacterial vaginosis demonstrates perfectly the dead end of isolation based reductionism. Vaginal bacteria are polymicrobial. To understand polymicrobials, we have to investigate them as a structure-functional unity. One helpful approach is the ribosomal-gene based fluorescence in situ hybridization or FISH. Each bacterium possesses 10^{3-5} ribosomes. Each ribosome includes a RNA copy. Some of the regions of the ribosomal RNA are strain-specific, others are universal for groups, domains, or even kingdoms. Oligonucleotides, synthesized complimentary to sequences of interest and labelled with fluorescent dye, are called FISH probes. When added to samples containing bacteria, FISH probes hybridize with the RNA of the bacterial ribosomes. No additional enhancement of fluorescence is necessary and bacteria can be directly visualized because of the large number of ribosomes in each bacterium (Amann et al., 1995).

Structured polymicrobial *Gardnerella* biofilms

The following presentation summarizes our FISH studies of the vaginal microbiom. The names of the FISH probes are listed according to abbreviations of probeBase online resource for rRNA targeted oligonucleotid probes (Loy et al., 2007).

The results revealed by structure-functional investigations of vaginal microbiota strongly oppose accepted "facts", often overturning traditional views. To demonstrate that the inconsistency stems from limitations of previously used methods, we introduce each result section with a short overview, explaining the history and origin of some (mis)interpretations.

In the last years a lot of literature has been dedicated to biofilms, each of the publications used often its own terminology, inconsistent with the terminology of other investigators. Before starting with the description we need to explain the terminology. Visible plaque, membranes and slimes coating the surface of human organs and prostheses are called films. In case of their colonization or contamination one supposes microbial biofilms. This assumption is often unjustified and leads to confusion in use of the term bacterial biofilm. For example "protective" vaginal lactobacilli biofilms, or "pathologic" *Candida* biofilms are often mentioned in the literature without any reference to their structure or to a single picture demonstrating their existence. Even we described lactobacilli in slime in our first publication mistakenly with the term "biofilm" (Swidsinski et al., 2005a). One should however distinguish between microorganisms suspended within secretions, which they do not build, and bacteria building their own matrix and growing in confluent layers. We will further use the word biofilm only in connection with self-maintaining biofilms creating their own environment.

Experimental procedures

The investigations were performed between 2004 up to the present. The materials investigated were: vaginal biopsies, desquamated vaginal epithelial cells from urine sediments, tissues from uterine curettage, surgically removed fallopian tubes, sections of nylon membrane strips placed into the vagina overnight, and sections of adhesive tape attached to the anal region for 60 seconds.

Fixation was performed with modified non-aqueous Carnoy solution (6/6/1 vol. ethanol/glacial acetic acid/chloroform). The fixated materials could be stored in Carnoy at room temperature for up to 6 months. Usually, the time used was convenient for the investigated subject and laboratory staff.

Biopsies and tissue samples

Biopsies of about 3-5 mm diameter were taken from the middle sidewall of the vagina with biopsy forceps (Schubert, Aesculap, Tuttlingen, Germany, No. ER 058 R). They were immediately fixated in Carnoy solution for 2 hours. Surgically removed tissue samples were reduced to size of maximal 1x1 cm and fixated in 25 ml

of Carnoy solutions for at least 24 hours. Nylon membrane and adhesive tape strips were fixated overnight. Carnoy fixated material was processed and embedded into paraffin blocks using standard techniques. Four μm sections were placed on SuperFrost slides (R. Langenbrinck, Emmendingen, Germany) for FISH studies (Swidsinski et al., 2005a).

Desquamated epithelial cells of the vagina or prepuce in urine sediments

Two ml of urine were mixed with 8 ml Carnoy fixative in 12 ml Falcon tubes. The collection and fixation was performed by the investigated subject. When possible, to increase the number of desquamated epithelial cells in urine sediments, women were asked to use the first portion of the morning urine and not to wash the region beginning with the evening before sample collection. Males were asked to pull the foreskin back over the glans penis before voiding.

An aliquot of 1.5 ml urine/Carnoy mix was centrifuged in a 1.5 ml Eppendorf tube for 6 minutes at 6,000 G. The sediment was decanted; the tube was filled with 1 ml of Carnoy solution and left at room temperature. After 1-5 minutes, the sediment was centrifuged once more (6 min/9,000 G), decanted, 50 μl Carnoy solution was added, and then stored at 4°C. If more than 10 hybridizations were planned, larger urine aliquots were prepared in the same manner as described above.

Fish

Biopsies and tissue samples

Bacteria were assessed in a multi-colour analysis using a mix of specific and universal FISH probes stained with Cy3 (orange fluorescence), FITC (green fluorescence), Cy5 (dark red fluorescence) and DAPI counter stain (blue fluorescence) according to previously described protocols (Swidsinski et al., 2005a; Swidsinski, 2006). GardV (Swidsinski et al., 2005a), Ato (Harmsen et al., 2000), Lab (Harmsen et al., 1999), Bac 303 (Manz et al., 1996), Ebac (Bohnert et al., 2002) and Eub 338 (Amann et al., 1990) probes representing *Gardnerella*, *Atopobium*, *Lactobacillus*, *Bacteroides/Prevotella*, and *Enterobacteriaceae*, *Eubacteria* cluster were applied for analysis of each sample. In special cases up to 140 further group-specific FISH probes were applied in different composed sets (Loy et al., 2007).

Nikon e600 fluorescence microscope, Nikon DXM1200F camera and accompanying software (Nikon, Tokyo, Japan) were used. The enumeration of bacteria was performed when hybridization signals were clear and morphologically distinguishable as bacterial cells by triple colour identification with universal and group-specific FISH probes and DAPI stain, with absence of cross-hybridization with taxonomically unrelated probes (Swidsinski, 2006).

Structured polymicrobial *Gardnerella* biofilms

The conversion of the numbers within defined microscopic areas to concentrations of bacteria per ml was based on the calculation that a 10- μ l sample with a cell concentration of 10^7 cells per ml has 40 cells per average microscopic field at a magnification of x1000, the details of conversion were previously described (Swidsinski et al., 2005a).

Urine sediments

A 5x5 mm quadrant area of hybridization was marked with a PAP pen on a SuperFrost plus glass slide. The Carnoy fixed urine sediment was vortexed; 5 μ l aliquots were pipetted within the area of hybridization and dried for 30 minutes at 50°C just prior to the hybridization.

Five microliters of the final aliquot were used for single hybridizations and represented 30 μ l of the initial urine volume.

Concentrations of epithelial cells within the 5x5 mm area of hybridization (30 μ l of sample volume) were calculated and converted to numbers of epithelial cells per ml of urine. The maximal and mean numbers of adherent bacteria per epithelial cell were determined. The overall concentrations of adherent bacteria in the urine resulted from multiplication of mean number of bacteria per epithelial cell with the concentration of epithelial cells per ml of urine.

Results

Our previous knowledge on bacteria attached to the vaginal epithelium is mainly based on indirect culture data, microscopy of vaginal secretions and swabs. Ridiculously, in the last 200 years no systematic study on bacterial colonization of human vaginal tissues was published and we do not know whether it was ever performed. We found no direct data in the literature except for singular microphotographs that illustrated viral, fungal or bacterial (toxic shock syndrome) infections and for electron microscopy of vaginal epithelium in animals. Contrary to the widespread belief of abundant bacteria covering the vaginal surface, the

electron microscopy of healthy animals demonstrated, if at all, only attached single microorganisms. To clarify the real situation it was therefore important for us to start specifically at this point.

Spatial organization of bacteria on the epithelial surface of the vagina

To investigate in situ the spatial organization of vaginal microbiota in different conditions 146 vaginal biopsies (Table 1) were obtained from women with BV, vaginal candidosis, aerobic vaginitis, and healthy pre- and postmenopausal adult subjects (cancer surveillance). All women were gynaecologically examined and Gram stain of the vaginal smears was performed. Vaginal candidosis was diagnosed according to clinical symptoms and characteristic detections of yeast in the vaginal smear. Aerobic vaginitis was diagnosed in case of colpitis in absence of clue cells, lactobacilli and normal pH. The discrimination between BV and health was made according to the Amsel criteria after exclusion of vaginal candidosis.

Healthy women

No bacterial biofilms or any ordered bacterial adherence on the epithelial surface was seen in 54/56 healthy controls. Up to five singular lying bacteria of different bacterial groups could be seen irregularly scattered over the epithelial surface in some microscopic fields of 26/56 women, while most of their microscopic fields of view were completely free of bacteria (Fig. 1).

In 11/56 women islands of 5 to 20 clumped and irregular bacteria attached to the epithelial with a distance of one to two microscopic fields between each (300 μ m) were observed (Fig. 2).

Bacteria were in addition observed as clouds of diffusely scattered microorganisms in secretions covering the biopsy and not adherent to the epithelial surface in 17/56 women (Fig. 3, Table 1). The comparatively rare detection of bacteria in secretions covering the biopsy was in contradiction to the findings in the torn-out portion of all biopsies from healthy

Table 1. Occurrence of bacteria in vaginal biopsies as detected by FISH.

	Structured polymicrobial <i>Gardnerella</i> biofilms	Mixed islands of bacterial accumulations attached to the vaginal epithelium	Singular or no bacteria	
			Diffuse nonadherent bacteria in secretions	
Bacterial vaginosis N=68	66 (66)	*	2 (2)	0
Healthy controls premenopausal N=23	1 (1)	4 (1)	4 (2)	14
Healthy controls postmenopausal N=33	1 (1)	7	13 (2)	12
Aerobic vaginitis N=12	0		1	11
Vaginal candidosis N=10	3 (3)	6 (5)	1 (1)	0

Numbers of patients with positive *Gardnerella* detection are given in parenthesis. *: Bacteria in secretions and sporadic islands of other bacteria were not evaluated when a structured polymicrobial biofilm was present.

Structured polymicrobial *Gardnerella* biofilms

women, where bacteria were readily seen in considerable concentrations (10 to 50 per microscopic field) partially invading deeply the mechanically damaged tissues and suggesting that bacteria are present in the vaginal fluids and readily contaminate tissues as soon as their anatomic integrity is lost. However, the overall low occurrence of bacteria in secretions covering the vaginal epithelial surface may be due to difficulties to preserve vaginal slime while processing biopsies in Carnoy fixative and in paraffin blocks, sections and FISH. Bacteria within secretions were more often seen in post- than in premenopausal women. Except for this finding, there was no noticeable difference in adherent microbiota between pre- and postmenopausal healthy women.

Bacteria located in slime covering the vaginal biopsy of a healthy woman hybridized with Lab Cy3 (*lactobacilli*), yellow fluorescence Eub338 FITC (all

bacteria), green fluorescence and Gard Cy5 (*Gardnerella*) probes, dark red fluorescence, x1000.

In two women, who were initially characterized as healthy according to the Amsel criteria, a confluent bacterial biofilm was observed. The biofilm was identical to that found in women with BV (described below). No clinical reinvestigation of these women took place. We therefore cannot exclude that the initial diagnosis was wrong, and that the women had in fact an asymptomatic form of bacterial vaginosis.

Bacterial vaginosis

The appearance of bacteria in BV was markedly different to those in healthy women.

Confluent dense bacterial biofilm (Fig. 4) adhered to the epithelial surface of 66/68 women with BV.

