

NUTRITIONAL RISK FACTORS OF BACTERIAL VAGINOSIS

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Bacterial vaginosis (BV) is a highly prevalent vaginal condition that has been associated with a number of pregnancy complications, including spontaneous preterm births (sPTB). A number of risk factors for BV have been identified yet its etiology is not understood. Few studies have investigated the role of nutrition in the etiology of BV. Maternal iron and folate status may be important in the development of BV as they play key roles in host immunity. The purpose of this review is to critically evaluate the literature and provide support to the hypothesis that certain micronutrients, iron, and folate, are risk factors for BV early in pregnancy. Iron and folate may be important in both humoral and cell-mediated innate immunity, respectively. Both facets of innate immunity are important in the control of BV.

Preliminary data suggests that vitamin D is associated with BV early in pregnancy. In a prospective cohort study, pregnant women enrolled at <16 weeks were followed through delivery. Serum collected at enrollment was analyzed for 25-hydroxyvitamin D (25(OH)D) and Gram-stained vaginal smears were evaluated for BV using Nugent criteria. Multivariable logistic regression was used to determine the association between 25(OH)D and BV while adjusting for a number of confounders. The association between vitamin D and BV varied by race (likelihood ratio test, $p = 0.09$). A 50-nmol/l decrease in 25OHD was associated with a 4.2-fold (OR 4.2; 95% CI, 2.1 - 8.1) increase in the odds of BV in black women. Among white women, there was no association between maternal 25(OH)D and BV. These results indicate that maternal vitamin D deficiency is associated with BV in early pregnancy among black, but

not white women. Studies such as these are of great public health significance because maternal nutrition is modifiable, and interventions to improve maternal nutritional status can be safe, inexpensive, and readily acceptable to patients.

TABLE OF CONTENTS

1.0	INTRODUCTION.....	1
2.0	BACKGROUND AND SIGNIFICANCE	2
2.1.1	Relationship to pregnancy outcomes.....	2
2.1.2	Epidemiology	5
2.1.3	Microbiology.....	8
2.1.4	Immunology.....	9
2.1.5	Diagnosis	11
2.1.6	Treatment issues with BV and pregnancy outcomes.....	13
2.1.7	Biologic plausibility of iron and folate in bacterial vaginosis	14
2.1.8	Literature review: the association between nutrition and BV	18
3.0	PRELIMINARY DATA - MATERNAL VITAMIN D STATUS AND BACTERIAL VAGINOSIS DURING PREGNANCY.....	21
3.1.1	Introduction.....	21
3.1.2	Methods.....	22
3.1.2.1	Study population	22
3.1.2.2	Study design and procedures	22
3.1.2.3	Outcome assessment: Bacterial vaginosis.....	23
3.1.2.4	Exposure assessment: vitamin D status	23

3.1.2.5	Statistical analysis	23
3.1.3	Results	24
3.1.4	Discussion.....	31
4.0	RESEARCH PRIORITIES.....	34
4.1	FUTURE STUDY: NUTRITIONAL RISK FACTORS OF BACTERIAL VAGINOSIS.....	34
4.2	OTHER NUTRITIONAL RISK FACTORS OF BACTERIAL VAGINOSIS	35
4.3	RACIAL DISPARITY IN BACTERIAL VAGINOSIS.....	36
4.4	RESOLVING THE ISSUE OF PERSISTENT BACTERIAL VAGINOSIS	37
4.5	INTERACTION OF GENETIC POLYMORPHISMS AND NUTRITION	38
5.0	PUBLIC HEALTH APPLICATIONS AND SIGNIFICANCE	39
	BIBLIOGRAPHY.....	41

LIST OF TABLES

Table 1. Nugent's criteria for diagnosis of bacterial vaginosis; summation of scores (73).....	12
Table 2. Maternal characteristics collected at first study visit stratified by BV status	26
Table 3. Effect on vitamin D ([25(OH)D] <50 nmol/l) on the odds of BV, stratified by race	31

LIST OF FIGURES

Figure 1. Race stratified by vitamin D status. Vitamin D deficiency ($[25(\text{OH})\text{D}] < 37.5$ nmol/L), vitamin D insufficiency, ($37.5 < [25(\text{OH})\text{D}] < 80$ nmol/L), and vitamin D sufficiency, ($[25(\text{OH})\text{D}] \geq 80$ nmol/L) among 404 mothers at <16 weeks gestation. Number of women provided, percentages are given in parentheses. Post-hoc power analysis indicates that power achieved was 99.8%.....	27
Figure 2. Bacterial vaginosis status stratified by vitamin D status. Vitamin D deficiency ($[25(\text{OH})\text{D}] < 37.5$ nmol/L), vitamin D insufficiency, ($37.5 < [25(\text{OH})\text{D}] < 80$ nmol/L), and vitamin D sufficiency, ($[25(\text{OH})\text{D}] \geq 80$ nmol/L) among 404 mothers at <16 weeks gestation. Number of women provided, percentages are given in parentheses. Post-hoc power analysis indicates that power achieved was 99.8%.....	28
Figure 3. Lowess regression curve of unadjusted probability of bacterial vaginosis and concentrations of 25(OH)D in all mothers at <16 weeks gestation.....	29
Figure 4. Lowess regression curve of unadjusted probability of bacterial vaginosis and concentrations of 25(OH)D in white mothers at <16 weeks gestation.....	30
Figure 5. Lowess regression curve of unadjusted probability of bacterial vaginosis and concentrations of 25(OH)D in black mothers at <16 weeks gestation.....	31

1.0 INTRODUCTION

Bacterial vaginosis (BV) is a highly prevalent vaginal condition that is characterized by the imbalance of normal vaginal flora; a reduction in the number of resident lactobacilli accompanied by the overgrowth of several anaerobic or facultative bacteria (1, 2). Many authors have examined the relationship between BV and a number of pregnancy complications, most notably spontaneous preterm birth (sPTB). Although a number of risk factors for BV have been identified, including race, smoking, and chronic stress, its etiology is not fully understood (3). Local vaginal immunity, which presents the first line of defense against pathogens, may play an important role in the development of BV. Deficiencies of a number of micronutrients affect local immune function, and it has been hypothesized that BV may be related to nutrition (4-6). Few studies have investigated the role of nutrition in the etiology of BV (6, 7). The purpose of this review is to critically evaluate the literature and provide support to the hypothesis that certain micronutrients, iron, and folate in particular, are risk factors for bacterial vaginosis early in pregnancy. This hypothesis is supported in part, by the association between vitamin D and BV. In a cross-sectional study, the association between maternal vitamin D status at <16 weeks gestation and the presence of bacterial vaginosis was assessed using multivariable logistic regression. There was an association between vitamin D and BV however, this association varied by race. Should significant associations exist between other micronutrients and BV, it provides an opportunity to develop primary and secondary prevention measures that cannot only be directed towards BV, but towards the prevention of sPTB as well.

2.0 BACKGROUND AND SIGNIFICANCE

2.1.1 Relationship to pregnancy outcomes

Bacterial vaginosis (BV) is a polymicrobial condition that may be associated with various pregnancy complications. BV is a highly prevalent vaginal condition characterized by the reduction of normal flora (predominantly lactobacilli) and the subsequent overgrowth of several anaerobic or facultative bacteria such as *G. vaginalis*, *Bacteroides spp*, and *Prevotella/Porphyromonas* (2). Molecular diagnostic techniques have identified novel species that are also associated with BV, including *Atopobium vaginae*, *Megasphaera spp*, and *Leptotrichia spp* (8). BV affects 15 - 30% of non-pregnant women of reproductive-age, and up to 23% of pregnant women (9, 10). Conflicting information includes evidence that pregnant women with BV have a higher risk of pregnancy complications such as spontaneous abortions, chorioamnionitis, and post-partum endometritis (10, 11). BV however, has been most consistently associated with spontaneous preterm birth (sPTB, birth that follows either spontaneous labor or preterm premature rupture of the membranes (PPROM) prior to 37 completed weeks of gestation). sPTBs account for 75% of all preterm births (PTB) (12). PTBs account for 75% of perinatal mortality and 50% of perinatal morbidity (12). One recent meta-analysis showed that women with BV have a 2-fold greater odds of sPTB, compared to women without BV (OR 2.16; 95%CI, 1.56 - 3.0) (13). The analysis was based on cohort studies and

control groups of randomized clinical trials. The mean gestational age of BV screening ranged from 7 to 31 weeks. A near 3-fold increase in odds in sPTB is observed when the analysis was limited to BV that is screened at < 16 weeks gestation (OR 2.97; 95% CI, 1.48 - 5.98). Although a random effects model was used for analysis, the test for heterogeneity was highly significant (p-value <0.01), indicating that there is little validity to the study results. There is an increased rate of PTBs among black women compared to white women, suggesting some racial disparity (14). One author has estimated that BV may account for 30% of this disparity (15). This is based upon BV prevalence of 23% and 9% in black and white women, respectively, and a relative risk of 2.0 (16). In a study of vaginal infections, race, and preterm birth, BV was estimated to have an attributable risk of 12.5% in black women vs. 4.8% in non-black women with sPTB that occur < 35 weeks gestation (14). Reasons for the disparity in either PTBs or BV are not clearly understood. The mechanism by which BV is associated with sPTB is not entirely known. However, PTB are thought to be primarily of infectious origin. Vaginal microorganisms found in BV are thought to ascend into the uterus before or early in pregnancy. Infection ascends through the cervix to the amniotic fluid, fetal-maternal membranes, decidua, and placenta (17). In women with sPTB with intact membranes, commonly identified bacteria include *Mycoplasma hominis*, *G. vaginalis*, and *Peptostreptococcus*, the same bacteria often found in BV (17). As a result of the maternal and fetal response to bacterial invasion, preterm labor and delivery may occur (18). It is postulated that vaginal microorganisms ascend the uterus into the choriodecidual space. Bacteria produce endotoxins and exotoxins which activate the decidua and fetal tissues to produce a number of proinflammatory cytokines (19). Bacterial endotoxin and exotoxins also stimulate prostaglandin synthesis, which stimulates uterine contractions. Increased production of cytokines also increases chemotaxis of neutrophils, which

increases the release of metalloproteases, leading to cervical ripening (20). Metalloproteases can also attack the chorioamnion, leading to its rupture. BV is also thought to confer a higher risk of PPRM, which is the rupture of the amniotic sac prior to the onset of labor that occurs before 37 weeks of gestation are completed (21). PPRM occurs in 3% of all pregnancies; however it is associated with one-third of sPTB (22).

