4-24-2020

The Relationship Between the Cervical Microbiome and Cervical Cancer Risk in Sub-Saharan Africa

Cameron Klein
University of Nebraska - Lincoln, kleincamerong@gmail.com

THE RELATIONSHIP BETWEEN THE CERVICAL MICROBIOME AND CERVICAL CANCER RISK IN SUB-SAHARAN AFRICA

by

Cameron G. Klein

A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Biological Sciences

(Genetics, Cellular, and Molecular Biology)

Under the Supervision of Professor Peter C. Angeletti

Lincoln, Nebraska

May, 2020

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Cameron G. Klein, Ph.D.

University of Nebraska, 2020

Advisor: Peter C. Angeletti

Despite ongoing efforts, sub-Saharan Africa faces a higher cervical cancer burden than anywhere else in the world. Besides HPV infection, definitive factors of cervical cancer are still unclear. Dysbiosis of the cervicovaginal microbiota, particularly involving sexually transmitted infections, is associated with increased cervical cancer risk. Notably, HIV infection, which is prevalent in sub-Saharan Africa, greatly increases risk of cervicovaginal dysbiosis and cervical cancer. To better understand and address cervical cancer in sub-Saharan Africa, a better understanding of the regional cervicovaginal microbiome is required. In this study, I establish the relationship between cervical cancer, HPV, HIV, cervicovaginal infections, and the cervicovaginal microbiome in sub-Saharan Africa.

To investigate the role of the bacterial microbiome in cervical dysplasia, cytobrush samples were collected directly from cervical lesions of 144 Tanzanian women and analyzed using 16s metagenomic sequencing. I found that cervical microbiota varied significantly depending on HIV infection, HPV infection, and the presence of cervical lesions. The bacterial family '*Mycoplasmataceae*' in particular was associated with the presence of pre-cancerous cervical lesions.

Mycoplasmataceae infection in sub-Saharan Africa is not well understood, especially when considering the differences between sexually transmitted species. To

establish the prevalence of common *Mycoplasmataceae* cervical infections and evaluate their relationship with risk factors for cervical cancer, a cohort of 1160 Tanzanian women responded to an epidemiological questionnaire and were tested for HIV, HPV, cervical lesions, *Mycoplasma genitalium*, *Mycoplasma hominis*, *Ureaplasma spp.*, and *Lactobacillus iners*. I found that *Mycoplasmataceae* infection was present in 66% of women tested. In particular, *Mycoplasma hominis* was associated with HPV and HIV infection, and significantly increased in relative abundance among women with pre-cancerous cervical lesions.

The results of this study suggest that intracellular, inflammatory infections like *M. hominis* are prevalent and relate to the development of pre-cancerous cervical lesions. The prevalence of cervical cancer in sub-Saharan Africa may be partially attributed to the high prevalence of cervical infections like *M. hominis*.

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LIST OF COMMON ABBREVIATIONS

ART	Antiretroviral therapy
ASC-H	Atypical squamous cell, cannot exclude high-grade lesion
ASC-US	Atypical squamous cells of undetermined significance
BV	Bacterial vaginosis
CST	Community state type
Db-RDA	Distance-based redundancy analysis
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSIL	High-grade squamous intraepithelial lesion
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
LSIL	Low-grade squamous intraepithelial lesion
MANOVA	Multivariate analysis of variance
NILM	Normal for intraepithelial malignancy
OR	Odds ratio
ORCI	Ocean Road Cancer Institute
OTU	Operational taxonomic unit

PID	Pelvic inflammatory disease
STI	Sexually transmitted infection
VIA	Visual inspection with acetic acid
WGS	Whole genome sequencing

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

In sub-Saharan Africa, cervical cancer risk is far greater than in developed countries. Human Papillomavirus (HPV) is a major health concern worldwide, contributing to an estimated 4.8% of all cancers (Formana et al., 2012). This percentage drastically increases in less developed regions of the world, with HPV attributing to 14.2% of all cancers in sub-Saharan Africa (Formana et al., 2012). It is well-established that HPV is the causative agent of cervical cancer (De Vuyst et al., 2013). In 2018, 570,000 women were diagnosed with cervical cancer, and 311,000 deaths were attributed to cervical cancer (Arbyn et al., 2020). It is estimated 1 in 70 women worldwide will develop cervical cancer before reaching 79 years of age (De Vuyst et al., 2013; Fitzmaurice et al., 2015). Cervical cancer disproportionately affects sub-Saharan Africa, where 9% of the world's female population accounts for 14% of the world's incident cervical cancer and 18% of cervical cancer related deaths (De Vuyst et al., 2013). This results in a cervical cancer mortality risk of 2.7%, about 70% higher than the second highest region: South-Central Asia (De Vuyst et al., 2013). In 2013, cervical cancer was the most common cause of cancer death in women in 46 of 54 sub-Saharan African countries (85%) (Fitzmaurice et al., 2015). Only 5 countries outside of sub-Saharan Africa count cervical cancer as the most common cause of cancer death in women. Despite current efforts against cervical cancer in sub-Saharan Africa, it is estimated the number of cervical cancer cases will continue to rise, highlighting the need to bring sub-

Saharan Africa up to modern standards for HPV treatment and prevention, and to understand the factors contributing to cervical cancer in the region (Williamson, 2015).

sub-Saharan Africa faces many unique issues regarding cervical cancer. Screening and prevention practices, sociocultural aspects, HPV genotype prevalence, HIV prevalence, HIV treatment, sexually transmitted infections (STIs), and the composition of the cervicovaginal microbiome are all important factors, which differ in sub-Saharan Africa compared to more developed regions. While some of these factors have been correlated with increased cervical cancer incidence, it is unclear how or if they contribute to HPV pathogenesis.

