Normal flora and bacterial vaginosis in pregnancy: An overview

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Abstract

The female genital tract is an intricate, yet balanced ecosystem that hosts a variety of different residential microflora. The physiological changes that occur during pregnancy may disrupt this balanced ecosystem and predispose women to a potentially pathogenic microbiota. Bacteria that are associated with bacterial vaginosis (BV) are opportunistic pathogens that frequently form part of this microbiota. The overgrowth of and infections with these bacteria are linked to poor obstetric outcomes and increased transmission of other reproductive tract infections (RTIs). These infections increase women's susceptibility of acquiring HIV, the rates of HIV shedding and the development of Acquired Immune

Deficiency Syndrome (AIDS) in HIV infected patients. It is unknown how the plethora of bacterial species associated with BV contributes to the dynamics of this condition. The use of high-throughput methods have led to the in-depth investigation of different BV-related bacterial species and the functional capabilities of these species. However, the pathogenesis of BV is still poorly defined and the role of individual BV-related bacterial species in specific pregnancy complications is unclear and controversial. The majority of BV infections are asymptomatic and successful diagnosis is complicated by the lack of reliable and standardized diagnostic tests.

1. Introduction

The human vaginal environment is an extremely dynamic, nutrient-rich milieu for bacteria that develop into a unique microbiota (Mirmonsef *et al*., 2011). It is an intricate and diverse ecosystem, which determines vaginal health (Diaz *et al*., 2010; Danielsson *et al*., 2011). This bionetwork mainly comprises a wide spectrum of aerobic and anaerobic bacterial genera and species in healthy asymptomatic women, with the *Lactobacillus* genus dominating (Donati *et al*., 2010; Aagaard *et al*., 2012). With the aid of culture-independent methods, a cross-sectional analysis of the vaginal microbiota of healthy asymptomatic women is reported to reveal at least six community state types (CSTs), including CST I, II, III, IV-A, IV-B and V (Ravel *et al*., 2011; Romero *et al*., 2014). Each one of the four most common vaginal *Lactobacillus* species, including *L. crispatus, L. gasseri, L. iners* and *L. jensenii* dominate a CST (Ravel *et al*., 2011). The other two CSTs have a deficiency of *Lactobacillus* species and may comprise an assorted collection of anaerobic bacteria, including *Prevotella* spp., *Peptostreptococcus* spp., *Megasphaera* spp., *Gardnerella vaginalis, Sneathia* spp., *Parvimonas* spp., *Mobiluncus* spp. and *Atopobium vaginae* (Cauci, 2004; Ravel *et al*., 2011; Romero *et al*., 2014).

The vaginal microbiota, especially by the production of lactic acid, is believed to protect pregnant women against reproductive tract infections (RTIs) (Donati *et al*., 2010; Witkin *et al*., 2007a). Endogenous and exogenous influences, such as pregnancy (hormonal status), the host's age, foreign bodies, state of health and geographical variation may allow the composition of the vaginal ecosystem to transform over time (Kiss *et al*., 2007; Ryckman *et al*., 2009). During pregnancy there is a typical vaginal discharge because of increased levels of serum estrogen (Omole-Ohonsi and Nwokedi, 2011). This discharge will be heavier and contain more cervical mucus as the pregnancy progresses and may predispose women to RTIs/STIs, such as bacterial vaginosis (BV) (Omole-Ohonsi and Nwokedi, 2011).

Bacterial vaginosis is a dysbiosis of the vagina, which increases the transmission of other RTIs and human immunodeficiency virus (HIV) proliferation (Sha *et al*., 2005; Hooven *et al*., 2012). In pregnant women, this polymicrobial condition has been linked to various poor obstetric outcomes, including preterm labour (PTL) and preterm delivery (PTD) (Hillier *et al*., 1995; Guerra *et al*., 2006). In developed countries, the use of antimicrobial agents in pregnancy is one of the main reasons for a decline in maternal and perinatal morbidity (WHO, 2005; Lockitch, 2004). In the case of BV, treatment success is countered by recurrent BV, a condition where treated women relapse within three months and redevelop this condition (Hay, 2000).

2. Normal vaginal flora dominated by *Lactobacillus* species

The microbiological fluctuations that typically occur during the course of the menstrual cycle are suspended when females fall pregnant (Genc and Onderdonk, 2011). During pregnancy, estrogen levels are elevated and glycogen synthesis is increased (Lin *et al*., 2011). Lactobacillary activity and proliferation are favored by the increased glycogen availability,

which leads to an enhanced epithelial tropism (Donati *et al*., 2010). Lactobacilli, especially hydrogen peroxide (H_2O_2)-producing strains, are the most eminent markers of normal flora and are important indicators of a healthy vaginal milieu (Donders, 2007; Genc and Onderdonk, 2011). Lactobacilli manifest themselves in the vagina by attaching to glycolipid receptors of the epithelia *via* pili that act as ligands and block the attachment of other bacteria to the vaginal epithelium (Donders, 2007; Zodzika *et al*., 2011). In addition, lactobacilli kill off and prevent the proliferation of other bacteria by the production of bacteriocins, antibiotic toxic hydroxyl radicals, H_2O_2 and probiotics (Donders, 2007; Zodzika *et al*., 2011). *Lactobacillus crispatus* strains in particular (up to 95%) are known to produce H_2O_2, while *L. iners* strains are weak H_2O_2 producers (Antonio *et al*., 1999). Wilks and colleagues (2004) reported the production of H_2O_2 by several *Lactobacillu*s species during pregnancy and found that *L. jensenii* and *L. vaginalis* produced the highest levels of H_2O_2. The ability of different *Lactobacillus* species to produce varying amounts of H_2O_2 is said to reduce the risk of BV during pregnancy (Onderdonk *et al*., 2003; Wilks *et al*., 2004). Consequently, H_2O_2-producing lactobacilli may decrease the incidence of ascending infections of the uterus and the complications of such infections, including chorioamnionitis (Wilks *et al*., 2004).

Aagaard and colleagues (2012) used high-throughput sequencing technologies to characterize the vaginal microbiome signature in pregnancy. This study found that the overall microbial diversity and richness were diminished in pregnancy. *Lactobacillus* species were dominant and the most enriched species included *L. crispatus, L. iners, L. jensenii* and *L. johnsonii* (Aagaard *et al*., 2012). Interestingly, *L. johnsonii* is predominantly found in the upper gastrointestinal (GI) tract (Pridmore *et al*., 2004). Rectal lactobacilli can reach amounts of up to 10^7 to 10^8 per gram vaginal fluid since growth is supported by the elevated concentration and low pH of glycogen in the stratified vaginal epithelium (Danielsson *et al*., 2011). Instead

of *L. johnsonii*, another study reported an increased richness of *L. vaginalis* during pregnancy (Romero *et al*., 2014), similar to Wilks *et al*. (2004).

