BASIC SCIENCE: GYNECOLOGY

An adherent *Gardnerella vaginalis* biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole

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OBJECTIVE: The purpose of this study was to determine the efficacy of standard treatment with oral metronidazole in the eradication of the bacterial vaginosis biofilm.

STUDY DESIGN: We conducted an interventional follow-up study in which 18 patients with bacterial vaginosis were treated with oral metronidazole during 1 week and subsequently had a single random follow-up assessment at 1-week intervals, up to 5 weeks, with 3 patients representing each point in time. Follow-up assessment included conventional scoring of the vaginal microflora and determination of bacterial biofilm characteristics on a vaginal biopsy through bacterial 16/23S recombinant DNA–based fluorescence in-situ hybridization.

RESULTS: Although all patients recovered, we consistently observed the resurgence with treatment cessation of a dense and active bacterial biofilm on the vaginal mucosa, primarily consisting of *Gardnerella vaginalis* and *Atopobium vaginae*.

CONCLUSION: A large reservoir of the core bacteria to bacterial vaginosis persists as a biofilm after metronidazole treatment.

Key words: Antibiotic resistance, bacterial biofilm, bacterial vaginosis, *Gardnerella vaginalis*, metronidazole

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Bacterial vaginosis is the single most common infection of the reproductive tract among women of childbearing age.[1] Besides a nuisance problem, bacterial vaginosis is associated with a tremendous disease burden of adverse obstetric and gynecologic outcome.[1] As a state of diminished colonization resistance, bacterial vaginosis also renders women particularly vulnerable to the acquisition of sexually transmitted diseases that include gonorrhoea,[2] chlamydiosis,[2] genital herpes,[3] and HIV-1.[4] Moreover, bacterial vaginosis propagates viral replication and vaginal shedding of the HIV-1[5-7] and HSV-2[8] viruses, thereby further enhancing the spread of pandemic sexually transmitted diseases.

Although control of bacterial vaginosis has emerged as a global issue of concern,[4,9] treatment abilities for bacterial vaginosis are limited. Standard treatment regimens, as recommended by the Centers for Disease Control and Prevention,[10] achieve primary cure rates of 60%-70%, whereas 20%-30% of those women who initially were treated successfully will experience relapse within 3 months.[1,4,11]

As a putative explanation, we recently documented that bacterial vaginosis is not only an overgrowth condition but also involves the presence of a dense, adherent bacterial biofilm on the vaginal mucosa, which is an obligate finding in bacterial vaginosis, and is absent among healthy control subjects.[12] Bacterial biofilms recently have been associated with several recalcitrant infections (eg, involving *Escherichia coli*, *Helicobacter pylori*, and *Pseudomonas aeruginosa*).[13] The biofilm is a prominent virulence mechanism that enhances bacterial attachment to epithelial surfaces, allows bacteria to reach much higher concentrations than in luminal fluids, and, as a immune subversion mechanism, prevents antimicrobial agents to reach the bacteria that often reside as nonmultiplying, latent colonies within the biofilm.[14]

In the present study, we aimed to study the efficacy of standard treatment with oral metronidazole in the eradication of bacterial vaginosis biofilm and assessed the persistence and the activity of the biofilm up to 5 weeks after treatment. Bacteria within the biofilm were visualized with 16/23S ribosomal DNA (rDNA)–based fluorescence in situ hybridization (FISH) of vagi-

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nal biopsy specimens that were obtained during therapy and at 1-week intervals after treatment.

MATERIALS AND METHODS

Study subjects

Eighteen white women with bacterial vaginosis (mean age, 26.3 ± 5.8 years) who attended the Vivantes Clinic for Obstetrics and Gynecology as out-patients from June 2005-March 2006 were enrolled. Initial diagnosis of bacterial vaginosis was confirmed by both standard clinical (Amsel)[15] and microbiological (Nugent)[16] criteria (ie, patients were considered eligible if they had at least 3 of the 4 Amsel criteria [increased vaginal discharge with a homogeneous appearance, a pH of > 4.5, presence of clue cells on wet mount, and/or a fishy amine odor on the addition of 10% potassium hydroxide) in addition to a Nugent score of $\geq$7 on a Gram-stained vaginal smear. Analyses of wet mounts and Gram stains were performed by a single investigator. Gram-stained vaginal smears were photographed and Nugent scores were confirmed by a second investigator. In addition, culture of the vaginal microflora was performed as outlined later. All women agreed to participate in the study through written informed consent, and the study protocol was approved by the Institutional Review Board of the Charité Hospital.