Screening is the key to early detection and treatment of cervical cancer and identifying at-risk populations. Cervical cancer rates in developed countries with screening and treatment programs, have cervical cancer rates below 10 per 100,000 women. In The USA, coverage of these screening programs within a 48 month period is 94% of women ages 25 to 29 years, decreasing at older ages to 69% at 45 to 49 years and 55% at 60 to 64 years (Cuzick et al., 2014). In countries without screening programs, cervical cancer rates are significantly higher (Fitzmaurice et al., 2015). In sub-Saharan Africa, screening methods and their efficiency vary significantly. Because of this, coverage is difficult to estimate and largely based on speculation. The most common cervical cancer screening method in sub-Saharan Africa is visual inspection with acetic acid (VIA) rather than the pap smear, the preferred method in developed countries. VIA

is cost effective, but is known to be less specific since it depends on visual recognition of lesions, whereas the pap smear identifies abnormalities at the cellular level (Gaffikin et al., 1999). The major factor contributing to the high incidence of cervical cancer in sub-Saharan Africa is the lack of reliable cervical cytology screening. Historically, introduction of population screening programs has reduced cervical cancer incidence by 25–77% (Gustafsson et al., 1997). Establishing better screening programs is a necessary step toward reducing the burden of cervical cancer in sub-Saharan Africa, however this alone is not enough to address the issue. Ignoring the contribution of current screening efforts in sub-Saharan Africa, even the most drastic decrease in cervical cancer after implementation of population screening seen historically (77%) would not bring cervical cancer rates as low as those in developed countries with population screening. This emphasizes the importance of understanding and addressing what other factors in sub-Saharan Africa are contributing to cervical cancer.

Besides screening, most factors correlated with developing cervical cancer relate to the cervical immune microenvironment. Recent research into the cervicovaginal microbiome has uncovered intricate relationships between the bacterial microbiota, HPV, HIV, and cervical cancer (Godoy-Vitorino et al., 2018; Huang et al., 2018; Klein et al., 2019). These relationships suggest that certain cervicovaginal microbes, or the microenvironment created by certain microbes, are cofactors of cervical cancer progression. HIV is a well-studied factor in sub-Saharan Africa, which influences the cervical microbiota (Curty et al., 2017). Cervical cancer is classified as an AIDS-defining cancer due to greatly increased risk among HIV positive individuals with low T cell

count. Despite extensive study of the prevalence and impact of HIV in sub-Saharan Africa, the exact mechanism by which HIV infection contributes to HPV driven cervical cancer remains unclear. A better understanding of other correlated factors will help clarify the mechanisms which drive cervical cancer, and address how to bring sub-Saharan Africa in line with other regions. STI screening of genital tract infections like chlamydia and gonorrhea has found they are much more prevalent in sub-Saharan Africa, while, metagenomic studies of the cervicovaginal microbiome have shown significant differences between the commensal and non-commensal components of sub-Saharan African microbiomes when compared with low cervical cancer risk areas. Considering such infections have been associated with pre-cancerous lesions, it is likely these differences, in part, account for sub-Saharan Africa's increased cervical cancer risk (Onywera et al., 2019).

Defining differences in cervical microbiota by geographic location, HIV status, and cervical cytology using compiled published data is difficult due to major differences in cohort makeup, cohort size, sampling and sequencing techniques, and other issues. The cervical microbiota varies greatly between individuals. Factors such as age, race, menstrual phase, and lifestyle have all been shown to affect the microbiome. Controlling for such a large number of factors is difficult, which has hindered the discovery of definitive microbiota. Furthermore, the microbiota of the cervix has been shown to be significantly different than that of the vagina, thus studies which sample the cervicovaginal microbiome do not best represent the microenvironment at the site of cervical transformation (Koedooder et al., 2019). For these reasons, the results of studies

are considered individually in this review, so that each speaks only for the niche represented by its cohort. To better understand and address the relationship between HPV, HIV, cervical microbiota, and increased cervical cancer risk, a better understanding of the unique sub-Saharan African environment is needed. Here, we discuss current knowledge in each of these areas, highlighting factors especially prevalent in sub-Saharan Africa which may drive HPV-dependent cervical cancer.

HPV GENOTYPES

The HPV family includes more than 200 genotypes, over 45 of which are known to infect the anogenital region. The regional prevalence and oncogenic potential of HPV genotypes varies significantly. Fifteen anogenital HPVs are classified as high-risk for development of cervical cancer (Guan et al., 2012). Among these, HPV16 and 18 are the predominant oncogenic genotypes, causing approximately 70% of cervical cancer cases globally (Ogembo et al., 2015). The relative oncogenic potential of HPV 16 and 18 has been shown to be markedly higher than that of other genotypes, followed by 45, 69, 58, 31, 33, 34, 67, 39, 59, 73, and 52 by decreasing oncogenic potential (Bernard et al., 2013). Of the global HPV burden, 22.5% of HPV infections are estimated to be produced by HPV-16, however, a significant inverse correlation has been observed between overall HPV prevalence and the contribution of HPV-16, with the lowest HPV16 proportions in the regions with the highest HPV prevalence (Bruni et al., 2010). As such, sub-Saharan Africa has been shown to have the lowest HPV-16 contribution to total HPV infections in women with normal cervical cytology when compared to other regions, with estimates of 13.7, 11.3, and 11.1% for Southern, Eastern, and Western Africa, respectively (Bruni et