The decreased diversity and richness of the vaginal microbiota in pregnancy are supported by Walther-António *et al*. (2014) who studied the vaginal microbiome of pregnant Caucasian women. The authors reported that *L. crispatus* and *L. iners* are the two species that dominated the microbial content of the women studied throughout the course of pregnancy. Throughout pregnancy, *L. iners* was found to dominate the vaginal microbiomes of women who were significantly older (35 ± 0 years), whereas *Lactobacillus crispatus* dominated the vaginal microbiomes of younger women (28 ± 3 years). Hyman and colleagues (2013) documented similar findings, with *L. crispatus* abundant in the vaginal microbiomes of Caucasian women, while *L. iners* dominated in the vaginal microbiomes of African-American and Hispanic women.

It is reported that the vaginal bacterial community structure between pregnant women may be more stable than the community structure between non-pregnant women (Romero *et al*., 2014; Walther-António *et al*., 2014). Romero and colleagues (2014) suggested that during pregnancy, vaginal microbial communities dominated by *Lactobacillus* species do shift from one CST to another CST, dominated by another *Lactobacillus* species, but rarely to a CST dominated by anaerobic bacteria.

3. Normal vaginal flora dominated by bacteria other than lactobacilli

In some healthy women (ranging from 7% to 33%), a dynamic vaginal ecosystem is still maintained where bacterial species other than lactobacilli fill the niche (Zhou *et al*., 2004; Witkin *et al*., 2007a). Comparable to the lactobacilli, *Atopobium*, *Leptotrichia* and

Megasphaera produce lactic acid and are able to retain a normal (moderately acidic) vaginal pH (Zhou *et al*., 2004; Ravel *et al*., 2011). It has been suggested that strains of *A. vaginae* have the potential to produce varying amounts of lactic acid but not to such an extent to protect the normal vaginal flora (Marconi *et al*., 2012). Non-*Lactobacillus* bacteria take part in mixed acid fermentation where other organic acids, such as acetic, linoleic and mydriatic acid are typically produced along with lactic acid (Huggins and Preti, 1976). Consequently, the presence of potentially pathogenic microbes, such as *Escherichia coli, Gardnerella vaginalis, Mycoplasma* spp., *Peptostreptococcus, Prevotella, Pseudomonas,* group B *Streptococcus* (GBS; *S. agalactiae*) and *Ureaplasma* spp. does not represent an anomalous state (Zhou *et al*., 2004; Genc and Onderdonk, 2011). These bacteria may be present in relatively low numbers and concentrations under the normal acidic conditions (≤ pH 4.5) of the vagina and do not cause any apparent (communicable) complications (Genc and Onderdonk, 2011). This is supported by the identification of non-lactobacilli species in CSTs IV-A and IV-B (in both pregnant women and non-pregnant women) by means of high-throughput, culture-independent methods (Romero *et al*., 2014). Romero and colleagues (2014) found several phylotypes (*Ruminococcaceae, Sneathia, Parvimonas, Gardnerella, Prevotella, Mobiluncus* and other taxa associated with BV) in pregnant women, albeit in significantly lower abundance. Ravel *et al*. (2011) noted that group IV communities not dominated by *Lactobacillus* species, had a great quantity of genera known to produce lactic acid. This indicates that a vital biological function, i.e. the production of lactic acid, appears to be conserved in all communities associated with the vaginal milieu of healthy women (Ravel *et al*., 2011).

4. Bacterial vaginosis

It is known that the imbalanced vaginal flora, the replacement of lactobacilli and the subsequent rise in pH create a more permissive milieu for elevated concentrations of endogenous aerobes and anaerobes and HIV acquisition and proliferation (Schmid *et al.* 2000; Sha *et al*., 2005; Bradshaw *et al*., 2013). Standardizing the diagnosis and treatment of BV is complicated by its polymicrobial nature; molecular studies revealed that the collection of bacteria related to BV can differ substantially between individuals (Pereira *et al*., 2005; Fredricks *et al*., 2009).

Epidemiology of bacterial vaginosis

The prevalence of BV ranges from 3.5% to more than 50% in pregnant women worldwide (Tolosa *et al*., 2006; Akinbiyi *et al*., 2008; Krauss-Silva *et al*., 2014). The prevalence of BV varies considerably over the course of pregnancy and is suggested to decrease as pregnancy progresses (Hay *et al*., 1994; Waters *et al*., 2008; Krauss-Silva *et al*., 2014). Waters *et al.* (2008) reported that BV is the most prevalent in the first trimester of pregnancy (43.9%) but less prevalent in the second (21.6%) and third trimesters (18.9%). The authors reported a stable intermediate vaginal flora (IVF) in roughly 12% of pregnant women throughout pregnancy (Waters *et al*., 2008). Intermediate vaginal flora is reported in up to 22% of pregnant women (Goffinet *et al*., 2003). Krauss-Silva and colleagues (2014) found a prevalence of 28.1% among asymptomatic pregnant women <20 weeks' gestation, which decreased with almost 40% after an 8-week follow-up to either BV-negative or IVF. This could be ascribed to the spontaneous resolution of BV infection, which is associated with higher concentrations of lactobacillus morphotypes and lower pH (Klebanoff *et al*., 2004).

Ethnicity is an imperative determining factor in vaginal colonization by various bacteria and women of African ethnicity are at an increased risk of developing BV (Hay, 2010). A survey from 2001 to 2004 revealed that the prevalence of BV among African-Americans was 3.31 times higher than among Caucasians (Livengood, 2009; Klatt *et al*., 2010). This may partially be elucidated by host genetics that play a role in the occurrence of BV; however, the apparent reasons still remain unclear (Danielsson *et al*., 2011). A study by Royce *et al*. (1999) documented that black women are 9.26 times more likely to harbor *Mobiluncus* spp. as compared to white women. Paternal black race has also been suggested to be an independent risk factor for BV during pregnancy (Simhan *et al*. 2008). Other risk factors for BV include (i) the concurrent use of medications, (ii) low socioeconomic status, (iii) increasing age, (iv) cigarette smoking, (v) young age of coitarche, (vi) precarious practices, such as vaginal douching, (vii) the use of intrauterine devices, (viii) a new sexual partner and (ix) multiple sexual partners (CDC, 2010; Zodzika *et al*., 2011).