Interventions and sample collection

After diagnosis, all women received standard oral therapy with 500 mg metronidazole twice daily for 7 days.[10] Study subjects subsequently were assigned randomly for a single follow-up assessment during early treatment (day 3) or at 7, 14, 21, 28, or 35 days after treatment, respectively; 3 women were considered for evaluation at each point in time. At each follow-up visit, before biopsy, vaginal microflora status was reassessed through wet mount and clinical criteria, Gram stain, and culture. Then, a vaginal biopsy specimen (1-3 mm diameter) was taken from the lateral wall of the mid portion of the vault with a biopsy forceps (Nr. ER 058 R; Aesculap, Tüttlingen, Germany) without any pretreatment, fixed in nonaqueous Carnoy solution (6/3/1 vol ethanol/glacial acetic acid/chloroform) for 2 hours, and processed and embedded into paraffin blocks by standard techniques. Microtome biopsy sections (4 μm thick) were placed on SuperFrost slides (R. Langenbrinck, Emmendingen, Germany) for FISH analysis.

Vaginal biopsy assays

FISH was applied to the slides. To this purpose, the oligonucleotide probes that targeted bacterial 16/23S rDNA genes were labeled with fluorescent dye markers (ie, carbocyanine at the 3′ end (Cy3), fluorescein isothiocyanate, or carbocyanine at the 5′end [MWG Biotech, Ebersberg, Germany]). To enhance visualization after hybridization, bacteria were always counterstained with 4, 6-diamidino-2-phenylindole (DAPI).

Bacterial diversity was assessed in a multicolor analysis by use of a set of different primers that were targeted at the major clusters of bacterial species in the biofilm.[17] The labeled, species-specific probes included *GardV,*[12] *Ato,*[18] *Lab,*[19] *Bac 303,*[20] and *Ebac*[21] targeted at the *Gardnerella*, *Atopobium*, *Lactobacillus*, *Bacteroides/Prevotella,* and *Enterobacteriaceae* clusters, respectively. FISH assays with these probes were performed by including the universal *Eub 338*- fluorescein isothiocyanate probe,[22] which targets virtually all bacteria.

The overall amenability of bacterial cells within the biofilm to the FISH assay was assessed subsequently with the use of the *Eub* 338-Cy3 probe at 46°C without formamide. It has been established that the number of ribosomes in a metabolically active bacterial cell may reach 10^8 ribosomes/cell, which permits an intense fluorescence signal.[22] The intensity of the hybridization signal fades with declining metabolic activity, decreasing numbers of ribosomes, or degradation of cell structures after cell death. Because all probes were handled in a standard manner, the amenability of bacteria to FISH and other DNA stains is therefore a parameter that characterizes the activity state and integrity of bacteria and is not caused by differences in applied protocols or changing permeability of the cell wall.[22] Hence, the overall amenability of the biofilm was estimated under fluorescence microscopy (as outlined later) as the number of active bacteria that bind to the universal Cy3-labelled *Eub* 338 probe relative to the overall number of bacteria that could be visualized through DNA-staining with DAPI.

Quantification of bacteria on FISH was performed with a fluorescence microscope (Nikon e600; Nikon, Tokyo, Japan) by enumerating bacteria within a 10×10 μm area that lines the epithelial surface in 10 adjacent microscopic fields. The quantification of bacteria was based on the assumption that a 10-μL suspension of bacteria with a concentration of 10^7 cells per milliliter applied to a glass surface in a 1-cm diameter circle contains 40 cells, on average, in a microscopic field at a magnification of $\times$1000.[23] High power (magnification, $\times$1000) photographs were made with a camera and processed with the accompanying software (Nikon DXM1200; Nikon).