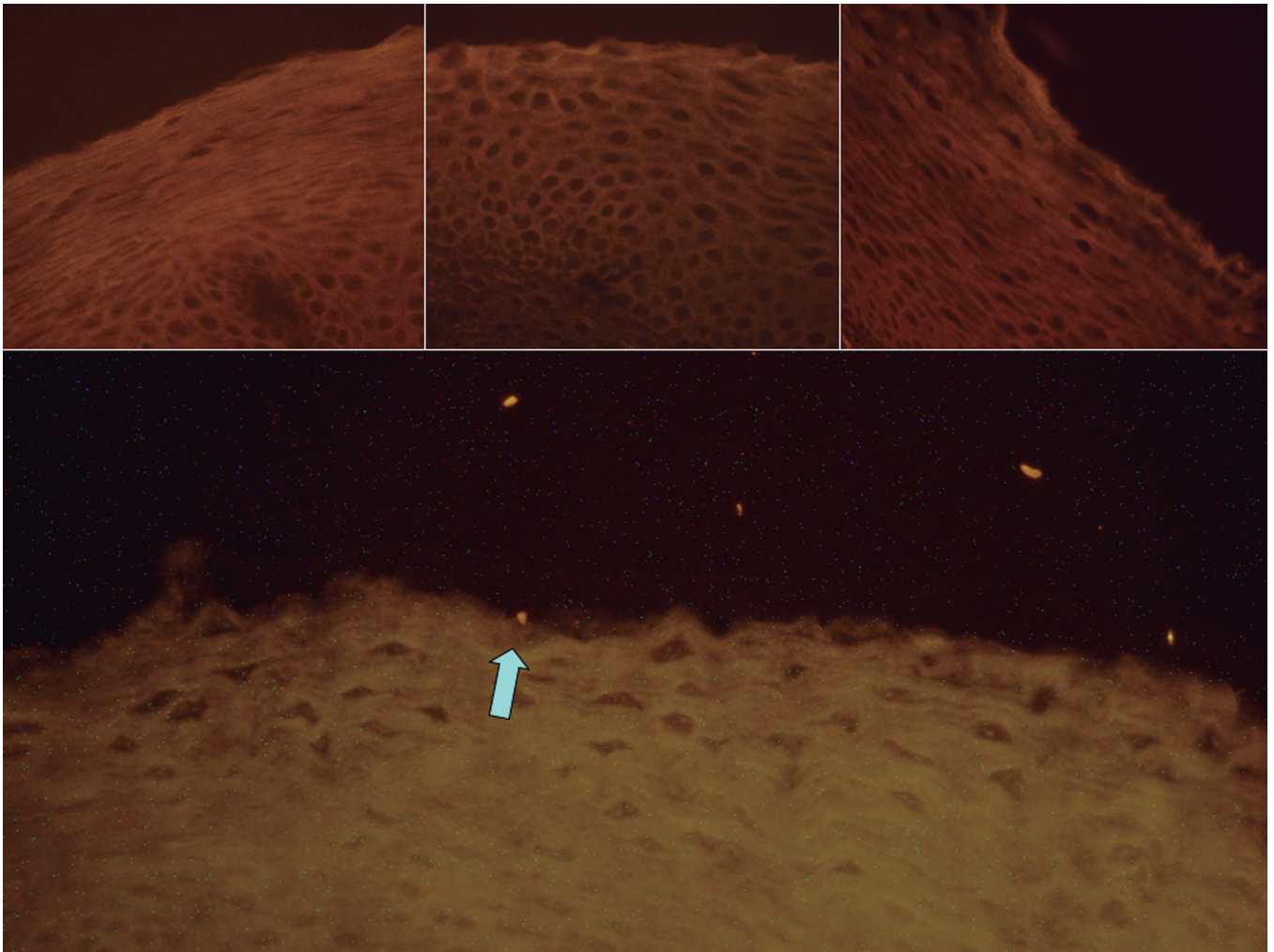


Fig. 1. Healthy vaginal epithelium, hybridization with universal for bacteria Eub338 Cy3 probe, yellow fluorescence: only single bacteria can be seen in slime over the vaginal surface or attached to the surface (arrow), no bacteria are detectable on the surface of most microscopic fields. x 1000; insertions above, x 400.

Structured polymicrobial *Gardnerella* biofilms

The biofilm covered the epithelial biopsy surface completely in 35/68, to at least 50% of the surface in 20/68, and to at least 30% in 11/68 women. The epithelial surface available for examination in the two women diagnosed with BV according to the Amsel criteria and negative for biofilm was very small (less than two microscopic fields, Table 1). The negative findings in these two women could be due to the small biopsy size.

Bacteria within the biofilm were tightly packed with no free space between single microorganisms and closely attached to the vaginal epithelium (Figs. 4, 6). The biofilm was polymicrobial and similarly composed (structured) all over the biopsy (Table 2). Single bacterial groups composing biofilms did not grow as isolated islands, but were organized. We called the BV biofilm - structured polymicrobial (StPM) biofilm because of the homogeneous appearance of structure.

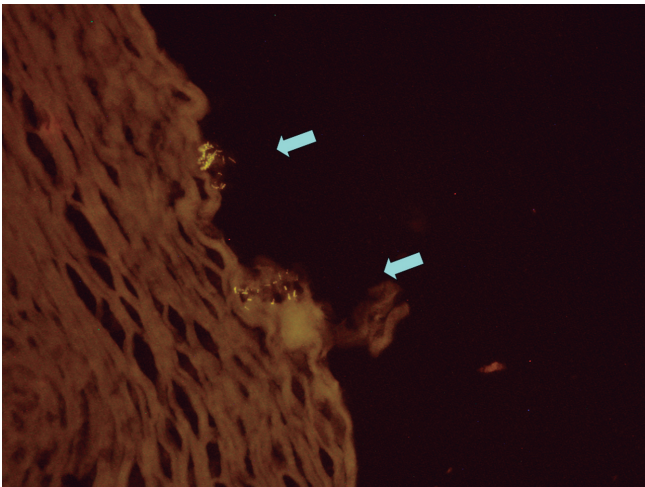


Fig. 2. Islands of irregular bacteria attached to the vaginal epithelium, Eub338 Cy3 probe, yellow fluorescence. x 1000

Gardnerella built the matrix of the biofilm and was numerically predominant. Within the *Gardnerella* matrix different less numerous bacterial groups were incorporated. Although these non-*Gardnerella* bacterial groups could occasionally reach considerable concentrations, which were never observed in healthy women, none of them outnumbered *Gardnerella* and none of these bacteria were present in all cases of BV (Table 2).

Atopobium was the second highest concentrated bacterial group after *Gardnerella* (Table 2, Fig. 5). It was observed in 60% of women compared to 8% of healthy controls. Concentrations of many other bacterial groups, which were marginal in healthy women, were massively increased in women with bacterial vaginosis (Table 2).

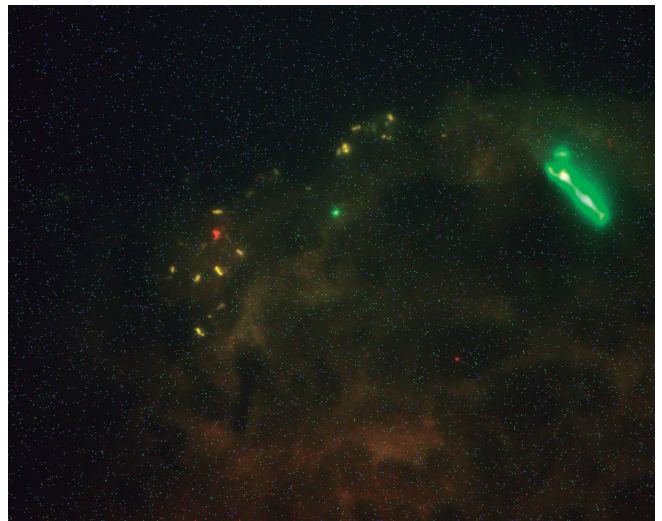


Fig. 3. Bacteria located in slime covering the vaginal biopsy of a healthy woman hybridized with Lab Cy3 (*Lactobacilli*), yellow fluorescence Eub338 FITC (all bacteria), green fluorescence and Gard Cy5 (*Gardnerella*) probes, dark red fluorescence. x 1000

Table 2. Occurrence and mean concentrations of bacteria in the vaginal biopsies as detected by FISH.

	Healthy N=51 (49)**		BV N=68	
	Mean±SD*/Max. Concentration/ml	Occurrence	Mean±SD*/Max. Concentration/ml	Occurrence
<i>Gardnerella</i> (Gard)	0.03±0.04 / 0.1x10 ⁸	14% (7)	31.8±21.8 / 120x10 ⁸	97% (66)
<i>Atopobium</i> (Ato)	0.17±0.26 / 0.5x10 ⁸	8% (4)	5±4.1 / 30x10 ⁸	60% (41)
<i>Lactobacillus</i> (Lab)	0.7±1 / 4x10 ⁸	39% (20)	2.8±3.8 / 20x10 ⁸	81% (55)
<i>Enterobacteriaceae</i> (Ebac)	0.01 / 3x10 ⁸	4% (2)	0.5±0 / 3x10 ⁸	12% (8)
<i>Coriobacterium</i> (Cor)	0.01 / 0.1x10 ⁸	6% (3)	1.9±1.7 / 4x10 ⁸	15% (10)
<i>Cytophaga-Flavobacteria</i> (CF)	0	0	1±1 / 3x10 ⁸	13% (9)
<i>Veillonella</i> (Veil)	0	0	0.18±0.35 / 1x10 ⁸	12% (8)
<i>Bacteroides</i> (Bac)	0	0	0.17±0.19 / 0.5x10 ⁸	10% (7)
<i>Clostridia</i> (Clit, Chis, Erec)	0	0	0.12±0.19 / 0.5x10 ⁸	9% (6)
<i>Fusobacteria</i> (Fus)	0	0	0.2±0.05 / 0.1x10 ⁸	6% (4)

*: Mean values were calculated exclusively for positive samples within the subgroup; **: The two women with confluent *Gardnerella* dominated biofilm in the healthy group were excluded from quantitative evaluation of mean concentrations and occurrences.

Structured polymicrobial *Gardnerella* biofilms

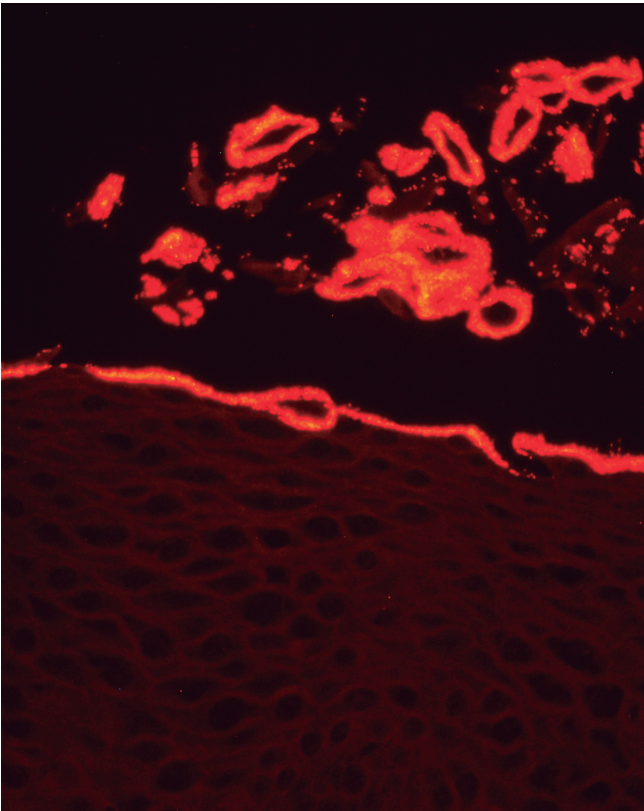


Fig. 4. *Gardnerella* dominated biofilm covering the surface of the vaginal biopsy in a woman with BV. The desquamated epithelial cells are enveloped with StPM biofilm. Hybridizations with Gard Cy5 probe, dark red fluorescence. x 400

Groups including *Clostridia*, *Fusobacteria*, *Veillonella*, *Cytophaga-Flavobacteria* incl. *Bacteroides*, were completely absent in biopsies of healthy controls, while *Enterobacteriaceae* and *Coriobacteria* were present just at the detection limit. However, these bacteria flourished within StPM-*Gardnerella* dominated biofilms.

