Vaginal sialoglycan foraging by *Gardnerella*: Mucus barriers as a meal for unwelcome guests?

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Key words: Bacterial vaginosis/*Gardnerella*/sialidase/sialic acid/microbiome

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Running Title: Glycobiology of vaginal dysbiosis

Abstract

Bacterial vaginosis (BV) is a condition of the vaginal microbiome in which there are few lactobacilli and abundant anaerobic bacteria. Members of the genus *Gardnerella* are often one of the most abundant bacteria in BV. BV is associated with a wide variety of poor health outcomes for women. It has been recognized since the 1980s that women with BV have detectable and sometimes markedly elevated levels of sialidase activity in vaginal fluids and that bacteria associated with this condition produce this activity in culture. Mounting evidence collected using diverse methodologies points to the conclusion that BV is associated with a reduction in intact sialoglycans in cervico-vaginal secretions. Here we review evidence for the contributions of vaginal bacteria, especially *Gardnerella*, in the processes of mucosal sialoglycan degradation, uptake, metabolism, and depletion. Our understanding of the impacts of vaginal sialoglycan degradation is still limited. However, the potential implications of sialic acid depletion are discussed in light of our current understanding of the roles played by sialoglycans in vaginal physiology.

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Introduction to bacterial vaginosis

Bacterial vaginosis (BV) is a condition characterized by low levels of lactic acid-producing bacteria in the vagina. Instead there are higher levels of diverse taxa that are often strict or facultative anaerobic bacteria. BV is associated with increased risks of sexually transmitted infections (Brotman, R.M., Klebanoff, M.A., et al. 2010, Wiesenfeld, H.C., Hillier, S.L., et al. 2003), endometritis (Watts, D.H., Krohn, M.A., et al. 1990, Wiesenfeld, H.C., Hillier, S.L., et al. 2002) and pelvic inflammatory disease (Sweet, R.L. 1995). In pregnancy, BV has been associated with complications such as preterm birth (Hillier, S.L., Martius, J., et al. 1988, Hillier, S.L., Nugent, R.P., et al. 1995, Holst, E., Goffeng, A.R., et al. 1994, Leitich, H. and Kiss, H. 2007, McGregor, J.A., French, J.I., et al. 1994), late pregnancy loss (Leitich, H., Bodner-Adler, B., et al. 2003, Leitich, H. and Kiss, H. 2007), preterm premature rupture of membranes (McGregor, J.A., French, J.I., et al. 1994), delivery of a low birth weight infant (Hillier, S.L., Nugent, R.P., et al. 1995, Holst, E., Goffeng, A.R., et al. 1994, Svare, J.A., Schmidt, H., et al. 2006), and infections of the placenta and amniotic fluid (Hitti, J., Hillier, S.L., et al. 2001, Leitich, H. and Kiss, H. 2007, Rezeberga, D., Lazdane, G., et al. 2008, Silver, H.M., Sperling, R.S., et al. 1989, Svare, J.A., Schmidt, H., et al. 2006). Importantly, many BV-associated bacterial species have been detected in invasive infections of the placenta and amniotic fluid (Berardi-Grassias, L., Roy, O., et al. 1988, DiGiulio, D.B. 2012, DiGiulio, D.B., Romero, R., et al. 2010, Hillier, S.L., Martius, J., et al. 1988, Holst, E., Goffeng, A.R., et al. 1994, Silver, H.M., Sperling, R.S., et al. 1989, Watts, D.H., Krohn, M.A., et al. 1990).

In the clinic, a woman is diagnosed with BV if she has three of the four Amsel's criteria: thin consistency of vaginal fluids, fishy odor upon potassium hydroxide treatment, elevated pH

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(>4.5), and >20% of the exfoliated epithelial cells being studded with bacteria ("clue cells") in wet mounts (Amsel, R., Totten, P.A., et al. 1983, Gardner, H.L. and Dukes, C.D. 1955). In the laboratory, BV is determined by the Nugent system of scoring Gram-stained vaginal smears. Briefly, the Nugent scoring scale is from zero to ten; lower scores (0-3) indicate normal vaginal microbiome (No BV) with abundant Gram-positive (purple) elongated rods. Higher scores (7-10) indicate BV with few lactobacilli, abundant Gram-negative/variable bacteria, and often the presence of curved rods (*Mobiluncus* and other bacteria) (Hillier, S.L., Krohn, M.A., et al. 1993, Holst, E., Goffeng, A.R., et al. 1994, Nugent, R.P., Krohn, M.A., et al. 1991) (Figure 1). DNA sequencing technologies and other molecular tools have provided finer resolution of the diversity and longitudinal variability of vaginal bacterial communities (Gajer, P., Brotman, R.M., et al. 2012, Ravel, J., Gajer, P., et al. 2011, Srinivasan, S., Liu, C., et al. 2010). However, the mechanisms linking BV to adverse reproductive outcomes are largely unknown.

A cadre of taxa have been associated with BV including one particularly abundant microbe, *Gardnerella vaginalis*. *G. vaginalis* was first identified as the causative agent of BV (Gardner, H.L. and Dukes, C.D. 1955); however, its role as the primary etiological agent of vaginosis has been argued and remains elusive (Hickey, R.J. and Forney, L.J. 2014, Schwebke, J.R., Muzny, C.A., et al. 2014, Swidsinski, A., Loening-Baucke, V., et al. 2014). Several studies that have evaluated the fundamental yet ambiguous roles of *G. vaginalis* in BV were reviewed recently (Morrill, S., Gilbert, N.M., et al. 2020, Schellenberg, J.J., Patterson, M.H., et al. 2017) and will not be discussed extensively here. We here focus on sialidase enzymes present in BV and their possible roles in the pathophysiology of the condition. Emphasis is placed on sialidases encoded

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by *G. vaginalis* as these are the best studied, but we also touch on sialidases, sialic acid transport and catabolic machinery that have been studied in other vaginal bacteria (see **Table I**).

Sialidases in vaginal fluids during bacterial vaginosis

Women with BV were first reported to have elevated levels of sialidase activity in vaginal fluids compared to women without the condition in a 1992 publication (Briselden, A.M., Moncla, B.J., et al. 1992). This is a reproducible finding in human specimens (Cauci, S., Thorsen, P., et al. 2003, Howe, L., Wiggins, R., et al. 1999, Lewis, W.G., Robinson, L.S., et al. 2012, Smayevsky, J., Canigia, L.F., et al. 2001) and it was later shown can be recapitulated in mice upon experimental vaginal colonization by *G. vaginalis* (Gilbert, N.M., Lewis, W.G., et al. 2013). Isolation and identification of bacterial strains from BV vaginal specimens has demonstrated that certain species displayed sialidase activity *in vitro*, including isolates of *Prevotella*, *Bacteroides*, and *Gardnerella*, but *not* the tested isolates of *Mobiluncus (curtisii* or *mulieris)*, or *Peptostreptococcus (asaccharolyticus, anaerobius, magnus,* or *prevotii*) (Briselden, A.M., Moncla, B.J., et al. 1992). Indeed, an earlier study reported purification and biochemical characterization of a sialidase from *G. vaginalis* (von Nicolai, H., Hammann, R., et al. 1984). Further studies have linked sialidase enzyme activity in vaginal fluids with increased likelihood of adverse outcomes including premature rupture of membranes and placental infection (Zhang, X., Xu, X., et al. 2002), miscarriage and late pregnancy losses (Cauci, S. and Culhane, J.F. 2011), preterm birth (Zhang, X., Xu, X., et al. 2002), as well as BV recurrence (McGregor, J.A., French, J.I., et al. 1994). Many other studies have demonstrated that sialidase activity is not only associated with BV, but can be used as a diagnostic biochemical indicator of the condition

(Bradshaw, C.S., Morton, A.N., et al. 2005, Myziuk, L., Romanowski, B., et al. 2003, Smayevsky, J., Canigia, L.F., et al. 2001, Wu, S., Lin, X., et al.*et al.* 2019).