al., 2010). This correlation is even more pronounced in cervical cancer, where HPV16 and 18 are less frequent in sub-Saharan Africa than in the rest of the world (49.4 vs. 62.6%), while HPV18 and HPV 45 are two times more frequent (19.3 vs. 9.4% and 10.3 vs. 5.6%) (Ndiaye et al., 2012). After HPV16 and 18, the most prevalent genital HPV genotypes vary between sub-Saharan Africa countries. Overall, HPV 52, 35, 58, 33, 31, 45, 53, and 51 are the most prominent non-16/18 genotypes in sub-Saharan Africa (Formana et al., 2012; Abate et al., 2013; Olesen et al., 2013; Adler et al., 2014; Boumba et al., 2014, 2015; McDonald et al., 2014; Mihret et al., 2014; Padalko et al., 2015; Pirek et al., 2015; Van Aardt et al., 2015; Bateman et al., 2015; Lebelo et al., 2015; Okonko and Ofoedu, 2015). When comparing prevalence with high-income regions, HPV 52, 58, 33, and 45 stand out as especially prevalent in sub-Saharan Africa (Human Papillomavirus and Related Diseases Report WORLD). Because these HPV genotypes are only common in sub-Saharan Africa, they have not been as well-researched as globally prevalent HPVs such as 16 and 18. Potential differences in pathogenesis in such genotypes may contribute to increased cervical cancer in sub-Saharan Africa, where a larger percentage of cervical cancer cases are attributed to non-HPV16/18 genotypes.

Accurate detection and identification of HPV genotypes depends upon the genotyping method used. Most large studies use one of several established genotyping assays, however more recent studies using sequencing-based identification of HPV genotypes have found that genotyping assays may only detect as little as 49% of those able to be detected with sequencing (Ndiaye et al., 2012). The bias introduced by genotyping assays may downplay the significance of certain HPV genotypes in sub-

Saharan Africa, especially those which have not been well-researched, such as HPV 34, 67, 69, and 73. Additionally, the extensive sequence variation within HPV genotypes, which has been demonstrated to be especially severe in sub-Saharan Africa, is not accounted for by genotyping assays as it is in sequenced-based approaches, and may be of clinical importance. Sequence variation of HPV may also contribute to reduced efficacy of HPV vaccination in sub-Saharan Africa, while increased genotypic diversity of HPV almost certainly does.

Concurrent cervical infection with multiple HPV genotypes is common in sub-Saharan Africa, however it is not clear if this represents a specific mechanism driving pathogenesis. Data from developed regions suggests multiple infection with HPV decreases in cervical cancer cases, however studies in sub-Saharan Africa suggest coinfection is more prevalent in cervical cancer and may exacerbate HPV pathogenesis. A study of South African women with cervical cancer found that 65% were coinfected with at least two HPV genotypes (Lebelo et al., 2015). Of the coinfected cervical cancer cases, 90.4% included HPV16, suggesting infection with other HPV genotypes may contribute to HPV16 driven cervical cancer. Similar results were found in a study of women in the Democratic Republic of Congo (Boumba et al., 2014). Further work has shown higher HPV16 viral loads in 70.3% of HPV16 coinfected samples (Lebelo et al., 2015). In a separate study, Cameroonian women with normal cervical cytology and multiple HPV infections were found to be about 10% more likely to develop cervical lesions within a year when compared to women infected with a single HPV type (Pirek et al., 2015). These results suggest a synergistic effect driving HPV replication and cervical cancer pathogenesis in cases of multiple HPV infection.

In contrast, other publications have suggested HPV16 may be more sensitive to attack from other genotypes, and thus may be at higher risk of competition when there is more immune suppression (Menon et al., 2016). A meta-study examining global HPV prevalence found that multiple HPV infections were, on average, 6% more common in women with normal cytology than in those with cervical cancer (Bernard et al., 2013). Sub-Saharan Africa was the least represented region by studies included in this analysis, allowing for the possibility that synergistic effects in cases of multiple infection are primarily found in the genotypes most prevalent in sub-Saharan Africa. Further research focusing on the long-term oncogenic potential of different combinations of HPV genotypes, is necessary.

HIV

HIV is the best studied co-factor to cervical cancer and has been strongly linked to severe HPV pathogenesis. The association between severe HIV pathogenesis and cervical cancer has classified cervical cancer as an "AIDS-defining cancer." In addition to increasing cervical cancer risk, evidence suggests that high HIV prevalence also contributes to increased prevalence and circulation of HPV (Williamson, 2015). Similarly, the widespread prevalence of multiple HPV infections has been shown to contribute to the spread of HIV by increasing susceptibility of HIV acquisition. Multiple studies have shown that immune response to HPV increases HIV-susceptible cells in both male and female genital tracts, increasing the opportunity for an initial infection to occur

based on the local immune microenvironment (Averbach et al., 2010; Tobian et al., 2013; Williamson, 2015). The regional relationship between HIV prevalence and cervical cancer is shown in Figure 1.1, which demonstrates associations between the two globally, highlighting the exceptionally high rates seen in sub-Saharan Africa.

