

RESEARCH ARTICLE

# The Effects of Hormones and Vaginal Microflora on the Glycome of the Female Genital Tract: Cervical-Vaginal Fluid

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## Abstract

In this study, we characterized the glycome of cervical-vaginal fluid, collected with a Catamenial cup. We quantified: glycosidase levels; sialic acid and high mannose specific lectin binding; mucins, MUC1, MUC4, MUC5AC, MUC7; and albumin in the samples collected. These data were analyzed in the context of hormonal status (day of menstrual cycle, hormonal contraception use) and role, if any, of the type of the vaginal microflora present. When the Nugent score was used to stratify the subjects by microflora as normal, intermediate, or bacterial vaginosis, several important differences were observed. The activities of four of six glycosidases in the samples from women with bacterial vaginosis were significantly increased when compared to normal or intermediate women: sialidase,  $P = <0.001$ ;  $\alpha$ -galactosidase,  $P = 0.006$ ;  $\beta$ -galactosidase,  $P = 0.005$ ;  $\alpha$ -glucosidase,  $P = 0.056$ . Sialic acid binding sites as measured by two lectins, *Maackia amurensis* and *Sambucus nigra* binding, were significantly lower in women with BV compared to women with normal and intermediate scores ( $P = <0.0001$  and  $0.008$  respectively). High mannose binding sites, a measure of innate immunity were also significantly lower in women with BV ( $P = <0.001$ ). Additionally, we observed significant increases in MUC1, MUC4, MUC5AC, and MUC7 concentrations in women with BV ( $P = <0.001$ ,  $0.001$ ,  $<0.001$ ,  $0.02$  respectively). Among normal women we found that the membrane bound mucin MUC4 and the secreted MUC5AC were decreased in postmenopausal women ( $P = 0.02$  and  $0.07$  respectively), while MUC7 (secreted) was decreased in women using levonorgestrel-containing IUDs ( $P = 0.02$ ). The number of sialic acid binding sites was lower in the postmenopausal group ( $P = 0.04$ ), but the number of high mannose binding sites, measured with Griffithsin, was not significantly different among the 6 hormonal groups. The glycosidase levels in the cervical-vaginal mucus were rather low in the groups, with exception of  $\alpha$ -glucosidase activity that was much lower in the postmenopausal group ( $P < 0.001$ ). These studies present compelling evidence that the vaginal ecosystem responds to the presence of different vaginal microorganisms. These effects were so influential that it required us to remove subjects with BV for data interpretation of the impact of hormones. We also suggest that certain changes occurring in

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vaginal/cervical proteins are due to bacteria or their products. Therefore, the quantitation of vaginal mucins and lectin binding offers a new method to monitor bacteria-host interactions in the female reproductive tract. The data suggest that some of the changes in these components are the result of host processing, such as the increases in mucin content, while the microflora is responsible for the increases in glycosidases and the decreases in lectin binding. The methods should be considered a valid marker for insult to the female genital tract.

## Introduction

Secreted cervical mucus coats the vaginal epithelium and cervix, forming a protective physical and biochemical barrier. The mucus is composed of 2% to 5% mucin glycoproteins and 1% other secreted proteins, including antibodies, antibacterial proteins, and peptides. Secreted mucins, the gel-forming component of mucus, are large, highly glycosylated proteins (10–40 MDa) that form a viscoelastic gel [1–6]. Branched carbohydrate chains (3–10 sugars in length) consisting of N-acetyl-glucosamine, N-acetyl-galactosamine, galactose, fucose, and sialic acid account for up to 80% of the weight of mucins [7, 8].

Glycosidic and proteolytic degradation of gastrointestinal mucus by enteric bacteria is well documented and has been linked to the pathogenesis of inflammatory conditions in the gastrointestinal tract [9, 10].

Mucins and the viscoelastic nature of mucus gels protect epithelial surfaces by providing lubrication, by creating an unstirred layer through which pathogens must pass, and by physically trapping pathogens. There are two different general types of mucins, gel-forming mucins and membrane bound mucins. The functions of these proteins are diverse and complex. Examples of their roles in biology include: cell adhesion and bacterial attachment; cellular differentiation; and the pathogenesis of cancer [11–14]. One of the most important characteristics of mucins and other glyco-molecules is the presence of sialic acid. There are about 50 different types of sialic acids (SA). Both the type of sialic acid and its linkage to subordinate sugars are important in the determination of how the SA functions [15, 16]. One of the best examples is the hemagglutinin proteins of the Influenza A viruses. The human Influenza A virus targets sialic acid in the  $\alpha$  2-6Gal(NAc) configuration while the avian Influenza hemagglutinin binds sialic acid in the  $\alpha$  2-3Gal $\beta$ -1 configuration. If the avian virus were to mutate so it recognized the  $\alpha$  2-6Gal(NAc) configuration, it could jump species and it may start infecting humans [17]. It is hemagglutinin specificity that defines the tissue to which the viruses may attach and subsequently infect, for review see [15, 16].