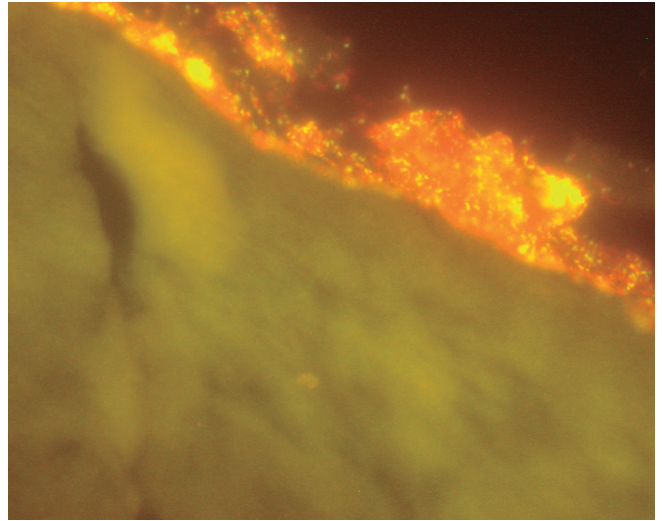


Fig. 5. Structured polymicrobial StPM-*Gardnerella* dominated biofilm in women with BV hybridized with Ato Cy3 yellow fluorescence, and Gard Cy5 dark red fluorescence, x1000. *Atopobium* is embedded in the matrix of *Gardnerella*.

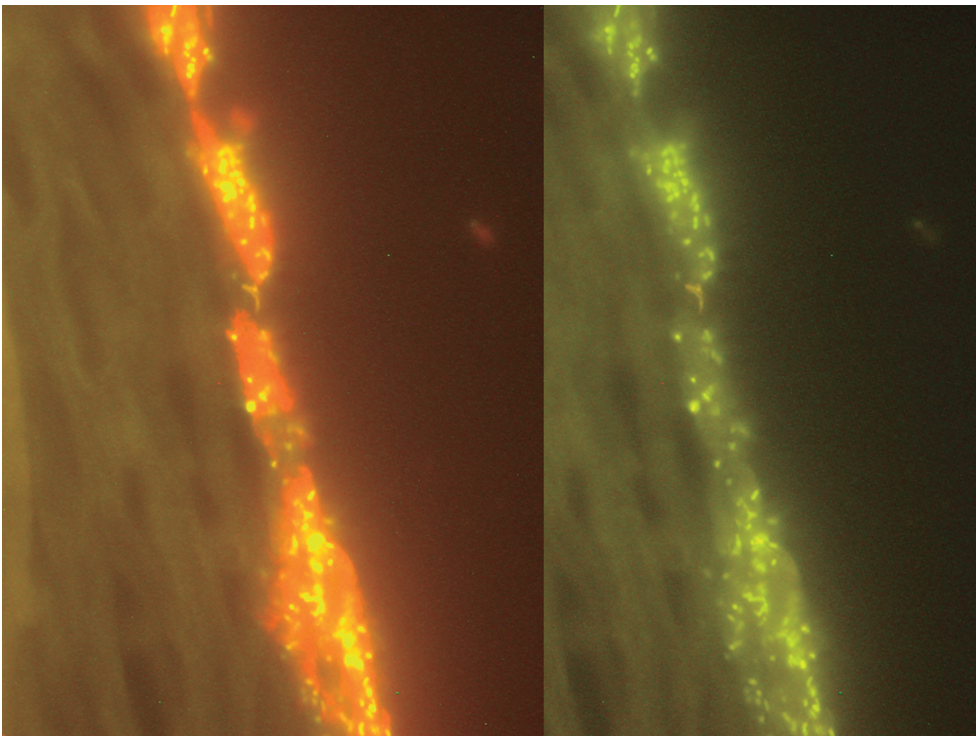


Fig. 6. Structured polymicrobial *Gardnerella* dominated biofilm in woman with BV hybridized with Lab Cy3 probe (*Lactobacilli*), yellow fluorescence and Gard Cy5 probe (*Gardnerella*), dark red fluorescence. The left panel shows *Gardnerella* and *Lactobacilli*, on the right is shown the same microscopic field in Cy3 fluorescence, demonstrating only the *Lactobacilli*.

Structured polymicrobial *Gardnerella* biofilms

Obviously the StPM biofilm creates conditions which dramatically facilitate the growth of different microorganisms. Despite this facilitation, not all bacterial groups were present in each case. On the contrary, the composition of StPM biofilms was (with the exception of obligate *Gardnerella*) highly individual. Either within individual StPM biofilms not all guests are equally welcome, or the components of StPM biofilm build a functional unity which determines and maintains its main frame. In the latter case, it would be more correct to speak about StPM-*Gardnerella* biofilms, rather than a biofilm. The individual structure of the StPM-*Gardnerella* biofilm is maintained over the whole biopsy surface (Figs. 5, 6) even after detachment of desquamated epithelial cells that are enveloped in situ (Fig. 4). The biofilm grows per continuum, the desquamated epithelial cells carry it unchanged.

We observed no depletion of lactobacilli, different to the widespread belief based on analysis of gram stains of vaginal smears. Both occurrence and concentrations of lactobacilli were markedly higher in BV than in healthy controls. Structured polymicrobial *Gardnerella* dominated biofilms enabled lactobacilli to attach and

reach concentrations on the vaginal epithelium which they never achieve in healthy women (Table 2, Fig. 6).

However, lactobacilli in BV had either form of coccoid, short rods, or slim rods. Lactobacilli with such appearance are probably difficult to recognize in Gram stains, because of this atypical morphology and because of their subordinate arrangement within the prolific StPM-*Gardnerella* dominated biofilm. Moreover, *L. iners* which is the predominant *Lactobacillus* type in BV (Srinivasan and Fredricks, 2008) stains gram-negative. Presently, available FISH probes do not allow to exactly distinguish between single lactobacilli species; we can therefore not exclude that different strains of lactobacilli are involved in BV and healthy women as previously postulated (Srinivasan and Fredricks, 2008).

Aerobic vaginitis

No bacterial biofilms were observed in women with aerobic vaginitis. Specifically, the occurrence of bacteria in women with aerobic vaginitis was lowest when compared to all other investigated patient groups (Table 1). Bacteria in slime were seen only in one of 12 biopsies.

Candidosis

In women with candidosis, fungi in different concentrations invaded the superficial areas of the vaginal epithelium. They could also be observed dispersed within secretions covering the biopsy surface and were surrounded by numerous bacteria. In 4 women, the concentration of infiltrating fungi was considerably high (more than 40 cells per microscopic field) as

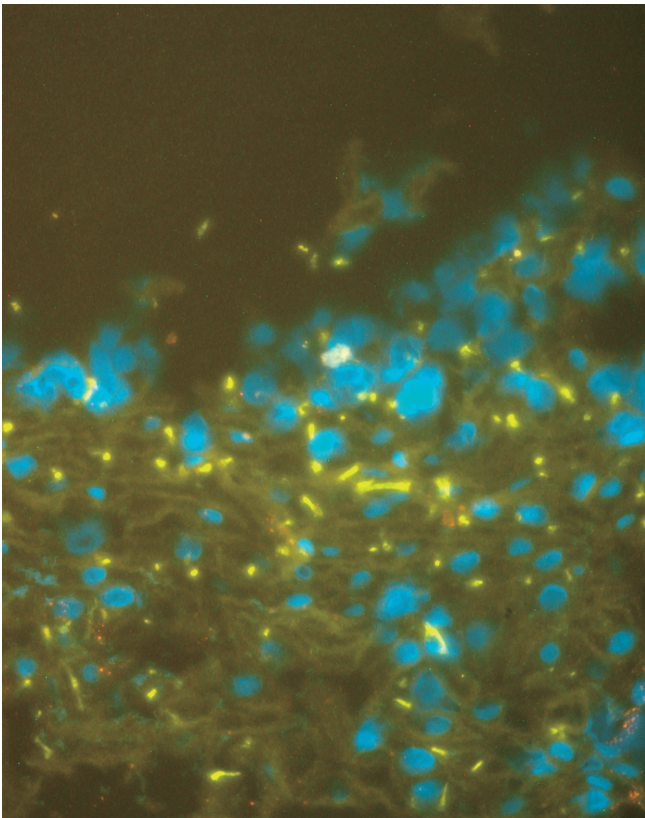


Fig. 7. Woman with vulvovaginal candidosis. Fungi are located mainly within the epithelial layer and not accompanied by invasion of bacteria. Fungal probe Cy3 orange fluorescence, DAPI counterstain of vaginal epithelial cells (blue fluorescence). x 1000

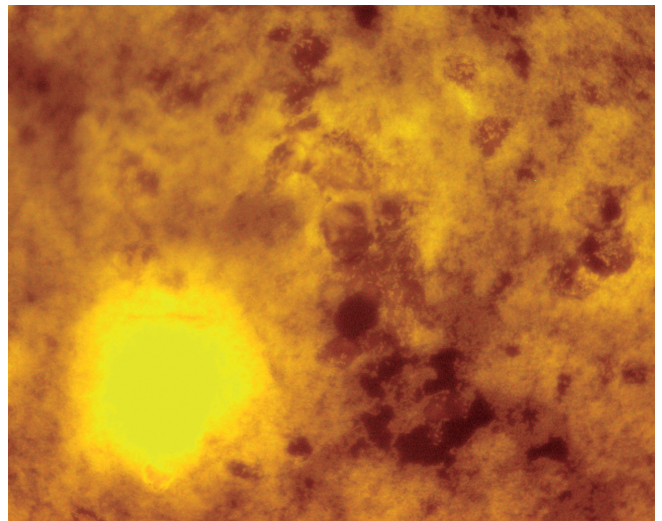


Fig. 8 Massive urinary infection with *E. coli* (EBAC Cy3 probe, orange fluorescence). Bacteria cover the whole surface of the microscopic field. Desquamated vaginal epithelial cells appear as holes.

Structured polymicrobial *Gardnerella* biofilms

presented in Fig. 7, however, they were never confluent. In vaginal secretions, the fungi were represented by up to 6 cells per microscopic field or less without any connection to each other. Contrary to our expectation, based on the opinion common in the literature, we observed in none of the women a confluent fungal biofilm or something approximately resembling it.

Gardnerella was found in all but one biopsy (Table 1). In 3/10 women a StPM-*Gardnerella* biofilm typical for BV was observed. These women could be described as having a StPM-*Gardnerella* biofilms with fungal superinfection. Candidosis could however occur also in absence of StPM-*Gardnerella* biofilms and even surrounded by highly concentrated lactobacilli, indicating an autonomous role of fungal infection and lacking protection through lactobacilli.