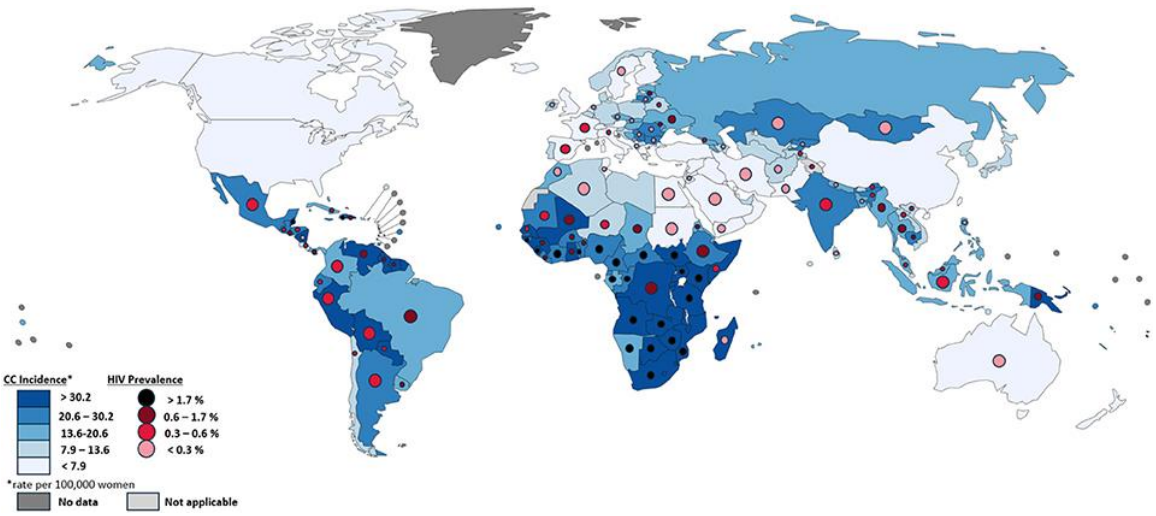

Figure 1.1 Comparison of cervical cancer incidence and HIV Prevalence by country. Each country is colored by cervical cancer incidence per 100,000 women, as described in the bottom left, based on data from GLOBOCAN 2012. Circles within each country's borders are colored by HIV prevalence, as described in the bottom left, based on data from UNAIDS (2016). Differences in the size of circles within countries is only for visibility and does not signify anything meaningful. Countries without HIV prevalence circles did not have such data available. Map produced by IARC.

Risk factors and predictors of cervical cancer are also increased in HIV+ individuals. HPV infection, abnormal Pap smears, and high-grade lesions are significantly more common in HIV+ women (Adler et al., 2014; Salazar et al., 2015). In addition to the increased rate of productive HPV infection, HIV is associated with a higher risk of progression from subclinical to clinical HPV disease (Williamson, 2015). Higher HPV viral loads are associated with increased risk of abnormal cervical cytology, and are seen among those co-infected with HIV, indicating this may in part be due to an undefined mechanism by which HIV infection influences HPV viral replication (Depuydt et al., 2012; Wang et al., 2013; Hanisch et al., 2014; Mbulawa et al., 2014). A likely factor is a decrease in T-cell surveillance controlling HPV replication with decreasing CD4+ cell count as a result of more severe HIV infection. Multiple studies have shown an increase in HPV detection, squamous intraepithelial lesions, and cervical intraepithelial neoplasia in individuals with AIDS (less than 200 CD4+ cells per μl serum) (Hanisch et al., 2013; Ezechi et al., 2014; Memiah et al., 2015; Menon et al., 2016). Identifying which aspects of the local and systemic effects of HIV infection contribute to progression from chronic HPV infection to cervical cancer is crucial to understanding the burden of cervical cancer in sub-Saharan Africa. Current knowledge suggests effects on the cervical immune microenvironment may be key in this process.

In HIV+ populations, there is a shift in prevalence of HPV genotypes, favoring high-risk HPVs (Ezechi et al., 2014). The reasoning for greater prevalence of certain HPV genotypes in HIV+ individuals is not currently well-understood. The influence of HIV may help explain why coinfection of multiple HPV genotypes is associated with

cervical cancer in sub-Saharan Africa, but not elsewhere. Studies from several sub-Saharan African countries have identified a greater number of multiple HPV infections among HIV-positive women (Akarolo-Anthony et al., 2013; Maranga, 2013; Adler et al., 2014; McDonald et al., 2014; Van Aardt et al., 2015). A study of South African females with cervical cancer found multiple HPV infections in 8% of HIV- women and 27% of HIV+ women (Van Aardt et al., 2015). In a study of South African adolescent females, the prevalence of multiple infections was found to be much higher in both HIV positive and negative individuals, with 22% percent prevalence in HIV- and 68.6% in HIV+ (Adler et al., 2014). Only 18.8% of all adolescents in this study had an abnormal pap smear, and none of them were diagnosed with cervical cancer. This supports the idea that exposure to many HPV types occurs early after sexual debut, with certain genotypes becoming dominant by the time HPV pathogenesis reaches cervical cancer. Potentially, infection with "accessory" HPV genotypes contributes to the early pathogenesis of a primary high-risk HPV either directly or through manipulation of the immune microenvironment, leading to increased replication and eventual faster or more frequent development of cervical cancer. These "accessory" HPV infections may then be cleared by a competent immune response, which may explain why multiple infection decreases in HIV- cervical cancer cases, but not in HIV+. Based on current evidence, it is yet unclear whether early multiple HPV infections expedite progression to cervical cancer. A short-term longitudinal study (16 months) was unable to find any additive or synergistic effect of multiple infection on development of cervical lesions, noting that increased frequency of cervical lesions was associated with infection of a single high-risk HPV. Cervical cancer development occurs over a period of decades however; looking at such a narrow

time frame means these results may be a consequence of only observing cytological effects in infections in which a high-risk HPV was already established and progressing toward cervical cancer, not early interactions which eventually contribute to lesions (Salazar et al., 2015). Further study focusing on interactions and outcomes in HPV coinfection, especially among young HIV+ women who have not yet developed cervical dysplasia, is desirable to clarify this relationship.