BV has been the target of a number of intervention trials to reduce incidence of sPTB. However, studies have yielded inconsistent results. In a recent meta-analysis, treatment did not result in a significant reduction of sPTB or PPRM <37 weeks (Peto OR 0.91; 95% CI, 0.78 - 1.06, 15 trials, 5,888 women and Peto OR 0.88; 95% CI, 0.61 - 1.28, 4 trials, 2,579 women, respectively) (23). However, treatment delivered prior to 20 weeks gestation may reduce the incidence of sPTB (Peto OR 0.63; 95% CI, 0.48 - 0.84, 5 trials, 2,387 women). There may be a number of reasons why BV treatment does not appear to reduce the risk of adverse pregnancy outcomes. One reason may be the timing of treatment delivery. BV is a strong risk factor for sPTB when screening occurs prior to 16 weeks gestation (13). Yet in a number of studies, treatment begins slightly before or after the 20th week of gestation (24-28). This suggests that the antibiotics may not have been administered in a timely fashion to treat infection early in pregnancy, making it probable that they were unable to prevent the bacteria's ascension into the uterus. Another issue to consider is that of subgroups. BV is quite prevalent in pregnant women, however only certain subgroups may benefit from treatment. One example of such women with BV that may benefit from treatment are those that have a pH >5.0 and a higher number of neutrophils (> 5 per oil field) (29). These findings were highly associated with PPRM at 24 – 32 weeks, compared to women who had PPRM at 32 to 36 weeks (p-value = 0.004). Genetic polymorphisms of pro-inflammatory cytokines have also been associated with

sPTB (30). However, BV may play a role in women with certain polymorphisms. For example, TNF- α allele 2 is a single nucleotide polymorphism that has been associated with increased gene transcription (31). Women who carry the TNF- α allele 2 are at a higher odds of sPTB, compared to women who do not carry the TNF- α allele 2 (OR 2.7; 95% CI, 1.7 - 4.5). However, women with this allele and symptomatic BV, had a substantially higher odds of sPTB (OR 6.1; 95% CI, 1.9 - 21.0), suggesting that a gene-environment interaction plays some role. This instance of increased gene transcription of TNF- α coupled with symptomatic vaginitis, would lead to a hyper-immune response that is associated with sPTB. Cytokine polymorphisms such a TNF- α allele 2 and others may provide a link as to why BV is associated with sPTB.

2.1.2 Epidemiology

There are several risk factors associated with BV. However, its etiology is not yet fully understood. Smoking, stress, and low socioeconomic status are associated with an increased risk of BV (3). Intrauterine device use has also been associated with BV but results have been inconsistent (32, 33). Vaginal douching is associated with BV (34). Douching may wash away the normal vaginal flora thus permitting pathogen overgrowth. BV protective factors include hormonal contraceptive use and condom use (35-37). Use of hormonal contraceptives is associated with increased estrogen, which increases glycogen produced by epithelial cells (33). The glycogen is used by lactobacilli to produce lactic acid, which is needed to inhibit the growth of BV-associated microorganisms. Use of condoms suggests that some part of BV is sexually associated. There is a consistent racial disparity that currently exists with BV (38). Using data from the 2001-2004 National Health and Nutrition Examination Survey, the prevalence of bacterial vaginosis among women ages 14-49 in the United States was estimated (39). Overall,

the prevalence of BV in women was approximately 29%. The odds of being BV positive among non-Hispanic black women was over 3 times greater compared to non-Hispanic white women (OR 3.13; 95%CI, 2.58 – 3.80). This disparity still existed even after controlling for lifestyle and behavioral risk factors (39). BV has been associated with sexual behavior. BV is not considered a sexually transmitted infection (STI) even though it is commonly found with other STIs (40). BV is associated with a number of sexual behaviors, including an increasing number of sexual partners and early age of sexual intercourse (41). High BV prevalence rates have been reported in lesbians and their sexual partners (42). Sexual practices that involve the transmission of vaginal fluid (oral sex, failure to clean sex toys after use) imply the sexual transmission of some unknown agent associated with BV (42). Other studies suggest that BV is not a STI. Although more commonly found in sexually active women, rates of BV are comparable in both virgins and non-virgin adolescent girls (43). Recurrence of BV in women continues even after the treatment of their male sexual partners (41).

BV recurrence/persistence may be attributable to a number of different factors. It may be the result of antibiotic resistance. Anaerobic organisms associated with BV may become resistant to metronidazole or clindamycin, treatments currently recommended by CDC (44). Recurrent BV itself has been associated with metronidazole resistant *A. vaginae* (45). Women treated with metronidazole who had a BV event during a year of follow-up had higher loads of both *A. vaginae* and *G. vaginalis*, compared to pre-treatment levels, indicating some level of resistance or an inability of metronidazole to effectively clear anaerobes loads (45). There is evidence that biofilms are associated with BV and may be important in its pathogenesis. Biofilms are groupings of bacteria entrenched in glycocalyx and are attached to epithelial surfaces. The biofilms effectively act as a coating and protect bacteria from the effects of

antibiotics because of decreased antibiotic penetration. Biofilms are primarily composed of *G. vaginalis* and to a lesser extent, *A. vaginae* and other microorganisms (46). One study found that a *G. vaginalis*-biofilm can be cleared *in vitro* by displacing it with *L. reuteri* RC-14 and *L. iners* (47). Studies have looked at the utility of reconstituting the Lactobacillus concentrations in women with BV by using probiotics (48).

Recurrence may also be due to re-infection with BV-associated pathogens, suggesting that some element of BV is sexually transmitted. Re-infection may explain the high recurrence rate often found in women who undergo BV treatment. A higher cure rate was found among women who either abstained from sex or used condoms, lending support to the STI hypothesis (49). BV occurs more often in heterosexual women who report new or an increasing number of male sexual partners (50). It has also been associated with a recent change of male partner (or a new partner), RR 1.6, 95%CI (1.3 - 2.5) (51). However, treatment of sexual partners does not result in reduction of recurrence rates (41). Molecular techniques such as 16s rRNA polymerase chain reaction (PCR) have been used to characterize microorganisms that are currently not cultivatable. These include *A. vaginae*, *Megasphaera*, *Leptotrichia*, and BV-associated bacterium 1 (BVAB1), BVAB2, and BVAB3 (52). Women with BV have a higher count of these microorganisms as well as a higher degree of species diversity, compared to women without BV (8, 52). Many of these microorganisms are specific for BV and their potential as STIs should be the focus of future research. In a nested case-control study of women who were cured vs. women with persistent BV (women were treated with intravaginal metronidazole gel), concentrations of a number of microorganisms were assessed by quantitative PCR (53). *G. vaginalis*, *A. vaginae*, BVAB1, BVAB2, BVAB3, *Megasphaera*, and *Leptotrichia* concentrations decreased in the cured group after treatment. However, in women with persistent BV after

antibiotic treatment, there were higher concentrations of BVAB1, BVAB2, *Megasphaera*, and *G. vaginalis* (53). Further studies should consider these microorganisms and their role in recurrent BV.

High recurrence rates may also be due to certain risk factors such as douching. Women who sustain behaviors that are highly associated with BV may continue to experience BV. Results from a douching cessation pilot study showed that women who ceased douching were 24% less likely to have BV compared to when they were not douching, (aOR 0.76; 95%CI, 0.33 - 1.76) (54).

2.1.3 Microbiology

The microbiology of bacterial vaginosis is quite complex and little is known about precipitating factors. However, the presence of normal vaginal flora is quite important in deterring BV. The normal vaginal flora consists of a number of genera and species. However, the dominant flora consists of several species of *Lactobacillus*: *L. crispatus*, *L. fermentum*, *L. gasseri*, and *L. jensenii* (55). Lactobacilli interfere with pathogen growth in two main fashions. Firstly, they provide a physical barrier by adhering to the mucosal membrane. This prevents pathogens from adhering to vaginal epithelial cells. Researchers have observed *in vitro* that *Lactobacillus* strains can successfully displace *G. vaginalis* and *Candida albicans* from adhesion to epithelial cells (56). The second method is through the production of several antimicrobial products. Lactobacilli produce hydrogen peroxide (H₂O₂), which inhibits the growth of non-catalase producing bacteria. Hydrogen peroxide-producing strains of lactobacilli were detected in 96% of women with normal flora but in about 6% of women with BV (57). Lactobacilli also produce lactic acid, which helps maintain a low pH environment. *In vitro* studies have shown

that acidification by lactobacilli can inhibit growth by pathogens *G. vaginalis* and *Mobiluncus* (58).