A shift from normal vaginal flora to one indicative of BV does not necessarily result in symptoms. Many BV cases are either paucisymptomatic or completely asymptomatic (± 50% of cases) (Livengood, 2009; Donati *et al*., 2010). However, when a patient is BV positive, clinical symptoms generally include a thin, grey, malodorous (fishy) discharge that may include local irritation (Srinivasan *et al*., 2009). The characteristic 'fishy odor' is the result of amines (cadaverine, putrescine and trimethylamine) produced by the anaerobes present (Livengood, 2009). These symptoms are aggravated when the vaginal pH increases, for instance after sex (Livengood, 2009). The alkaline buffering action of semen nullifies the acidity of the vagina for several hours after intercourse and it is likely that this brief loss of acidity is permissive for anaerobic bacterial overgrowth and leads to the probable up-regulation of amines (Cherpes *et al*., 2008; Zodzika *et al*., 2011).

High vaginal pH values are associated with CSTs IV-A and IV-B, community states dominated by bacterial species other than lactobacilli (Ravel *et al*., 2011). These potentially pathogenic bacteria may overgrow and lead to BV when bacterial numbers increase uncontrollably to reach 100- to 1000 fold the normal vaginal levels (Eschenbach, 1993). Novel bacterial species, which are highly specific for BV, have been identified (Fredricks *et al*., 2005). Additional to *A. vaginae*, these bacterial species include *Leptotrichia/Sneathia* spp., bacteria closely related to *Megasphaera* spp. and three bacteria of the order *Clostridiales*, including BV-associated bacteria (BVAB) types 1, 2 and 3 (Fredricks *et al*., 2005; Aagaard *et al*., 2012; Bradshaw *et al*., 2013). The concentration of *A. vaginae* in vaginal fluid is positively associated with pH and Nugent scores (Marconi *et al*., 2012). By investigating the transcriptome of a vaginal sample from a BV positive patient, Twin *et al*. (2013) found *Prevotella amnii* to be the most metabolically active species in the sample. Constituting the rest of the sequenced 16S rRNA reads, *Megasphaera* made up 19% of the reads, *Leptotrichia/Sneathia* 8% of the reads and *Fusobacterium* also 8% of the reads (Twin *et al*., 2013). High-throughput sequencing analysis of the vaginal microbiome of women with BV has revealed that *G. vaginalis, A. vaginae, Eggerthella, Prevotella*, BVAB2 and *Megasphaera* type 1 were highly predictable for BV (Shipitsyna *et al*., 2013).

About 15% to 60% of treated women will relapse within three months to develop recurrent bacterial vaginosis (Hay, 2000; Bradshaw *et al*., 2006). The risk factors for recurrent BV are mostly the same as for initial BV infection (Klatt *et al*., 2010; Bradshaw *et al*., 2013). Behavioral and contraceptive practices may alter the efficacy of BV treatment (Bradshaw *et al*., 2013). Intrauterine device use, a history of BV infection and the presence of bacterial species *Facklamia, Corynebacterium* and *Veillonella* are factors associated with BV treatment failure (Wang *et al*., 2014). Treatment of BV fails in up to 50% of cases

(Schoeman, 2002; Hay, 2010). Wang *et al*. (2014) proposed that BV treatment failure is essentially due to the failed restoration of the lactobacilli population instead of antimicrobial resistance. It is suggested that for the effective treatment of BV, the restoration of *Lactobacillus* species to dominance alone will not suffice and that a decrease in BV-associated anaerobe concentrations below an unknown threshold is also required (Lambert *et al*., 2013a).

Bacterial vaginosis is considered to be sexually associated instead of sexually transmitted due to its association with sexual activity; however, sexual activity is not the sole determinant for its occurrence (Morris *et al*., 2001; Guédou *et al*., 2013). The concept of male-to-female heterosexual transmission is opposed by (i) the treatment of male partners that is not beneficial as it does not result in a decline in BV prevalence (Verstraelen *et al*., 2010) and (ii) the fact that there is no solitary etiological agent responsible for BV (Turovskiy *et al*., 2011). Bacterial vaginosis can be sexually transmitted in women who have sex with women (Berger *et al*., 1995; Bradshaw *et al*., 2013). This is supported by the Gardner and Dukes study where healthy young women who were inoculated with the fluid of BV positive women, had resulting symptoms characteristic of BV (Gardner and Dukes, 1955). However, BV has also been reported in 18% of sexually inexperienced women (Yen *et al*., 2003).

Swidsinski and colleagues (2010) documented that *Gardnerella* may be present in two forms: cohesive and dispersed. Cohesive *Gardnerella* appears as brickwork-like structures, characteristic of biofilms, and is most concentrated on epithelial cells (Swidsinski *et al*., 2010). Dispersed *Gardnerella* surrounds leukocytes instead of epithelial cells and is occasionally concentrated to small clusters of 10 to 20 bacteria (Swidsinski *et al*., 2010). Cohesive *Gardnerella* is significantly present in BV positive patients and their partners and is

sexually linked as opposed to dispersed *Gardnerella*, which is not (Swidsinski *et al*., 2010). *Gardnerella* biofilms are suggested to be an entity entirely different from BV (Swidsinski *et al*., 2010). The carriage of *G. vaginalis* in the urethra and prepuce of males has been reported (Kinghorn *et al*., 1982). Schwebke (2009) established that carriage is directly associated with condom use. This supports the theory of female-to-male transmission of *G. vaginalis* and other BV-related bacteria instead of the opposite (Holst *et al*., 1990; Schwebke *et al*., 2009). Sexually active and heterosexual males are significantly more colonized than prepubertal boys and homosexual men, respectively (Verstraelen *et al*., 2010).

The composition of the female reproductive tract makes women two times more likely than men to acquire HIV (Turovskiy *et al*., 2011). Bacterial vaginosis enhances viral replication and BV-related bacteria directly up-regulate the replication of HIV through a heat-stable HIV-inducing factor (Zariffard *et al*., 2005; Johnson and Lewis, 2008). Vaginal shedding of HIV is propagated by BV and women who are affected by both HIV and BV can shed virus particles up to six times more *versus* those who are BV negative (Coleman *et al*., 2007; Taha *et al*., 1998). Bacterial vaginosis can act as a co-factor for HIV and conversion to seropositivity (Cohen *at el*., 1995).