Multifaceted sources of sialidase activity in vaginal specimens

Sources that yield the sialidase activity detected in the vaginal fluids of women with BV have not been fully characterized. Early studies of sialidase activity produced by vaginal bacterial isolates, from women with BV, concluded that sialidase is microbial in origin. A study by Briselden *et al* showed that women with sialidase-positive vaginal fluids harbor multiple sialidase-producing bacteria including *Prevotella bivia, P. oralis, P. loeschii, Bacteroides fragilis and G. vaginalis* (Briselden, A.M., Moncla, B.J., et al. 1992). Early studies showed that all strains of *P. bivia*, but only a subset of *G. vaginalis* isolates produce sialidase activity; later studies have largely confirmed these findings (Moncla, B.J., Braham, P., et al. 1990, Moncla, B.J., Chappell, C.A., et al. 2016, Santiago, G.L., Deschaght, P., et al. 2011). However, the production of sialidase activity by *Gardnerella* and *Prevotella* in culture does not rule out the participation of sialidases from other potential origins *in vivo*. Early studies seem to conclude that because all *Prevotella* isolates produced sialidase *in vitro*, while only a fraction of *Gardnerella* isolates did, the former must be the main source of sialidase activity in women (Moncla, B.J., Braham, P., et al. 1990). Recent studies in mouse models, which reflect some but not all features of BV in women, show that *Gardnerella* and *Prevotella* colonization both result in an increased sialidase activity compared to a mock-infected control group. However, *Prevotella* seemed to require 100-fold higher bacterial levels to result in similar levels of sialidase activity as seen in *Gardnerella* colonized C57BL/6 mice (Gilbert, N.M., Lewis, W.G., et al. 2019). These data suggest that there may be other factors such as different expression levels of sialidase among bacterial

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strains/species that in some cases may be more important than the levels of bacteria themselves. Future studies would benefit from the development of genetic tools to make mutants in *Gardnerella*, as would the entire field of *Gardnerella* biology. Studies have shown that multiple strains of *Gardnerella* can concurrently occupy the vagina (Balashov, S.V., Mordechai, E., et al. 2014, Hilbert, D.W., Schuyler, J.A., et al. 2017, Hill, J.E., Albert, A.Y.K., et al. 2019, Shipitsyna, E., Krysanova, A., et al. 2019) and may therefore also contribute to the heterogeneity of sialidase sources in individual samples (Schellenberg, J.J., Paramel Jayaprakash, T., et al. 2016). Finally, it has not (to our knowledge) been studied whether host sialidases (at least 4 are known) (Miyagi, T. and Yamaguchi, K. 2012) might also contribute to the enzyme activity seen in BV.

Endogenous microbiota-derived sialidase activity has also been reported in laboratory mice (C57BL/6) from specific vendors (e.g. Charles River/NCI, Envigo, but not Jackson) (Agarwal, K., Robinson, L.S., et al. 2020, Gilbert, N.M., Lewis, W.G., et al. 2013). In these studies, sialidase-positive colonies of *Bacteroides spp.* or *Enterococcus gallinarum* were isolated from vaginal washes of Envigo mice (202-A Indianapolis facility, IN, USA) (Agarwal, K., Robinson, L.S., et al. 2020), and bacteria of *Eubacteria consortium* or *Enterococcus spp.* were isolated from Charles River/NCI mice with vaginal sialidase activity (Gilbert, N.M., Lewis, W.G., et al. 2013). In the presence of potential sialidase-producing microbiotas, another consideration is that the addition of BV bacteria to existing microbial ecosystems could itself trigger changes in the naturally occurring sialidase producers, which could also influence sialidase levels. An example of this was shown in a recent study in which (sialidase-negative) *Fusobacterium nucleatum* addition to *ex vivo* cultures of mouse, as well as human, vaginal microbiotas led to marked increases in sialidase activity

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(Agarwal, K., Robinson, L.S., et al. 2020). As with other models of mucosal colonization/infection using conventionally raised animals, indirect effects of exogenously added microbe(s) on the endogenous microbiota may contribute to sialidase activity. The use of antibodies, proteomics, bacterial genetic tools to make mutants in fastidious vaginal anaerobes, and/or gnotobiotic models may help further clarify the sources of sialidase activity *in vivo*.

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Vaginal pH may also play an important role in determining which bacterial sialidases contribute to sialidase activity in BV or which targets they act on. The human vaginal microbiome is unique among mammals studied to date, with *Lactobacillus* dominance often contributing to a low pH (<4.5) (Miller, E.A., Beasley, D.E., et al. 2016). A recent study measured an even lower vaginal pH in women with *Lactobacillus* dominated microbiota, utilizing methods to maintain physiologically relevant hypoxic and high CO_2 conditions, estimating an average pH of 3.5 when equilibrated at 5% CO_2 (O'Hanlon, D.E., Moench, T.R., et al. 2013). A higher pH is observed in women with BV that may be closer to the pH optima of many sialidases (~pH 5.5) (von Nicolai, H., Hammann, R., et al. 1984, Yamamoto, T., Ugai, H., et al. 2018). *In vitro* studies suggest that the pH optimum for purified *Gardnerella* sialidase using glycoprotein substrates (Robinson, L.S., Schwebke, J., et al. 2019, von Nicolai, H., Hammann, R., et al. 1984) and recombinant *B. fragilis* (Yamamoto, T., Ugai, H., et al. 2018) sialidase using a small molecule fluorogenic substrate is between 5.0 to 5.5. However, the pH optimum may also depend on the substrate; for example - the sialidase purified from *B. fragilis* (same strain as the above study, SBT3182) was reported to have optimal activity at pH 6.1 with colominic acid (α2-8-linked polymer) (Tanaka, H., Ito, F., et al. 1992). Thus, vaginal pH might help determine which vaginal bacteria contribute

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to sialidase activity and/or which potential sialoglycoprotein substrates may become targets; this requires further study.

Can *G. vaginalis* trigger features of BV?