Finally, for each biopsy, aerobic and anaerobic microbial cultures were made from the corresponding vaginal smears, and colony-forming units were scored in a semiquantitative manner. A cotton-tipped swab was rolled against the lateral wall of the vagina at the mid portion of the vault and then placed into *Amies* transport medium (Transystem, HAIN Lifescience, Nehren, Germany). Samples were plated on the culture medium and incubated in anaerobic and in a 5% CO_2 atmosphere at 36°C within 2 hours after collection. Culture media included Schaedler/KV agar, V agar (for *Gardnerella vaginalis*), Columbia blood agar, MacConkey II agar, and CHROMagar *Candida* (Becton Dickinson, Paris, France) by which the preponderance of vaginal species were allowed to grow. Small translucent colonies that were betahemolytic on *Gardnerella* agar (V agar), were catalase negative, and exhibited Gram-variable, pleiomorph, coccobacillary morphology on Gram stain, were determined as *G vaginalis*. Metronidazole sensitivity of cultured *Gardnerella* isolates was tested with 50 μg metronidazole discs.

Outcome measures

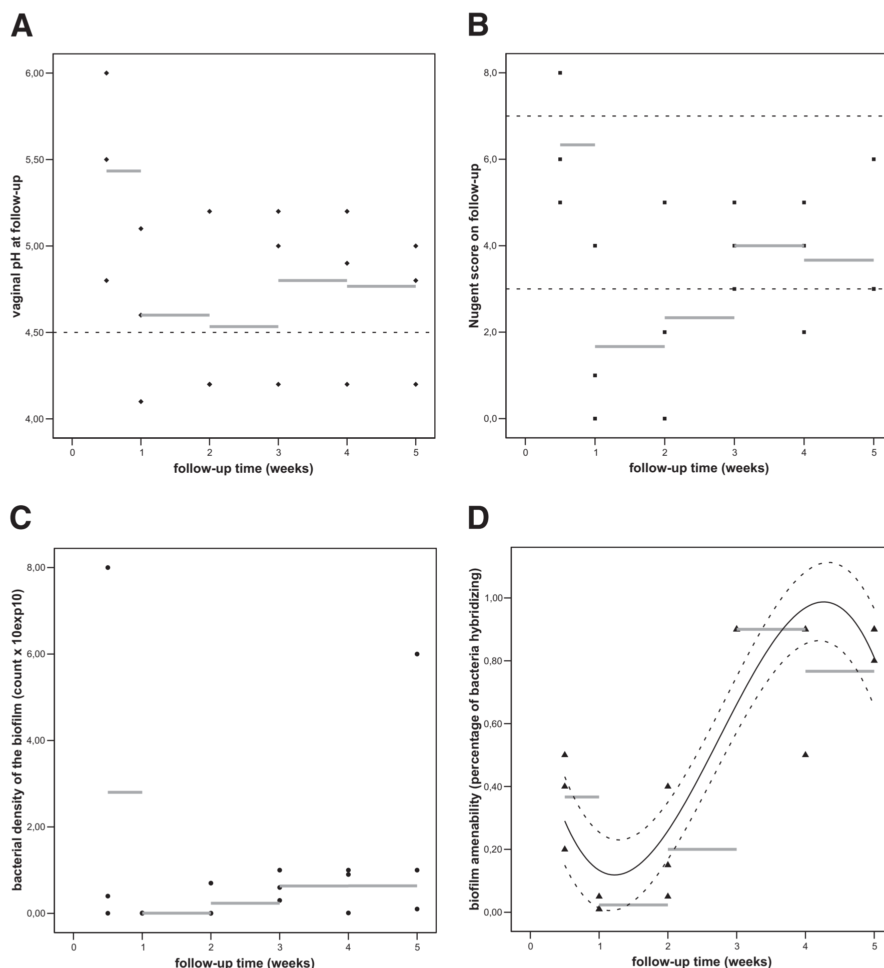
In each patient, the following outcome measures were evaluated at follow-up evaluation: (1) Amsel criteria and Nugent score that indicated vaginal microflora status; (2) bacterial density of the biofilm that was expressed as the corresponding bacterial count in log[10] units; (3) bacterial diversity of the biofilm that was expressed as the relative abundance of the species clusters under study; and (4) the amenability of the biofilm as an indicator of bacterial activity that was expressed as the proportion of DAPI-stained bacteria binding the Cy3-labelled universal *Eub* 338 probe.

Statistics

All data were analyzed according to a repeated measures design under the nonparametric assumption. Accordingly, trends were assessed through the Friedman's test, under the assumption that all observed values are mutually independent, thereby accounting for the study design. On account of the sample size, statistical significance was estimated through Monte Carlo simulations that were based on 10,000 reiterations, rather than on asymptotic probability values. Statistical significance was accepted if the 2-tailed *P* value was < .05. All analyses were performed with SPSS (v 12.0; SPSS Inc, Chicago, IL).