Pathogenesis of bacterial vaginosis

Infection by potentially pathogenic microorganisms is not only prevented by the activity of the normal vaginal microbiota, but also by a finely tuned innate and adaptive immune response (Witkin *et al*., 2007a; Danielsson *et al*., 2011). The vaginal mucosa is the primary point of interaction between microorganisms and the host's genital tract and the innate immune response at this epithelial lining play an integral role against microorganism invasion (Witkin *et al*., 2007a; Genc and Onderdonk, 2011). The innate immune system identifies

microbial intruders instantly *via* the pathogen-associated molecular patterns (PAMPs), while the adaptive immune system produces cell-mediated and antibody-mediated immunity, which are antigen-specific (Witkin *et al*., 2007a). Innate immune system components functioning in the vagina may include membrane-bound Toll-like receptors (TLR), surfactant protein A, lactoferrin, complement component, β-defensins, secretory leukocyte protease inhibitor (SLPI), mannose-binding lectin (MBL), heat shock proteins and nitric oxide (Giraldo *et al*., 1999; Genc *et al*., 2006). The antigen-specific B lymphocytes, which are predominantly present in the endocervix and vagina of the female lower genital tract, produce IgG and IgA antibodies locally that are secreted into the mucosa (Witkin *et al*., 2007a; Hickey *et al*., 2011).

There are at least two suggested models explaining the possible pathogenesis of BV (Srinivasan and Fredricks, 2008). The *Lactobacillus* depletion model proposes that there is an initial reduction in H_2O_2-producing lactobacilli, allowing the overgrowth of facultative anaerobes, which results in BV (Srinivasan and Fredricks, 2008). The primary pathogen model proposes that the entry of facultative anaerobes causes the displacement of lactobacilli, thereby resulting in BV (Srinivasan and Fredricks, 2008).

The *Lactobacillus* depletion model is supported by the notion that a rise in the vaginal pH occurs first with subsequent anaerobic bacterial overgrowth (Kiss *et al*., 2007; Cherpes *et al*., 2008). Udayalaxmi and colleagues (2012) support the primary pathogen model as it is postulated that BV-related bacteria first adhere to the vaginal epithelium, proliferate and then create a dense biofilm (Udayalaxmi *et al*., 2012). The biofilm is by no means affected by the increase in pH, which may be the result of metabolic events of the amplified bacterial population (Udayalaxmi *et al*., 2012). A biofilm is an intricate collection of sessile bacterial

cells, which is covered by an extracellular matrix of biopolymeric substances (García-Castillo *et al*., 2008). The pathogenic function of a biofilm is to allow the bacteria to repel the host's immune defenses and tolerate higher concentrations of antimicrobial agents, explaining the recurrence rates of BV (Danielsson *et al*., 2011). The formation of biofilms may be due to certain properties that some *Gardnerella* strains possess, such as pathogenicity islands, virulence factors and plasmids or it may be a polymicrobial synergism between bacteria, e.g. *Gardnerella* and *Atopobium* spp. (Swidsinski *et al*., 2010). High vaginal concentrations of *G. vaginalis* and *A. vaginae* indicate that these two species are most strongly associated with BV (De Backer *et al*., 2006; Menard *et al*., 2012). A study by Bradshaw *et al*. (2006) supports a synergism between *G. vaginalis* and *A. vaginae*. Several researchers suggested that infection with *A. vaginae* is even more specific (and a diagnostically more valuable marker) for BV than infection with *G. vaginalis* (Bradshaw *et al*., 2006; Trama *et al*., 2008). These two species are strongly associated with bacterial biofilms (Swidsinski *et al*., 2005). Bacterial vaginosis-associated bacteria one (BVAB1) have been found to adhere to vaginal epithelial cells similar to clue cells, which are epithelial cells covered with Gram-variable pleomorphic rods and are desquamated cells from a biofilm (Fredricks *et al*., 2005; Swidsinski *et al*., 2005). Alves and colleagues (2014) suggested that G. vaginalis has the highest virulence potential, as defined by greater initial adhesion to epithelial cells and the cytotoxicity thereof. Even though the authors showed that most of the 30 investigated BV-related bacteria had a tendency to form a biofilm, *G. vaginalis* was found to have a greater inclination of forming biofilms. *Gardnerella vaginalis* biofilms have a greater tolerance to H_2O_2 and lactic acid as compared to planktonic cells (Patterson *et al*., 2007). It is possible that each of the two forms of *Gardnerella* may be responsible for a different pathogenesis model (Swidsinski *et al*., 2010). However, as Hickey and Forney (2014) stressed, the correlation between *G. vaginalis*

and BV does not necessarily indicate causation and hence responsibility of this bacterium for either of the two pathogenesis models (Verstraelen *et al*., 2010).

Castro and colleagues (2013) demonstrated reciprocal interference between *Lactobacillus* spp. and *G. vaginalis* on initial adherence to epithelial cells. A pathogenic *G. vaginalis* strain (isolated from a woman with BV) was shown to have greater ability to adhere to cervical epithelial cells than a strain isolated from a healthy woman. The non-pathogenic *G. vaginalis* strain is suggested to have no differential effect on *L. crispatus* but not *L. iners*. Conversely, the pathogenic *G. vaginalis* strain displaced *L. crispatus* but not *L. iners*, which enhanced adhesion of the pathogenic strain. This is in agreement with the fact that *L. iners*, a weak H_2O_2-producer, colonize women irrespective of BV status (Fredricks *et al*., 2005; Macklaim *et al*., 2013). Macklaim *et al*. (2013) documented a different expression profile of *L. iners* in BV positive and healthy women. The work of these authors seems to support the primary pathogen model. In a BV environment, *L. iners* increases its expression of a cholesterol-dependent cytolysin and of mucin and glycerol transport and related metabolic enzymes (Macklaim *et al*., 2013). The intricate microbial structure of BV is said to result in increased cell lysis and subsequent carbohydrate availability. Carbohydrates may be converted to succinate and other short-chain fatty acids that can regulate host immunity and increase vaginal pH (and most likely decrease the lactobacilli population) (Macklaim *et al*., 2013). This may be accompanied by an increase in the bacteriophage load and an environment where BV associated organisms cooperate to cause symptomatic disease (Macklaim *et al*., 2013).

Despite the lack of a definitive pathogenesis model, several components have been identified to act as virulence factors. Some *G. vaginalis* strains have anti-IgA activity and similar to

sialidases and cleavage enzymes produced by some bacteria, attenuate the defensive action of *G. vaginalis*-specific IgA (Cauci, 2004; Donders, 2007). Additional bacterial enzymes believed to play a role in the pathogenesis of BV include proteases, *G. vaginalis* hemolysins and mucinases (McGregor *et al*., 1994). Mucinase and sialidase, two hydrolytic enzymes, may enhance placental inflammation and weakening of the chorioamniotic membrane (Howe *et al*., 1999). These enzymes may promote increased ascending of lower genital tract organisms and sialidase increases the probability of PTB (Cauci, 2004). Marconi *et al*. (2013) showed that women with BV and elevated loads of *Leptotrichia* spp., *Megasphaera* spp. and *A. vaginae* have increased sialidase activity. Protease activity may lead to intrauterine death by stimulating the production of pro-inflammatory cytokines and premature rupture of membranes (PROM) and/or PTL by stimulating phospholipase A2 production (Govender *et al*., 1996; Nelson *et al*., 2007). Vaginolysin (VLY), a human-specific cytolysin produced by *G. vaginalis*, is responsible for lysing erythrocytes and epithelial cells at higher pH levels (Hooven *et al*., 2012).