The evidence for *G. vaginalis* as a trigger of the characteristic features of BV following vaginal inoculation in women, non-human primates, and animal models has been recently reviewed (Morrill, S., Gilbert, N.M., et al. 2020) and will therefore not be extensively covered here. The causal role of *Gardnerella* in triggering features of BV in mice (including sialidase activity in vaginal fluids) and the potential metabolic functions of sialidase action were studied in two 2013 papers (Gilbert, N.M., Lewis, W.G., et al. 2013, Lewis, W.G., Robinson, L.S., et al. 2013). In these studies, *G. vaginalis* strain JCP8151B was introduced into β-estradiol treated C57BL/6 mice (Charles River/NCI) reproducing BV features, including vaginal sialidase activity (Figure 2A), mucus sialoglycan degradation and depletion (Figure 2B-C), increased numbers of shed epithelial cells in vaginal washes (Figure 2D), *Gardnerella* adhering to shed epithelial cells (Figure 2E), and no evidence of inflammation by histopathology (Gilbert, N.M., Lewis, W.G., et al. 2013, Lewis, W.G., Robinson, L.S., et al. 2013). As discussed below, two of the prominent phenotypes commonly seen in BV – the increase in pH and a fishy amine odor – are believed to be due to organisms other than *G. vaginalis* in BV (Nelson, T.M., Borgogna, J.L., et al. 2015, Srinivasan, S., Morgan, M.T., et al. 2015, Wolrath, H., Forsum, U., et al. 2001) and were not studied in the mouse model.

First, the acidity of the human vagina (a human-specific trait) is likely produced by dominant lactobacilli, which produce lactic acid. When lactobacilli are in short supply, as in women with

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BV, there is typically a higher pH due to lower levels of lactic acid. Studies suggest that vaginal microbiotas in mice are rarely dominated by lactobacilli (Agarwal, K., Robinson, L.S., et al. 2020, Jasarevic, E., Howard, C.D., et al. 2017, Vrbanac, A., Riestra, A.M., et al. 2018). For example, in studies by Vrbanac et al. only 1 out of 20 mice was found to have a *Lactobacillus*-dominant microbiome at day 0, which by day 6 had transitioned to a *Staphylococcus* dominated vaginal microbiota. It is not known whether the rare instance of a *Lactobacillus*-dominant mouse vaginal microbiome coincides with a low vaginal pH. From these data, we conclude that the pH shift observed in BV is not a feature that can be modeled starting with the endogenous microbiota in mice.

A second feature of BV not reproducible in mice through introduction of *Gardnerella* alone is the characteristic fishy amine odor in some women with BV. A bioinformatic analysis of vaginal taxa revealed that only a few members of the vaginal microbiome encode the predicted pathways for synthesizing the amines, including putrescine, cadaverine, and trimethylamine (TMA), associated with "fishy" odor (Nelson, T.M., Borgogna, J.L., et al. 2015). For example, *E. coli* 83972, *P. mirabilis, and Janthinobacterium spp.* were the only taxa found encoding the pathway for cadaverine biosynthesis, while *Prevotella*, *Dialister*, *Veillonella* spp. and a few others were found to encode pathways for putrescine production. Although, prior studies in women have revealed an association between a positive whiff test and the presence of *Gardnerella* (in addition to other organisms) (Srinivasan, S., Hoffman, N.G., et al. 2012), *Gardnerella* has not been shown to encode the predicted pathways for biosynthesis of these amines (Nelson, T.M., Borgogna, J.L., et al. 2015), nor produce these compounds in culture (Chen, K.C., Forsyth, P.S.,

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et al. 1979). Further studies are required to determine roles and inter-relationships of vaginal bacteria in reproductive health problems associated with BV physiology.

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Genetic and biochemical basis for sialidase activity in BV bacteria

More recently, studies have begun to explore the genes underlying sialidase activity in bacterial species associated with BV, including members of the genera *Bacteroides*, *Prevotella* and *Gardnerella*. Although the genes for sialidase activity among species and strains of *Bacteroides* and *Prevotella* from the vagina have not been studied to our knowledge, many studies have investigated the genes (as well as the biochemical and biological functions of sialidases) for sialidase activity from members of the *Bacteroidetes* that reside in the gut (Juge, N., Tailford, L., et al. 2016, Ng, K.M., Ferreyra, J.A., et al. 2013, Robinson, L.S., Lewis, W.G., et al. 2017). Indeed, the sialidase gene from *Bacteroides fragilis* was first identified more than 3 decades ago by heterologous expression studies in *E. coli* (Russo, T.A., Thompson, J.S., et al. 1990). Recently, Yamamoto et al. have also characterized a sialidase from *B. fragilis* YCH46 that preferentially hydrolyzes α2-8-linked sialic acids (Yamamoto, T., Ugai, H., et al. 2018). Sialidase activity in vaginal strains of *Gardnerella* have been under investigation for approximately three decades. However, the genetic machinery underlying sialidase activity in laboratory-cultured *Gardnerella* was not understood until 2019 when recombinant sialidase homologs from *G. vaginalis* were investigated for their biochemical activity and substrate specificity (Robinson, L.S., Schwebke, J., et al. 2019).

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To date, three sialidase homologs have been reported in *G. vaginalis*, namely NanH1 (also known as sialidase A) (Janulaitiene, M., Gegzna, V., et al. 2018), NanH2 and NanH3 (Robinson, L.S., Schwebke, J., et al. 2019). Sialidases NanH2 and NanH3 from *G. vaginalis* were recently identified and named based on their homology to the NanH2 *Bifidobacterium longum* sialidase, to which they share ~60 % identity. *Gardnerella* NanH1 is more closely related to *Bifidobacterium* NanH1 than to NanH2. The genome of *G. vaginalis* strain JCP8151B contains all three homologs in different regions. NanH2 and HanH3 are 49% identical, while NanH1 is 30% identical to both NanH2 and NanH3, with closest homology at the active domain (Robinson, L.S., Schwebke, J., et al. 2019). Recombinant NanH1 had little to no activity against most of the tested substrates, while NanH2/NanH3 cleaved sialic acid from nearly all tested substrates (Robinson, L.S., Schwebke, J., et al. 2019).

Similar to bifidobacteria (Kiyohara, M., Tanigawa, K., et al. 2011, Sela, D.A., Chapman, J., et al. 2008, Sela, D.A., Li, Y., et al. 2011), strains of *Gardnerella* can access sialic acid from different linkages and a wide range of substrates. Recombinant NanH1 from gut-associated *Bifidobacterium longum* subspecies *infantis* had 140-fold lower turnover rate (k_{cat} = 1.0 ± 0.1 s^{-1} i.e. 1 molecule of sialic acid generated per molecule of enzyme per second) than NanH2 (k_{cat} = (1.4 ± 0.1) × 10^2 s^{-1}) for substrate containing α2-3-linked sialic acid, and 175-fold lower turnover on α2-6-linked substrate (Sela, D.A., Li, Y., et al. 2011). As with *B. longum* NanH1, *Gardnerella* NanH1 had little activity when tested on a wide variety of plausible mucosal substrates. Consistent with a prior report that NanH1 (sialidase A) had activity when 200 mM of 4-methylumbelliferyl (MU)-Neu5Ac was used as substrate (Janulaitiene, M., Gegzna, V., et al. 2018), recombinant NanH1 was found to release small amounts of Neu5Ac from 4-MU-Neu5Ac

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used at lower substrate concentrations (250 µM) (Robinson, L.S., Schwebke, J., et al. 2019). NanH1 also released small amounts of Neu5Ac from bovine submaxillary mucin (BSM), and colostrum IgA. However, it was completely unable to access Neu5Ac from α2-3- or α2-6-linked sialyllactose, nor was it able to access 7-*O*- or 9-*O*-acetylated sialic acids from BSM under the conditions tested. In contrast, recombinant NanH2 and NanH3 were able to cleave sialic acids in a variety of contexts (e.g. α2-3- and α2-6-linked sialic acids as well as *N*- and *O*-linked sialoglycans found on secreted IgA and mucin) (Robinson, L.S., Schwebke, J., et al. 2019). Similarly, strains of *G. vaginalis* encoding NanH2 or NanH3 were also able to deplete α2-3- or α2-6-linked sialyllactose added to culture media (Robinson, L.S., Schwebke, J., et al. 2019).