Upper respiratory tract mucosal secretions are known to trap many microorganisms, which aids in their removal [18]. Cervical mucus also traps pathogens as small as herpes simplex virus, suggesting that cervical mucus may also play a critical role in the innate immunity of the female reproductive tract [19–21]. Conversely, some pathogens such as influenza virus produce sialidases that alter the local environment by exposing the sub-terminal galactose or N-acetyl galactosamine that facilitates colonization by opportunistic bacteria [15, 16].

We have evaluated vaginal antimicrobial peptides, pH, and viscosity as they relate to innate immunity and the use of microbicides. Chappell et al., using the same group of women used in this report, found significant differences in pH, viscosity and protein content were associated with hormonal status or type of hormonal contraceptive used [22]. A subsequent report detailed the studies of innate anti-HIV, HSV-1 and HSV-2 activity among women with different hormonal status and found anti-HIV activity was significantly lower in post-menopausal

women [22, 23]. The lower innate immunity we observed in post-menopausal women was correlated to lower protein content in the CVF of the post-menopausal group. Post-menopausal women samples had about 40% less protein than all of the reproductive age women combined ( $P < 0.001$ ) [22, 23].

Genetic studies have identified about 20 genes for mucin production, but the heterogeneity observed when mucins are purified suggests that many post-production modifications occur such as the addition of carbohydrate side chains [24, 25]. The glycoproteins' sugar side-chains may function as lectin-like receptors for immunological events, modulate microbial flora and vaginal fluid viscosity, and vary with estrogen levels and allow for survival and migration of spermatozoa [1, 5, 6, 26]. We have previously reported on the cervical vaginal lavages (CVLs) of our cohort of women and also observed decreased viscosity among the hormonal groups in addition to changes in the sugar side-chains detected by lectin binding, but the impact of the hormonal status was much less than that of the vaginal microflora [22, 23, 27, 28].

The carbohydrate side chains protect the protein core from proteolytic attack but are subject to cleavage by glycosidases. In the vagina, endogenous microflora produce glycosidases and sulfatases that slowly alter the mucus proteins; this leads to their eventual turnover [20, 29]. Many times the carbohydrate chain terminates in a sialic acid molecule that carries a negative charge. This charge is important in determining the function of the molecule and what message it will send. It also is important for the tertiary structure of many different types of molecules, for review see [12, 16, 30, 31]. Intra-vaginal products may affect the protective mucus gel directly or indirectly. Some may directly absorb the mucus gel proteins and carry them away or alter the endogenous enzymes to increase or decrease their respective activities in the vagina, thus changing the delicate equilibrium and altering glycoprotein structure and function (unpublished observations, [22, 23, 27, 28]).

We know that women with bacterial vaginosis are at increased risk of HIV acquisition and low birth weight babies [32–34]. The vaginal microbiome shifts from a normal aerobic flora consisting of gram positive rods and cocci to a flora of women with BV that consists primarily of anaerobic gram negative rods and cocci [34]. We have already reported the changes observed in the vaginal glycome and demonstrated that some of these changes are related to the presence and increase of bacterial enzymes present in the CVL [22, 23, 27, 28].

We are developing intra-vaginal products for the prevention of HIV transmission. One of the parameters is the composition of the mucus layer of the female reproductive tract. However, there are several factors that confound these studies before a baseline can be established. It was also quite likely our samples could be affected by the stage of the menstrual cycle, the type of birth control used, or type of microbiota present [1, 6, 35]. We set out to characterize the protein structures of the cervical-vaginal proteins and to measure the impact of hormonal status and vaginal microbiota on these proteins. We have previously shown the changes in vaginal enzymes and their correlation to changes in lectin binding to the proteins in CVL [27, 28].

Bacterial enzymes, which degrade mucins and other glycoproteins, may play an important function by altering the glycoproteins/mucins, which in turn may impact their turnover intra-vaginally. Many of these enzymes are also known to be associated with bacterial vaginosis and low birth weight babies [7, 36–43]. Others have reported differences in composition of the various samples taken from different locations in the lower female genital tract [44] as well as differences in sampling methods [45, 46]. In this study we report the changes in membrane bound (MUC1 and MUC4) and gel-forming mucins (MUC5AC and MUC7) relative to the influence of hormones and the presence of the abnormal vaginal flora of bacterial vaginosis.

## Material and Methods

### Study Population

This was a secondary analysis of samples collected as part of a study of the physical properties of vaginal fluid, and the complete methods describing the study populations are described elsewhere [22]. Written informed consent was obtained following a protocol approved by the University of Pittsburgh IRB. Women were excluded if they were: breastfeeding or pregnant; presented vaginal symptoms; had a hysterectomy; had been diagnosed with any cervical or vaginal infections or had used any antimicrobials in the past 14 days; had used any vaginal devices or vaginally-applied products (excluding tampons) in the past week. Upon enrollment the women had: an OraQuick advance rapid HIV test; a pregnancy test; their demographic information recorded; height and weight taken and medical, gynecologic and sexual histories taken. Cervicovaginal fluid (CVF) was collected from 165 women and characterized as: post-menopausal; first 14 days of cycle, (1–14 days of menstrual cycle); second 14 days of cycle, (15–30 days of menstrual cycle); oral contraceptives; depo- medroxyprogesterone acetate (DMPA); or women using the Mirena intrauterine device (IUD). Vaginal smears were Gram stained and evaluated using the Nugent criteria [34].