When available, treatment for individuals infected by HIV in sub-Saharan Africa is primarily antiretroviral therapy (ART). Unlike high-income regions, a significant number of HIV infected sub-Saharan African individuals go without treatment. ART coverage of HIV infected individuals across sub-Saharan Africa ranges from 9-92% (UNAIDS, 2019). Studies examining the effects of ART on HPV pathogenesis have had mixed results. While previous studies suggest ART has no significant effect on HPV genotype detection, more recent studies suggest modern ART reduces the prevalence of high-risk HPV's in HIV infected women (Palefsky, 2003; Ezechi et al., 2014; Zeier et al., 2015). This reduction in high-risk HPV prevalence grows with duration of ART use. Besides a reduction of HIV, the effects of ART on cervical microbiota are currently unknown, but may be significant, as several studies have found that ART affects the gut microbiota. ART does not appear to have a significant effect on cervical lesions and tumor development, and only minor effects on limiting progression of lesions and preventing recurrence (Ahdieh-Grant et al., 2004; Paramsothy et al., 2009; Dryden-Peterson et al., 2015; Memiah et al., 2015). A study of Kenyan women found that the spread of ART has been accompanied with a decrease in age-specific cancer risk,

however an increase in the number of HPV cancers, which is attributed to an aging HIV+ population rather than to any effect of ART (Memiah et al., 2015). Compared to the risk reduction after ART seen in other AIDS-defining cancers like Kaposi's sarcoma and non-Hodgkin's lymphoma, the risk of cervical cancer is not significantly affected, and recurrence rates remain high with or without treatment (Foulot et al., 2008; Mungo et al., 2013; Russomano et al., 2013; Cobucci et al., 2015). This suggests that HPV depends on immunological status of the host such that ART is only able to indirectly affect HPV pathogenesis, potentially through an effect on circulatory CD4+ cell count and microbiota composition.

NON-VIRAL MICROBIOTA

Several studies have proposed that the cervicovaginal microbiota is a co-factor of the development of cervical lesions (Guijon et al., 1992; Mitra et al., 2016; Kyrgiou et al., 2017). The precise mechanism, and the microbes responsible have not been identified, but several common STIs have been associated with cervical cancer individually. Health of the lower female reproductive tract, and its ability to defend against dysbiosis and infection, is directly related to the microbiota present. Its defense mechanisms include antimicrobial peptides, a microbiome dominated by *Lactobacilli*, and a pH of <4.5. An imbalance in these defenses can result in physiochemical changes, which produce histological alterations of the vaginal mucosa and cervical epithelium (Audirac-Chalifour et al., 2016). Communal differences in the cervical microbiome between sub-Saharan Africa and developed regions have not been well-established, however the prevalence and incidence of pathogenic cervicovaginal microbiota is much higher in sub-Saharan

Africa. Among factors associated with preventing or developing cervical cancer, cervicovaginal pathogens are second only to HPV vaccine coverage when comparing differential rates in sub-Saharan Africa and North America (Table 1.1). Nearly all studies of the cervicovaginal microbiome in sub-Saharan Africa to date have used sequencing of the ribosomal RNA 16s amplicon, which only includes bacteria. Because of this, little is known of the virome or other non-bacterial members of the microbiome outside of targeted screening. Whole genome sequencing (WGS) allows characterization of the microbiome in its entirety and has been shown to be more accurate at the detection of bacterial species and diversity than 16s (Ranjan et al., 2016). RNA sequencing (RNASeq) is another powerful approach to characterize gene expression, which is now being used in microbiome studies. Large scale metagenomic studies of sub-Saharan African populations using WGS is needed to more fully characterize the microbiome and address potential bias introduced by 16s sequencing. These newer methods are likely to improve our understanding of these complex microbial networks.

Table 1.1 Comparison of factors which may influence cervical cancer in sub-Saharan Africa and North America

	North America	Sub-Saharan Africa	% Difference in sub-Saharan Africa	Fold difference
HPV Vaccine Coverage[a]	35.6%	1.2%	3%	29.7–
Neisseria gonorrhoeae Incidence	259.3	5,500	2,121%	21.2+
HIV Prevalence	0.3%	4.38%	1,460%	14.6+
Smoking Prevalence[b]	15.67%	2.3%	15%	6.8–
Trichomonas vaginalis Prevalence[c]	3.1%	20.2%	652%	6.5+
cervical cancer Incidence[d]	6.6	35.18	533%	5.3+
Chlamydia trachomatis Incidence	456.1	2160	474%	4.7+
Proportion of Adenocarcinoma cervical cancer	18%	5.5%	30.6%	3.3–
Fertility[e]	1.9	4.7	247%	2.8+
HPV Prevalence (NILM)	13.8%	22.9%	166%	1.7+
Anti-Retroviral Therapy Coverage	59%	40.2%	68%	1.5-
Bacterial Vaginosis	29.2%	41.4%	142%	1.4+
HPV 16/18 Prevalence in cervical cancer	71.4%	62.8%	88%	1.1–

[a] *Women aged 10–20, full-course.*
[b] *In surveyed females 15+.*
[c] *Women ages 14+.*
[d] *Age standardized rate per 100 k women per year.*
[e] *Children per woman.*

NON-COMMENSAL MICROBIOTA

Chronic inflammation of the cervix is closely associated with developing cervical cancer (Giraud et al., 1998; Skapinyecz et al., 2003; Ilhan et al., 2019). Cervicitis can result from several different conditions, which often are attributed to infection with non-commensal microbes. Pelvic inflammatory disease (PID) in women usually results from bacterial infection of the cervix ascending to the uterus and oviducts, wherein certain bacteria express antigens which induce a chronic inflammatory state. The association observed between PID and cervical cancer is thought to be due to development of a microbiome rich in inflammation-inducing bacteria at the cervix, causing cervicitis. Not surprisingly, PID is more prevalent in HIV-infected women than uninfected (Dehon et al., 2016). The overall prevalence of PID is difficult to define in sub-Saharan Africa, however diagnosis of PID is more than twice as likely to be attributed to a bacterial infection when compared to rates in the developed world (Ross, 2008). This suggests that bacterial infections more often contribute to HPV pathogenesis in sub-Saharan Africa.