Prior studies observed that the sialidase activity present in human clinical specimens could access a broad range of exogenously provided sialoglycan substrates relevant to the mammalian mucosa (Lewis, W.G., Robinson, L.S., et al. 2012). Recombinant NanH2 and NanH3, as well as cultured strains of *Gardnerella*, also cleave sialic acid from the same range of substrates as accessed by sialidases in human vaginal samples (Robinson, L.S., Schwebke, J., et al. 2019). These broad and overlapping substrate preferences suggest that the sialidase activity observed in human vaginal specimens is plausibly derived from *Gardnerella* sialidases NanH2 and NanH3.

Molecular characterization of the three *G. vaginalis* sialidase homologs showed that sialidase activity in cultured *G. vaginalis* has been misattributed to the gene encoding "sialidase A" (*sldA*, renamed *nanH1*) (Robinson, L.S., Schwebke, J., et al. 2019). In the biochemical studies conducted by Robinson et al., substantial levels of NanH1 were successfully expressed (Coomassie and anti-His Western), and the need for metal cations was also ruled out. However,

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it remains possible that NanH1 was incorrectly folded for optimal activity, or that it acts by a mechanism or on a substrate that has not yet been tested. We note that the multiple sialidases of *Streptococcus pneumoniae* yield different sialic acid products (Xu, G., Kiefel, M.J., et al. 2011); however, this has not been studied extensively for members of the *Bifidobacteriacea*. NanH1 was originally believed to encode the *Gardnerella* sialidase since it bore homology to other sialidases, predicted catalytic residues were conserved (Robinson, L.S., Schwebke, J., et al. 2019), and is encoded immediately adjacent to the predicted sialic acid catabolic gene cluster in *Gardnerella vaginalis* strain JCP8151B (Lewis, W.G., Robinson, L.S., et al. 2013). All of these facts indicate its central role in sialic acid biology. However, *nanH1* is often present in strains of *Gardnerella* that do not have detectable sialidase activity in culture. Some *Gardnerella* strains encode all three *nanH* homologs and some encode none; most of the strains encode either encode only *nanH1* or they encode *nanH1* along with either *nanH2* or *nanH3* (Robinson, L.S., Schwebke, J., et al. 2019). The presence of a predicted sialic acid catabolic pathway has only been evaluated in a limited number of *Gardnerella* strains, therefore it is not clear if *nanH2* or *nanH3* are encoded only in strains that have *nanH1* and the sialic acid catabolic pathway (and vice versa). Although *nanH1* appears to be present in all sialidase-positive *G. vaginalis* strains, at least 3 different studies have reported that many strains have the *nanH1* gene while being negative for sialidase activity in culture (Hardy, L., Jespers, V., et al. 2017, Pleckaityte, M., Janulaitiene, M., et al. 2012, Schellenberg, J.J., Paramel Jayaprakash, T., et al. 2016). In one strain set (Schellenberg, J.J., Paramel Jayaprakash, T., et al. 2016), less than 50% of 77 *nanH1* positive strains produced sialidase activity that could be detected in culture. In another set of 34 divergent *Gardnerella* strains, sialidase activity corresponded exactly with the presence of *nanH2* or *nanH3* by PCR (Robinson, L.S., Schwebke, J., et al. 2019). In contrast, 16 of 19

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sialidase-negative strains of *Gardnerella* were PCR positive for *nanH1*, but not *nanH2* or *nanH3*. Conversely, *Gardnerella* strains encoding only *nanH1* had no detectable sialidase activity in culture. In conclusion, NanH1 appears unlikely to contribute significantly to the sialidase activity seen in *Gardnerella* cultures. These data strongly suggest that "sialidase A" is a misnomer, at least as it refers to the sialidase activity measured in laboratory cultures of *Gardnerella*.

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If the primary role of NanH1 (aka sialidase A; sldA) is not as a sialidase, then what is its function?

Although NanH1 does not appear to be responsible for the sialidase activity seen in *Gardnerella* cultures, several studies nevertheless point toward carbohydrate or sialic acid-related roles of this protein in the biology of *Gardnerella*-host interactions. *Gardnerella* NanH1 appears to be an ortholog of the NanH1 protein encoded by *B. longum* (note that both *Gardnerella* and *Bifidobacterium* belong to the *Bifidobacteriales)*. NanH1 in *B. longum* and *G. vaginalis* share the following features: 1) very low but detectable levels of sialidase activity when expressed as recombinant proteins (Robinson, L.S., Schwebke, J., et al. 2019, Sela, D.A., Li, Y., et al. 2011), 2) an *N*-terminal putative lectin domain, 3) proximity to putative sialic acid catabolic operons, 4) lack of canonical signal sequences (for secretion by the Sec apparatus) or membrane anchoring regions, 5) a C-terminal CBM40/GH33 sialidase domain with apparently intact active site residues. Interestingly, recent studies have shown that some CBM40-containing sialidase domains can bind to sialic acid residues of glycans, promoting adherence to the mucosa. For example, a CBM40 containing *Ruminococcus gnavus* sialidase (RgNanH) was recently shown to bind broadly to sialoglycans with a preference for α2-3-linked sialic acids on sialoglycans and to

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mediate interactions with intestinal mucus (Owen, C.D., Tailford, L.E., et al. 2017). Sialic acid binding was also demonstrated for the CBM40-containing sialidase of *Vibrio cholerae* (Moustafa, I., Connaris, H., et al. 2004). Additionally, the extracellular sialidase from *Bifidobacterium bifidum*, SiaBb2, was found to bind α2,6-linked sialic acids to facilitate sialoglycan foraging (Nishiyama, K., Yamamoto, Y., et al. 2017). Together, these features suggest that *G. vaginalis* NanH1 may be involved in bacterial adherence to sialoglycans on mucus or epithelial cells; however, this requires further investigation.

Several translational studies have tested associations between clinical parameters and the presence/absence (contingency, 2 studies) or abundance of the *nanH1* gene (quantitative, 2 studies). Two small studies evaluated whether presence/absence of the *nanH1* gene (based on PCR detection) was associated with the BV status of women from whom the strains had been isolated, with neither finding a positive association (Castro, J., Alves, P., et al. 2015, Mohammadzadeh, R., Sadeghi Kalani, B., et al. 2019). However, another larger study went beyond presence/absence of *nanH1* to measure the genomic load of *nanH1* in relation to BV. This study identified a strong association between a high *nanH1* load and BV diagnosis. There was also a strong correlation between high levels of *nanH1* and the presence of adhesive sheets of bacteria dominated by *Gardnerella* as shown by fluorescence *in situ* hybridization (Hardy, L., Jespers, V., et al. 2017). This finding further supports the idea that NanH1 might afford adherence traits. Another study investigated the relationship between *Gardnerella nanH1* gene abundance and the persistence of high risk Human Papillomavirus (HPV), showing a strong correlation between high levels of the *nanH1* gene and HPV persistence (as opposed to clearance) (Di Paola, M., Sani, C., et al. 2017). Since *nanH1* is not found among all *G. vaginalis*

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strains, the association with HPV persistence may indicate relationships with specific subtypes of *Gardnerella*.