### Sample Collection

Cervical-vaginal fluid (CVF) was collected by inserting an Insteada Softcup<sup>®</sup> to the base of cervix. The fluid is allowed to collect for 45 min in place and the cup removed and placed into a 50 mL conical centrifuge tube and stored at 4°C until processing. The samples were transported to the laboratory within one hour for processing.

### CVF processing

The CVF samples were collected by centrifuging the cups at 2,000 x g for 10 minutes and the protein laden material scraped out and the volumes estimated. These samples were stored at -80°C.

### ELISA and ELLA assays

We used the same methodology for both the enzyme-linked immunosorbent assay (ELISA, used to measure mucins and human serum albumin) and the enzyme-linked lectin assay (ELLA, used to measure the carbohydrate structures). CVFs were diluted to give a final SDS (Sigma-Aldrich, St. Louis MO) concentration of 1% in 50mM sodium carbonate buffer. The samples were heated in a boiling water bath for 5 min, added to flat bottom 96 well clear microtiter plates (Nunc, Thermo Fisher, 75, Panorama Creek Dr., Rochester, NY 14625), at a concentration of 300–500 ng protein /100µL and allowed to air dry in an oven set to 40°C overnight. Plates were washed 4 times in phosphate buffered saline (PBS), blocking buffer was added (PBS plus 0.5% polyvinyl alcohol 30–50 KDa (Sigma-Aldrich, St. Louis, MO) and incubated at room temperature for 1 hr. Plates were washed 4 X with PBS. Horse radish peroxidase labeled lectins or antibodies (Vector Laboratories, Burlingame, CA 94010 Abcam, Cambridge, MA 02139, Novus Biologicals, Litton CO 80120) were diluted to the optimum concentration in PBS containing 0.5% PVA and 0.05% Tween 20 and 50 µL was added to each well. Plates were covered with aluminum foil and incubated on an orbital shaker at 300 rpm for 1 hour. Plates were washed 4 X with 0.05% Tween 20 in PBS. The plates were developed using 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma-Aldrich, St. Louis, MO, 63178) and incubated for an appropriate time. The reactions were stopped by the addition of 1N H<sub>2</sub>SO<sub>4</sub> and the optical densities read with a DTX880 Multimode Detector (Beckman-Colter,

Fullerton, CA 92834–3100 USA). Each plate included dilutions of MCF7 cell lysate (Santa Cruz Inc. Santa Cruz, CA.) as a mucin standard and an appropriate dilution of cervical mucus that was derived from a pool of four different women (Lee Bio-Solutions, St. Louis, MO). ELISA data are presented as ng/ng protein. Griffithsin binding was performed as the other lectins except the HIV glycoprotein gp120 (Protein Sciences Corp. Meriden, CT 06450) was used to generate the standard curve. Griffithsin and anti-Griffithsin antibodies were a generous gift from Ken Palmer (University of Louisville, College of Medicine, Louisville, Kentucky).

### Glycosidase assays

Glycosidase assays were performed exactly as previously described, in triplicate, using a modification of the methods in Moncla et al. [15,39]. Briefly, 4-methylumbelliferyl- sugar derivatives (Sigma-Aldrich, St. Louis, MO) ( $\alpha$ -D-glucoside,  $\beta$ -D-glucoside,  $\alpha$ -L-fucoside,  $\beta$ -D-fucoside,  $\alpha$ -D-galactoside,  $\beta$ -D-galactoside, and  $\alpha$ -D-N-acetyl neuraminic acid) were suspended in ultra-pure water Milli-Q (Millipore, Billerica, MA) to a final concentration of 0.2 mM. Cervical vaginal fluid was diluted to 2mg protein per ml saline and 80  $\mu$ L placed into the bottom of a glass tube. The samples then received 10  $\mu$ L of 0.1 M sodium acetate buffer, pH 5.0 and incubated at 37 C in a reciprocating shaker for ten minutes. Reactions were initiated by the addition of 10  $\mu$ L of the substrate. The reactions were stopped, at appropriate intervals, (from 20 to 60 min) by the addition of 300  $\mu$ L 0.1M borate buffer pH 9.2 prepared fresh daily. Precipitates were removed by centrifugation at 10,000 x g for 5 min and 100  $\mu$ L of the clear supernatants transferred to microtiter plate and read in a DTX880 Multimode Detector (Beckman-Coulter, Fullerton, CA), excitation light 365nm emission 450nm.

### Protein concentrations

Protein concentrations were determined using a modification of the Lowry assay [47, 48].

## Results and Discussion

A total of 165 women were enrolled in our study; of these, two samples were lost and not fully analyzed. The samples used are the same samples as used in previous reports [22, 23, 27, 28] and the population's characteristics are given in Chappell et al. [22].

We estimated the volume of CVF recovered (0.6–0.8 mL/participant) and the average protein concentration of the all samples was 1.4 mg/mL. We analyzed the albumin content of the entire sample since we believed it should remain constant and it did, see Tables 1 and 2.