FIGURE 1

Vaginal pH, Nugent score, bacterial count, and amenability of the biofilm at 1-week intervals after treatment of bacterial vaginosis with metronidazole



A, The *dots* represent individual observations; the *horizontal bars* denote the mean vaginal pH at each point in time, and the *Y-axis reference line* denotes the established pH cut-off value of 4.5. **B**, The *dots* represent individual observations; the *horizontal bars* denote the mean Nugent score at each point in time, and the *Y-axis reference lines* denote the estblished Nugent score cut-off values of 3 and 7, respectively. **C**, The *dots* represent individual observations; the *horizontal bars* denote the mean bacterial counts at each point in time. **D**, The *dots* represent individual observations, and the *horizontal bars* denote the mean amenability at each point in time; the overall trend approximates a cubic trend ($R^2 = 0.777$); the *solid curve* represents the cubic fit line, and the *dashed lines* denote the bounds of the 95% CI to the curve fit.

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RESULTS

At initial assessment all patients (n=18) were positive for the 4 Amsel criteria and had a median Nugent score of 9 (interquartile range, 8-9).

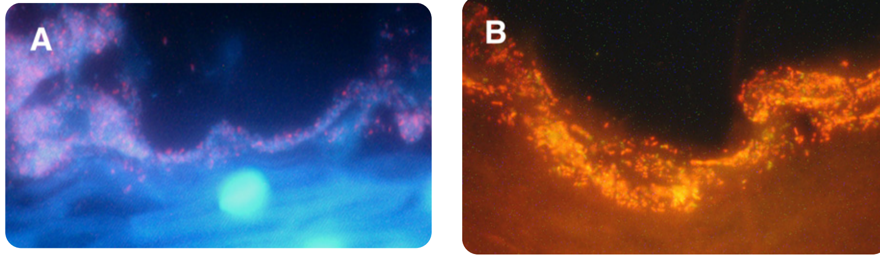
After completion of the 7-day treatment course, patients remained free of vaginal discharge, malodor, and clue cells throughout the study period, although a moderately elevated vaginal pH persisted among 9 of the 15 patients (60%; Figure 1A). There was a statistically significant trend in vaginal pH at follow-up evaluation (Friedman χ^2 = 9.94; *P* = .002; 95% CI, 0.001-0.002).

Similarly, Nugent scores after completion of the treatment course remained consistently <7 throughout the study period. Seven of 15 patients (47%) still had intermediate flora (Nugent score, 4-6) on follow-up evaluation (Figure 1B) without a significant trend in Nugent scores over time (*P* = .422; 95% CI, 0.412-0.431).

Numbers of bacteria adherent to the vaginal epithelium as assessed by FISH microscopy showed a significant increase over time (Friedman χ^2 = 5.56; *P* = .030; 95% CI, 0.027-0.033). In particular, a significant increase (Friedman χ^2 = 11.27; *P* < .001) in bacterial counts was observed after treatment cessation (Figure 1C).

The most significant finding, however, was the time-dependent change of the amenability of the biofilm (Friedman χ^2

FIGURE 2
FISH microscopy of the bacterial biofilm during and after treatment with metronidazole



A, Bacterial biofilm (original magnification, ×400) that was observed in a patient on day 3 of the therapy with metronidazole; a prolific, adherent bacterial biofilm can be seen in *DAPI (DNA) stain blue*, although the *GardV*-Cy5 probe (*Gardnerella* cluster) elicits *red fluorescence*. The biofilm was targeted with various probes and counterstained with DAPI. The overall amenability of this biofilm with the universal *Eub* 338 probe is rather low at this stage (20%, not shown) of antibiotic treatment; therefore, only a small part of the bacteria within the biofilm hybridizes with the probe for *G vaginalis* (*GardV*) as the primary constituent, although not with the less abundant species such as *A vaginae*. **B**, Prolific bacterial biofilm (original magnification, ×400) that was observed in a patient on day 35. The bacterial biofilm has completely recovered. The amenability of the biofilm with the universal *Eub* 338 probe is as high as 90% (not shown), and intense fluorescence signals are observed that resulted from the hybridization of bacteria from the *Gardnerella* and *Atopobium* clusters as the major constituents of the biofilm (*Ato*-Cy3 probe binding *Atopobium* [*orange*] and *GardV*-Cy5 probe binding *Gardnerella* [*red*]). Counterstaining was performed with DAPI (*blue*).