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***Gardnerella* sialidase: tethered to the cell surface, excreted, or both?**

Sialidase activity can be measured in the cell-free supernatant of *G. vaginalis* cultures in significant quantities. However, most of the *G. vaginalis* sialidase activity appears to be cell-associated (Lewis, W.G., Robinson, L.S., et al. 2013, Lewis, W.G., Robinson, L.S., et al. 2012). When *G. vaginalis* cells were incubated with secreted IgA (SIgA, containing the highly glycosylated secretory component), free sialic acid was liberated into the extracellular environment followed by apparent uptake and catabolism (as evidenced by the disappearance of free sialic acid from the supernatant) (Figure 3A). This is perhaps the best piece of biochemical evidence that the sialidase is tethered to the bacterial surface facing outward. At present, it is unclear if *G. vaginalis* actively secretes the sialidase enzyme(s) observed in culture supernatants or if this activity is released upon bacterial lysis or proteolytic cleavage from the cell surface. The discovery of *Gardnerella* NanH2 and NanH3, which bear homology to *Bifidobacterium* sialidases, revealed predicted transmembrane α-helices at their C termini (Robinson, L.S., Schwebke, J., et al. 2019). Studies with recombinant *G. vaginalis* sialidases also provide insight into the plausible cellular location of these enzymes. When constructs encoded a C-terminal His-tag just after the predicted transmembrane regions, recombinant protein could not be detected in soluble fractions or supernatants of *E. coli* lysates using anti-His Western blot, even though sialidase enzyme activity could be detected in both (Robinson, L.S., Schwebke, J., et al. 2019). These data suggested that the full-length proteins were insoluble due to hydrophobic α-helices. Moreover, the presence of sialidase activity in culture supernatant and soluble lysates, together

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with its absence in identically processed fractions from *E. coli* containing the empty vector, suggested that some sialidase activity was being released by cell death or proteolysis into soluble and secreted fractions. In contrast, truncation of both *nanH2* and *nanH3* to remove the predicted transmembrane regions, but still containing the C-terminal His-tag resulted in soluble proteins that could be readily purified using the intact His-tag. Together these and other published observations suggest that NanH2 and NanH3 are embedded in the *G. vaginalis* cell surface but may be released into the supernatant following cell death or proteolytic cleavage. Likewise, the cell surface sialidase of *Streptococcus pneumoniae* (NanA) can also be solubilized by proteolysis and released without substantial loss of activity (Lock, R.A., Paton, J.C., et al. 1988). In some bacteria hydrolytic enzymes are also carried in spherical membranous structures that are released from the outer membrane, commonly known as outer membrane vesicles (OMVs), and provide a protective environment for dissemination of the encapsulated cargo (Caruana, J.C. and Walper, S.A. 2020). For example, *B. fragilis,* sialidase activity has been reported in the OMVs (Elhenawy, W., Debelyy, M.O., et al. 2014). While membrane vesicles have recently been reported for *Gardnerella* (Shishpal, P., Kasarpalkar, N., et al. 2020), it is unclear if sialidase is present in these because the study used a sialidase-negative type strain.

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Sialoglycan foraging by *Gardnerella* and other vaginal bacteria

Sialidase activity is linked with the ability of *Gardnerella* to liberate sialic acids from bound sources (mucus sialoglycans) (Lewis, W.G., Robinson, L.S., et al. 2013, Lewis, W.G., Robinson, L.S., et al. 2012). The incubation of *Gardnerella* with exogenous sialoglycan substrates revealed that sialidase action occurs extracellularly, liberating an increased pool of free sialic acid, which is then depleted from the extracellular milieu (Figure 3A). The predicted sialic acid catabolic

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gene cluster in *G. vaginalis* ATCC14019, contains both transporters and an *N*-acetyl neuraminate lyase that are required for the uptake and utilization of sialic acid (Lewis, W.G., Robinson, L.S., et al. 2013) (Figure 3B). *Gardnerella* are capable of cleaving both Neu5Ac and Neu5Gc from sialoglycans such as BSM, although Neu5Ac was cleaved preferentially (Lewis, W.G., Robinson, L.S., et al. 2013, Robinson, L.S., Schwebke, J., et al. 2019). The depletion of Neu5Gc by *G. vaginalis* cultures is interesting in that Neu5Gc is not synthesized by humans, though it can be found in various human tissues due to metabolic incorporation from other sources (Dhar, C., Sasmal, A., et al. 2019). Despite the capacity of *G. vaginalis* sialidase to liberate Neu5Gc, the nonhuman sialic acid had an inhibitory effect at high concentrations on the ability of *Gardnerella* to take up Neu5Ac. In contrast, neither sialidase activity nor Neu5Ac lyase activity were affected by excess Neu5Gc (Lewis, W.G., Robinson, L.S., et al. 2013) (Figure3A). This appears to be consistent with the observation that *Gardnerella* are not generally found at high levels in other nonhuman primates tested, which could have higher levels of Neu5Gc in the reproductive tract.

The broad substrate preferences of *Gardnerella* sialidases (NanH2 and NanH3) suggest that their main functional difference is the improved capacity of NanH2 compared to NanH3 to cleave 9-*O*-acetylated sialic acid substrates (Robinson, L.S., Schwebke, J., et al. 2019). It has not been systematically studied whether the female reproductive tract in mammals contains *O*-acetylated sialic acids. Analyses of the rapidly expanding number of *Gardnerella* genomes has revealed wide genetic diversity among the strains so far isolated and suggest that there are multiple species yet to be adequately classified or studied. Only a subset of the sialidase-positive strains of *Gardnerella* encode *nanH2*. Moreover, foraging studies revealed that the presence of *nanH2* in the genome predicted a greater capacity to forage on 9-*O*-acetylated sialic acids compared to

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strains with only *nanH3* (Robinson, L.S., Schwebke, J., et al. 2019). In the human colon, 9-*O*-acetylation of sialic acids are highly abundant and these modifications restrict the action of many of the sialidases ubiquitously found in the colonic microenvironment (Corfield, A.P., Wagner, S.A., et al. 1992, Robinson, L.S., Lewis, W.G., et al. 2017). While *Gardnerella* has been considered to be a vaginal bacterium, studies of anal swabs have shown that the bacterium is also present in the distal gastrointestinal tracts of men, women, and young children (Cox, C., Watt, A.P., et al. 2017, Marrazzo, J.M., Fiedler, T.L., et al. 2012, Myhre, A.K., Bevanger, L.S., et al. 2002). If NanH2 confers increased capacity for growing on 9-*O*-acetylated sialic acids, this may allow strains encoding this sialidase to colonize the colon. However this possibility has not been investigated.