### Impact of microbial flora

The Nugent criteria were used to characterize the vaginal microflora as *Lactobacillus*-predominant, intermediate or consistent with BV. Post-menopausal women are excluded from these analyses because the Nugent score has only been validated for use in women of reproductive age. When we stratified the data by glycosidases and lectin binding in the CVF across the Nugent categories, we observed differences in both glycosidase activities and glycan patterns.

These changes are significantly associated with the changing bacterial composition (Table 1). We have recently demonstrated that these changes include the increases in bacterial glycosidases that are concurrent with conversion of the vaginal microflora from normal to abnormal. The glycosidases have long been studied but it is only recently that their impact on the vaginal proteins has been demonstrated. The changing microflora is also correlated with increases in certain mucins and changes in the carbohydrate structures of the vaginal proteins [27, 28].

**Table 1. Summary of enzyme activity, lectin binding and mucin content of women stratified by microflora type, Nugent criteria.**

CVF	Nugent Score			P-value <sup>3</sup>
	Normal (n = 89)	Intermediate (n = 23)	Bacterial Vaginosis (n = 23)	
Glycosidases <sup>1</sup>				
Sialidase	0 (0–0.14) <sup>2</sup>	0 (0–0.17)	0.08 (0–0.25)	<0.001
α-fucosidase	0.007 (0–0.75)	0.005 (0–0.02)	0.006 (0–0.17)	0.51
α-galactosidase	0 (0–0.16)	0 (0–0.04)	0.004 (0–0.07)	0.006
β-galactosidase	0 (0–0.43)	0 (0–0.03)	0.003 (0–0.03)	0.005
α-glucosidase	0.12 (0–12.86)	0.16 (0–0.71)	0.28 (0–1.16)	0.056
β-glucosidase	0 (0–0.07)	0 (0–0.02)	0 (0–0.01)	0.23
Lectin binding				
<i>Maackia amurensis</i>	0.20 (0.06–0.60)	0.17 (0.07–0.50)	0.12 (0.07–0.26)	<0.001
<i>Sambucus nigra</i>	0.15 (0.06–1.03)	0.18 (0.09–0.85)	0.12 (0.07–0.29)	0.008
Griffithsin	186.32 (14.98–500)	171.29 (9.76–500)	69.13 (9.87–247.32)	<0.001
HSA	0.07 (0.05–0.10)	0.07 (0.06–0.09)	0.07 (0.06–0.10)	0.25
Mucins				
MUC1	0.35 (0.09–4.77)	0.42 (0.20–1.70)	1.13 (0.19–45.68)	<0.001
MUC4	0.49 (0.11–1.32)	0.53 (0.25–1.72)	0.72 (0.31–2.12)	0.001
MUC5AC	0.22 (0.10–0.49)	0.25 (0.15–0.40)	0.30 (0.17–0.60)	<0.001
MUC7	0.17 (0.04–0.70)	0.18 (0.06–0.44)	0.23 (0.06–0.71)	0.02

<sup>1</sup>Enzyme activity is expressed as μM hydrolyzed (4MU-glycosides) per min per mg of protein at pH 5.0. SNA and MAL binding is expressed as ng lectin bound per ng protein. Mucin content is reported as μg mucin per μg protein. Griffithsin binding is in pg bound/ng protein.

<sup>2</sup>Data presented as median (range)

<sup>3</sup>P-value from Kruskal-Wallis

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### Impact on glycosidase

The levels of glycosidases, in specific activity, in the CVFs of normal and intermediate women were very low; however, there were statistically significant increases in: sialidase, α-galactosidase, β-galactosidase and α-glucosidase ( $P = <0.001, 0.006, 0.005$  and  $0.056$  respectively), but not α-fucosidase or β-glucosidase in women with BV, see [Table 1](#). We saw the same enzymes increasing in the CVLs from these women with BV, but the median enzyme values for the CVLs were much higher and ranges were greater [27]. We believe most enzymes appear to be of bacterial origin. Bacteria are seldom observed in cervical mucus Gram stains, but CVLs usually contain  $>10^9$  bacteria per mL [49]. It is therefore highly likely that the higher enzymatic activity observed in CVFs is of bacterial origin. Further, the activity of the CVF could be the result of contamination upon removal of the Catamenial cup. Attempts to remove the bacteria and yeast from the samples have been unfruitful as the mucins sediment with the bacteria and yeast. We estimate that as much as one third of the CVL protein may be bacterial. Even this amount of bacterial protein should not affect the lectin binding assays since the quantities of sialic acid bearing bacteria would be a small part of the whole; however, bacterial enzymatic activities probably represent most or all of the enzyme activities present. These glycosidases are sufficient to alter the endogenous glycoproteins or cause changes in the glycosylation patterns or both.