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= 8.00; $P = .007$; 95% CI, 0.005-0.009). As displayed in Figure 1D, the percentage of DAPI-stained bacteria in the biofilm that bound the FISH-labeled oligonucleotide probes dropped sharply after treatment with a median amenability of 1.0% (interquartile range, 1.0%-5.0%) after 7 days of treatment. Figure 2A shows the limited access of the DAPI-stained bacteria to *Gardnerella*-targeted FISH probes during treatment with metronidazole. The amenability gradually increased thereafter (Friedman $\chi^2 = 15$; $P < .001$) over a median value of 15.0% (interquartile range, 5.0%-40.0%) after 2 weeks up to a median amenability of 90.0% (interquartile range, 85.0%-90.0%) during further study follow-up. Figure 2B shows extensive layers of conglomerates of *G vaginalis* and *A vaginae* on the vaginal epithelium already at 4 weeks after treatment cessation.

The precise composition of the biofilm on the species level could not be assessed reliably during early study follow-up period because of the initially limited amenability of the bacteria. In biopsies in which the amenability was at least 5% (n = 16), however, bacteria belonging to the *Gardnerella* cluster were found consistently as the primary species (16/16). Bacteria belonging to the *Atopobium* cluster gave positive hybridizations signals in one-half of the patients and accounted for 5%-40% of the hybridizing bacteria (8/16). Bacteria from the *Lactobacilli* and *Bacteroides* clusters were found in less than one-half of the patients and, in the latter, consistently constituted only a minor fraction of the biofilm (<10% of hybridizing bacteria). Overall, we found that of all bacteria that were targeted, *G vaginalis* and *A vaginae* were the primary constituents of the recovering biofilm.

Hence, within 3 weeks after clinically successful standard therapy of bacterial vaginosis, we consistently found a pronounced accumulation of the core bacteria to bacterial vaginosis in an adherent biofilm.

All *G vaginalis* isolates that were obtained showed proper metronidazole susceptibility on standard testing with the 50-μg disc assay.

COMMENT

We performed a follow-up study in a series of patients with microbiologically and clinically documented bacterial vaginosis according to standard diagnostic criteria[15,16] and evaluated the presence and the activity of the *G vaginalis* biofilm over time, after standard therapy with oral metronidazole according to Centers for Disease Control and Prevention guidelines.[10] We were able to document that, although all patients apparently successfully converted to normal or intermediate vaginal microflora, the bacterial vaginosis biofilm[12] actually was suppressed only temporarily by metronidazole and rapidly regained its activity after treatment cessation.

Our study provides evidence on a persistent alteration of the vaginal ecosystem after an episode of successfully treated bacterial vaginosis. In particular, our data point at the putative role of a Trojan horse–like attribute of virulence in bacterial vaginosis; that is, it could be shown that the biofilm accumulates high numbers of bacteria and constitutes a persistent herd of *G vaginalis* and to lesser extent *A vaginae,* although apparently being relatively inaccessible to metronidazole as the drug of choice. It is therefore plausible that the persistent biofilm may contribute to the recurrence of bacterial vaginosis,[1,4,11] albeit speculative at this time, because time of follow-up was obviously too short in our study to document.

Bacterial biofilms have been shown to be involved in several recalcitrant bacterial infections (such as endocarditis, otitis media, periodontitis, and chronic prostatitis) and to complicate chronic conditions, that include inflammatory bowel disease, chronic obstructive pulmonary disease, and cystic fibrosis.[13,14,23] In bacterial vaginosis, the formation of a biofilm by *G vaginalis* was initially shown on shed epithelial cells through electron microscopy as a dense and tight web that consisted of bacterial cells that were encased within a fibrillar exopolysaccharide network, further conferring strong adherence to the vaginal epithelium.[24,25] More recently, we described the bacterial biofilm, which pri-

marily consists of *G vaginalis* and *A vaginae* as an obligate finding in bacterial vaginosis through bacterial rDNA fluorescence in situ hybridization of vaginal biopsy specimens.[12]