Structured polymicrobial (StPM) *Gardnerella* dominated biofilms in samples other than biopsies

Historic overview on data based on wet mounts and gram stains of vaginal smears

Gardner and Dukes first described epithelial cells coated with bacteria in vaginal smears of BV women as a specific feature of the disease (Gardner and Dukes, 1955). Although they misinterpreted bacterial attachment as secondary to desquamation, they recognized the appearance of such cells in vaginal secretions as a decisive sign of bacterial vaginosis and named them for this reason “clue-cells”. The diagnostic significance of clue cells remains probably the only unquestioned feature of BV, its relevance grew with time even further, as hopes connected with microbial culture, PCR or sequencing techniques for diagnosis of bacterial vaginosis vanished.

Because desquamated vaginal epithelial cells and bacteria attached to them (clue cells) or bacteria loosely scattered within vaginal secretions are apparent in vaginal smears already in native microscopy, the diagnostic microscopy found a broad application in gynaecologic practice. Many of our opinions about vaginal microbiome are based on interpretations of native or gram stained microscopic pictures. Bacterial numbers are comparatively low in wet mounts of vaginal smears from healthy women. While most constituents are morphologically unremarkable, prominent large lactobacilli rods attracted attention, being a marked feature of vaginal well-being. Large rods are lacking in BV, and desquamated epithelial cells are covered with masses of polymorph mainly gram labile bacteria. The affiliation of these bacteria in native microscopy can only be assumed, but since large rods are lacking, the lactobacilli are thought to be depleted.

Although gram stain refines the picture, the taxonomic accuracy of microscopic detection is not principally different to the times of Leeuwenhoek. Only some marked morphotypes like *Mobiluncus* can be recognized within all other bacteria. Despite obvious

limitations, gram stain is nevertheless often regarded as an attainable diagnostic benchmark and is recommended for scientific quantification of the pathologic changes. This view is questionable. The bacterial load of desquamated epithelial cells in vaginal smears is not uniform. Some of the epithelial cells within the smear have low or no bacteria on the surface, others are heavily loaded. The variation in abundance of different morphotypes of bacteria and their attachment to epithelial cells is even higher in smears taken from different women at different times. The marked differences in level and appearance of free and cell bound microbiota imposes the assumption that bacterial adherence gradually advances from low to massive. The gram stain based Nugent score and its modifications order “good” and “bad” morphotypes into nine to eleven formally arranged aggravation steps (Nugent et al., 1991). We must be aware however that the Nugent arrangement is formal and the postulated aggravation is not backed by reference to specific pathomechanism, or clinical/taxonomic feature. Strictly speaking, the gram score is inconsistent and pseudo quantitative. The assumption of disease-related gradual changes in composition of microbiota is wrong. The availability of bacterial morphotypes on desquamated epithelial cells mirrors at least two contrary processes. In healthy women, cells which loose contact with the epithelial layer became increasingly susceptible to bacterial adherence and are progressively covered with saprophytes, using them as shelter and for nutrition. The number of attached bacteria grows with time elapsed since desquamation, and depends on the abundance of saprophytes in the vaginal secretions. The situation is the

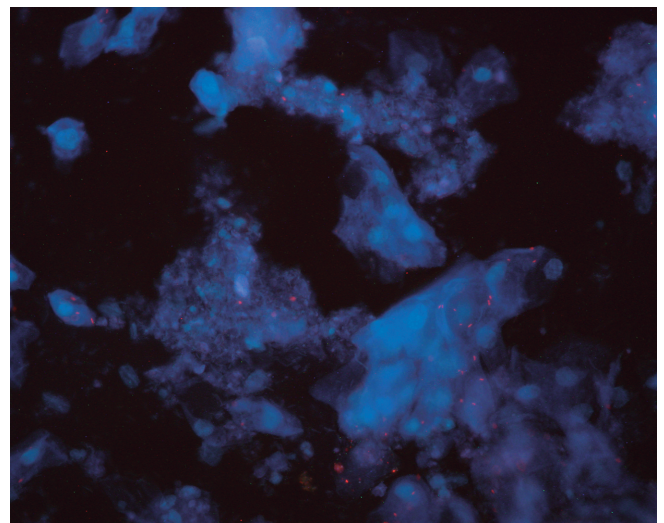


Fig. 9. DAPI stain of urine sediment from a woman with urinary infection (*Proteus* sp.). Multiple bacteria can be seen organized in islands of bacterial sludge in DAPI stain (blue fluorescence). Hybridization with Gard Cy5 probe (*Gardnerella*), dark red fluorescence, visualizes single *Gardnerella* bacteria located within the bacterial sludge (red dots). x 400

opposite in BV. The bacterial cover of the clue cells is an extension of StPM-*Gardnerella* biofilm of the vaginal surface. The bacterial coat is therefore most prolific on freshly detached cells and gets progressively dissolved and lost within the vaginal fluid with time (Fig. 4). Both growing bacterial attachment (healthy) and detachment (BV) are an epiphenomenon of low practical value, which adds no substantial information to the detection of clue cells in native preparations. Graduating two opposite processes into a single pathologic progression is wrong.

Multicolour FISH enables the identification of potentially all bacterial groups in structural relation to each other and in relation to anatomic structures of the host. Other than vaginal smears biological material can be investigated for in situ presence of bacteria and bacterial biofilms, including vaginal biopsies, surgically removed material of vagina and ovary, curettage material from the uterus, sperm samples, medical devices, foreign bodies removed from vagina or uterus, and desquamated epithelial cells in the urine.

Desquamated vaginal epithelial cells in urine

Vaginal smears were until now the main source of data for vaginal microbiota. Each smear is, however, unique with regard to the investigated area of the vagina, thickness and distribution of material on the glass surface, leading to considerable restrictions of the evaluation. First, it is impossible to prepare two smears exactly the same. FISH affords multiple hybridisations with probes covering a wide range of bacterial species. The sets of these hybridisations must be exactly reproducible. Vaginal smears are highly inappropriate

for this purpose. Second, vaginal smears afford a gynaecologic examination, and multiple examinations are inconvenient for the women. Third, women visiting a gynaecologic practice clean the vaginal opening shortly before the visit and only in exceptions is the natural environment available.

Fortunately, smears are dispensable. Desquamation of epithelial cells is a continuous natural process. While voiding, cells are washed from the epithelial surface in large numbers. Sample collection can be performed by the patient herself at any time of the day, independent from doctor visit and before the cleaning procedure. Urine sediments can be fixated, stored over long periods of time, and portioned. This permits a practically unlimited number of standardized hybridizations with any imaginable combination of FISH probes.

The fixated urine sediments allow to longitudinally follow-up polymicrobial infection in different groups of patients and healthy subjects, revealing chains of infection, monitoring natural cause of disease, its response to antibiotic therapy, impact in changes of life style, sexual activity, pregnancy, age etc. and performing this monitoring practically in real time.

Appearance of bacteria and desquamated epithelial cells in urine sediments

Bacteria in urine sediments were either associated with epithelial cells and leucocytes or were unattached.

Epithelial cells in urine sediments were easy to recognize by their large size and bright nuclei in DAPI counter stain, they lay singular or in agglomerates of two to 20 cells or more (an example can be seen in Fig. 12A

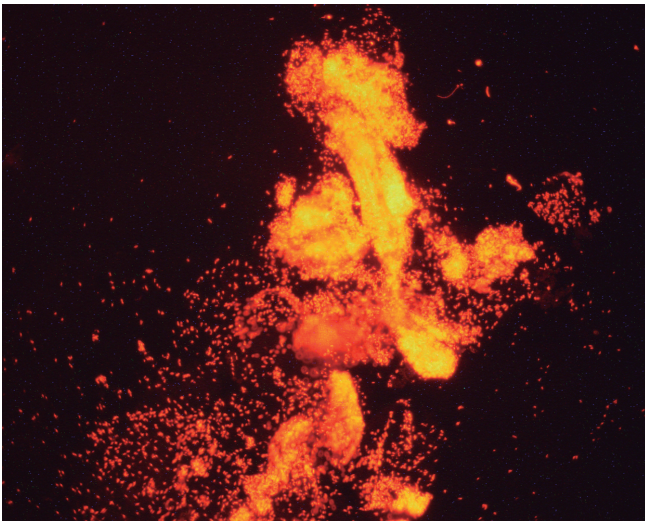


Fig. 10. Brick-wall-like organization of StPM-*Gardnerella* biofilms coating the vaginal desquamated epithelial cells. The disintegration of the bacterial coat with increasing distance to the epithelial cells is apparent, Gard Cy5 probe, dark red fluorescence. x 400.

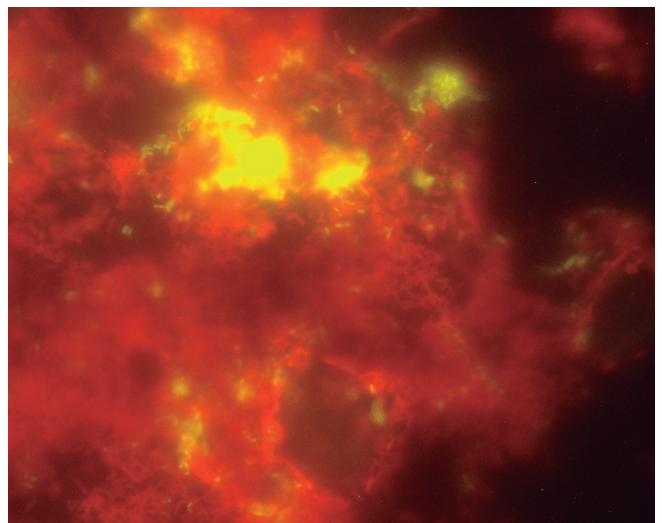


Fig. 11. Lactobacilli within StPM-*Gardnerella* biofilm covering the clue cells. Hybridization was performed with Gard Cy5 probe (dark red fluorescence) and Lab Cy3 probe (orange fluorescence). Accumulations of lactobacilli in similarly high concentrations were not observed in healthy women.

Structured polymicrobial *Gardnerella* biofilms

which is discussed later in the text). Generally, the concentrations of epithelial cells were higher in the morning than in the evening samples and in BV women than in healthy controls.

Urine bacteria non-associated with desquamated epithelial cells

Bacteria freely suspended in urine and not associated with desquamated epithelial cells were most abundant in symptomatic urinary infections. They were either diffusely distributed over the microscopic field (Fig. 8) or concentrated to islands of bacterial sludge (Fig. 9). Diffuse distribution is most typical for *Enterobacteriaceae*.

In the case of bacterial sludge, one urinary pathogenic group dominates numerically. The sludge, however, incorporates multiple other, not necessarily urinary infection related groups of bacteria. *Gardnerella* can also be occasionally seen as a single bacteria embedded in masses of other bacterial groups (Fig. 9).

Bacteria and StPM biofilms associated with desquamated vaginal epithelial cells in longitudinal investigations of urine sediments

Bacterial vaginosis

Urine samples from 4 women with BV were investigated daily for one week and weekly for 8 weeks, resulting in 60 samples. All samples were positive for StPM-*Gardnerella* dominated biofilm, although in 4 samples no epithelial cells were seen during the initial microscopy. These sediments were centrifuged once

more to obtain visible numbers of epithelial cells, which proved then to be positive for StPM-*Gardnerella* biofilm.