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Sialoglycan foraging in the vagina may further extend to potential pathogens that do not encode a sialidase but may derive benefits from sialidase producers like *G. vaginalis* (Figure 4). For example, with assistance from exogenous sialidases produced by other bacteria, *F. nucleatum* can uptake and utilize free sialic acid for growth under nutrient limiting conditions, a behavior that promoted persistence within a sialidase-positive vaginal niche (Agarwal, K., Robinson, L.S., et al. 2020). Similarly, *in vitro* studies have shown that sialidases from *B. fragilis* and *B. thetaiotaomicron* can facilitate sialic acid utilization by *E. coli* that does not encode its own functional sialidase and cannot access sialic acid bound to glycoconjugates (Robinson, L.S., Lewis, W.G., et al. 2017). While *B. thetaiotaomicron* only encodes sialidase, *B. fragilis* encodes both a sialidase and an *O*-acetyl esterase (EstA) that can convert 9-*O*-acetylated sialic acids to non-acetylated sialic acids. This is important because *O*-acetylated sialic acids are often resistant to sialidases and therefore *O*-acetylation limits the scavenging of this carbohydrate residue by

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microbes. Sialic acid *O*-acetyl esterase activity facilitates foraging of *O*-acetylated sialoglycans that were otherwise inaccessible to the sialic acid consumers. It is plausible that similar metabolic interactions may occur among bacteria in the vaginal microbiota. For example, presence of *B. fragilis* in the vaginal microbial community may provide access to 9-*O*-acetylated sialic acids to *G. vaginalis* strains that do not encode *nanH2* or other sialidase-producing bacteria. Though the presence of *O*-acetylated sialic acids has not been documented in the reproductive tract, *Gardnerella* might also benefit from the presence of esterase-producing bacteria when colonizing other niches such as the gastrointestinal tract (Cox, C., Watt, A.P., et al. 2017, Marrazzo, J.M., Fiedler, T.L., et al. 2012, Myhre, A.K., Bevanger, L.S., et al. 2002). Indeed, cross-feeding of host-derived sialic acids has been shown to support persistent colonization of polymicrobial communities associated with dysbiosis in the gastrointestinal and reproductive tracts (Agarwal, K., Robinson, L.S., et al. 2020, Ali, S.R., Fong, J.J., et al. 2014, Huang, Y.L., Chassard, C., et al. 2015). Mutualistic and antagonistic interactions among vaginal bacteria involving other metabolic pathways have been reviewed earlier (Pybus, V. and Onderdonk, A.B. 1997, Pybus, V. and Onderdonk, A.B. 1999).

Evidence that bacterial sialidase acting on host glycans leads to mucosal sialic acid depletion in BV

Several studies have provided experimental evidence that significant degradation of sialoglycans occurs in vaginal specimens from women with BV compared to women with normal *Lactobacillus*-dominant vaginal microbiomes. The first of these studies measured levels of free (already pre-liberated within the sample) and total (released by mild acid hydrolysis) sialic acids fluorescently derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB) for HPLC separation and measurement (Lewis, W.G., Robinson, L.S., et al. 2013) (Figure 2). Compared to

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women without BV, women with the condition had ~3-fold lower levels of total sialic acids and 3.5-fold higher levels of free/liberated sialic acid. These data suggest that sialidase enzymes present in BV specimens catalyze the degradation of sialoglycans, leading to the liberation of free sialic acid and the simultaneous depletion of intact sialoglycans. Consistent with these findings, another group used metabolomics to compare small molecule metabolites present within the vaginal microbiome in the setting of *Lactobacillus*-dominant microbiotas or diverse BV microbiotas (Srinivasan, S., Morgan, M.T., et al. 2015). This study confirmed the earlier finding that women with BV have higher levels of free/liberated sialic acid compared to women with *Lactobacillus*-dominant microbiotas.

Finally, lectins (carbohydrate-binding proteins) have been used to evaluate differences in the presence and context of intact sialoglycans present within vaginal secretions in women with and without BV. These lectin binding studies were done utilizing samples from the same cohort of women, collected in two different ways, (i) cervical vaginal fluids were collected by inserting a softcup to base of cervix (Moncla, B.J., Chappell, C.A., et al. 2016) and (ii) cervicovaginal lavages (CVLs) were collected by washing the ectocervix and vaginal vault with saline (Moncla, B.J., Chappell, C.A., et al. 2015, Wang, L., Koppolu, S., et al. 2015). In the cervical cup samples from women with BV, there was an increase in sialidase activity compared to women without BV and a reduction in binding of *Maackia amurensis lectin* (MAL-II) and *Sambucus nigra agglutinin* (SNA), which interact with α2-3- and α2-6-linked sialic acids respectively (Moncla, B.J., Chappell, C.A., et al. 2016). These data are consistent with biochemical data showing that sialidase enzymes in BV specimens can efficiently access sialic acids bound by both these linkages (Lewis, W.G., Robinson, L.S., et al. 2012). In an earlier study using CVLs, Moncla et

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al. (2015) observed a significant reduction in binding of SNA (α2-6), but not MAL-II (α2-3), in BV positive women. Similarly, in a glycomic analysis (using lectin microarray) of CVLs, reduced binding was observed for SNA in BV samples, but no significant changes were observed with MAL-I (another variant of *Maackia amurensis* that also binds to α2-3 linked sialic acids) (Wang, L., Koppolu, S., et al. 2015). While both variants of *Maackia amurensis* lectin (MAL-I and MAL-II) selectively recognize α2-3 linked sialic acids, they can also bind to sulphated galactose residues in different contexts (Geisler, C. and Jarvis, D.L. 2011). Therefore, different results observed in binding of MAL (α2-3 linked sialic acids) in the above studies remains ambiguous and need to be verified using other biochemical approaches. Moncla et al. note that cervical cup samples had lower overall glycosidase activity as compared to CVL; however, these sample types were not directly compared in either study (Moncla, B.J., Chappell, C.A., et al. 2016, Moncla, B.J., Chappell, C.A., et al. 2015). The authors noted this was consistent with the additional unpublished observation that cervical cup samples have lower concentrations of bacteria compared to bacterial levels previously reported in CVL. It is noteworthy that in spite of the differences in the observed range of glycosidase activity and bacterial concentrations, depletion of α2-6-linked sialic acids was similar in both types of specimens in women with BV. Mucins are heavily glycosylated proteins and one of the most prominent sources of sialoglycans in the reproductive tract. Interestingly, in the cervical cup samples sialic acid-containing glycans were depleted in spite of a reported increase in mucins that are expressed in the reproductive tract, including MUC1, MUC4, MUC5AC, and MUC7.