The biofilm may further explain the apparent metronidazole paradox in the treatment of bacterial vaginosis. In vitro resistance of *Gardnerella* strains has been reported occasionally,[26,27] though overall, the preponderance of clinical isolates that were obtained from women with bacterial vaginosis seems to be susceptible to metronidazole. Beigi et al,[28] for instance, determined metronidazole susceptibility of vaginal cultures up to 3 months after treatment of bacterial vaginosis and found that <1% of all anaerobic isolates demonstrated metronidazole resistance. This convincing in vitro observation is in sheer contrast with clinically observed cure rates with metronidazole,[11] and this discrepancy may be explained in part by the persistence of the *G vaginalis* biofilm in vivo. It remains to be determined whether prolonged treatment courses and/or higher doses of metronidazole could eradicate the biofilm in bacterial vaginosis, although unlikely to serve as a treatment option. Increasing doses of metronidazole have been shown to inhibit the indigenous lactobacilli,[29] although preserving and restoring the vaginal lactobacilli is already a critical element in the convalescence from bacterial vaginosis to healthy vaginal microflora with standard doses.[30]

Finally, our observations on the persistence of a *G vaginalis* biofilm may challenge current diagnostics. Indeed, the presence of a considerable active *G vaginalis* reservoir was not quite paralleled by conventional indicators of disturbed vaginal microflora, even though we documented that high numbers of *G vaginalis* and *A vaginae* were present on the vaginal epithelium at follow-up examination.

We recognize that our study had several limitations and that our results should be interpreted with caution. First, our sample size was rather limited. However, because the pronounced resistance of the biofilm to metronidazole therapy became apparent during the conduct of this study, we considered it unethical to further extend the study within the lines of the study protocol in agreement with the institutional review board. Second, some selection bias is likely to have been introduced, which may impinge on the generalizability of our results, in as much that we may have recruited volunteer women who were already more prone to relapsing bacterial vaginosis. Third, we handled our observations as a longitudinal data set, whereas each point in time was actually represented by 3 different patients.

Nonetheless, it may be acknowledged that our primary findings on the persistence of the bacterial biofilm hardly can be played down by these methods considerations. In particular, although our observations relied on 3 different patients at each point in time, there was hardly any dispersion to the estimated value of the primary outcome measure (ie, the biofilm amenability); indeed, in the second half of the study timeframe, we found that the median amenability was approximately 90%, with a very narrow confidence interval (interquartile range, 85%-90%), even though this estimate was based on 9 different patients at 3 different weekly intervals.

Other limitations include the lack of controls (ie, women with bacterial vaginosis who did not receive therapy) and the lack of biofilm assessment at baseline. Although accounting for the latter methods issues could have strengthened our findings, we are confident that women consistently had a bacterial vaginosis biofilm at baseline as previously shown[12] and is also apparent from early follow-up data (week 0) in this study. Hence, we believe that the persistence of the bacterial vaginosis biofilm is a genuine phenomenon, although an extended period of follow-up with repeated assessments would be desirable to firmly document the postulated role of the biofilm in the pathogenesis of bacterial vaginosis.

In summary, it can be stated that primary and long-term treatment failure of bacterial vaginosis has been a longstanding frustration to both doctors and patients and has been attributed generally to our very limited understanding of the pathogenesis of this particularly common condition.[11] Failure to control the high prevalence of bacterial vaginosis has now become a global issue of concern, considering the increasing body of knowledge that points at the consistent cooccurrence between sexually transmitted diseases and bacterial vaginosis.[4,9] It has been estimated, for instance, that, in endemic areas, nearly one-third of all new HIV cases might be prevented if all cases of bacterial vaginosis could be cured.[9] Very recent studies that have involved genomic fingerprinting of the vaginal microflora seem to have turned the wheel, and we slowly are gaining insight into the mechanisms that are involved in the refractory nature of this condition, which include the detection of the metronidazole-resistant *A vaginae*[31] as a prominent indicator of the recurrence risk of bacterial vaginosis[32] and the discovery of a *G vaginalis*/*A vaginae* biofilm,[12] which we found to resist standard treatment. This increasing knowledge may pave the path for novel therapeutic strategies in the control of bacterial vaginosis. In particular, we suggest that forthcoming studies on the efficacy of prebiotics, probiotics, and antibiotics in the treatment of bacterial vaginosis may account for the ability of these therapeutics to eradicate the bacterial vaginosis biofilm. ■

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