Changes in innate immunity are partially liable for triggering the conversion of a vaginal microflora controlled by lactobacilli to one that resembles BV (Genc and Onderdonk, 2011). The suggested mechanisms may include: (i) the insufficient release and/or function of mannose-binding lectin, (ii) reduced TLR activation, (iii) amplified production of extracellular heat shock protein 70 (Hsp70) as well as (iv) the reduction in vaginal SLPI (Genc *et al*., 2005; Witkin *et al*., 2007b). These factors may lead to the disruption of controlled inflammation that inhibits the overgrowth of microorganisms in the vagina (Genc *et al*., 2005; Koumans *et al*., 2007; Witkin *et al*., 2007b).

A characteristic of BV is the absence of inflammation as there is no increase in the number of circulating leukocytes; there is a very low production of interleukin 8 and a slight rise in interleukin 1 levels (Donati *et al*., 2010). Nevertheless, a subgroup of women produces a local pro-inflammatory response (Genc and Onderdonk, 2011). Toll-like receptors transduce an inflammatory signal in cells upon recognition of microbial products (Genc and Onderdonk, 2011), which leads to the production of pro-inflammatory cytokines and induction of the adaptive immune response (Witkin *et al*., 2007a). Pregnant women with BV and women who are heavily colonized with *G. vaginalis* and anaerobic Gram-negative rods, contain elevated levels of pro-inflammatory cytokines and are at an increased risk for PTB (Genc and Onderdonk, 2011). Genital mycoplasmas, *Prevotella* spp. and *Bacteroides* spp. are all microorganisms associated with PTB (Genc and Onderdonk, 2011). Marconi *et al*. (2013) established that *Atopobium vaginae*, *Megasphaera* spp. and *Leptotrichia* spp. do not affect the local innate immunity.

Complications associated with bacterial vaginosis

The pathogenesis of BV in several pregnancy complications is not defined, but BV has been reported as a non-specific marker for these complications (Leitich *et al*., 2003; Cauci and Culhane, 2011). Theoretically, the inception of pregnancy complications as a result of BV is due to its potential to favor ascending infections (from the vagina to the chorioamnion) to cause inflammation of the choriodecidual space and activating pathways of labor and subsequently PTD and PTB (Guerra *et al*., 2006; Denney and Culhane, 2009; Guédou *et al*., 2013). BV-related bacteria and the toxins produced by these bacteria can cross the placenta and result in fetal complications (Turovskiy *et al*., 2011).

It is reported that women with BV diagnosed in the first trimester of pregnancy (up to 10 weeks gestation) are at an increased risk for adverse pregnancy outcome, such as second trimester pregnancy loss (<26 weeks gestation) and PTD (<37 weeks' gestation) (McGregor *et al*., 1995; Guerra *et al*., 2006; Nelson *et al*., 2007). Nelson and colleagues (2007) found that only severe BV conditions, i.e. absence of *Lactobacillus* spp., are the most at risk to experience a second trimester pregnancy loss. Conversely, Donders *et al*. (2000) suggested that the levels of *Lactobacillus* spp. are not related to the risk of pregnancy loss. The risk for adverse outcome is 10-fold increased if women have a history of pregnancy loss (Hay *et al*., 1994; Guerra *et al*., 2006). While this is supported by the finding that BV is associated with very preterm delivery (<32 weeks gestation) (Goffinet *et al*., 2003), it is contradicted by other studies that found no association of BV with PTD (Povlsen *et al*., 2001; Harper *et al*., 2012). It is suggested that the association of BV with PTD is inconsistent and must be interpreted differently in different populations (Goffinet *et al*., 2003).

The results of a meta-analysis by Leitich and Kiss (2007) established that BV is associated with PTB and late miscarriage. The first 16 weeks of pregnancy possibly marks a critical stage during which BV enters the upper genital tract because women in this gestation period are at highest risk for PTB (Guaschino *et al*., 2006). The reason why some women with BV are more prone to deliver preterm can in part be explained by genotype-environment interactions (Denney and Culhane, 2009). The hypothesis is that only women who have a genetic predisposition to generate pathological inflammatory responses to BV will result in having PROM and/or going into PTL (Denney and Culhane, 2009). Accordingly, the abnormal vaginal flora generally associated with BV would result in different lengths of gestation in susceptible women (Pretorius *et al*., 2007). Children may present with long-term neurological effects, such as cerebral palsy, hyperactivity, developmental delays, severe

handicaps and preventricular leucomalacia (Eschenbach, 1997; Grether and Nelson, 2000; Ling *et al*., 2004). The risk of PTB is reported to increase considerably in women with the most antagonistic vaginal flora (i.e. Nugent score of 9 or 10 and/or vaginal pH > 5) (Hauth *et al*., 2003).

Bacterial vaginosis has also been linked with other complications, including intrauterine infection, chorioamnionitis and postoperative abortive infections (Kurki et al., 1993; Govender *et al*., 1996; Guaschino *et al*., 2006). Women with pelvic inflammatory disease (PID) are more commonly affected by BV but this disease entity alone does not result in pruritus, dysuria, burning or any inflammation in the vagina (Klebanoff *et al*., 2004; Sobel *et al*., 2012).

Diagnosis of bacterial vaginosis

Screening for BV during pregnancy is inexpensive and sample collection is non-invasive. Collecting a sample is as simple as obtaining a self-collected vaginal swab from the patient and preparing a vaginal smear on a slide. Self-collected vaginal swabs are comparable to practitioner-collected swabs for routine diagnostic purposes (Boskey *et al*., 2004; Menard *et al*., 2012). However, many countries lack routine screening protocols for BV. Guise *et al*., (2001) evaluated several studies and concluded that there is no significant benefit to routine BV screening and treatment of asymptomatic pregnant women and that only a small group of high-risk women may benefit from screening. Nonetheless, it is recommended that all pregnant women should be screened for BV if there is any history of a spontaneous abortion, PTD or a low birth weight (LBW; <2 500 g) infant, regardless of symptoms, and be treated if BV positive (WHO, 2005; CDC, 2013). In addition, it is recommended that women with symptoms be screened and treated for BV (CDC, 2013).