Bacterial vaginosis (BV) is a dysbiosis of cervicovaginal bacteria which, like PID, is associated with cervicitis (Lehtinen et al., 2011; Ogembo et al., 2015). BV alters the cervicovaginal microenvironment, which may increase cervical dysplasia as a result of anaerobic infection producing nitrosamines, which cause cervical inflammation (Lazenby et al., 2014). The microenvironment created by BV has also been identified as a cofactor in the persistence of HPV infection (Gillet et al., 2011; Clarke et al., 2012; Guo et al., 2012; Vriend et al., 2015). Several of the causal bacteria of BV are associated with cervical lesions and/or inflammation. The most common causes of BV are: *Gardnerella*

vaginalis, Atopobium vaginae, Mobiluncus curtisii, Mobiluncus mulieris, Megasphaera type 1, Megasphaera type 2, Sneathia sanguinegens, Mycoplasma hominis, Mycoplasma genitalium, Ureaplasma urealyticum, Bacteriodes fragilis, and bacterial-vaginosis-associated-bacteria (BVAB) 1-3 (Signat et al., 2011; Audirac-Chalifour et al., 2016). Comparative genomic analysis has shown that the shift in microbial diversity as a result of BV is more pronounced in women infected with HIV, suggesting BV is more severe in this population and thus more likely to drive HPV pathogenesis (Spear et al., 2008). In sub-Saharan Africa, BV prevalence is estimated to range from 20 to 50% in reproductive aged women, making it the most common cause of cervicovaginal dysbiosis. This prevalence suggests BV, or the cervical microenvironment created by BV, could be a major contributor to increased malignant HPV pathogenesis in the region, especially among HIV+ women (Msuya et al., 2002; Lewis, 2011; Swanepoel et al., 2013). Further study is necessary to determine if general inflammation caused by conditions like BV and PID is sufficient to promote HPV pathogenesis, or if specific microbes which contribute to the diseases are responsible.

STIs also alter the cervicovaginal microenvironment. Several sexually transmitted microbes have been associated with cervicitis and persistence of HPV infection (Gillet et al., 2011; Lehtinen et al., 2011; Clarke et al., 2012; Guo et al., 2012; Ogembo et al., 2015; Vriend et al., 2015). Among these, *Neisseria gonorrhoeae, Chlamydia trachomatis*, *Trichomonas vaginalis*, and *Syphilis* are particularly common in sub-Saharan Africa. Sub-Saharan Africa accounts for a disproportionate 20, 9.9, 31.7, and 32.2% of worldwide cases of the aforementioned infections respectively, resulting in significantly

higher incidence than in high-income regions (Table 1.1) (Guijon et al., 1992; Lewis, 2011; Rodriguez-Cerdeira et al., 2012; Swanepoel et al., 2013). This issue is exacerbated by the large HIV+, immunocompromised population, which is more readily infected. Screenings of sub-Saharan African women estimate 70–80% of those infected with discharge-causing infections remain asymptomatic (Sylverken et al., 2016; Barnabas et al., 2018). This contributes to increased transmission, but also allows infections to persist for longer before treatment, meaning persistence of a cervical microenvironment conducive to cervical lesions and HPV persistence. Less screened infectious microbes such as *Mycoplasma spp.*, *Ureaplasma spp.*, and *Leptotrichia amnionii* have been shown to be involved in cervicitis in HIV+ women (Linhares et al., 2000; Dehon et al., 2016; Mitra et al., 2016). *Mycoplasma* infection has also been directly associated with pre-cancerous cervical lesions (Klein et al., 2019). When screening for STIs, *Mycoplasma genitalium* is more often included, however *Mycoplasma hominis*, which is similar to *M. genitalium* in pathogenesis, is rarely screened for. Thus, a good estimate of *Mycoplasma* prevalence in sub-Saharan Africa is not well-established. In sub-Saharan African cohort studies which include *M. hominis* detection, prevalence of *M. hominis* ranges from 17 to 67.5%, far exceeding *M. genitalium* (Agbakoba et al., 2007; Redelinghuys et al., 2013; Kouegnigan Rerambiah et al., 2015a; Sylverken et al., 2016). This is significantly higher than what has been shown in North America and may be due to the HIV+ population acting as a reservoir for *M. hominis* infection (Djigma et al., 2011).