### Impact of flora on lectin binding

We observed increased sialidase and glycosidases activities in the women with BV and hypothesized that we would observe significantly lower quantity of α-2,6 and α-2,3 linked sialic acids

**Table 2. Summary of enzyme activity, lectin binding and mucin content of women stratified by hormonal status.**

CVF <sup>1</sup>	Postmenopausal (n = 23)	Premenopausal, Proliferative (n = 19)	Premenopausal, Follicular (n = 23)	OCP (n = 24)	DMPA (n = 19)	IUD (n = 25)	P <sup>3</sup>
<b>Glycosidases<sup>2</sup></b>							
• Sialidase	0 (0–2.44)	0 (0–6.21)	0 (0–6.90)	0 (0–0.07)	0 (0–0.22)	0 (0–1.16)	0.08
• α-fucosidase	0.006 (0–0.02)	0.008 (0–0.46)	0.006 (0–0.02)	0.006 (0–0.01)	0.008 (0–0.75)	0.007 (0–0.05)	0.4
• α-galactosidase	0 (0–0.05)	0.001 (0–0.16)	0 (0–0.09)	0 (0–0.10)	0 (0–0.10)	0 (0–0.05)	0.58
• β-galactosidase	0 (0–0.15)	0.001 (0–0.16)	0 (0–0.43)	0 (0–0.08)	0 (0–0.09)	0 (0–0.05)	0.6
• α-glucosidase	0.005 (0–1.51)	0.15 (0.001–12.86)	0.11 (0–0.68)	0.15 (0–0.69)	0.19 (0–5.96)	0.10 (0–0.71)	<0.001
• β-glucosidase	0 (0–0.10)	0 (0–0.05)	0 (0–0.07)	0 (0–0.003)	0 (0–0.05)	0 (0–0.02)	0.39
HSA	0.07 (0.05–0.09)	0.08 (0.06–0.10)	0.08 (0.06–0.10)	0.07 (0.05–0.09)	0.06 (0.05–0.08)	0.07 (0.06–0.09)	0.03
<b>Lectin binding</b>							
<i>Maackia amurensis</i>	0.15 (0.07–0.37)	0.24 (0.07–0.54)	0.18 (0.06–0.60)	0.19 (0.09–0.35)	0.21 (0.10–0.53)	0.17 (0.08–0.45)	0.045
<i>Sambucus nigra</i>	0.21 (0.06–0.54)	0.19 (0.07–1.03)	0.15 (0.06–0.75)	0.13 (0.09–0.33)	0.20 (0.11–0.85)	0.15 (0.09–0.51)	0.043
Griffithsin	155.26 (14.20–500)	188.71 (18.99–500)	195.06 (9.76–500)	156.49 (23.76–500)	217.46 (43.62–500)	200.07 (19.11–285.09)	0.28
<b>Mucins</b>							
MUC1,	0.29 (0.05–0.98)	0.44 (0.13–1.70)	0.35 (0.13–3.75)	0.34 (0.14–4.77)	0.43 (0.22–4.21)	0.33 (0.09–1.07)	0.26
MUC4,	0.32 (0.33–1.83)	0.52 (0.29–1.72)	0.44 (0.25–1.32)	0.50 (0.11–0.76)	0.48 (0.31–1.25)	0.50 (0.24–0.72)	0.02
MUC5AC	0.19 (0.07–0.29)	0.25 (0.16–0.49)	0.23 (0.15–0.43)	0.22 (0.10–0.40)	0.27 (0.15–0.40)	0.20 (0.10–0.43)	0.07
MUC7	0.24 (0.09–0.69)	0.19 (0.04–0.42)	0.18 (0.06–0.70)	0.17 (0.06–0.53)	0.18 (0.07–0.44)	0.13 (0.04–0.24)	0.02

<sup>1</sup>Data from women with BV have been omitted from these analyses.

<sup>2</sup>Data presented as median (range), enzyme activity, μM hydrolyzed per min per mg of protein; SNA and MAL binding is expressed as ng lectin bound per ng protein. Mucin bound is reported as μg bound per μg protein. Griffithsin binding is in pg bound/ng protein.

<sup>3</sup>P-value from Kruskal-Wallis test

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in these women with BV. Comparing the lectin binding among the women stratified by microflora type, we found a significant decrease in the binding of three lectins, *Maackia amurensis* (MAL, preferentially binds to α-2,3 linked sialic acid,  $P = <0.001$ ), *Sambucus nigra* (SNA, preferentially binds to α-2,6 linked sialic acid,  $P = 0.008$ ) and Griffithsin (GRFT, binds high mannose structures,  $P = <0.001$ ) among women with BV compared to normal or intermediate women (Table 1). Although 4-methylumbeliferone derivatives assays are quick and easy for the detection of glycosidase enzymes, they do not provide information about the specificity of the enzyme. However, others working with vaginal swabs have demonstrated a sequential carbohydrate hydrolysis of glycoconjugates including SIgG and IgG [50, 51].