Little is known regarding factors that influence the vaginal microbiota. There is no one single associated agent of BV although *G. vaginalis* is present in up to 95% of cases (59). *Mobiluncus* is Some of the other commonly found anaerobic organisms include *Prevotella/Porphyromonas*, and *Bacteroides* (2). Only a certain number of bacteria present in the vaginal flora are cultivatable. Molecular identification using 16S rRNA reveals additional bacteria that have a high specificity for BV (8). These include *Megaphaera*, *A. vaginae*, and BV-associated bacteria (BVAB) 1, BVAB-2 and BVAB-3 (52). Metronidazole resistance is often found in recurrent BV and its presence may explain why metronidazole treatment trials do not yield any significant results (60). *A. vaginae* is associated with metronidazole resistance (37). Potentially, the imbalance of vaginal flora or the etiology of BV recurrence could be the result of the failure of H₂O₂-producing Lactobacilli to reestablish itself.

2.1.4 Immunology

BV is known as a “microbial/mucosal immunity disorder”, due not only to the changes in relative numbers of microbes but also to changes in the vaginal immune response as well (21). In the normal vaginal immune response, immune cells produce IL-1 β , a proinflammatory cytokine, in response to infection. An increased concentration of IL-1 β induces secretion of IL-8, IL-6, and TNF- α . IL-8 is a chemokine that is responsible for the recruitment of neutrophils. Neutrophils are an important tool in the host response to vaginal mucosal pathogens (61). IL-6 stimulates B-cell differentiation, particularly the maturation of B-cells into immunoglobulin-producing cells. TNF- α is another proinflammatory cytokine that activates various white blood

cells (macrophages, lymphocytes) in response to microbial exposure and other cytokines (62). In the vaginal response of a women with BV, the vaginal fluid has a high concentration of IL-1 β which indicates some level of host response does indeed exist (63). However, higher concentrations of IL-8, IL-6, or TNF- α are not seen. Neutrophil count is strongly correlated with IL-1 β and IL-8 concentration. However, while IL-1 β concentration is much higher in women with BV compared to those without, neither neutrophil count nor IL-8 concentration are significantly higher in women with BV (63). Neither increased concentrations of IL-6 nor TNF- α are seen in women with BV compared to those without BV (64).

In pregnancy, there are higher concentrations of pro-inflammatory cytokines IL-1 β , IL-6, and IL-8 compared to nonpregnant women with BV (65). However, the balance of pro-inflammatory to anti-inflammatory cytokines may be responsible for conferring a higher risk of PTB. Women with low concentrations of IL-1 β , IL-6, and IL-8 found early in pregnancy are at a higher risk of developing chorioamnionitis vs. those who do not have low concentrations of these cytokines (66). In a similar vein, women with high levels of anti-inflammatory cytokines IL-4, IL-10, IL-13, relative to the level of pro-inflammatory cytokines IL-1 α , IL-1 β , IL-6, have a higher risk of PTB. This is compared to women with either a high pro-inflammatory/low anti-inflammatory profile or balanced profiles (67). Pro-inflammatory and anti-inflammatory cytokines play important roles in the regulation of innate immunity. An appropriate balance between the two is required to keep the immune system functioning properly. An over-production of pro-inflammatory coupled with an under-production of anti-inflammatory cytokines can lead to tissue destruction. The adverse scenario of under-production of pro-inflammatory cytokines and over-production of anti-inflammatory cytokines can enhance susceptibility to infections. Some factor is disrupting proper immune system function.

Nutrition, which has a demonstrated role in cell-mediated and humoral immunity, may play a role in this improper balance of inflammatory cytokines.

2.1.5 Diagnosis

There are several diagnostic criteria for BV and choosing the most appropriate criteria is important for its study. Oftentimes in clinical settings BV is diagnosed by a set of criteria defined by Amsel et al (68). Three of the four criteria must be present for an accurate diagnosis of BV: 1) thin, homogenous white discharge that adheres to the vaginal wall; 2) vaginal fluid pH > 4.5; 3) a whiff test which yields an amine odor with the addition of 10% potassium hydroxide to vaginal fluid; and 4) the presence of clue cells on a microscopic examination of vaginal smear. Vaginal cells that are heavily coated with bacteria are known as clue cells; they are formed when there is a high number of *G. vaginalis*. Of the four criteria, the presence of clue cells is the most reliable predictor of bacterial vaginosis (69). However, the sensitivities and specificities of some of the remaining criteria contribute to the variability of BV diagnosis. The specificity of the pH assessment is in question as an increase in vaginal pH may be due to other lower genital tract conditions. The whiff test lacks sensitivity because it is subjective for each individual clinician.

Another diagnosis method, Spiegel criteria, uses gram-stained vaginal smears for the diagnosis of BV (70). Bacteria are grouped into morphotypes, with *Lactobacillus* characterized as elongated bacteria and *Gardnerella* characterized as short bacteria. BV is diagnosed by the relative numbers of Lactobacilli compared to numbers of other morphotypes. This method of diagnosis was used for many years but was criticized for its inability in distinguishing the severity of BV (71).

Nugent et al expanded upon Spiegel's method to a scoring system (71, 72). Gram stains are used to score the amount of three morphotypes: *Lactobacillus* (large uniform gram-positive rods), *G. vaginalis* (small pleomorphic Gram-variable rods) or *Prevotella/Bacteroides* (small Gram-negative rods), and *Mobiluncus* (curved Gram-variable rods) (73). Nugent scores 0 to 3 are considered healthy normal vaginal flora, scores 4 to 6 are classified as intermediate flora, while scores ranging from 7 to 10 are diagnosed as BV (74). This set of criteria has an advantage over Spiegel because it evaluates vaginal flora on an ordinal scale (increasing positivity of disturbed flora). Nugent showed a sensitivity of 89% and a specificity of 83%, while Spiegel criteria have sensitivity and specificity of 62% and of 95%, respectively compared to Amsel criteria (75, 76). As such, Nugent's criteria have become the current preferred method of BV diagnosis in research studies. Calculation of Nugent scores is illustrated in the table below.

Table 1. Nugent's criteria for diagnosis of bacterial vaginosis; summation of scores (73)

Nugent score	Lactobacillus types	Gardnerella or Prevotella/Bacteroides types	Mobiluncus
0	4+	0	0
1	3+	1+	1+
2	2+	2+	2+
3	1+	3+	
4	0	4+	

Total score = *Lactobacilli* + *G. vaginalis* or *Prevotella/Bacteroides* + *Mobiluncus*. 0, no morphotypes present; 1, <1 morphotype present; 2, 1 - 4 morphotypes presents; 3, 5 - 30 morphotypes present; 4, 30 or more morphotypes present. Morphotypes of bacteria are scored and classified as normal (0 - 3 points), intermediate (4 - 6 points), or bacterial vaginosis (7 - 10 points).

2.1.6 Treatment issues with BV and pregnancy outcomes

Treatment of BV is a complex issue. The benefits of treating BV in nonpregnant women are generally to relieve vaginal symptoms and reduce complications of infection after surgeries (e.g. abortion, hysterectomy) (38). Other benefits of treatment may include risk reduction of other STDs and HIV (77). There are current recommendations from the Centers for Disease Control and Prevention (CDC) for the treatment of symptomatic nonpregnant (44). Separate recommendations exist for symptomatic BV treatment during pregnancy. The U.S. Preventive Services Task Force (USPSTF) currently recommends against BV screening in asymptomatic pregnant women who are at low-risk and average-risk for PTB (78, 79). This is because there is no evidence that screening of asymptomatic women at low and average-risk for PTB reduces adverse pregnancy outcomes. Other groups make similar recommendations regarding screening of low-risk or average-risk, asymptomatic women including the CDC, American College of Obstetricians and Gynecologists (ACOG), and the American Academy of Family Physicians (AAFP). An assessment for high-risk women reveals that there is insufficient evidence to conclude any substantial benefits or harms exist after BV treatment during pregnancy (79). CDC, ACOG, and AAFP surmise that there may be some benefit to screening and treating high-risk women during pregnancy.

Treatment trials report 1-week post-treatment cure rates as high as 80 - 90% (80). However, cure rates for BV tend not to exceed past 70% four weeks following treatment (81). Recurrent BV is quite common; BV will recur within 30 to 90 days among 15 - 30% of women treated for (82). Treatment of sex partners does not appear to have any bearing upon relapse or recurrence of BV, so management of sex partners is not recommended (83). Treatment of BV appears to be more successful during pregnancy. This may be because women do not menstruate

during pregnancy as BV has been known to recur during menstruation (84). Spontaneous cure during pregnancy has been noted but has been observed in non-pregnant women as well (85). BV was spontaneously cured in 42% - 48% of women who were followed for up to 12 weeks (86, 87). Not much is known as to why BV spontaneously recurs and cures. Local vaginal immunity is thought to play an important role in the development of BV and may play a role in both its cure and recurrence. Micronutrient deficiencies can affect local immune function (4, 5). It has previously been hypothesized that nutrition and BV may be related. Few studies have investigated this relationship. Given what information there is regarding the role of nutrition in immunity and the role that immunity plays in maintaining vaginal homeostasis, it makes this association worth exploring.