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Implications of sialic acid depletion and sialidases in vaginal secretions

Negatively charged sialic acid residues present at the terminus of carbohydrate chains of mucin provide a rigid conformation to these molecules and ionic interactions between the carbohydrate chains allows for a specific arrangement of these glycoproteins in the mucus layer (Elstein, M. 1978). The presence of sialic acids at the terminal ends of glycan chains has also been proposed to protect underlying glycans from other glycosidase activities (Moncla, B.J., Chappell, C.A., et al. 2015), that would result in successive deglycosylation of cervico-vaginal glycoproteins in BV (Lewis, W.G., Robinson, L.S., et al. 2012), and consequently protects the underlying protein backbone from proteolysis (Lewis, W.G., Robinson, L.S., et al. 2012). For example, higher levels of terminal galactose and *N*-acetylgalactosamine were evident on glycans within BV vaginal secretions using lectin probes (Wang, L., Koppolu, S., et al. 2015). In addition to higher levels of sialidase in BV, it has been shown that women with BV have higher levels of other glycosidase activities (including β-galactosidase) (Moncla, B.J., Chappell, C.A., et al. 2016, Moncla, B.J., Chappell, C.A., et al. 2015, Olmsted, S.S., Meyn, L.A., et al. 2003). Also, metabolic profiling revealed increased levels of free galactose in women with BV, further supporting the action of β-galactosidas(s) on host glycans (Srinivasan, S., Morgan, M.T., et al. 2015). Studies by Deman *et al.* suggested that removal of charged sialic acid residues from cervico-vaginal mucins impacts the arrangement of mucin molecules in the presence of EDTA when pH is titrated below 3.0 (Deman, J., Mareel, M., et al. 1973, Rabouille, C., Aon, M.A., et al. 1989). As such, it is not surprising the viscosity of the mucus gel is significantly reduced in BV (Chappell, C.A., Rohan, L.C., et al. 2014, Olmsted, S.S., Meyn, L.A., et al. 2003). Thus, changes in physical properties of mucus during BV might lead to greater susceptibility to invading genital tract pathogens.

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Studies suggest that sialidase could be important in BV pathophysiology by enabling further breakdown of mucus components. One study used BV vaginal specimens as a source of enzyme activity, incubating them along with a heavily glycosylated model protein (the secretory component of IgA) to better understand the relationship of deglycosylation and proteolysis in BV (Lewis, W.G., Robinson, L.S., et al. 2012). Incubation of exogenous sIgA in BV specimens resulted in lower molecular weight products that were recognized by the mannose-binding lectin ConA, similar to patterns observed when adding 3 exogenous enzymes: sialidase, β-galactosidase, and hexosaminidase as a positive control. In contrast, after incubation of secretory component with vaginal specimens of women without BV, significantly lower levels of ConA reactivity were observed compared to BV specimens. These results suggest that enzymes found in the vaginal milieu during BV are engaging in processive deglycosylation of *N*-glycans, revealing underlying mannose residues within these glycans. In further biochemical experiments, it was shown that partial *N*-deglycosylation of secretory component led to enhanced bacterial proteolysis of secretory component.

Among other Gram-positive pathogens, *Streptococcus pneumoniae* shares two features in common with *Gardnerella*: it encodes multiple sialidases and can also catabolize sialic acids. In addition to this, *S. pneumoniae* is able to bind to underlying carbohydrate residues, once exposed by sialidase (Blanchette, K.A., Shenoy, A.T., et al. 2016). It is possible *Gardnerella* could similarly benefit from the exposure of cryptic receptors. *Gardnerella* sialidase could also modify the properties of mucus secretions, leading to greater capacity for bacteria to gain proximity to the epithelium or invade the upper reproductive tract. Indeed, several lines of investigation suggest that sialic acids may be an important driver of niche specificity and pathogenic potential

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for *Gardnerella*. In addition to potential metabolic benefits of sialic acid catabolism (Lewis, W.G., Robinson, L.S., et al. 2013), the addition of sialidase inhibitors reduced (by half) *G. vaginalis* attachment and invasion of HeLa cells *in vitro* (Govinden, G., Parker, J.L., et al. 2018). Preliminary studies by Cook et. al. revealed that *G. vaginalis* is most frequently found adhering to clue cells present in vaginal fluid of women with BV (Cook, R.L., Reid, G., et al. 1989). More recently, investigation of vaginal biopsy specimens show that *G. vaginalis* forms adherent biofilms on the vaginal epithelium, along with some other BV associated bacteria (Swidsinski, A., Mendling, W., et al. 2005, Swidsinski, A., Loening-Baucke, V., et al. 2014). So far, it is unclear how *G. vaginalis* attaches to the vaginal epithelial cells and the mechanisms underlying epithelial cell damage remain unknown. Future studies in this direction may evaluate the role of sialidases and carbohydrates in attachment to vaginal epithelial cells by different strains of *G. vaginalis*. This will provide new avenues for development of therapeutics such as carbohydrate analogs, which may act as substrate decoys or competitive inhibitors of bacterial attachment to vaginal epithelium, for BV.

Several sialic acid-binding proteins known for their immune-modulatory functions have been described in the female reproductive tract, in particular, sialic acid binding immunoglobulin-like lectins (Siglecs) have been reported on amniotic membranes, the cervical endothelium, and on immune cells throughout the urogenital system (Ali, S.R., Fong, J.J., et al. 2014, Brinkman-Van der Linden, E.C., Hurtado-Ziola, N., et al. 2007, Patras, K.A., Coady, A., et al. 2017, Tecle, E., Reynoso, H.S., et al. 2019). The removal of sialic acids within cervicovaginal secretions suggests that these immune-modulating receptors might be regulated inappropriately. This could have myriad effects on bacterial-host interactions in an array of different cell types and might help explain the overgrowth of particular subsets of bacteria, as well as the enhanced inflammatory

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potential described in women with a diverse BV-like microbiota (Farcasanu, M. and Kwon, D.S. 2018). In fact, sialidases have been shown in other contexts to regulate immune states through Siglec receptors (Chang, Y.C., Uchiyama, S., et al. 2012, Chen, G.Y., Brown, N.K., et al. 2014).

The widespread roles of sialic acids and sialoglycans in human reproduction include aspects of sperm migration in the female reproductive tract (through mucus), formation of the sperm oviductal reservoir, sperm capacitation- a required process after ejaculation for sperm to become capable of fertilization (Tecle, E., Reynoso, H.S., et al. 2019), as well as fertilization itself (Lassalle, B. and Testart, J. 1994). One of the most well-known sialoglycans, glycodelin, affects embryo implantation, placental development, and immune regulation. This topic has been recently reviewed (Lee, C.L., Lam, K.K., et al. 2016), with recent studies suggesting a role for Siglec-6 in glycodelin-mediated fetal trophoblast invasion into the maternal decidua (Lam, K.K., Chiu, P.C., et al. 2011).

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In conclusion, the female reproductive tract has a wide array of important functions that might be disrupted in the setting of BV-associated sialoglycan depletion of vaginal secretions. More study is needed to understand the key functions of sialoglycans and sialic acid binding receptors in the female reproductive tract and to understand how microbes endanger reproductive health by interfering with these functions.