The reduction of the sialic acid binding sites in BV is consistent with the association of increased sialidase activity that has long been associated with the syndrome [39, 52, 53]. The correlation of sialidase activity with reduction in SNA and MAL binding supports our earlier

observations, demonstrating that sialidase activity is inversely correlated with the number of sialic acid binding sites [27]. The decreases in sialic acid specific lectin binding suggest that the sialidases are active against the glycoproteins in the female reproductive tract. By extension, the other enzymes detected and associated with BV are most likely as active against the glycoproteins as the sialidase. We have previously reported an increased level of two of the epitopes that would likely result from sialic acid removal, terminal  $\beta$ -galactosides and  $\beta$ -N-acetylgalactosides [28]. There are many potential contributors to the enzymatic milieu of the vagina, each of which shape the environment in its own way; for example, two bacteria associated with BV are *Gardnerella vaginalis* and *Prevotella bivia*. Sialidase is produced by all the *P. bivia* isolates but only one in four *G. vaginalis* isolates [40, 42, 54]. The *Prevotella* sialidase is produced by all strains tested and most active against  $\alpha$ -2,6-linked sialic acid; it is cell bound while the *Gardnerella* enzyme is only produced by 1 of 4 isolates and most active against  $\alpha$ -2,3-linked sialic acids. *G. vaginalis* also excretes large amounts of sialidase into the environment [27]. The two organisms have the same enzyme but given the differences between the two, we would expect them to have very different effects in modeling the vaginal environment. Secreted sialidases have the capability of reaching locations distal to the organisms themselves and in doing so, may remove sialic acid residues from carbohydrate chains far from the organism. This would not only make sialic acids available to other organisms capable of catabolizing them but would open the carbohydrate chains making them vulnerable to exo- and endo-glycosidase attack. Having many different organisms secreting glycosidases with different specificities is advantageous to the microbiome as a whole, not only for nutritional reasons or in invasive processes but also because the necessary genes may be distributed among different members of the population. The sugar moieties may come in many different forms, for example, sialic acids are often linked in an  $\alpha$ -2-3, 2-4 or 2-8 linkage to another sugar. Accordingly, many different sialidases are specific for one or more of these linkages. When we use some synthetic substrates for the measurements of glycosidases, we cannot directly observe the subtle nuances that are occurring.

Thus, there are many factors controlling the very complex lectin-glycan interactions. Work with *Streptococcus* spp. and other organisms that have exoglycosidases with exquisite substrate specificities and have shown that these enzymes are important in both promoting colonization and infection [55–57]. King et al. elegantly demonstrated that there is a sequential hydrolytic attack on the sugars on human glycoproteins by exoglycosidases produced by streptococci [56, 58–60]. Thus, it is important to realize that many of these glycosidases must act in concert and in some cases in sequence to effect carbohydrate removal.

The reduction in binding of all three lectins in women with BV was comparable in magnitude, suggesting the underlying mechanism may be the same. There is an impressive reduction of the high mannose present in the cervical mucus of women with BV. Since the high mannose binding is part of the innate immune system, this would lower women's defenses against pathogens that utilize such sugar motifs such as HIV and HSV [61, 62]. This is one possible explanation of why women with BV have an increased risk of HIV acquisition.

### Impact of flora on MUCs

When we stratified the data according to the Nugent score we omitted the postmenopausal women because the Nugent criterion is not accurate for these women. We found the median values for MUC1, MUC4, MUC5AC and MUC7 were significantly higher in women with BV (N = 23) than women with normal or intermediate Nugent scores (N = 112) ( $P$  values =  $<0.001$ ,  $0.001$ ,  $<0.001$  and  $0.02$  respectively), see Table 1. These values are consistent with our unpublished data of the levels of mucins in cervical-vaginal lavage.



These data indicate that the MUC proteins' synthesis is increased in women with BV. In contrast, we believe the data for the changes in the enzyme levels are due to the changes in the microbiota that results in a milieu rich in enzymes not found in the normal healthy vagina. These enzymes remove MAL, SNA and possible GRFT binding sites. However, the mucins are derived from the cervix. This increase in mucin production is supported by the *in vitro* work that demonstrates that bacterial components stimulate increased mucin production [63, 64]. In BV, the microflora shifts from a *Lactobacillus* dominant type flora to one where the gram negative anaerobic bacteria dominate.

It would seem easy to visualize the predominantly anaerobic gram negative organism associated with BV releasing lipopolysaccharides and other cellular components that would activate cytokines and so on to increase mucin production [63, 65–68]. Mucins also function as attachment sites and decoys for binding. Bacteria may attach to a specific sugar or sugar motifs that are part of the mucins; for example, *Helicobacter pylori* binds to the sialyl-lewis antigen of MUC7, as do *Pseudomonas aeruginosa* and *Haemophilus influenzae* [66, 69–71]. The increased quantities of mucin we measured may be the result of increased biosynthesis of these proteins or an increased rate of shedding, the purpose of which is unknown, but it may be to present the flora of BV with binding site decoys or to release already bound organisms [66].

Cytokines have been used to monitor microbicide safety as indicators of toxicity [72–75]. They are small glycoproteins and may be difficult to measure. There is considerable evidence that pro-inflammatory cytokines up-regulate the mRNAs of some mucins [67, 76]. Because the interactions of the many cytokines *in vivo* are so complicated, we decided to quantify the changes in the some of the end products of the pro-inflammatory cascade, for example the mucins, rather than the cytokines themselves [77, 78].