2.1.7 Biologic plausibility of iron and folate in bacterial vaginosis

Maternal iron and folate status may be important for the development of BV.

Micronutrient deficiencies suppress innate immune function by affecting both cell-mediated and humoral responses (4). This leads to immune dysfunction, resulting in an unbalanced host response. When the host response is compromised, there is an increased susceptibility to infections. Iron plays an integral part in many proteins and enzymes and has a demonstrated role in innate immunity (88, 89). Iron deficiency is the reduction in total body iron to the point that iron stores are fully exhausted and some level of tissue iron deficiency is present. Insufficient dietary iron intake predicts iron deficiency. Folate appears to play a role in proper immune function as well. It is thought to serve as a coenzyme in amino acid metabolism, purine and pyrimidine synthesis, as well as DNA and amino acid methylation (90). Folate deficiency results primarily from the insufficient intake of dietary folate.

Iron and folate deficiencies are two of the most common micronutrient deficiencies that occur during pregnancy. Iron deficiency is one of the most commonly recognized nutritional deficiencies in the world, affecting an estimated 42% of all women (91). Iron-deficiency anemia comprises the majority of anemias that are diagnosed during pregnancy (92). During the first two trimesters of pregnancy, iron-deficiency anemia has been associated with a 2-fold and 3-fold increased risk of PTB and delivery of a low birth weight baby, respectively (93). Iron requirements in the first trimester are very low but the need increases as iron requirements for fetal growth increase, reaching its apex in the third trimester (94). Data suggests that women during pregnancy do not appear to adjust their iron intake to the recommended daily intake requirements. The National Health and Nutrition Examination Survey, 1988 – 1994 (NHANES III) estimates the median iron intake among pregnant women is 15 mg/day. This is less than the Estimated Average Requirement (EAR) of 22 mg/day, which suggests that pregnant women consume inadequate amounts of dietary iron (95). Iron absorption increases with gestational length but the appropriate amount of iron stores is needed prior to the start of pregnancy to support iron requirements (96). More than 500 mg of storage iron is required to prevent iron deficiency during pregnancy. Data from the 1999 – 2000 NHANES indicated that 12% U.S. women of reproductive age (12 - 49y) were iron deficient (97). This means that a considerable number of women carry an iron deficit when they enter pregnancy. This iron deficit, coupled with the growing needs of the fetus, may not provide enough iron to maintain normal immune functions. Folate deficiency is one of the most common vitamin deficiencies in the world and is associated with a number of adverse pregnancy events, including neural tube defects (98), low birth weight, preterm delivery, and placental abruption (99, 100). Second to iron deficiency anemia, folate deficiency anemia is the most common micronutrient

deficiency to occur during pregnancy (92). Folate demands increase during pregnancy due to increased folate needs of fetal and uteroplacental organ growth, decreased folate absorption, and hemodilution (101-104). For pregnant women, the EAR increases from 320 µg/day to 520 µg/day (92). An estimated 36% of pregnant women have dietary folate intakes below the EAR, indicating a significant number of women are at risk for nutritional inadequacy (105). Unlike iron, folate absorption does not appear to change during pregnancy (98). Even though low serum levels are observed in mothers, most newborns have normal folate status levels (106). This suggests that the mother's body ensures that the fetus has adequate levels of folate at the expense of the mother. As a result, there may not be enough folate to maintain proper immune function, which is necessary to stave off invading pathogens.

Iron and folate play key roles in host immunity. These nutrient deficiencies may allow susceptibility to BV through cell-mediated and humoral immunity. Insufficient iron levels compromise neutrophil activity. One study has shown that iron deficient rats have neutrophils with reduced myeloperoxidase activity, leading to a lowered capacity for the intracellular destruction of pathogens (107). Iron deficiency also affects intestinal mucosal immunity by reducing the number of intestinal cells that secrete IgM and IgA (108). Iron may also have an influence on the level of pro- and anti-inflammatory cytokines. The iron chelating agent desferrioxamine suppresses TNF- α production and IL-1 production is suppressed by iron deficiency in rats (109, 110). Iron deficiency with underfeeding of a diet containing 5 mg/kg of ferrous sulfate to mice decreases the production of both IFN γ and IL-10. However, iron deficiency alone did not have a significant effect on IL-10 levels, compared to pair-fed mice (111). This finding suggests that iron deficiency affects the relative balance of pro- and anti-

inflammatory cytokines, an outcome that has been associated with PTB risk (67). Iron levels may affect normal vaginal flora as well.

Regarding the biologic plausibility of iron's role in BV, an iron deficient state would provide fewer resources to maintain cellular and humoral immunity. Iron deficiency may affect IgM and IgA secretion, impairing one of the first response mechanisms to a microbial invasion. Iron deficiency may also impair neutrophil activation as well, allowing for pathogen growth without eliciting an adequate response from the host. An environment with an imbalance of pro- and anti-inflammatory cytokines may also result from iron deficiency. There is a host response that occurs, as concentrations of IL-1 β are increased in the presence of BV (63). However, the relative concentration of anti-inflammatory cytokines may be higher than that of pro-inflammatory cytokines. In an environment where the resources are limited (especially during pregnancy where more iron is being utilized for fetal growth), a compromised host response is mounted. Higher concentrations of anti-inflammatory cytokines will counteract the effects of pro-inflammatory cytokines in an effort to control local inflammation. The consequence of such an action would increase susceptibility to infection and permit large numbers of abnormal flora to flourish.

Folate deficiency modulates immune response by affecting cell-mediated immunity. Rapidly dividing cells, including hematopoietic cells (stem cells that give rise to all blood cell types including macrophages and neutrophils) are most vulnerable to irregularities in DNA production. One of the first clinical manifestations of folate deficiency is neutrophil hypersegmentation (90). They are of particular importance because they are a major player in the host response to vaginal mucosal pathogens (61). A significant positive correlation ($r = 0.426, p < 0.01$) between neutrophil phagocytosis and serum folate levels has been observed in 92

patients with protein-calorie malnutrition (112). Improvement in neutrophil phagocytosis was observed 1 week after intravenous administration of 10 mg folinic acid (113).

2.1.8 Literature review: the association between nutrition and BV

Few studies have examined the relationship between nutrient intake and the development of BV. Verstraelen et al. investigated the role of subclinical iron deficiency as a predictor of BV in early pregnancy in a cross-sectional study of 18 cases of BV and intermediate flora and 80 women with normal flora (7). BV and iron biomarkers were assessed at the first antenatal visit (gestational age < 14 weeks). The investigators collected a number of erythrocyte and serum iron biomarkers, including hemoglobin, serum ferritin, and serum soluble transferrin receptors (sTfR). sTfRs have become the preferred biomarker for measuring tissue iron deficiency levels (114). Other than the increase in erythroid mass precursors, tissue iron deficiency is the only determinant of sTfR concentration, which increases as tissue iron deficiency increases. Additional measures used were \log_{10} transformations of serum ferritin and sTfR concentrations ($\log_{10} [\text{sTfR}]/\log_{10}[\text{ferritin}]$ and $\log_{10} [\text{sTfR}/\text{ferritin}]$). These measures are highly sensitive and specific for iron deficiency with $\log_{10}[\text{sTfR}/\text{ferritin}]$ considered as the most precise composite measure of iron deficiency available today (115). BV was diagnosed using Nugent criteria. Intermediate flora and BV (respective numbers not reported) were pooled and categorized as disturbed vaginal flora for the purposes of the study. The authors did not find an association between most iron biomarkers and disturbed vaginal flora, possibly due to an underpowered study. However, an association between the presence of BV and tissue iron deficiency (sTfRs) was observed. Concentrations of sTfRs greater than 1.45 mg/L were associated with a 4-fold increased odds of having disturbed vaginal microflora (aOR 4.5; 95%CI, 1.4 – 14.2) after

adjustment for smoking, pre-pregnancy BMI, gravidity, parity, maternal age, gestational age, and C - reactive protein level. A significant difference between the mean values of $\log_{10}[\text{sTfR}/\text{ferritin}]$ for healthy vs. disturbed vaginal flora was also observed (1.57 ± 0.30 versus 1.08 ± 0.56 , $p\text{-value} = 0.003$). This difference between mean values of sTfR concentrations of normal and disturbed vaginal flora suggests that deficient iron stores are associated with the presence of BV in early pregnancy. One limitation of the study is its small sample size, which would not have provided enough statistical power for some of the measures used. Another limitation of the study is its use of a homogeneous population; all the women were of white Caucasian origin. Very little mention was made regarding economic status and education, factors that are often associated with BV and iron deficiency (116). Due to the homogeneity of the population, a number of the biomarkers under study did not show a great deal of variance, thus limiting the study's ability to demonstrate any significant differences. The percentage of women in the study were classified as iron deficient, anemic, and iron deficient anemic were 10.2%, 4.1% and 1.0%, respectively. Given this limited variation, results may have been biased towards the null. The authors also neglected to collect and control for sexual behavior, contraceptive use, and douching practices; these are behaviors that are associated with BV and may be associated with iron deficiency. However, given that a significant association was seen with preferred measures of iron deficiency, this study suggests that iron may play a role in BV in early pregnancy and warrants further study.