The gold standard for the detection of BV is based on clinical and laboratory diagnoses, Amsel's criteria and the Nugent scoring system, respectively (Amsel *et al*., 1983; Nugent *et al*., 1991). Although these two methods often do not agree (Menard *et al*., 2010), these tests still perform better than alternative diagnostic tests. This is supported by a comparison of three tests for the diagnosis of BV during pregnancy in the one study population (Hogan *et al*. 2007). The tests that were compared included the Nugent scoring system, Amsel's criteria and a commercial amine and pH test card [FemExam TestCard (CooperSurgical, Inc., Trumbull, CT)]. The prevalence of BV was 55% with the Nugent scoring system, 28.5% with Amsel's criteria and 12.6% with the commercial test.

The Nugent scoring system

The laboratory diagnosis of a clinical condition with Gram-stained smears was first done by Spiegel *et al*. (1983) but was refined by Nugent *et al*. (1991) who established the Nugent scoring system. The Nugent scoring system is a system where Gram-stained slides are microscopically analyzed, which is mainly based on the presence or absence of lactobacilli (Figure 1) (Nugent *et al*. 1991). The different cell types are counted (*Mobiluncus* spp., *G. vaginalis/Bacteroides* spp. and *Lactobacillus* spp.) and a score between zero and ten is obtained; whereby a score of seven to ten corresponds to BV (Figure 1B), a score of four to six is considered intermediate (partial BV) and a score of zero to three indicates an undisturbed vaginal microflora (Figure 1A) (Nugent *et al*., 1991). Intermediate scores may indicate the development of BV or a woman that is being cleared of this disease entity; however, these 'intermediate flora' remains contentious (Guédo *et al*., 2013).

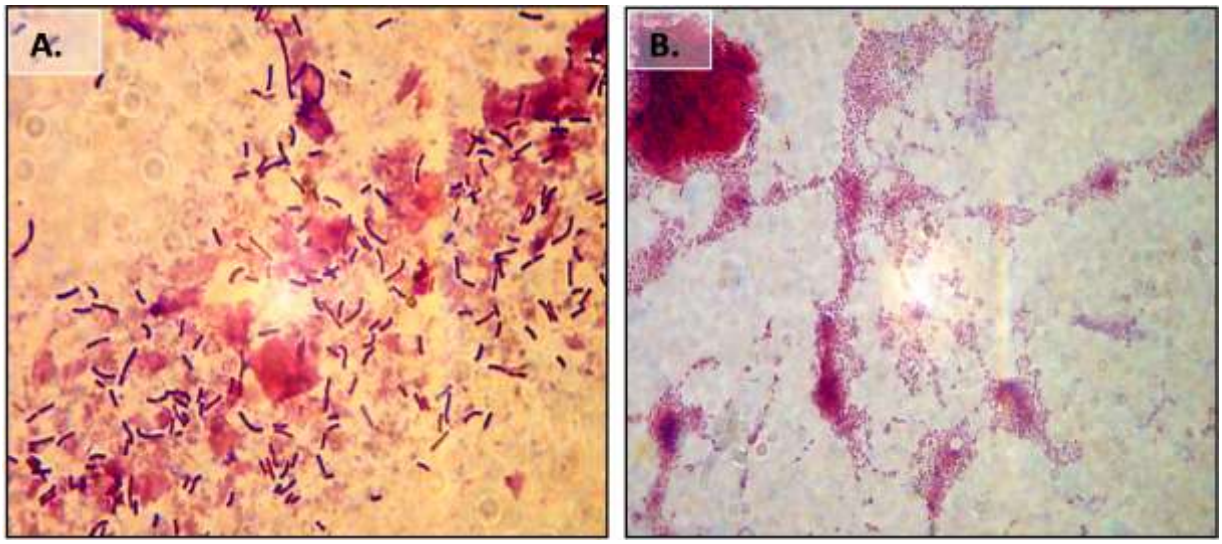

Figure 1: Microscope images of Gram-stained vaginal smears from (A) a healthy woman (Nugent score = 0) with a *Lactobacillus* dominated vaginal environment (100X objective) and (B) a BV-affected woman (Nugent score = 10) with *G. vaginalis/Bacteroides* spp. morphotypes dominating the vaginal environment, appearing as a granular flora pattern on the slide (10X objective)

Cultivation-independent methods indicated that many healthy women lack high numbers of lactobacilli and the healthy environment is maintained by other bacteria (Zhou *et al*., 2004; Ravel *et al*., 2011). This may give rise to misleading Nugent scores and incorrect diagnosis. Another drawback of the Nugent scoring system is that it lacks sensitivity when *A. vaginae* is investigated because this bacterium is not readily detected by Gram-staining due to its erratic morphology (Brotman and Ravel, 2008).

Ison and Hay scoring system

Ison and Hay (2002) established another grading system for Gram-stained vaginal smears. Instead of grading and allocating a score to individual bacterial species, the Ison and Hay grading system assigns a grade to a mixed group of bacteria, depending on the numerical contribution of individual morphotypes (Ison and Hay, 2002). Where only *Lactobacillus* morphotypes (normal flora) are present, a smear will be graded as grade I. Grade II

comprises intermediate flora, which include reduced *Lactobacillus* morphotypes with diverse bacterial morphotypes, whereas grade III (BV) contains mixed bacterial morphotypes with few or no *Lactobacillus* morphotypes (Ison and Hay, 2002). Grade 0 will be smears that contain epithelial cells with no bacteria and in which case antibacterial agents in the vagina might be present (Ison and Hay, 2002). Grade IV contains epithelial cells enclosed in Gram-positive cocci only (Ison and Hay, 2002).

The Ison and Hay criteria have been refined by Verhelst *et al*. (2005). Verhelst and colleagues (2005) subdivided the grade I category into grade Ia, grade Ib and grade Iab and proposed a new category called grade I-like. Specimens containing only *L. crispatus* cell types (short, plump, darker-stained rods) were categorized as grade Ia, those containing only other *Lactobacillus* cell types (smaller/elongated and less stained than in grade Ia smears) were categorized as grade Ib and specimens containing both *L. crispatus* and other lactobacilli were categorized as grade Iab (Verhelst *et al*., 2005). The grade I-like category contains short Gram-positive rods that are unevenly shaped with curved ends and may appear as Chinese letters (diphtheroid cell types) (Verhelst *et al*., 2005). An interesting comparison would be to evaluate this grading system with high-throughput NGS technologies and measure agreement with the different CSTs in terms of species type and relative species numbers. Such an evaluation will reveal the potential of this grading system as an alternative screening test to the Nugent scoring system.