Our results suggest there is communication between the vaginal microflora and the mucin synthesizing apparatus of the upper female genital tract. Our data support the notion that as the microflora shifts from healthy to abnormal the message shifts to one that results in the increased production of mucin proteins. Conversely, a message could be from the *Lactobacillus* maintaining a normal healthy ecosystem.

After the mucus matures on the surface of the cervix it is shed and descends into the lower reproductive tract where it mixes with vaginal fluids, bacteria and so on. The material from the cervix is produced at a rate of 0.5 to 1 ml per hour. During its residency in the vagina, bacterial and any endogenous enzymes would result in hydrolysis of mucus components. When we lavage the women we should see a mixture of the altered mucus in the vagina and unadulterated cervical mucus that comes directly from the cervix.

## Impact of microflora on evaluation of hormonal status

The data for the effects of the microflora on the mucins tested compelled us to re-evaluate the data stratified by hormonal status without the effect of BV. Those results are presented here. Postmenopausal women had significantly less MUC4 than the women of reproductive age, [Table 2](#) ( $P = 0.02$ ). Among all the women of reproductive age, women using IUDs had lower levels of MUC7 in the CVF ( $P = 0.02$ ). There were no other statistically different results with any of the analytes we tested.

Numerous studies of cervical mucus have used indirect tools such as PCR and RNA for quantitation; however, there are considerable post-synthesis modifications to the basic proteins, thus making these methods indirect as they fail to actually measure what is actually present [5, 11, 26, 35, 63, 76].

We were somewhat surprised that we did not observe more differences when the values were compared between women in the proliferative versus the follicular phases; only MUC1

and MUC4 values were statistically significantly different ( $P = 0.04$  MUC1 and MUC4 versus all others).

The most interesting and surprising conclusion from these studies is that the influence of hormones is much less far reaching than that of the microflora. Both gel-forming and secreted mucins increase significantly in women with BV. These observations are consistent with many of the *in vitro* tissue models that demonstrated mucin production is up-regulated by bacterial metabolic products [63, 67, 76]

It was believed that MUC1 was released from the cell surface to allow attachment of the fertilized egg [1, 79, 80] but recently, it was determined that it is MUC16 that prevents cellular adhesion [1, 79–81]. Our results suggest that the three different contraceptives act differently on the control of mucin in the reproductive tract. MUC1 CVF values in the Depo-Provera group are higher than all the other groups except the pre-menopausal (days 1–15), in which we expect MUC1 to be higher [1, 38, 80, 82]. Mirena IUD users present values that differ from the other groups. Of the women using hormonal contraception, the Depo-Provera group demonstrated the highest levels of MUC1 and MUC4.

MUC1 and MUC4 are cell surface associated mucins and MUC5AC and MUC7 are gel-forming mucins; all increased significantly during the transition from normal to intermediate to bacterial vaginosis. The levels of MUC1 protein were three to nine times higher in CVF samples from women with BV.

The strong influence of the microflora on the expression of the mucins suggested that the inclusion of women with BV influences some of the observations of the cohort and when they were stratified by hormonal status (data not shown). Therefore, we analyzed the data excluding women with BV. These are novel observations, but are consistent with other works that reported that MUC5AC and MUC4 transcripts are upregulated *in vitro* by gram-positive and gram-negative organisms in respiratory explants [63]. MUC4 genes are important as they code for a sialoglycoprotein complex in the female genital tract [83, 84]. Increasingly, mucins are recognized as elements of an inflammatory response [85]. MUC5AC, Toll Like Receptors, broad spectrum antimicrobial peptides, IL-6, IL-8 and TNF- $\alpha$  in other systems, are upregulated by lipopolysaccharide and other bacterial components [21, 37, 63, 85–87].

MUC1, MUC4, and MUC5B mRNAs have been studied relative to the menstrual cycle [6, 35] and their role in fertility there has been extensively studied [6, 35, 88, 89]. Because we lacked a reliable antibody, we have not studied the MUC5B mucin. However, MUC5AC appears to behave as it does in other systems, where it responds to inflammatory cytokines and bacterial endotoxins [26, 81, 89, 90], and did not appear to be regulated by methods of contraception or hormonal levels in our study. This suggests a different role for these mucins in the vaginal vault and cervix that is separate from reproduction (unless the mucins play a dual role). The increased quantities of MUC7 observed in women with BV suggest this protein may play a protective role of some sort, as it appears to do in the respiratory system [91].

In this study, we are able to distinguish two distinct sets of phenomena. First, we see the shift of the microflora from the normal flora of primarily gram positive rods and cocci to a BV type of flora dominated by gram negative anaerobic rods. With this shift, we see increases in glycosidase activity, probably of bacterial origin, which is correlated with alterations of the proteins found in the vagina and cervix. We also see how women respond to the shift in their microflora by increasing the levels of specific mucins. These data suggest that MUC5AC and MUC7 function as they do elsewhere in the body, where they are part of the inflammatory response. This is consistent with other observations showing inflammatory cytokines in BV [21, 87, 92].