In one of the most rigorous nutrition – BV studies to date, Neggers et al. conducted a prospective study of 1521 non-pregnant women, measured the dietary intake of 21 different micro-and macronutrients, including iron and folate, using a validated FFQ (6). The objective was to determine if an association exists between these nutrients and the prevalence and

incidence of BV. Women were monitored at baseline and quarterly for a year but study analysis concentrated on visits 2 and 3. FFQs were administered at visit 2 and the derived nutrient intakes were divided into quartiles. Vaginal samples were taken at visits 2 and 3. Vaginal flora was evaluated by gram stain using the Nugent criteria. BV was defined as a Nugent score ≥ 7 and severe BV a Nugent score ≥ 9 . A significant inverse association between severe prevalent BV and the highest quartile of folate (aOR 0.4; 95% CI, 0.2 – 0.8) was found in their cross-sectional analysis. This association was independent of confounding by energy from fat, carbohydrate, or protein, as well as age, race, income, education, douching, and contraceptive pill use. No association was found between BV and folate in the prospective analysis. There was no association found between BV and iron in either their cross-sectional or prospective analyses. This study lends partial support to the hypothesis that certain nutrients play a role in the development of BV and the extent of its severity. This study also suggests that women who report a high intake of folate were less likely to have BV. The limitations of this study include the questionable generalizability of the study since the majority of the study population was African-American (86%) who are primarily of low socioeconomic status and are generally from one geographic area. This may have contributed to a limited variance in iron intake, as 75% of the study population had iron intakes less than the Recommended Daily Allowance (RDA). The lack of variation in iron intake does not allow any differences to be detected. This would allow the results to be biased towards the null. There was no assessment of multivitamin or dietary supplement intake, which can have a huge impact on the intake amount of various nutrients. Misclassification of dietary intake, due to measurement error and inaccurate recall, may also have been possible in this study.

3.0 PRELIMINARY DATA - MATERNAL VITAMIN D STATUS AND BACTERIAL VAGINOSIS DURING PREGNANCY

3.1.1 Introduction

One of the micronutrients assessed through FFQ by Neggers et al was vitamin D. The investigators neglected to find an association between vitamin D and either prevalent or incident BV. Even though vitamin D can be obtained through a number of dietary sources (fatty fish, fortified food products and vitamin supplements), most vitamin D is synthesized in the skin in response to UVB light exposure (117). As such, dietary intake of vitamin D as measured by a FFQ will underestimate the amount of vitamin D exposure. Serum 25 hydroxyvitamin D (25(OH)D) is a good biomarker to represent vitamin D sufficient status, as it reflects both vitamin D dietary intake and vitamin D synthesized as a result of sunlight exposure (118).

Vitamin D has been associated with a number of different pregnancy and birth outcomes including preeclampsia, birth weight, and small-for-gestational age (119-121). Vitamin D deficiency is more common among individuals with heavily pigmented skin (117). One study reports that early in pregnancy, 29% of black mothers, and 5% of white mothers are vitamin D deficient (122). With both vitamin D insufficiency and BV common amongst pregnant women, there may be a possible association between maternal vitamin D status and BV in early pregnancy.

3.1.2 Methods

3.1.2.1 Study population

Data for the analysis comes from the Study of Nutrition and Pregnancy (SNAP, R01 HD052732, PI: H Simhan), an ongoing prospective cohort study in Pittsburgh, PA. The objective of the study was to investigate the extent of nutritional status and genetics as risk factors of prematurity. SNAP provides an opportunity to study BV in a cohort of high-risk African-American and Caucasian pregnant women who seek prenatal care at Magee-Womens Hospital (MWH) prenatal clinics. The population who seeks care at MWH is 55% African-American and 44% Caucasian. Over 80% of the women seen at the clinics receive Medicaid. Inclusion criteria for the study were: 1) self-reported race of either black or white, 2) a single gestation, and 3) gestational age (GA) <16 weeks at enrollment (123). Exclusion criteria included reports of vaginal bleeding, autoimmune disease (inflammatory bowel disease, systemic lupus erythematosus, rheumatoid arthritis, scleroderma), immunocompromisation (HIV+, use of post-transplant immunosuppressive medications, or use of systemic steroids within the 6 months), pre-gestational diabetes, and use of illegal drugs. The Institutional Review Board of the University of Pittsburgh approved this study.

3.1.2.2 Study design and procedures

Women were enrolled at GA <16 weeks. GA was determined by either the first day of the last menstrual period or ultrasound examination. Information on sociodemographics, medical history, drug/alcohol use, and health behaviors were collected at the first visit with the use of a standardized questionnaire. Self-reported race was used to classify participants as either black or white. At this first visit, vaginal swabs were used to sample the vaginal flora for BV

classification and to assess concurrent sexually transmitted infections (*Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*).

3.1.2.3 Outcome assessment: Bacterial vaginosis

Two vaginal swabs were used for the culture and identification of vaginal flora at the first visit, GA < 16 weeks. Swabs were rolled across the vaginal wall and then a vaginal smear was produced by rolling the swab onto a glass slide. Vaginal flora was evaluated by Gram stain using Nugent criteria. Nugent scores 0 to 3 were classified as healthy normal vaginal flora, scores 4 to 6 as intermediate flora, while scores ranging from 7 to 10 were diagnosed as BV (71). Intermediates were left out of the analysis.

3.1.2.4 Exposure assessment: vitamin D status

Maternal sera collected at enrollment were analyzed for 25-hydroxyvitamin D [25(OH)D] plus 25-hydroxyvitamin D₃ [25(OH)D₃]. Quantification of 25(OH)D was performed using a DiaSorin RIA kit. The RIA can detect 100% of 25(OH)D and 100% of 25(OH)D₃. The RIA could detect 25(OH)D in the range of 3.75 to 250 nmol/L. 25(OH)D concentrations were categorized as follows: deficiency (25(OH)D < 37.5 nmol/L), insufficiency (25(OH)D 37.5 to < 80 nmol/L) , and sufficiency, (25(OH)D ≥ 80nmol/L).

3.1.2.5 Statistical analysis

Descriptive, univariate, and multivariable statistics were performed with Stata software (version 10; Stata Corporation, College Station, TX). Pearson's X² statistics, Fisher's exact, and Student t-tests were used to assess significant differences between cases and controls. Multivariable logistic regression was used to determine the independent association of serum 25

hydroxyvitamin D and BV. Covariates maternal age, race, education, pre-pregnancy body mass index (BMI), marital status, smoking before and during pregnancy, parity, presence of sexually transmitted diseases and family income were assessed for confounding. Covariate importance in these models were determined by their removal; if its removal induced a significant change in the BV β -coefficient ($\geq 10\%$ variation), then it was considered an important confounder. The relationship between serum 25(OH)D and BV was assessed using non-parametric regression. Effect modification of race on vitamin D concentration was determined by likelihood ratio test. Effect modification was considered significant if the p-value < 0.10 .

3.1.3 Results

Cases of bacterial vaginosis were more likely to be unmarried, have less than a high school education, and more likely to report an income of less than \$10,000. Mothers with bacterial vaginosis were also more likely to be smokers before pregnancy, and were more likely to have given birth to more children (Table 1). There were no differences in age and body mass index (BMI) between cases and controls. Black women were more likely than white women to be diagnosed with BV early in pregnancy (70.9% vs. 58.2%, respectively, p-value < 0.01 , data not shown). Most mothers were either deficient or insufficient for vitamin D (< 80 nmol/l). However, black women were more likely than white women to be vitamin D deficient (71.6% vs. 28.1%, Figure 1a). A small number of white women were sufficient for vitamin D (13%, 25(OH)D ≥ 80 nmol/l) while none of the black women in the study population were vitamin D sufficient. The majority of BV cases were vitamin D deficient (25(OH)D < 37.5 nmol/L) early in pregnancy (62.6% vs. 46.0%, Figure 1b). A small percentage of both cases and controls were classified vitamin D sufficient (25(OH)D ≥ 80 nmol/L).

The dose-response relationship between serum 25(OH)D and the unadjusted probability of BV was examined using nonparametric regression. There was an inverse, linear relationship between concentrations of serum 25(OH)D and risk of BV (Figure 2a). When this relationship is stratified by race, serum 25(OH)D did not appear to be associated with BV in white women (Figure 2b). However, in black women, an inverse relationship between serum 25(OH)D and the probability of BV was observed (Figure 2c). The interaction between race and vitamin D was assessed using a likelihood ratio test ($\alpha = 0.09$).