Amsel's criteria

Amsel *et al*. (1983) described four criteria for the diagnosis of BV in clinical settings. A fulfillment of at least three of the four criteria for women is needed to be clinically diagnosed with BV (Amsel *et al*., 1983). Amsel's criteria include (i) vaginal pH >4.5, (ii) a thin

homogeneous vaginal discharge, (iii) a fishy odor on the addition of 10% potassium hydroxide (KOH; whiff test) and (iv) clue cells present on wet-mount microscopy (Amsel *et al*., 1983).

The shortcomings of Amsel's criteria are several. The whiff test may be subjective as a fishy odor is not always present, even after the application of KOH and interpretation may vary between investigators (Donders, 2007). A vaginal discharge has been reported to have low sensitivity (56%) and specificity (49%) and is present in only ±50% of BV positive women (Schwiertz *et al*., 2006; Donders, 2007). Additionally, a raised vaginal pH may be the result of several other lower genital tract conditions or due to vaginal and cervical secretions (Nelson and Macones, 2002). Clue cells may be difficult to recognize as these cells can be entirely, partially, or not at all covered by anaerobic flora and *G. vaginalis* morphotypes (Marconi *et al*., 2012). Marconi *et al*. (2012) highlighted that a granular flora pattern (Figure 1B) is more indicative of BV than to search for clue cells.

Culture and PCR detection of bacteria associated with bacterial vaginosis

Commercial media are available for the cultivation of BV-related bacteria, such as *Gardnerella* agar for *G. vaginalis* and Chocolate agar for anaerobes (*Bacteroides/Mobiluncus* spp.) (Goffinet *et al*., 2003). Cultures of bacteria, such as *G. vaginalis* are of no value for BV diagnosis as women who are merely colonized with this bacterium will also have positive cultures, whereas other bacteria, such as *A. vaginae* are fastidious, which makes cultivation difficult (Donders, 2007; Trama *et al*., 2008). Molecular detection methods may be more expensive than the gold standard but allow better characterization of the vaginal flora by targeting genes (mainly the 16S rRNA sequence) of specific bacterial genera or species (Fredricks *et al*., 2007). The molecular detection of fastidious bacterial species has been

shown to be a more consistent indicator of BV than the detection of bacteria present in patients without BV, such as *G. vaginalis* (Fredricks *et al*., 2007). *Atopobium vaginae, Leptotrichia* BVAB1-3, *Megasphaera* spp. and *Sneathia* spp. are examples of bacterial species that have been identified by means of molecular techniques (Fredricks *et al*., 2005; Verhelst *et al*., 2005). Multiplex quantitative PCR (qPCR) assays may aid in elucidating the pathogenic or protective roles bacteria play in health and disease (Mernard *et al*., 2010; Zozaya-Hinchliffe *et al*., 2010).

Other diagnostic tests

Alternative tests have either low sensitivity and/or specificity or are more expensive than the gold standard (Livengood, 2009). An example includes the Papanicolaou smear, which has been found to be a poor screening test with a sensitivity ranging from 50% to 89% and a specificity of around 90% (Greene *et al*., 2000; Sodhani *et al*., 2005). Wet-mount microscopy relies on a phase-contrast microscope and may be more rapid and accurate than performing Gram-staining by identifying the characteristic granular flora pattern of *G. vaginalis/Bacteroides* spp. morphotypes (Donders, 2007). Donders *et al*. (2000) suggested that the staining process damages some of the lactobacillary flora and by this means favors the non-lactobacillary flora. The normal vaginal lactobacillary flora is also better visualized with wet-mounts than with the Gram-stain (Donders, 2007). However, wet-mount microscopy is subjective due to inter-observer variability and the preparation of the wet-mount (Schoeman, 2002). Therefore, it would be of more value in the case of skilled microscopists (Schoeman, 2002).

Treatment and prevention of bacterial vaginosis

It is anticipated that if BV increases women's susceptibility to HIV infection, interventions to reduce the occurrence of BV will have an impact on the prevention of the spread of HIV at a population level (Myer *et al*., 2005). Currently, metronidazole and clindamycin (oral antimicrobial agents that are known to be active against anaerobic bacteria) are the preferred treatment for BV as recommended by the Centers for Disease Control and Prevention (CDC), with a cure rate of 80% to 90% within one week (Armstrong and Wilson, 2010; CDC, 2010). Even though the use of metronidazole in the first trimester was previously discouraged due to its potential for teratogenicity (Struthers, 1997; WHO, 2005), both antimicrobial agents are said to be safe for use in pregnancy (CDC, 2010; Sobel *et al*., 2012). The dose recommendations for the treatment of BV are (i) 400 mg or 500 mg oral metronidazole, twice daily for seven days, (ii) 300 mg oral clindamycin, twice daily for seven days, (iii) 5 g of 0.75% metronidazole gel intravaginally, twice daily for five days or (iv) 5 g of 2% clindamycin vaginal cream intravaginally, at night for seven days (CDC, 2013; WHO, 2014). Cure rates of up to 83% is attainable with these therapies one month after treatment (Ferris *et al*., 1995). The route of antimicrobial agent administration (orally/vaginally) has a minor impact on bacterial eradication in pregnant women with BV (Mitchel *et al*., 2009).

Due to difficulties related to the management of RTIs in developing countries, such as financial constraints, the World Health Organization (WHO) introduced syndromic management guidelines for treating RTIs. Women presenting with an abnormal vaginal discharge (i.e. abnormal in terms of quantity, colour or odour) are treated according to the vaginal discharge syndrome (VDS) flowchart (WHO, 2005). Combination therapy may be given according to specificity of discharge. According to the VDS flowchart, if a pregnant woman is presenting with a vaginal discharge without any abdominal pain then treatment

consists of: (i) an oral single 400 mg dose cefixime, (ii) 500 mg amoxicillin (orally), three times daily for seven days and (iii) oral 2 g metronidazole to cover all potential pathogens (Lewis and Maruma, 2010). Studies have shown that the prevalence of symptomatic RTIs, including BV may be reduced by syndromic management approaches but it has little influence on the prevalence of RTIs that typically present with no symptoms (Romoren *et al*., 2007; Johnson *et al*. 2011).