In 48 samples more than 80% of desquamated epithelial cells in the urine sediments were covered by a prolific structured polymicrobial biofilm (Fig. 10, Table 3). In 8 samples the percentage of coated epithelial cells was between 20 and 50%. *Gardnerella* was the only obligate and always numerically predominant constituent of this biofilm. *Gardnerella* bacteria had a short coccoid rod form, were tightly packed to a characteristic “brick-wall-like” structure and were most concentrated on the surface of the epithelial cell. They progressively dissolved in fluid surroundings resulting in increasing disintegration of the StPM biofilm with larger distance to the epithelial cell surface (Fig. 10).

Similar to the situation already described for vaginal biopsies from BV women (Table 2, Figs. 5, 6), the StPM-*Gardnerella* biofilm of the clue cells harboured a vast diversity of microorganisms. Lactobacilli were present in 82% of the samples (Table 3). The concentrations of lactobacilli were extremely high in some samples (Fig. 11). *Atopobium* was the second most frequent bacterium after *Gardnerella* within the StPM-*Gardnerella* dominated biofilms. It was detected in 42/45 urine samples from 3 women with BV and completely absent in all samples from one woman with BV (Table 3).

Healthy women

Urine samples from 10 healthy controls were investigated daily for one week and weekly for 8 weeks resulting in 150 samples. In healthy women, bacteria

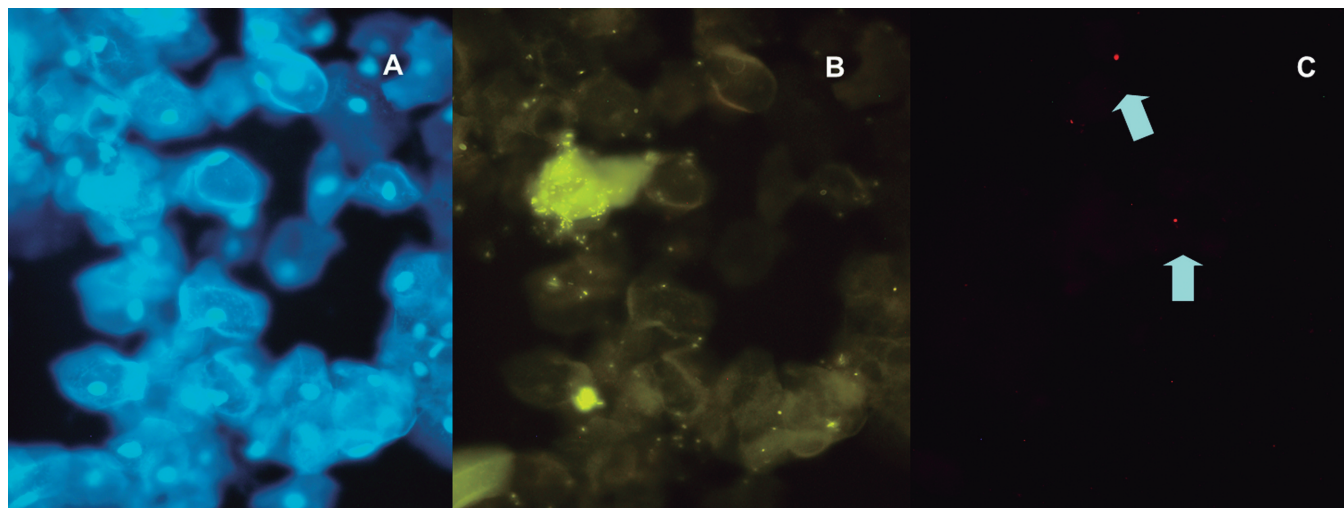


Fig. 12. Urine sediment of a healthy adult woman. Fluorescence of the same microscopy field with three different filter sets x 400. **A.** DAPI stain, mainly colouring desquamated epithelial cells of the vagina. **B.** orange fluorescence of Eub338 Cy3 probe representing all bacteria. **C.** Dark red fluorescence of Gard Cy5 probe representing *Gardnerella*. Most desquamated epithelial cells are free of bacteria, some of the epithelial cells adsorb clots of mixed bacteria. Single cells of *Gardnerella* can be seen dispersed between epithelial cells and other bacteria (arrows).

Structured polymicrobial *Gardnerella* biofilms

were seen mainly in association with desquamated epithelial cells, irregularly attached to them as single bacteria or in clots of up to 50 bacteria (Fig. 12B). The composition of these clots varied widely. No structural organization or stable assembly patterns were noticeable.

Lactobacilli were most common and occurred in 5 women in more than 80% of their urine sediments (N=68/75). Lactobacilli were present in about 50% (20/45) of the samples of 3 healthy women and in 5 of 30 samples of 2 healthy women. However, the overall number of lactobacilli attached to epithelial cells was often low. Lactobacilli were represented by one to a maximum of 5 bacteria per microscopic field in 37 of 93 Lab-probe positive samples.

Generally, the overall bacterial numbers were dependent on the number of epithelial cells and therefore highly variable from sample to sample. In three women, however, a maximum of 2 lactobacilli bacteria were seen per 50 desquamated epithelial cells in all 14 of the urine samples positive for lactobacilli. Despite low incidence and concentrations of lactobacilli, these women were free of any symptoms. Obviously, in contrast to the era of Döderlein, modern women have a much higher

standard of personal hygiene and substantially lower bacterial concentrations in the vagina, including lactobacilli, than 100 years ago. The lack of these did not cause any discomfort.

The occurrence of *Gardnerella* and *Atopobium* were 12% and 7% of samples, accordingly, and were typically represented through singular bacteria irregularly dispersed over the microscopic field (Fig. 12C). We called them non-StPM-biofilm-embedded- *Gardnerella* “dispersed *Gardnerella*”, because of their appearance and distribution. Dispersed *Gardnerella* were observed in 18/30 samples from 2 healthy women. Accumulations of *Gardnerella* of up to 10 bacteria were seen in 5 samples of one woman; however, these were not in contact with each other or with epithelial cells.

Most desquamated epithelial cells are free of bacteria, some of the epithelial cells adsorb clots of mixed bacteria. Single cells of *Gardnerella* can be seen dispersed between epithelial cells and other bacteria (arrows).

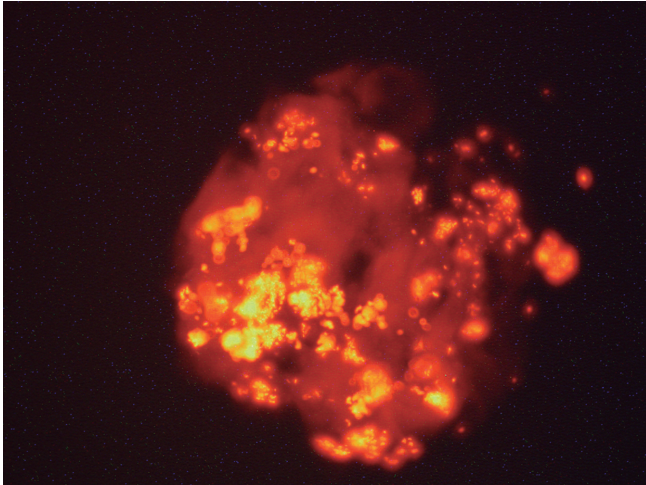


Fig. 13. StPM-*Gardnerella* biofilm in a 26-year old male, Gard Cy5 probe, dark red. x 1000

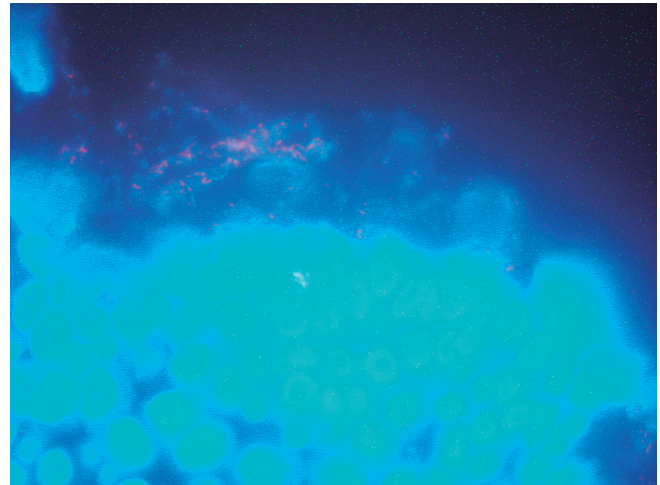


Fig. 14. StPM-*Gardnerella* biofilm after two days of metronidazole therapy. Bacteria are still seen in DAPI, but they do not hybridize with the FISH probes. Only single positive signals (Gard 5 probe Cy5, dark red fluorescence) can be seen within the framework of prolific biofilm. x 1000

Table 3. Occurrence and (concentrations) of selected bacterial groups associated with desquamated vaginal epithelial cells in urine sediments of women with bacterial vaginosis and healthy women.

N=samples	StPM- <i>Gardnerella</i>	Dispersed <i>Gardnerella</i>	Lab	Ato
Healthy 150	0/150 (0)	18/150 (2) 12%	93 (10) 62%	10/150 (3) 7%
BV 60	56/60 (4) 94/100%*		49/60 (4) 82%	42/60 (3) 70%

The number of patients positive for StPM-*Gardnerella*, dispersed *Gardnerella*, Lab (*lactobacilli*) and Ato (*Atopobium*) are given in parentheses. *: including urine samples centrifuged for a second time for higher concentration of epithelial cells.

Structured polymicrobial *Gardnerella* biofilms

Occurrence of structured polymicrobial Gardnerella biofilms and dispersed Gardnerella in patients admitted to a general hospital

Midstream urine samples of randomly selected 100 female, 100 male and 100 children (50/50 boy/girls) hospitalized in a general hospital for indications other than gynaecologic diseases and sent to microbiologic laboratory to exclude urinary tract infection were investigated. An aliquot of urine was fixated in Carnoy upon delivery to the routine microbiologic laboratory and used for FISH.

The occurrence of dispersed *Gardnerella* was 22% in female, 4% in male, and 10% in the paediatric population. The occurrence of structured polymicrobial *Gardnerella* biofilms was 13% in female, 7% in male and 0% in the paediatric population. StPM-*Gardnerella vaginalis* biofilms in males were undistinguishable from those in women with BV (Fig. 13).

Occurrence of structured polymicrobial Gardnerella biofilms and dispersed Gardnerella in the general population

We analysed 3700 urine sediments from 262 women and 69 men in randomly selected outpatients visiting a general practice with FISH. The patients were not uro-gynaecologically examined, attended the outpatient clinics of the Charite hospital for different mainly gastroenterologic indications (patients with inflammatory bowel disease IBD were not included) and can be regarded as representatives of the general adult population.

StPM-*Gardnerella* dominated biofilms were found in 22% (58/262) of women and 13% (9/69) of men, indicating that there is no principal difference between distribution of StPM-*Gardnerella* biofilms in the male and female population.

For comparative analysis of bacteria associated with desquamated epithelial cells, we randomly selected 150 women (50 negative for StPM in all samples and 100 positive for StPM in at least one of the delivered urine samples) of which at least three consecutive urine samples taken monthly were available. The selection resulted in altogether 683 samples.