Separate unadjusted odds ratios (OR) for BV were computed for black and white mothers whose serum 25(OH) levels were less than 50nmol/l. Black mothers were at a 2.8 greater odds of having BV when their 25(OH)D serum level was less than 50 nmol/l (OR 2.8; 95%CI, The Breslow-Day test of homogeneity was used to compare the homogeneity of the odds ratios between black and white women. The test for homogeneity was significant (p-value = 0.09), indicating a significant race by serum 25(OH)D concentration effect on the prevalence of BV. The results are presented in Table 2. Multivariable logistic regression models were used to determine the association between vitamin D and BV prevalence. The final model was adjusted for marital status, smoking before and during pregnancy, age, education, BMI, parity, and family income. White women with sufficient levels of vitamin D (80nmol/l) were used as the reference group. We found no association between vitamin D status and BV among white women. Black women with a serum 25(OH)D concentrations of less than 50 nmol/l had a 2-fold (OR 2.2; 95%CI, 1.0 - 4.8) increased odds of BV. Black women with deficient levels of vitamin D had more than a 4-fold increase odds of being diagnosed with BV, compared to white women with sufficient levels of vitamin D (OR 4.2; 95%CI, 2.1 - 8.1), data not shown).

Table 2. Maternal characteristics collected at first study visit stratified by BV status

	BV+ (n = 195)	BV- (n = 209)	p-value
Age (years), mean	24.7	25.1	0.4
Marital Status, %			
Unmarried	92	80.1	<0.01
Married	8	19.9	
Education, %			
High School Education or Less	92.5	84	0.01
More than HS	7.5	16	
Smoking status before becoming pregnant, %			
Non-smoker	33.5	51.5	<0.01
Smoker	66.5	48.5	
Smoking status since becoming pregnant, %			
Non-smoker	42.7	57.1	<0.01
Smoker	57.3	42.9	
Prepregnancy BMI (kg/m ²), %			
<18.5	1.1	3	0.39
18.5-24.9	36	33	
25.0-29.9	24.9	21.2	
≥30.0	38.1	42.9	
Income, %			
< \$10,000	48.4	37.6	0.02
\$10,000 - \$25,000	35	34	
>\$25,000	16.7	28.4	
Parity, %			
0	12.2	24	<0.01
1	39.9	44	
≥2	47.9	32	

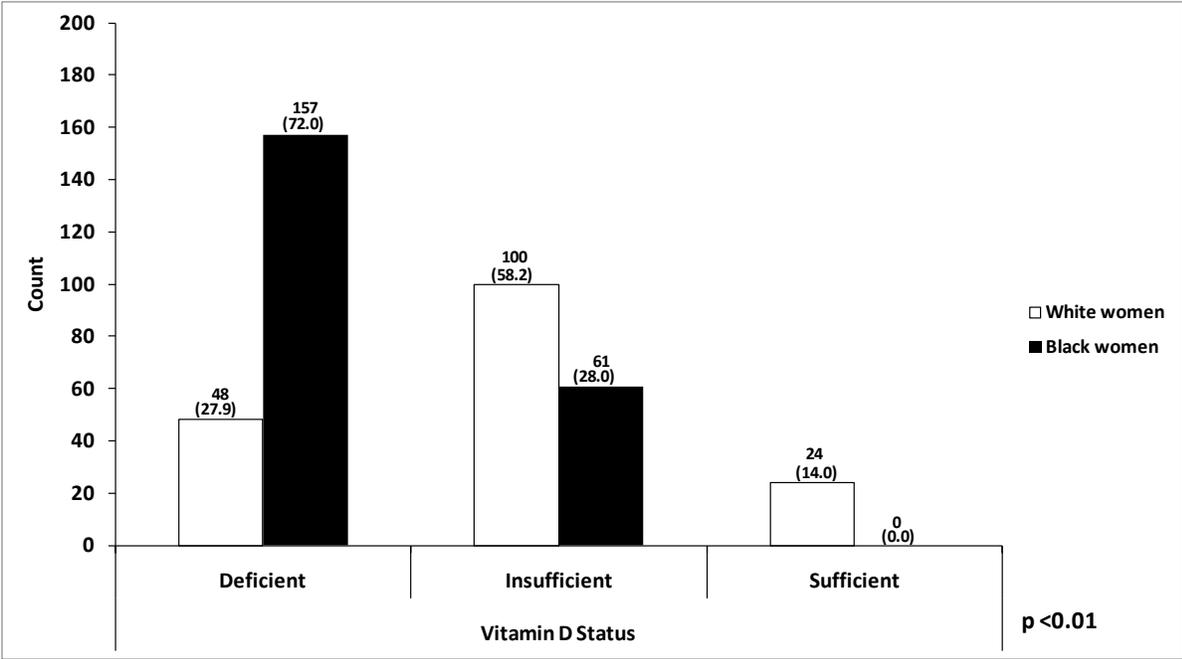


Figure 1. Race stratified by vitamin D status. Vitamin D deficiency ($[25(OH)D] < 37.5$ nmol/L), vitamin D insufficiency, ($37.5 < [25(OH)D] < 80$ nmol/L), and vitamin D sufficiency, ($[25(OH)D] \geq 80$ nmol/L) among 404 mothers at <16 weeks gestation. Number of women provided, percentages are given in parentheses. Post-hoc power analysis indicates that power achieved was 99.8%.

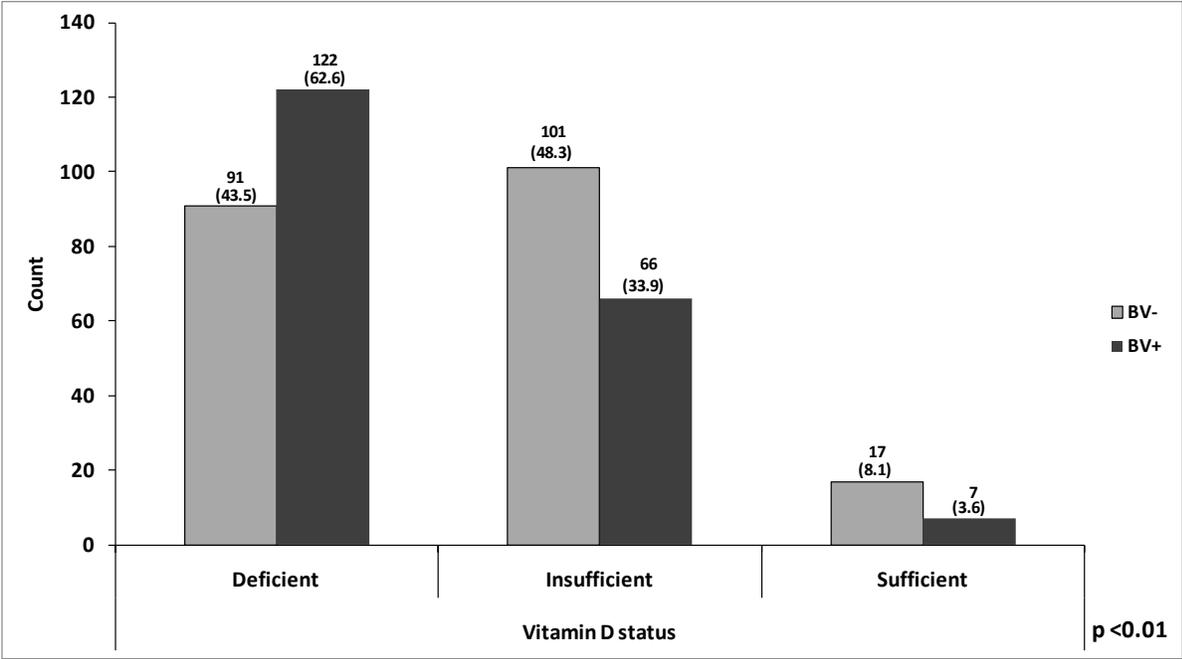


Figure 2. Bacterial vaginosis status stratified by vitamin D status. Vitamin D deficiency ($[25(\text{OH})\text{D}] < 37.5$ nmol/L), vitamin D insufficiency, ($37.5 < [25(\text{OH})\text{D}] < 80$ nmol/L), and vitamin D sufficiency, ($[25(\text{OH})\text{D}] \geq 80$ nmol/L) among 404 mothers at <16 weeks gestation. Number of women provided, percentages are given in parentheses. Post-hoc power analysis indicates that power achieved was 99.8%.

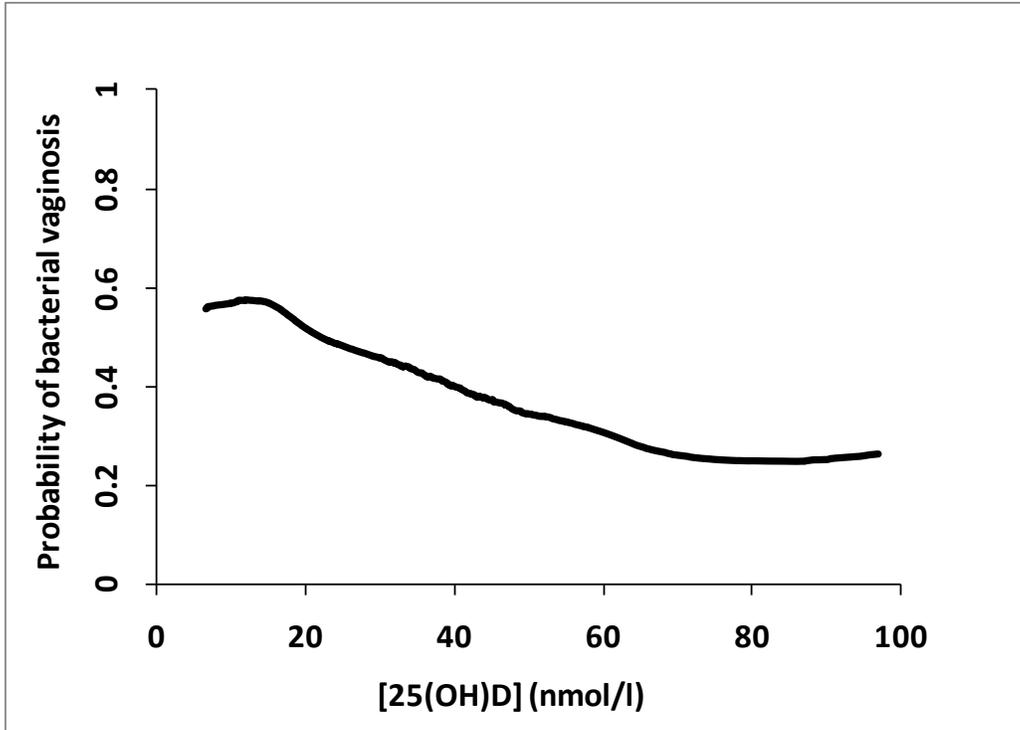


Figure 3. Lowess regression curve of unadjusted probability of bacterial vaginosis and concentrations of 25(OH)D in all mothers at <16 weeks gestation.