COMMENSAL MICROBIOTA

The cervicovaginal microbiome has been shown to most often fall into one of seven general community state types (CSTs) (Salas and Chang, 2014; Mitra et al., 2016). These CSTs are characterized by the relative abundance of various species of *Lactobacillus* and anaerobic bacteria and are separated into healthy and dysbiotic groups. The healthy CSTs are all *Lactobacillus* dominated; type 1 is dominated by *L. crispatus*, type 2 by *L. gasseri*, type 3 by *L. iners*, and type 5 by *L. jensenii*. Common dysbiotic CSTs are characterized by an abundance of anaerobic bacteria; type 6 is dominated by Gardnerella vaginalis, type 4 is characterized by a high abundance of anaerobic bacteria and low abundance of *Lactobacillus* species, and type 7 is characterized by high abundance of both Gardnerella vaginalis and *Lactobacillus* species. These cervicovaginal CSTs have been previously associated with significantly different prevalence of infecting HPV genotypes (Brotman et al., 2014; Mitra et al., 2015; Dareng et al., 2016; Reimers et al., 2016). *L. crispatus* dominated microbiomes (type 1) are considered to be the most protective against HPV and HIV, and have been shown to be significantly less likely to have HIV, HSV-2, any HPV, or high-risk HPV than other CSTs (Borgdorff et al., 2014). *L. crispatus* produces lactic acid, antimicrobial compounds, and inhibits inflammation (Graver and Wade, 2011; Hickey et al., 2012; Rose et al., 2012; Aldunate et al., 2013; Petrova et al., 2013). These represent likely mechanisms by which *L. crispatus*, and potentially other microbes, are able to influence HPV infection and progression of HPV-associated diseases. A study of Rawandan women found that women with the *L. crispatus* dominated CST had the lowest prevalence of HIV/STIs, with a slight increase in the *L. iners* dominated CST, and a significant increase in the dysbiotic CSTs (types 4,

6, and 7) (Borgdorff et al., 2014). Similar results have been found in studies of Nigerian and South African women, supporting an association between HPV pathogenesis and decreased abundance of *Lactobacillus spp.* (Dareng et al., 2016; Onywera et al., 2019). *L. crispatus* is much less abundant in sub-Saharan Africa than in high-income regions (Jespers et al., 2015a). Instead, *L. iners* is the most abundant "protective" cervicovaginal microbe, especially in HIV+ women without dysbiosis. *L. iners* dominated microbiomes have been shown to be less protective against cervicovaginal infections, and closer to dysbiotic CST rates of HPV infection and cervical dysplasia (Norenhag et al., 2020). This suggests that differences in commensal microbiota in sub-Saharan Africa are also contributing to the prevalence and transmission of cervicovaginal infection and dysbiosis in the region.

CONCLUSION

Cervical cancer, a preventable and treatable cancer, remains the cancer with the highest incidence in women in 27 countries, and the leading cause of cancer death in women in 45 countries, most of which are in sub-Saharan Africa. Efforts to determine the most cost-effective strategies to reduce cervical cancer burden through human papillomavirus vaccination and screening are ongoing and will hopefully lead to a continued decrease in cervical cancer incidence in the most affected areas of the world. However, it is expected that the number of women with cervical cancer in sub-Saharan Africa will increase as more women get access to HIV therapy, increasing the life expectancy of HIV+ women (Williamson, 2015). There is therefore an urgent need to roll out better cervical screening programs. To accomplish this, a better understanding of the

region's unique factors contributing to cervical cancer is necessary to improve identification and treatment of at-risk individuals before the onset of disease. Current screening and vaccination in sub-Saharan Africa is sparse, irregular, and does not consider factors beyond HIV and cervical lesion status. In considering the factors contributing to sub-Saharan Africa's disproportionate burden of cervical cancer, the disparity between HPV prevalence, HPV genotype prevalence, and sociocultural factors in sub-Saharan Africa and high-income regions is much less than the disparity seen in vaccination and cervicovaginal infection and dysbiosis, suggesting that disruption of the cervicovaginal microbiome may be the most significant factor and predictor of cervical cancer in sub-Saharan Africa. Research up to this point suggests that latent, basal layer infections of HPV's are activated when the host is immunocompromised, leading to increased HPV replication and development of abnormal cytology. HIV and dysbiosis of the cervicovaginal microbiome are the most common factors in sub-Saharan Africa able to activate HPV via effects on the cervical immune microenvironment. ART may help reduce these effects by restoring immune competence, however it does not return activated HPVs to full latency. Transition to cervical cancer is associated with a single HPV genotype becoming dominant, however data suggests coinfection with multiple HPV genotypes or other cervical pathogens plays an important role early on, potentially contributing to the early pathogenesis of a primary high-risk HPV either directly or through manipulation of the immune microenvironment, leading to increased replication and eventual faster or more frequent development of cervical cancer. This process is likely mediated by expression of inflammatory and wound healing cytokines such as IL1, IL6, TNFα, and IFNγ, leading to cell stress and genetic instability, increasing the risk of

mutation or integration of the episomal HPV genome (Figure 1.2). These infections are likely cleared by a competent immune response, which may explain why multiple infection decreases in HIV- cervical cancer cases, but not in HIV+, immunocompromised individuals. A better understanding of the early events influencing HPV control and persistence in the genital tract is needed to test this theory. In sub-Saharan Africa particularly, further research on the impact of HIV on these early events is desirable. Identification of the microbial risk factors for development of cervical cancer will allow for improved identification of those at elevated risk, while improving design and application of primary and secondary preventative treatment and screening.