Acknowledgements

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Figure Legends

Figure 1. The human vaginal microbiota. Images of Gram-stained vaginal smears depict some of the microscopic features of BV. Top: schematic magnification illustrating features of the Nugent scoring system (Nugent, R.P., Krohn, M.A., et al. 1991), in which an abundance of large Gram-positive (purple) rods contributes to a low score (0-3, No BV, left). In contrast, diverse morphotypes of Gram-negative (pink) and Gram-variable bacteria with low levels of long Gram-positive rods contributes to a high Nugent score (7-10, BV, right). Note the higher numbers of bacteria in BV specimens and their tendency to be concentrated around vaginal epithelial cells (VECs). Scale bars = 20 μm

Figure 2. Mouse model of *Gardnerella* infection replicates features of BV.
Representative data from multiple publications directly compare some of the phenotypes of *G. vaginalis* colonized mice to features of BV in women. **A.** Sialidase activity in vaginal fluids measured with the 4MU-Sia assay. **B-C.** Free and total sialic acids measured by fluorescent derivatization and HPLC resolution. **D.** Epithelial cells in vaginal washes counted by blinded observers. **E.** H&E-stained vaginal sections reveal bacteria (purple puncta indicated by arrowheads) on the epithelium (pink) of *Gardnerella* infected mice. *P<0.05, **P<0.01, ****P<0.0001. Adapted from data previously published (Gilbert, N.M., Lewis, W.G., et al. 2013, Lewis, W.G., Robinson, L.S., et al. 2013).

Figure 3. A model of *Gardnerella* sialoglycan foraging and depletion. A. Model based on biochemical evidence for *G. vaginalis* sialoglycan degradation and foraging in the vagina. 1) *G. vaginalis* strains with *nanH2* or *nanH3* in their genomes produce sialidase activity in culture. 2)

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Free sialic acid is released by sialidases NanH2 or NanH3 from mucosal sialo-glycoconjugates such as mucin and secretory IgA. 3) Extracellular free Neu5Ac can be transported into the bacteria, depleting them from the culture or assay supernatant. In *G. vaginalis* the transport of Neu5Ac is inhibited by presence of high concentrations of Neu5Gc. 4) Intracellular sialic acid (Neu5Ac) is converted to *N*-acetyl mannosamine (ManNAc) and pyruvate by a sialic acid lyase. **B.** The predicted sialic acid catabolic gene cluster of *G. vaginalis*. Predictions are based on homology and have not been functionally tested. Note that the sialidases of *G. vaginalis*, NanH2 and NanH3 are encoded elsewhere in the genome. There are some strains that encode putative catabolic machinery for sialic acid without encoding NanH2 or NanH3. Note that multiple BV bacteria encode sialidases and/or sialic acid catabolic machinery.

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Figure 4. A model of sialoglycan degradation and foraging by vaginal bacteria. 1) Sialidase-producing bacteria including members of the *Gardnerella*, *Prevotella*, and *Bacteroides* express sialidases, leading to sialidase activity in vaginal fluids, and 2) creating higher levels of free sialic acid. 3) Free sialic acid is taken up and catabolized by not only sialidase-producing bacteria, but taxonomic groups that do not encode their own sialidases, such as group B *Streptococcus*, *E. coli*, and *Fusobacterium nucleatum*. 4) Improved growth or colonization by sialic acid consumers can in turn lead to 5) Sialoglycan depletion and 6) benefits or costs for other community members (e.g. expansion of *Gardnerella*), and 7) increase in sialidase (Agarwal, K., Robinson, L.S., et al. 2020).

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Table I: Sialidase activity and predicted sialic acid transport and catabolic machinery among vaginal bacteria.

NK, none known to us

Name	Sialidase activity reported	Refs	Predicted sialic acid transport or catabolic pathway	Refs
Gardnerella vaginalis	Yes *clade 2, some *clade 1	(Briselden, A.M., Moncla, B.J., et al. 1992, Robinson, L.S., Schwebke, J., et al. 2019, Schellenberg, J.J., Paramel Jayaprakash, T., et al. 2016)	Yes	(Haines-Menges, B.L., Whitaker, W.B., et al. 2015, Lewis, W.G., Robinson, L.S., et al. 2013)
Peptostreptococcus asaccharolyticus	No	(Briselden, A.M., Moncla, B.J., et al. 1992)	NK	NK
Peptostreptococcus anaerobius	No	(Briselden, A.M., Moncla, B.J., et al. 1992)	NK	NK
Peptostreptococcus magnus	No	(Briselden, A.M., Moncla, B.J., et al. 1992)	NK	NK
Peptostreptococcus tetradius	No	(Briselden, A.M., Moncla, B.J., et al. 1992)	NK	NK
Peptostreptococcus prevotii	No	(Briselden, A.M., Moncla, B.J., et al. 1992)	NK	NK
Mobiluncus curtisii	No	(Briselden, A.M., Moncla, B.J., et al. 1992)	NK	NK

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Mobiluncus mulieris	No	(Briselden, A.M., Moncla, B.J., et al. 1992)	NK	NK
Mycolasma hominis	No	(Briselden, A.M., Moncla, B.J., et al. 1992)	NK	NK
Prevotella bivia	Yes	(Briselden, A.M., Moncla, B.J., et al. 1992)	Yes	(McDonald, N.D., Lubin, J.B., et al. 2016, Young, W., Egert, M., et al. 2015)
Prevotella oralis	Yes	(Briselden, A.M., Moncla, B.J., et al. 1992)	Yes	(Haines-Menges, B.L., Whitaker, W.B., et al. 2015)
Prevotella loeschii	Yes	(Briselden, A.M., Moncla, B.J., et al. 1992)	NK	NK
Prevotella disiens	Yes	(Briselden, A.M., Moncla, B.J., et al. 1992)	NK	NK
Bacteroides fragilis	Yes	(Briselden, A.M., Moncla, B.J., et al. 1992, Tanaka, H., Ito, F., et al. 1992, Tanaka, H., Ito, F., et al. 1994)	Yes	(Brigham, C., Caughlan, R., et al. 2009, Haines-Menges, B.L., Whitaker, W.B., et al. 2015)
Bacteroides vulgatus	Yes	(Briselden, A.M., Moncla, B.J., et al. 1992, Huang, Y.L., Chassard, C., et al. 2015)	Yes	(Haines-Menges, B.L., Whitaker, W.B., et al. 2015)
Fusobacterium nucleatum	No	(Agarwal, K., Robinson, L.S., et al. 2020, Moncla, B.J., Braham, P., et al. 1990)	Yes	(Agarwal, K., Robinson, L.S., et al. 2020, Haines-Menges, B.L., Whitaker, W.B., et al. 2015, Kumar, J.P., Rao, H., et al. 2018, Yoneda, S., Loeser, B., et al. 2014)

Escherichia coli	No	(Robinson, L.S., Schwebke, J., et al. 2019)	Yes	(Huang, Y.L., Chassard, C., et al. 2015, Kalivoda, K.A., Steenbergen, S.M., et al. 2013, Kalivoda, K.A., Steenbergen, S.M., et al. 2003, Vimr, E.R. and Troy, F.A. 1985a, Vimr, E.R. and Troy, F.A. 1985b)
Group B *Streptococcus*	No	(Yamaguchi, M., Hirose, Y., et al. 2016)	Yes	(Pezzicoli, A., Ruggiero, P., et al. 2012)

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*Four clades have been defined for *G. vaginalis* based on four sub-groups (A-D) that are defined by sequencing of a region of the chaperonin-60 (*cpn60*) gene: clade 1 corresponds to subgroup C, clade 2 corresponds to subgroup B, clade 3 corresponds to subgroup D, clade 4 corresponds to subgroup A (Schellenberg, J.J., Paramel Jayaprakash, T., et al. 2016).