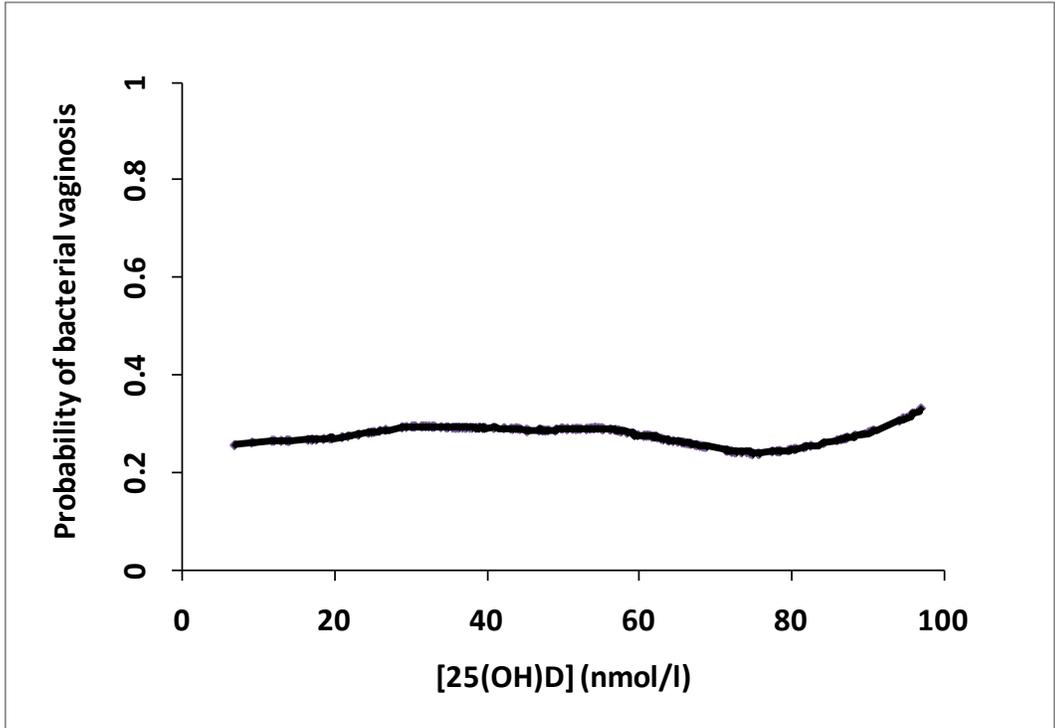


Figure 4. Lowess regression curve of unadjusted probability of bacterial vaginosis and concentrations of 25(OH)D in white mothers at <16 weeks gestation.

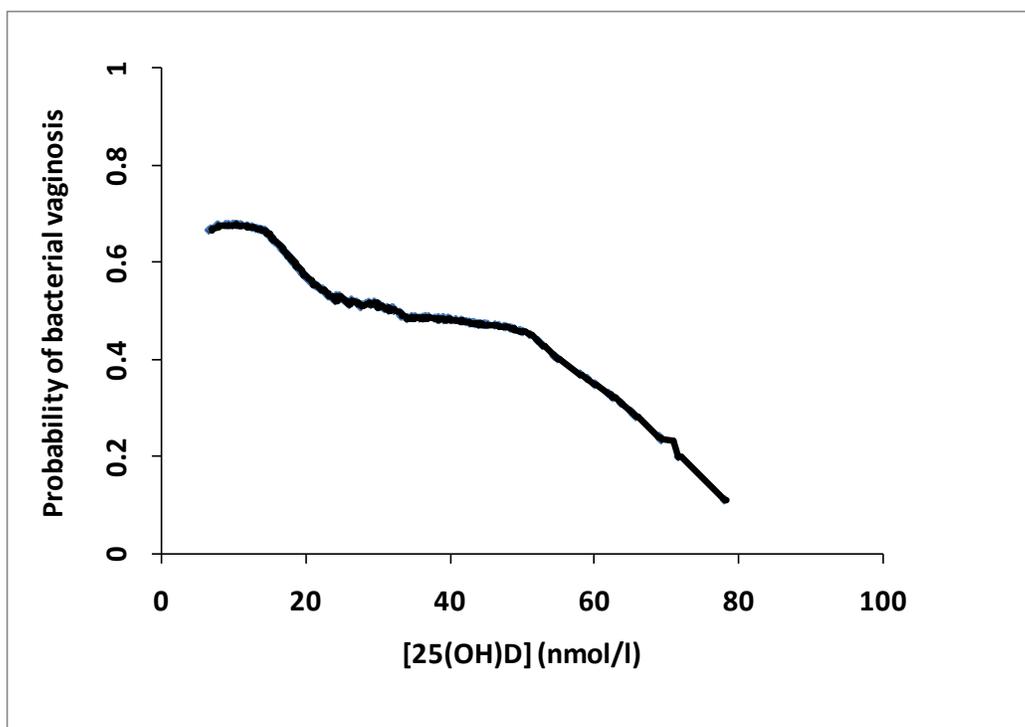


Figure 5. Lowess regression curve of unadjusted probability of bacterial vaginosis and concentrations of 25(OH)D in black mothers at <16 weeks gestation.

Table 3. Effect on vitamin D ([25(OH)D] <50 nmol/l) on the odds of BV, stratified by race

Race	OR (95% CI)	Breslow-Day* p-value
White mothers	1.2 (0.6, 2.3)	0.09
Black mothers	2.8 (1.1, 7.6)	

*Test of homogeneity; p-values <0.1 indicates significance.

3.1.4 Discussion

In a population of low-income black and white pregnant women, bacterial vaginosis was associated with vitamin D (as characterized by serum 25(OH)D), independent of a number of risk factors, early in pregnancy. BV was more prevalent among black women compared to white women, as was vitamin D deficiency. Vitamin D deficiency was significantly more common

among women with BV than women without BV. After adjustment for a number of confounders, there was a strong inverse dose-response relationship between 25(OH)D and BV among black women. Among white women however, there was no association between maternal 25(OH)D and BV after confounder adjustment.

Vitamin D₃ is the precursor to 25 hydroxyvitamin D₃, which is ultimately converted to 1, 25 dihydroxyvitamin D₃ (1, 25(OH)₂D₃) (117). 1, 25(OH)₂D₃ is the active metabolite in the body and its effects are mediated through vitamin D₃ receptors (VDR). VDRs are constitutively expressed by antigen presenting cells, and are inducibly expressed by lymphocytes following activation by 1, 25(OH)₂D₃, suggesting vitamin D's role as an immunomodulator. 1, 25(OH)₂D₃ itself has been shown to increase the expression of cathelicidin antimicrobial peptide (CAMP) in neutrophils and macrophages (124). CAMP is a peptide that is capable of destroying microorganisms such as *Mycobacterium tuberculosis*. When 25(OH)D serum levels fall below 50 nmol per liter, macrophages are prevented from eliciting a response (125). This may explain why African-Americans are more prone to *M. tuberculosis* compared to Caucasians. This may also explain why African-Americans are also at a higher risk for BV.

This study has several limitations. The analysis is hampered by its cross-sectional design. As such, the nature of the temporal relationship between vitamin D and BV is unknown. In this study population, the low variation in vitamin D exposure may have also affected analyses. Both black and white women had limited 25(OH)D serum levels (geometric means 26.29 nmol/l, 95%CI (24.77, 27.90) and 48.32 nmol/l 95%CI, (44.90, 52.00), respectively). This limited variability may be the reason why many of the multivariable results were non-significant. Although a strong relationship between vitamin D and BV has been observed in this study, the relationship between vitamin D, much less nutrition and BV in general is largely unknown.

There may be other important confounders that have not been accounted for in this study and may undermine the accuracy of the derived estimates. However, there are several strengths to this study. The large sample size of the cohort provided enough power to identify a significant association between vitamin D and BV. Nugent criteria, the gold standard, were used to diagnose BV. A large prevalence of the outcome provided enough power to conduct the analysis. This work may provide support to BV treatment trials since many women have BV recur after antibiotic treatment (82). To conclude, this study suggests that vitamin D and BV found at <16 weeks gestation were associated and that the relationship was modified by race. Vitamin D sufficiency disparities between black and white women may partially explain why black race is a strong risk factor of BV status. Future research is needed to determine whether maternal vitamin D status is associated with incident BV and understand why the racial disparity in the association between vitamin D status and BV exists.