The data on occurrence and concentrations of selected bacterial groups associated with vaginal desquamated epithelial cells in urine sediments (Table 4) did not principally differ from data raised while investigating vaginal biopsies (Table 2), indicating that the investigation of desquamated epithelial cells in urine samples can to some degree substitute the vaginal biopsies. The occurrences of *Gardnerella*, *Atopobium* and *Lactobacilli* were qualitatively similar between biopsies and desquamated epithelial cells (Table 2 versus Table 4). Especially the visual appearance of the StPM-*Gardnerella* biofilms was marked and unmistakable for diagnostic purposes. However, the availability of desquamated epithelial cells in the urine from the same

subjects was irregular and highly fluctuated, probably contributing to the lack of detection of StPM-*Gardnerella* in 27/292 of the repeated samples from women otherwise positive in at least one of the consecutive urine samples. Furthermore, the pollution of desquamated epithelial cells by bacteria was high in the urine sediments, resulting in a higher incidence of concomitant bacterial groups when compared to biopsy results. Because of this pollution and inhomogeneous attachment of bacteria to single epithelial cells in the urine sediments, the quantification of less numerous bacterial groups was difficult and unreliable.

StPM-Gardnerella biofilm in couples awaiting childbirth and visiting a medical practice

The incidence of StPM-*Gardnerella vaginalis* biofilms in urine sediments was evaluated in unrelated female and male patients, who were hospitalized or attended a general practice. Because it is important to follow the link between sexual partners, we evaluated 72 married couples awaiting childbirth. They delivered urine samples when returning for routine evaluations. Women were additionally gynaecologically investigated and a Nugent score was determined.

The occurrence rate of cohesive *Gardnerella* in unselected pregnant women was 17% (Graph 1, Table 7D).

While there was no congruence between findings of dispersed *Gardnerella*, the sexual partners shared the presence or absence of StPM-*Gardnerella* biofilms (Swidsinski et al., 2010a).

All male partners of pregnant women, who were negative for StPM-*Gardnerella* biofilms (N=60) were also negative (N=60). The urine samples from male partners of pregnant women positive for StPM-*Gardnerella* biofilms (N=12) were either positive for StPM-*Gardnerella* (N=8) or were non-analyzable because of the low number of desquamated epithelial cells (N=4). None of the women negative for StPM-*Gardnerella* had symptoms consistent with BV according to the Amsel criteria. Two of the 53 women negative for StPM-*Gardnerella* had a Nugent score >6, three had a Nugent score between 4 and 6, and all others a score of ≤3. Seven of the 12 women positive for StPM-*Gardnerella* biofilm fulfilled the Amsel criteria for BV. The Nugent scores in 10 of the 12 pregnant women with cohesive *Gardnerella* were >6. Five women were asymptomatic or oligosymptomatic (Amsel=2) indicating that bacterial vaginosis is a symptomatic form of StPM-*Gardnerella* biofilms.

The male partners were not clinically investigated and no clinical symptoms were available.

The anatomic location of StPM-*Gardnerella* in women is obvious. However, what could be an equivalent of the vagina in male and why did some of the urine samples from male partners of the women positive for the StPM-*Gardnerella* biofilm contain no epithelial cells?

Impact of the prepuce on the number of epithelial cells in urine samples

Urine sediments from randomly selected women visiting a general practice contained 10 to 10,000 desquamated epithelial cells per area of microscopic evaluation (approximately 25 microscopic fields at magnification of 100). In men the variation was similar (0 to 10,000 epithelial cells). However 18% of the male and none of the female urine samples contained less than 5 visible cells.

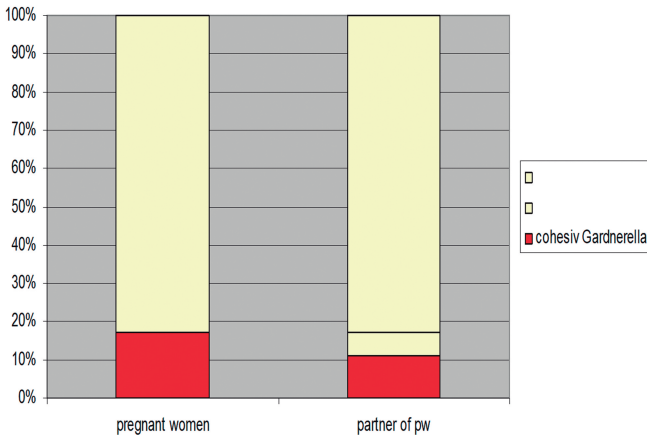
To explain the difference we asked three of the healthy male volunteers to deliver two urine samples per day for 10 days. One sample had to be obtained with the foreskin left over the glans penis, the other with the foreskin pulled back.

The difference was marked. When the prepuce was pulled back, the number of epithelial cells was low and 65% of the samples were non-analyzable. When the prepuce was covering the glans penis, the number of epithelial cells was significantly higher and only one urine sample was non-analyzable (Graph 2).

We therefore recommended to the male probands to leave the prepuce over the glans penis for sample collection. From this point on we also recommended to both males and females to avoid cleaning the genitals on the evening before investigation and to use the first portion of the morning urine.

How long may the StPM-Gardnerella biofilms persist after the end of the sexual partnership?

One of the BV patients participating in the longitudinal investigation of the urine samples had married for a second time 11 years ago. She reported that the BV complaints started with the second marriage. We collected urine samples from both partners. The husband from the first marriage was found negative for *Gardnerella*, while the second husband was positive for StPM-*Gardnerella* biofilm. The second husband was divorced 15 years ago from his first wife, who had no children and who remained unmarried. We found this woman. The investigation of a urine sample from this woman proved positive for StPM-*Gardnerella* biofilm. The *Gardnerella vaginalis* strains were isolated from all three affected persons and sent for genotyping to Ghent, Belgium. The genotyping used arbitrarily primed PCR (RAPD) with fluorescent primer OPM1 followed by



Graph 1. Occurrence of cohesive *Gardnerella* (StPM-G) in unselected pregnant women and their partner.

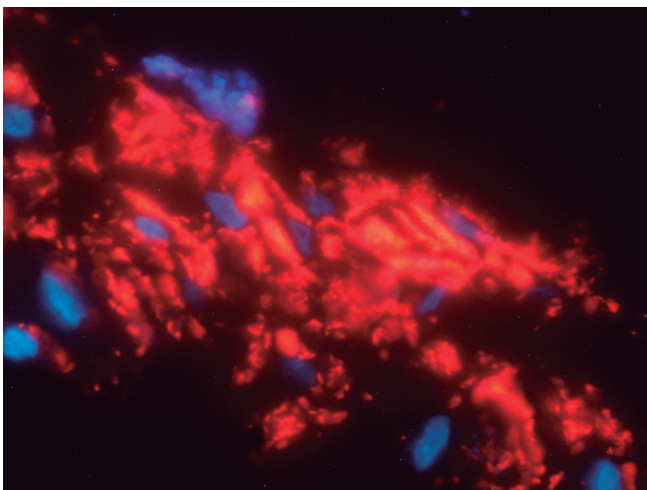


Fig. 15. StPM-*Gardnerella* biofilm growing on a membrane strip inserted overnight into the vagina of a woman with BV. Hybridization with Gard Cy5 probe, dark red fluorescence, and DAPI counterstain, blue fluorescence. x 400

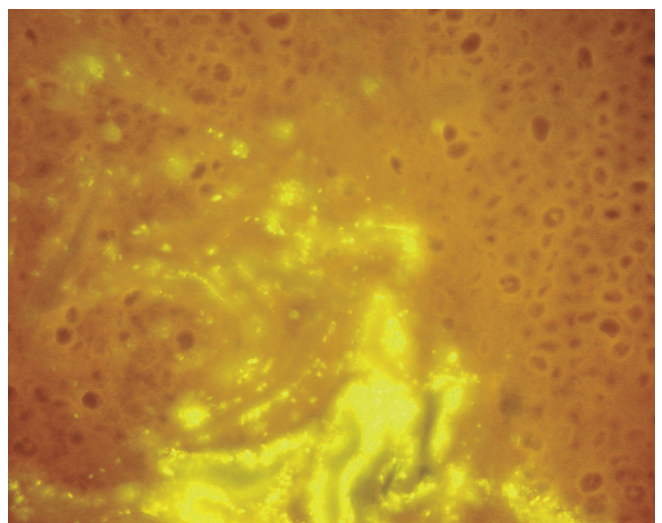


Fig. 16. Structured polymicrobial *Gardnerella* biofilm in material of uterine curettage Prolific *Gardnerella* dominated biofilm within the endometrium. Hybridization is performed with Gard Cy3 orange fluorescence FISH probe, at magnification x1000. The anatomic location of bacteria within the endometrium excludes the possibility of contamination.

Structured polymicrobial *Gardnerella* biofilms

capillary electrophoresis. The strains were identical indicating a branched chain of infection (Swidsinski et al., 2010a). Obviously StPM-*Gardnerella* biofilms can be acquired by sexual contact and prevail over years.

Interestingly, two female children of the second marriage with both parents positive for StPM-*Gardnerella* biofilms, and living together in the same home, were all negative. No transfection of the StPM-*Gardnerella* biofilm took place despite close body contact.

Donor semen as a vehicle for bacterial vaginosis

A 46-year old woman with recurrent bacterial vaginosis reported that her vaginal complaints started after fertilization attempts four years ago and had continued since then.

To evaluate if StPM-*Gardnerella* biofilms could be transmitted during fertilization, we investigated 20 μ l aliquots of cryopreserved semen samples from 20 men (Swidsinski et al., 2010b). Epithelial cells in low numbers were present in all semen samples during microscopy. In 3 of the 20 semen samples the epithelial cells were covered with a distinctive StPM-*Gardnerella* biofilm. In one sample, epithelial cells were covered with bacterial biofilm, but the bacteria were not amenable to FISH evaluation. The suppression of fluorescence could be the result of freezing or of concomitant antibiotic therapy. Epithelial cells were free of bacteria in 16 samples.

Effects of therapy on the StPM-*Gardnerella* biofilms

Systemic antibiotics

Antibiotics/antibacterials are presently the only known drugs with proven efficacy in BV. Metronidazole and clindamycin are often used. Metronidazole is preferred because of lower side effects and theoretically lower impact on lactobacilli. The efficacy of antibacterials is high, and reaches 80-90% as long as clinical criteria are used for evaluation (Verstraelen and Verhelst, 2009), although the recurrence rates are as high as 50% three months after the end of therapy.

Different to the clinical data, FISH monitoring of the StPM-*Gardnerella* biofilms on the vaginal surface

during therapy was disappointing with regard to cure rates. In our first interventional study (Swidsinski et al. 2008), vaginal biopsies from women with BV were taken at different time points after the start of therapy with metronidazole. The biopsies were taken only once in order to reduce the inconvenience for the women.

Starting with the first day of therapy, the numbers of adherent bacteria dropped below 10^9 , which are typical for BV. However in none of the biopsies at any time point did the adherent bacteria disappear completely. The biofilm remained preserved in all cases in concentrations of higher than 10^7 bacteria/ml, except in one patient and at one time point of investigation.

Although the bacterial layer could still be seen with DAPI stain, which stains all DNA rich structures, most bacteria within the biofilm layer stopped hybridizing with bacteria specific FISH probes, indicating low metabolic activity or loss of ribosomes (Fig. 14).

In Graph 3 each dot represents a single vaginal biopsy plotted on a time scale (horizontal axis) and related to concentrations of bacteria found on the vaginal surface (vertical axis). The colour of dot expresses the pH in the vagina, the number on the side expresses the presence of bacteria identifiable in DAPI stain that still hybridized with the Eub 338 probe universal for all bacteria. The characterization of the biofilm with group specific FISH probes was impossible during this global suppression.