Ferris and colleagues (2004) demonstrated that *A. vaginae* has *in vitro* resistance to metronidazole but remains susceptible to clindamycin. With *A. vaginae* being one of the major pathogenic contributors of BV, it is expected that clindamycin would be more effective in the treatment of BV positive women (Bradshaw *et al*., 2006). However, it has been shown that metronidazole and clindamycin have equal short-term effectiveness in the treatment of BV (Koumans *et al*., 2007). A possible reason is that following treatment with metronidazole, the decline in metronidazole-sensitive species can lead to a concurrent decline in metronidazole-resistant species due to the possible activity of the hydroxy metabolite [1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole], also known as the alcohol metabolite (Bradshaw *et al*., 2006).

It is reported that treatment of BV later in pregnancy is more successful (Klebanoff *et al*., 2004). It may be directly linked to the spontaneous resolution of up to 27% of BV infections as gestational age progresses (Hay *et al*., 1994; McDonald *et al*., 1994; Klebanoff *et al*., 2004). Although the etiology of recurrent BV is unknown, it has been found that retreatment with the same antimicrobial agent produces a higher cure rate (Bunge *et al*., 2009). This suggests that antimicrobial resistance is not the principal etiology for treatment failure (Bunge *et al*., 2009). Klebanoff and colleagues (2004) documented that two 2 g doses of

metronidazole, administered 48 hours apart, eliminated BV in 72% of women and Gram-stain scores were restored to normal in 55% of women. The effectiveness of this treatment regimen remained steady over the course of 2 to >10 weeks. Suppressive antimicrobial therapy with twice-weekly, 0.75% metronidazole vaginal gel has been shown to reduce recurrence rates considerably (Sobel *et al*., 2006). However, this has been associated with secondary vaginal candidiasis (Sobel *et al*., 2006). Bacterial vaginosis can be prevented by limiting the number of sexual partners and avoiding douching and thereby not disrupting the normal vaginal flora (CDC, 2013). The occurrence of BV may be reduced by the use of condoms (Fethers *et al*., 2008) and the completion of a course of antibiotics may prevent relapse (CDC, 2013).

Previous reviews combining and analyzing the results of a large collection of studies mainly report that the routine screening of asymptomatic pregnant women for BV and the administration of antimicrobial agents may successfully reduce BV infection but does not necessarily decrease the risk of PTB or PROM (Guise *et al*., 2001; McDonald *et al*., 2007; Brocklehurst *et al*., 2013). However, treatment may reduce the risk of PTB < 37 weeks' gestation in women with abnormal vaginal flora (i.e. intermediate flora or BV). Intermediate vaginal flora has been shown to have almost the same correlates as BV flora (Guedo *et al*., 2013).

Tinidazole is an alternative antimicrobial agent that has been used in different doses to treat BV (Armstrong and Wilson, 2010). Metronidazole is however still the antimicrobial agent of choice as it has a low cost, favourable pharmacokinetic and pharmacodynamic properties and is effective against pathogenic anaerobic bacteria (Löfmark *et al*., 2010). Rifamixin, a semi-synthetic rifamycin derivative, has recently been proposed as an alternative treatment

regimen for BV and the prevention of recurrence (Cruciani *et al*., 2013; Donders *et al*., 2013). It has a good safety profile due to negligible systemic absorption (Rivkin and Gim, 2011). A dose of 25 mg for five days has been shown to be the most effective in treating BV and maintaining a healthy flora (Donders *et al*., 2013; Laghi *et al*., 2014). Rifamixin treatment is associated with an increase in the lactobacillus/BV-related bacteria ratio, lactic acid concentration and a reduction in metabolites normally produced by BV-related bacteria (Laghi *et al*., 2014). Retrocyclin 101 (RC-101), a cyclic antimicrobial peptide, has been shown to strongly inhibit the cytolytic activity of vaginolysin and biofilm formation of *G. vaginalis in vitro* and is a potential candidate for the treatment and prevention of BV (Hooven *et al*., 2012). Briese and colleagues (2011) studied the efficacy and tolerability of a local antiseptic, octenidine hydrochloride/phenoxyethanol (OHP), for the treatment of BV and/or vaginal dysbiosis in pregnancy. This study found that OHP is indeed effective against BV/dysbiosis and well tolerated in pregnancy without side effects and suggested it could be used as a supplementary therapeutic option in the prevention of PTB (Briese *et al*., 2011). Rajan *et al*. (2014) studied and proposed the use of combining the antimicrobial peptide subtilosin within covalently cross-linked polyethylene glycol (PEG)-based hydrogels for vaginal administration. It was shown to inhibit the growth of G. vaginalis but not the growth of the four main *Lactobacillus* species. This study highlights the potential application of vaginal subtilosin-containing hydrogels for the prophylaxis of BV (Rajan *et al*., 2014).

5. Comment

The detection and early management of BV is essential to prevent complications and adverse outcomes in pregnancies. This might minimize the risk for complications at a later stage, which may require the patient to seek further medical attention and lead to a greater financial burden. It may also reduce the rates of neonatal morbidity and mortality. An accurate and

reliable molecular tool for BV diagnosis was proposed by Menard and colleagues (2010) based on the combination of high vaginal quantification of *A. vaginae* and *G. vaginalis*. The real-time qPCR assay was sensitive (100%) and specific (93%) compared to the Nugent score as the reference method (Menard *et al.*, 2010). Shipitsyna and co-workers (2013) suggested that a more accurate diagnosis of BV would be to measure the richness of normal and BV microbiota relative to total bacteria in vaginal fluid instead of qualitative detection or absolute counts of BV associated bacteria. Only more research on the plethora of BV-associated microorganisms will elucidate the value of such diagnostic tests. The advent of high-throughput technologies such as NGS have yet to be comprehensively applied and evaluated in studying BV in different populations. These approaches have the potential of revealing low-diversity species, the functional gene composition (for instance biofilm formation) and dynamics of ecosystems, including the metabolic co-dependencies and the pathogenesis of communities. Other approaches to characterize microbiomes are also being pursued and may be of value to study the vaginal microbiota and complement NGS techniques. These include 'culturomics' studies (Greub, 2012; Lagier *et al*., 2012) and phylogenetic branch-inclusive quantitative PCR (PB-qPCR) and *Lactobacillus* blocked/unblocked qPCR (Lb-qPCR) (Lambert *et al*., 2013b). The lack of defining the pathogenesis and a single causative organism for BV has led to the investigation and suggestion of BV and/or specific markers as predictors for pregnancy complications (Goffinet *et al*., 2003; Onderdonk *et a*l., 2003; Cauci and Culhane, 2011). The diverse findings emphasize the composite nature of the vaginal microbiota associated with BV and its exact role(s) in pregnancy outcome.

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Declaration of interest

The authors declare no conflicts of interests. The authors alone are responsible for the content and writing of this article.

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