The distribution of the women by hormonal status in our studies was: post-menopausal (N = 23); pre-menopausal, days 1–14 of cycle (N = 19); pre-menopausal, days 15–30 of cycle

(N = 23); those using oral contraceptives (N = 24): DMPA (medroxyprogesterone acetate, Depo-Provera, (N = 19), or the Mirena IUD (N = 25).

We found only one statistically different enzyme result:  $\alpha$ -glucosidase activity was lower in postmenopausal women ( $P = <0.001$ ) when compared to all reproductive age women. Because there is a decrease in *Lactobacillus* in postmenopausal women [93–95], we believe the low level of this enzyme activity is a reflection of lower levels of *Lactobacillus* and other microorganisms that secrete the enzyme to utilize poly-glucose polymers like glycogen for carbon and energy.

### Impact on lectin binding

Among the hormonal groups, postmenopausal women had increased SNA binding (detects  $\alpha$ -2,6-linked sialic acids) and decreased MAL binding (detects  $\alpha$ -2,3-linked sialic acids) bound sialic acid  $P = 0.043$  and  $0.045$ , respectively. There were no differences in the content of high mannose structures in any of the hormonal groups as determined by griffithsin binding (Table 1). In a previous study of the cervical-vaginal lavage samples from these same women, the quantities of SNA and MAL bound were comparable to the value we report here. However, in the previous study the quantities of GRFT bound ranged from 5 to 11 pg bound per ng protein, but here we report values ranging from 155 to 217 pg bound per ng protein. Thus, the CVF has about 18–20 fold higher concentrations of GRFT (binding sites) than does the CVL. The changes in sialic acid lectin binding were small and certainly less than we anticipated considering the mRNA work as well as our earlier studies with the CVL samples from the same women [1, 3, 5]. We did observe a major difference in the amount GRFT binding proteins. It is difficult to understand why these values did not change in concert. It at very least suggests there is a substantial loss of one or more of the proteins that have left the cervix to become part of the vaginal fluid and further indicates the extent to which microbial action is occurring. With MAL and SNA and our other lectins it is possible there may be some contribution by bacteria since it is well established that many bacteria display sialic acids on their surfaces [1, 3, 5, 32, 96, 97]. But as of yet high mannose, the GRFT binding structure, has not been identified in bacteria.

### Impact on MUC concentrations

We observed higher, but not highly statistically significant, levels of MUC1 in days 1–14 of cycle and DMPA-using women; MUC4 was lower in days 15–30 of cycle ( $P = 0.02$ ); MUC5AC was lower in postmenopausal women and women using IUDs; and MUC7 was lower in IUD-using women (Table 2).

Overall, when compared with our previous studies of CVLs, the values observed for the enzyme activities were much lower in the CVFs than the CVLs. The quantities of sialic acid binding lectins bound were almost identical in the CVL and CVF samples of postmenopausal women. This suggests that vaginal bacteria are responsible for the enzyme activity, as the concentration of bacteria in the CVLs are about  $10^8$  or more and there are very few organisms in or on the cervix of healthy women. Furthermore, it indicates that the cervical mucus samples collected are relatively free of bacteria, or that bacterial enzymes could contaminate the Catamenial cup during sample collection.

### Conclusions

We have demonstrated that in the CVFs of women with bacterial vaginosis, there are greater amounts of four of the six glycosidases studied and we think it highly likely these enzymes are of bacterial origin. These enzymes may be responsible for altering the vaginal and cervical glycomes. CVF sialidase activity is associated with decreases in the binding of sialic acid binding

lectins. Women with BV also had significantly less high mannose binding sites as well as increases in all four of the mucins measured, compared to normal or intermediate scoring women. The decrease amount of GRFT binding sites could be an important key in understanding the impact of BV on innate immunity.

When we stratified by hormonal status, we were able to detect a very subtle picture. The post-menopausal women bound more SNA (specific for  $\alpha$ -2-6 linked sialic acids) and less MAL (specific for  $\alpha$ -2-3 linked sialic acids), but there was no difference in GRFT binding. The mucin levels demonstrated very subtle differences that were affected by hormones. MUC1 was higher in the first 14 days of the menstrual cycle and in women using DMPA. MUC4 was lower in days 15–30 of the cycle. IUD users had lower MUC5AC and MUC7 and post-menopausal women had lower MUC7. Quantitatively, the data presented here are comparable to those present in our work with CVLs, except for the binding of GRFT that is far greater (20–30 fold) in the CVF than the CVL. Thus, the cervix and vaginal vault glycomes are dynamic systems that respond to internal signals such as hormones and to other signals such as the vaginal microbiome.

## Supporting Information

### S1 Table. Reagents used for the work presented.

(DOCX)

### S2 Table. Primary data used to derive the data presented.

(XLSX)

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## Author Contributions

Conceived and designed the experiments: BJM CAC. Performed the experiments: BMD BJM. Analyzed the data: BJM CAC LAM. Contributed reagents/materials/analysis tools: BJM CAC LAM. Wrote the paper: BJM CAC LAM. Overall concept: BJM. Clinical design: CAC. Statistical analysis: LAM.

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