4.0 RESEARCH PRIORITIES

4.1 FUTURE STUDY: NUTRITIONAL RISK FACTORS OF BACTERIAL VAGINOSIS

A future study of maternal nutrition and its role in BV early in pregnancy should be carried out with a focus on iron and folate exposure. The study design and population ideally would be similar to that of SNAP. The study would include three visits as opposed to the two in SNAP (one scheduled for each trimester with the last trimester visit, taking place before 32 weeks to avoid losing visits to possible PTBs). A similar study can be performed in non-pregnant women with arranged visits over the course of a year. Each visit would collect vaginal samples for BV diagnosis, as well as dietary intake and biomarker data. The study population would consist of a high-risk cohort of black and white pregnant women. Blood should be collected for the measurement of various nutritional biomarkers, including iron and folate. Study participants should be administered a validated FFQ for the assessment of dietary intake. Serum ferritin and serum soluble transferrin receptor (sTfR) would serve as iron nutritional biomarkers. Serum ferritin is indicative of depleted iron stores, and sTfR is an early indicator of iron deficiency. A ratio of sTfR to serum ferritin can be used to estimate total body iron (115). Folate biomarkers to represent folate status include serum folate and red blood cell folate. Serum folate has been used as an indicator of recent folate intake while RBC folate is an indicator of average folate

intake over the past 3 months (126, 127). FFQs capture usual, past dietary intake of numerous foods and nutrients with good accuracy and have been validated in a number of different populations including pregnant women (128-130). Information about dietary supplement use should also be considered. Dietary intake values would be validated by correlation with nutrient biomarkers. Cross-sectional and prospective analyses should be performed to determine association of nutrients and BV in both early and later terms of pregnancy. The relationship between dietary measures and BV should be evaluated using multivariable log-binomial regression to determine prevalence rate and incidence rate ratios. Ordinal logistic regression may also be used to determine the odds of the having intermediate vaginal flora, an outcome that studies often neglect to consider. Intermediate flora (Nugent scores 4-6) is often thought of as a transitional stage between normal flora and BV. Women in this group however, are also at an increased risk of preterm birth (131). Vaginal flora should be assessed early in gestation (<16 weeks) with Nugent criteria to be used for BV diagnosis. Vaginal flora should also be assessed for non-cultivable organisms using molecular techniques such as 16s rRNA. The current definition of BV by molecular techniques has not been established. However, organisms that are specific for BV (those are found in women who are BV-positive by Nugent criteria vs. women who have normal vaginal flora), most likely will include positive identification of organisms *A. vaginae*, BVAB1, BVAB2, and/or BVAB3.

4.2 OTHER NUTRITIONAL RISK FACTORS OF BACTERIAL VAGINOSIS

In addition to iron and folate, future studies should focus on other nutrients and their possible role in BV. Micronutrients under consideration should have an impact on innate immunity,

which is important in the control of BV (22). Vitamin A for example affects mucosal immunity by impairing mucosal epithelial cell regeneration (132). Vitamin A can also play a role in BV by affecting neutrophil development. Zinc is another micronutrient that may a role in BV. Zinc is a co-factor for enzymes that are involved in DNA replication and transcription, including DNA polymerase (133). Zinc deficiency has been associated with a reduced number of neutrophils (133). Similar studies to the one described above should be considered for studying new nutrients. There may be other nutrients that are important in the etiology of BV. Vitamin A nutritional status can be measured using serum retinol while zinc nutritional status can best be measured using FFQs.

4.3 RACIAL DISPARITY IN BACTERIAL VAGINOSIS

Black women still have a higher propensity for BV, even after adjustment for socioeconomic disparities. This difference currently goes unexplained. Nutrition is one such factor that may help to explain the gap. Candidate nutrients for study should also be considered as possible sources of the racial dichotomy found in BV. Both iron and folate are good nutrients to consider for this reason. The prevalence of iron deficiency among non-Hispanic reproductive-age black women was approximately twice that of non-Hispanic white women of the same age range (97). Non-Hispanic black women have significantly lower serum folate levels than non-Hispanic white women (134). Micronutrient disparities should also be considered for the varying cytokines levels found between black and white women with and without BV. A recent study found that black women with normal vaginal flora had lower concentrations of IL-1 α , IL-6, and IL-10 compared to white women with normal vaginal flora (123). Although there were no

significant differences noted in cytokines levels between black and white women with BV, the authors hypothesized that higher levels of cytokines found in white women make them less susceptible to BV. Multivariable linear regression should be used to identify factors independently associated with cytokine levels. Effect modification of race on micronutrient concentration would be determined by likelihood ratio test. Future research should consider disparities in nutrient intake as a possible explanation for cytokine level differences.

4.4 RESOLVING THE ISSUE OF PERSISTENT BACTERIAL VAGINOSIS

Another gap to be addressed is risk factors for persistent BV or recurrent BV. Few studies have considered risk factors for recurrent BV. Research thus far has identified previous history of BV, lack of hormonal contraceptive use, and a regular sex partner as significant risk factors (37). Although BV recurrence may be due to re-infection or antibiotic resistance, the possibility of recurrence due to either the failure of H₂O₂-producing Lactobacilli to reestablish itself or the failure of the vaginal immune response to help restore flora balance after some unknown insult should be considered. Using the parameters of the aforementioned study, the definition of a case would be a woman who tests positive for BV at 2 consecutive visits. A control would be a woman who tests either negative or one who tests positive for BV intermittently (i.e., on non-consecutive visits). The association between nutritional indicators and BV persistence can be determined through multivariable logistic regression. A study such as this should take care to adjust for confounding by sexual activity and douching. The study population should be restricted to women who have not had any antibiotic therapy for at least one month prior.

4.5 INTERACTION OF GENETIC POLYMORPHISMS AND NUTRITION

At times nutrient intake in an individual will be adequate; however, nutrient metabolism may be impaired due to the existence of nutrient-metabolizing gene polymorphisms. Methylenetetrahydrofolate reductase (MTHFR) for example, is an enzyme that catalyzes the reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (135). A common polymorphism, MTHFR 677 CT, changes cytosine to thymidine at nucleotide position 677 is associated with reduced enzyme activity and DNA hypomethylation. Polymorphisms such as this and in other nutrient-metabolizing genes may also be important in immune function homeostasis and in turn, increase one's susceptibility to BV. A gene-environment interaction of MTHFR 677 CT and low folate intake has been associated with neural tube defects (136). This and other gene-environment interactions should be considered in the etiology of BV. This analysis can be carried out within the context of the aforementioned study, with dietary intake assessed with a validated-FFQ. Nutrient-metabolizing gene polymorphisms would be assessed by genotyping DNA extracted from blood samples. Polymorphism-nutrient interactions would be examined on the multiplicative scale using multivariable log-binomial regression.

5.0 PUBLIC HEALTH APPLICATIONS AND SIGNIFICANCE

While nutritional risk factors for BV can be identified through observational studies, prevention strategies employing nutritional recommendations can be tested through randomized controlled trials (RCT). RCT is a strong experimental study design for measuring a desired “cause and effect” (137). By randomizing subjects to either a treatment or a control group, the design provides appropriate means of controlling confounding factors – both known and unknown. The use of techniques such as double-blinding are used to minimize bias in recording the outcome of such trials, making these designs ideal to use. Apart from the usual disadvantages of implementing RCT (expensive to operate, ethical considerations, limited generalizability), RCTs that evaluate nutritional interventions (whether they be treatment diets or dietary supplements) may not always be easy to institute. The optimal nutrient dosage is often hard to establish. A modest dose of a vitamin or supplement may not alter blood concentrations substantially while too high of a dosage may lead to toxicity. Adherence also becomes an issue in trials involving nutritional interventions. Dietary supplements for example, are often easy to obtain outside of the clinical trial (unlike experimental drugs), so adherence to dosage, especially in control groups may be difficult to manage. Even if an appropriate dosage can be established, it may take some time for the dose to result in a physiological effect. As such, clinical trials may need to long in duration. Issues such as these make RCT not only difficult to perform but make results difficult to interpret.

BV is a vaginal condition of interest, as it is associated with a number of adverse pregnancy outcomes including prematurity. Subsequent to birth, premature infants face a number of various health challenges such as, some of which may become chronic conditions (138). The annual economic burden of preterm births in the US is \$26.2 billion (139). Outside of pregnancy, BV has been associated with a number of gynecological conditions including pelvic inflammatory disease (PID) (140, 141). It affects 1 million women in the US annually and presents an economic burden of \$4.2 billion (142). BV also appears to increase one's risk of acquiring HIV (77, 143). An estimated 39.5 million (34.1–47.1 million) people are currently living with HIV worldwide. A hallmark of the current epidemic is the increasing burden of HIV infection in women, which has implications for mother-to-child transmission of the virus (144). Given BV's association with a number of gynecologic and obstetric outcomes culminating in massive economic and medical burdens, research into new BV prevention methods is highly warranted.

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