During the therapy and one week after the therapy, $\leq 5\%$ of all bacteria were amenable in 4/10 and $\leq 50\%$ in 6/10 samples. The suppression was temporary and already two weeks after treatment cessation, the amenability reached more than $\geq 90\%$ of bacteria within the biofilm in 8/10 and $\geq 40\%$ in remainder 2/10 biopsies.

Disappointed with the outcome of metronidazole treatment, we performed a similar trial with moxifloxacin in the hope it would be more efficient against *Bifidobacteriaceae*. Moxifloxacin was given over 5 days. The results were broadly similar to those observed with metronidazole. More than 50% of the biopsies during therapy with Avalox (moxifloxacin) and one week thereafter still showed adherent biofilms on their surface. Similar to metronidazole treatment, this biofilm was not amenable to the FISH probes during therapy, but was amenable 4 weeks after the end of

Table 4. Occurrence and concentrations of selected bacterial groups associated with desquamated vaginal epithelial cells.

	StPM- <i>Gardnerella</i>	Dispersed <i>Gardnerella</i>	Lab Lactobacilli	Ato <i>Atopobium</i>	Ebac <i>Enterobacteriaceae</i>	Str i Streptococci	Bac <i>Bacteroides</i>
No biofilms N=100	0%	51/391 (13%)	263/391 (67%)	30/391 (8%)	95/391 (24%)	48/391 (12%)	28/391 (7%)
StPM N=50	265/292 91%	8/292 (0.3%)	235/292 (81%)	142/292 (49%)	130/292 (45%)	121/282 (43%)	113/292 (39%)
Concentration 10^4 /ml*	2.8 \pm 4.9		0.8 \pm 1.9	0.3 \pm 1.2			

*: The overall concentrations of adherent bacteria in the urine resulted from multiplication of mean number of bacteria per epithelial cell with the concentration of epithelial cells per ml of urine.

therapy in a significant proportion of women (Swidsinski et al., 2011).

Local therapy

The incidence and the severity of side effects of antibiotic therapy increase with prolonged or repeated treatment. The decision for such should be carefully considered. Local therapy is less worrisome. Acigel[®], Octenisept[®], yoghurt or *Lactobacillus acidophilus* and hydrogen peroxide are in discussion. In Germany acigel (Relactagel[®]) and Octenisept[®] are available as standardized vaginal therapeutics and can be purchased as over-the-counter (OTC) products.

Relactagel[®]

10 women with BV were treated daily with local application of Relactagel[®] containing lactate and glycogen, for four weeks. The state of StPM-*Gardnerella* biofilms was evaluated weekly in urine sediments. Eight women reported improvement of the local vaginal discomfort. However, in none of the 40 urine samples did StPM-*Gardnerella* biofilms disappear. The density of StPM-*Gardnerella* biofilms covering the desquamated epithelial cells was lower in the first days of Relactagel[®] application, probably due to the dissolving ability of Relactagel[®], but the number of epithelial cells covered with StPM biofilm was not diminished and even increased during treatment.

Octenisept[®]

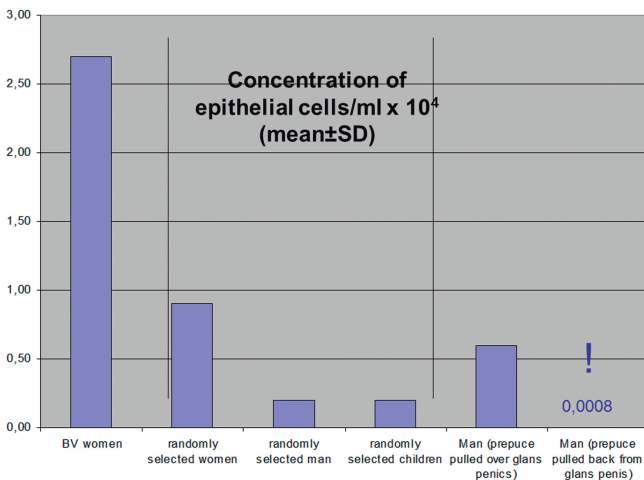
The Octenisept[®] vaginal therapeutic agent is specifically developed to treat both partners and contains the antiseptic octenidine dihydrochloride. The 24 women included in the trial were asked to treat their partner or

avoid sexual contact during the study. The initial therapy lasted 7 days. The effects of the therapy were monitored using urine sediments. The results were at first encouraging. In 21/24 women, the StPM-*Gardnerella* biofilm was undetectable after the end of therapy. The patients with positive response included the above discussed family in which the chain of infection could be followed during 17 years. In this family, the positive therapy effect remained preserved until now, 18 months later, indicating that the StPM can be cured even in cases of lacking previous responses to multiple systemic antibiotic therapies. However, the rate of therapy failures increased with time and higher patient numbers. In 3/24 women, the biofilm persisted after the 7-day therapy. Additionally, in 14/21 (67%) women with initial response, the StPM- *Gardnerella* was again detectable within one to 6 months after the end of therapy.

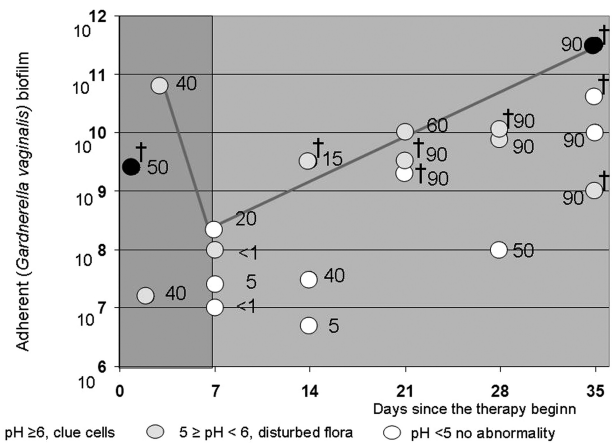
Three women without response and 14 women with relapse were again treated with Octenisept[®], this time for 28 days. No response was observed in 6/17. In 11/17 women the StPM-*Gardnerella* disappeared from the urine, but appeared again in 4 women within two months.

Four women with relapse after the second round of the therapy were treated with 28 day Octenisept[®] daily followed by weekly applications of Octenisept[®] for a duration of another 2 months. Only one of these women responded. The cumulative non-response rate after 3 rounds of the therapy was 38%.

Possible explanations for the antibiotic/antibacterial recalcitrance of structured polymicrobial *Gardnerella* biofilms may be the functional differentiation within polymicrobial communities or the anatomic spread of the biofilm. Because concentrations of drugs vary in tissues and fluids from vagina, urethra, intrauterine, intrapelvic etc., the biofilms, which spread over extended regions, may use these gradients in tissue



Graph 2. Concentrations of epithelial cells in urine sediments.



Graph 3. Changes in concentrations and amenability of adherent *Gardnerella* biofilm under metronidazole therapy. 1-90 Percent of DAPI stained bacteria, which positively hybridize with universal bacteria probe. † - *Gardnerella* dominated biofilm with all components can be clearly seen in FISH.

Structured polymicrobial *Gardnerella* biofilms

specific drugs concentrations for their survival strategies.

Horizontal extension of StPM-*Gardnerella* biofilms into the perianal region and rectum

The simultaneous occurrence of certain bacterial groups in the vagina and intestine fuels the theory that intestinal microbiota are responsible for assembly and maintenance of the vaginal microbiome. In this context, it was important to test whether StPM-*Gardnerella* biofilms can spread from vagina over the perianal region into the rectum or vice versa. Keratinizing epithelium of perianal region and intestinal mucosa are however different from vaginal epithelium, as are conditions in these habitats.

Rectal mucosa

The mucus barrier completely separates mucosa from the faecal compartment in healthy persons. No adherent bacteria can normally be found on the surface of rectal biopsies. However, this separation is impaired in a variety of diseases. Bacteria migrate through mucus and form biofilms on the mucosal surface (Swidsinski et al., 2005b). Our laboratory collected over the last 10 years more than 8000 colonic biopsies. In 500 of them a prolific adherent biofilm had been documented. We selected randomly 100 colonic biopsies and tested them for the presence of StPM-*Gardnerella* biofilm with FISH. We found no StPM biofilm or even solitary *Gardnerella* in all investigated biopsies, despite the presence of prolific biofilms when using a FISH probe universal for all bacteria.

Perianal skin

The microbiota of the perianal region were investigated using adhesive tapes. The 2x2 cm Tesa strips were spread over the perianal region of 10 women

with BV for 2 to 5 minutes and then fixated in Carnoy solution. Bacterial biofilm, adherent to the surface, stick to adhesive tape. Sections of these tapes allow the investigation of the biofilms. To be sure that bacterial biofilms attached to the tapes are comparable to those covering vaginal epithelium, we asked 5 of the 10 women to insert 2x2 cm nylon membrane strips overnight into the vagina, remove them in the morning and to put them into Carnoy solution.

Bacterial biofilms detected on nylon membrane strips inserted in the vagina demonstrated all features typical for StPM-*Gardnerella* biofilms (Fig. 15). The *Gardnerella* grew on the surface in structured polymicrobial biofilms building confluent, tightly packed layers.

No structures resembling StPM-*Gardnerella* biofilms were observed on the surface of adhesive tapes in all 10 investigated women with BV, including those with positive biofilms on vaginal inserted strips. Different intestinal bacterial groups could be found coating the adhesive tape and *Gardnerella* could be confirmed as a part of this coat in all women, but there was no resemblance between these bacteria and the StPM-*Gardnerella* biofilms of the vagina. Bacteria on the adhesive tape removed from the perianal skin were diffusely scattered over the surface and nonconfluent or structured (Swidsinski et al., 2010c).

Keratinized skin epithelial cells that were attached to the adhesive tape showed likewise no resemblance to clue cells. Obviously, keratinized epithelium of the perianal region does not promote the growth of StPM-*Gardnerella* biofilms.

Vertical extension of *Gardnerella* biofilms into the uterus and ovarian tubes

Normally, the cavity of the uterus is sterile. Intrauterine colonization is however neither rare nor irrelevant and is reported to be responsible for 25–40% of preterm births (Goldenberg et al., 2008). We

Table 5. Relationship between vaginal StPM-*Gardnerella* biofilm (urine sediment) and microbiota found in materials from uterus and tubes surgically removed for different indications.

N=68 Bacteria in tissues after uterine curettage or tubal resection																		
Curettage material								resected tube										
Missed abortion				Other indications				Pregnancy				Other indications						
All 19	Neg 9	Pos./G- 5	StPM 5	All 27	Neg 17	Pos 8	StPM 2	All 22	Neg	Pos/G-	StPM 1	Neg 17	Pos/G- 4	StPM 0				
Urines positive for vaginal StPM- <i>Gardnerella</i> , N=15																		
8	1 (12%)	2 (25%)	30	5 (62%)	<i>73810</i>	6	2 (33%)	2 (33%)	22	2 (33%)	35	4	0	0	1 (100%)	3 (100%)	0	0 (0%)
Urines negative for vaginal StPM- <i>Gardnerella</i> , N=53																		
11	8 (73%)	3 (27%)	22	0	21	15 (71%)	6 (29%)	0	18	0	0	0	14 (78%)	4 (22%)	4	0	0	0

Neg, no bacteria detected; Pos/G-, no StPM-*Gardnerella* biofilm detected while other bacteria were present; StPM, structured polymicrobial *Gardnerella* biofilm present; (...%), percent of women with specific bacterial findings in surgically removed tissues depending on the indication for intervention; Italics, demonstrate mean concentrations of bacteria x 10⁷/ml found in tissues of women positive for bacteria. No statistical evaluation of the bacterial concentrations was performed because of the small number of subjects within each subgroup.

Structured polymicrobial *Gardnerella* biofilms

compared microbiota found in surgically removed material from the uterus and tubes with vaginal StPM-*Gardnerella* biofilms. Seventy women hospitalized for curettage of the uterus, ovariectomy or tubal resection were consecutively enrolled. All women voided urine in the morning prior to surgery. The urine was fixated in Carnoy solution. Surgery was performed by a single doctor with precautions to avoid contamination with vaginal contents. The urines of two of the 70 enrolled women were lost during transport to the laboratory. These women were excluded from the evaluation. Of the remaining 68 women, 22 were laparoscopically operated on the tubes (one of them for missed abortion) and 46 women had uterine curettage (19 for missed abortion, 27 for other indications). Table 5 summarizes the findings of StPM-*Gardnerella* biofilms and other bacteria in the material from the uterus and tubes of women depending on the diagnosis, kind of surgical intervention, and presence and absence of vaginal StPM-*Gardnerella* biofilms. This resulted in 24 subgroups, which were too small for statistical analysis. However, they present unbiased results, and when pooled unravel many significant relationships.

Occurrence of StPM-*Gardnerella* biofilms on vaginal epithelium

Vaginal StPM-*Gardnerella* biofilms were detected in 18 (26%) of the 68 investigated women (Table 6).

Table 7 shows that StPM-*Gardnerella* was vaginally present in 43% of women with missed abortion. This is significantly higher when compared to nonpregnant women, to unselected cohorts of women with normal pregnancy, and to non-pregnant women hospitalized in a

general hospital or visiting a general practice for nongynaecologic disorders (Table 7, line C, E).

Bacterial colonization of uterus/tubes in relation to vaginal StPM-*Gardnerella* biofilm

Bacteria in the uterus or tubes were found in 25 of 68 women (37%, Table 6).

The impact of vaginal StPM-*Gardnerella* biofilms was suggestive for bacterial ascendance (Tables 5, 6).

While colonization of uterus/tubes in women positive for vaginal StPM-*Gardnerella* biofilms was 67% (12/18), the bacterial colonisation in women negative for vaginal StPM-*Gardnerella* biofilms was 26% (13/50), $P < 0.005$, Table 6.

Structured polymicrobial *Gardnerella* biofilms were found in the uterus of women positive for vaginal StPM-*Gardnerella* biofilms (7/14 or 50%), this is different to the perianal skin. Structured polymicrobial *Gardnerella* biofilms were completely absent in women negative for StPM-*Gardnerella* (0/32), Table 6. Obviously, vaginal StPM-*Gardnerella* biofilms can extend into the uterus.

Vaginal StPM-*Gardnerella* biofilms seem to have no impact on the bacterial colonization of the tubes in nonpregnant women. Three of four women with vaginal StPM-*Gardnerella* had no bacteria in their tubes (Table 6), while 4 of 18 women without vaginal StPM-*Gardnerella* biofilms had bacteria in their tubes other than *Gardnerella*. However, the fact that one woman with missed abortion operated for suspected extrauterine pregnancy had StPM-*Gardnerella* biofilms in her tubes and vagina indicates that under specific conditions vaginal StPM-*Gardnerella* biofilms may ascend to the

Table 6. Occurrence of bacteria in uterus/tubes in women with and without vaginal StPM-*Gardnerella* biofilm.

	Samples of uterus/tubes positive for bacteria
Vaginal StPM- <i>Gardnerella</i> biofilm negative (N=50)	13/50 (26%)
Vaginal StPM-Gard positive (N=18)	12/18 (67%) P <0.005
including StPM-Gard pos. non pregnant	4/9 (44%)
including StPM-Gard pos. pregnant	8/9 (89%)

Table 7. Occurrence of vaginal StPM-*Gardnerella* biofilms in patients with missed abortion and non-pregnant women undergoing tubal surgery or uterine curettage.

Group:	Vaginal StPM- <i>Gardnerella</i> biofilms	P compared to group A
A. Missed abortion (curettage)	9/20* (43%)	
B. Nonpregnant women with curettage of the uterus or tubal surgery	9/48 (19%)	P=0.05
C. Randomly selected women hospitalized for different nongynaecologic diseases (14)	13/100 (13%)	P=0.002
D. Randomly selected pregnant women (14)	12/72 (17%)	P=0.012
E. Samples from randomly selected outpatients from the general practice*	58/262 (22%)	P =0.04

* including one women with resected salpinx

Structured polymicrobial *Gardnerella* biofilms

tubes (Fig. 16).

Vaginal StPM-Gardnerella biofilms, missed abortion and colonisation of uterus/tubes

Despite the small number of patients in the subgroups of Table 6, a clear tendency to increase of bacterial occurrence in uterus and tubes of women with missed abortion and vaginal StPM-*Gardnerella* biofilms can be observed in all subgroups without exceptions. Within a statistical model which characterises each of the 68 patients by three characteristics, pregnant or not (independent 1), vaginal StPM-*Gardnerella* biofilm or not (independent 2), and upper genital tract biofilm or not (dependent), a subsequently performed stepwise LR-based binary logistic regression model, assuming that upper genital tract biofilm status can be predicted by the two independent features shows that both pregnancy and vaginal StPM-*Gardnerella* biofilms are highly significantly predictive of upper genital tract colonization.

When women with vaginal StPM-*Gardnerella* biofilms are removed from the evaluation, the occurrence of bacteria in uterus of women with missed abortion 3/11 (27%) and non pregnant women 6/21 (28%) is actually the same, indicating that the pathogenic significance of StPM-*Gardnerella* biofilm increases in the case of pregnancy and specifically by missed abortion.

Concluding hypothesis and outlook

It is broadly assumed that lactobacilli biofilm covers the vaginal epithelium in healthy women, with the lack of lactobacilli biofilm leading to reduced colonization resistance and ending in dysbiosis (Sobel, 2000; Marrazzo, 2011).

In fact, however, there is no study that proves this or even a single microphotograph published that demonstrates the existence of adherent confluent lactobacilli biofilms on healthy vaginal epithelium either in human or animal. Direct investigation of tissues from healthy human and animal vagina using electron microscopy or FISH demonstrates only single bacteria within a microscopic field, which are diffusely distributed over the vaginal surface.

Likewise, it has never been shown that the presence of lactobacilli in considerable quantities in the vagina is indispensable for the maintenance of health. The concentration of lactobacilli attached to desquamated epithelial cells is low on a permanent basis in many modern healthy women.

Lactobacilli are saprophytes digesting organic material of desquamated epithelial cells in vaginal secretions and therefore often found in vaginal smears of healthy women. They may be beneficial for maintaining and restitution of the vaginal milieu; however, the “positive” impacts should be demonstrated in direct interventional studies and not deduced from presence in healthy and absence in BV women, especially when the

detection is based mainly on wet microscopy or grams stains. Ontogenetically, the vagina originates from the cloaca. The high bacterial load of cloacae is an immense threat for reproduction. Reptiles and cloacal animals have developed tools to control bacteria in this critical region during evolution. Although only some of these tools are described thus far - their efficiency is beyond any doubt. We may assume that similar mechanisms are at work in the human vagina and hinder bacteria from permanently settling on the vaginal epithelium. The region remains however open for efflux from perianal skin that is rich with bacteria and is deeply polluted during sexual activity.

The vaginal self-cleansing hinders effluent bacteria from permanently settling. Only highly specialized species, which can exist at low pH and use desquamated epithelial cells as substrate and shelter, can temporarily survive.

The so-called “healthy vaginal flora” is not the result of a stable settlement but rather an equilibrium between vaginal self-cleansing and continuous recolonisation. It appears that the “stability” of the vaginal microbiota depends on bacteria that are ubiquitous, highly abundant and at the same time optimally specialized for this hostile environment. Repeated investigations of the vaginal flora over the menstrual cycle demonstrate a nearly complete change in the participants over short periods of time (Srinivasan et al., 2010; Santiago et al., 2011; Gajer et al., 2012).

The situation is different when bacteria, instead of adapting themselves to hostile conditions, deliberately reshape the environment. To succeed, they need a critical mass of biochemical power, a permanent anchor in the vagina, resistance to mechanisms of native and adoptive immunity, and the ability of quick response to abrupt changes during the menstrual cycle. These tasks cannot be achieved with single bacterial groups. The situation is different in the case of biofilms and even more so in the case of structured polymicrobial biofilms (StPM).

StPM-*Gardnerella* dominated biofilms in bacterial vaginosis are typical representatives. Cohesive *Gardnerella* is obviously a constitutive component: anchor and matrix. Other bacterial groups embedded within the matrix of *Gardnerella* biofilm impart the biofilm specific pathogenic properties and biochemical potentials, making it extremely variable and well equipped to any possible negative event. None of the single bacterial groups constituting StPM biofilm is sufficient for survival and propagation. All ingredients contribute to the success. Because the composition of the vaginal StPM-*Gardnerella* biofilm is in each subject individual, the term should be biofilms and not biofilm. The assembly to a functioning entity is obviously not trivial. A lack of vaginal StPM-*Gardnerella* biofilms in children, even from mothers with BV, indicates that the availability of single bacterial groups in the environment is not decisive. Despite close body contact between parents and children and exchange of single bacterial groups, no assembly of StPM biofilms takes place. Probably multicellular biofilms do not often arise anew,

but like multicultural organisms have a long evolutionary history during which different bacterial groups go together, co-evolve their structure and properties, maintain stability conserving their main constituents and exchange only subsidiary components.

StPM-*Gardnerella* biofilms grow within the anatomic region they are best adapted to, occupy niches of the vagina or prepuce, spread into the distal part of the urethra and ascend into the uterus or even into the tubes. Like the rain forest, which creates living space for biodiversity of different plants and animals, vaginal StPM-*Gardnerella* biofilm create niches for different pathogens, which normally have no access to the vaginal epithelium, making the human body susceptible to different secondary infections and jeopardizing pregnancy.

To be transmitted, the biofilm must maintain its structural integrity. Since the intact structure of the biofilm is indispensable, it is the polymicrobial biofilm and not the single constituents that are the true subject of the infection. The desquamated “clue cells” that carry the structurally organized polymicrobials are most appropriate as vehicles for StPM-*Gardnerella* biofilm transfection. The contamination takes place by intercourse or insemination with infected sperm samples. Presently, we have no usable culture techniques for growth of StPM-*Gardnerella* biofilms and cannot test their properties in vitro. However, the development of reliable, quick and cheap FISH methods for monitoring of StPM-*Gardnerella* biofilms on vaginal epithelial cells of urine sediments allows us to test in vivo the occurrence, to follow the individual chain of infection, and to investigate the response of StPM biofilms to different events, including therapy regimes.

Unfortunately, the diagnostic possibilities always run ahead of treatment options.

Similar to prior clinical trials, we were not able to reliably cure the disease with the use of antibiotic or local antiseptic therapies. Eradication occurred in about 30% of women. In the remainder, the StPM-*Gardnerella* biofilms down regulated their metabolic activity for the duration of therapy, recovered quickly, and became as prolific as they were before treatment (Swidsinski et al. 2008, 2011).

Future therapeutic trials should expand the therapy duration over longer time periods during which a complete eradication of the biofilms takes place. It appears reasonable to combine local intravaginal therapy directly impairing the StPM biofilm body with systemic antibiotics, which reach the intravaginal/tubal component of the biofilm. The treatment of the sexual partner and the monitoring of the effects at all stages of intervention are likewise necessary to terminate this otherwise probably life-long disease.

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