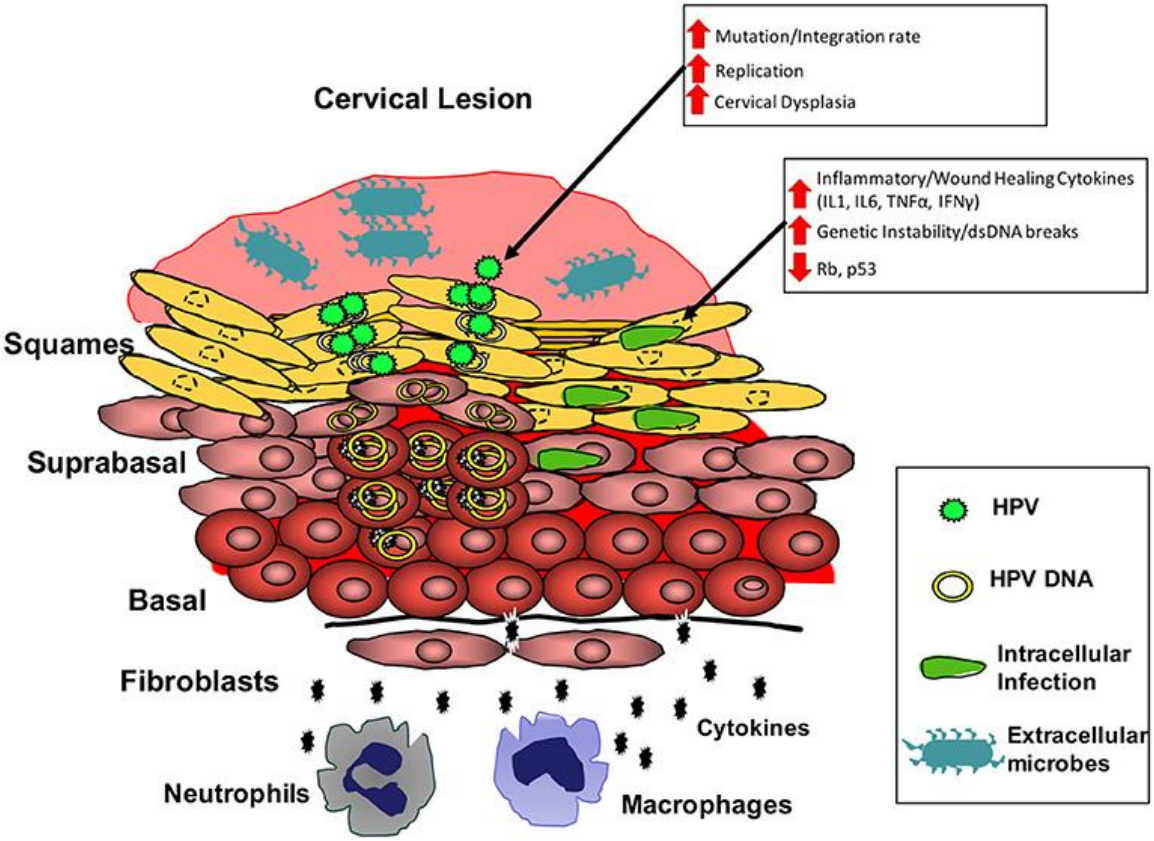

Figure 1.2 Model for the cervical microbial and immune microenvironment driving cervical cancer. Microbial dysbiosis and infection at the cervical epithelium results in increased local expression of inflammatory and wound healing cytokines (IL1 and IL6). Chronic expression of these cytokines can result in increased genetic instability and reduced tumor-suppressor protein function in infected cells. These conditions increase HPV replication, while also increasing risk of mutation and integration of the HPV genome. Thus, the cervical microbiota can increase the risk for events necessary in the transformation of cells by HPV.

CHAPTER 2

RELATIONSHIP BETWEEN THE CERVICAL MICROBIOME, HIV STATUS, AND PRECANCEROUS LESIONS

ABSTRACT

Nearly all cervical cancers are causally associated with human papillomavirus (HPV). The burden of HPV-associated dysplasia in sub-Saharan Africa is influenced by HIV. To investigate the role of the bacterial microbiome in cervical dysplasia, cytobrush samples were collected directly from cervical lesions of 144 Tanzanian women. The V4 hypervariable region of the 16S rRNA gene was amplified and deep sequenced. Alpha diversity metrics (Chao1, PD whole tree, and operational taxonomic unit [OTU] estimates) displayed significantly higher bacterial richness in HIV-positive patients ($P = 0.01$) than in HIV-negative patients. In HIV-positive patients, there was higher bacterial richness in patients with high-grade squamous intraepithelial lesions (HSIL) ($P = 0.13$) than those without lesions. The most abundant OTUs associated with high-grade squamous intraepithelial lesions were *Mycoplasmatales, Pseudomonadales,* and *Staphylococcus*. We suggest that a chronic *Mycoplasma* infection of the cervix may contribute to HPV-dependent dysplasia by sustained inflammatory signals.

IMPORTANCE

HPV is known to be the causal agent in the majority of cervical cancers. However, the role of the cervical bacterial microbiome in cervical cancer is not clear. To investigate that possibility, we collected cervical cytobrush samples from 144 Tanzanian women and performed deep sequencing of bacterial 16S rRNA genes. We found that HIV-positive patients had greater bacterial richness ($P = 0.01$) than HIV-negative patients. We also observed that women with high-grade squamous intraepithelial lesions (HSIL) had greater cervical bacterial diversity than women with cytologically normal cervices. Data from our precise sampling of cervical lesions leads us to propose that *Mycoplasma* contributes to a cervical microbiome status that promotes HPV-related cervical lesions. These results suggest a greater influence of the bacterial microbiota on the outcome of HPV infection than previously thought.

INTRODUCTION

Human papillomavirus (HPV) is the causative agent responsible for 99% of cervical cancers (Formana et al., 2012). HPV contributes to about 4.8% of all cancers (Formana et al., 2012). The disease burden of HPV is most dramatic in developing regions of the world, with HPV contributing to 14.2% of cancers in sub-Saharan Africa (Formana et al., 2012). Cervical cancer disproportionately affects sub-Saharan Africa, where 9% of the world's female population over 15 years old accounts for 14% of the world's incidence of cervical cancer and 18% of cervical cancer-related deaths (De Vuyst

et al., 2013). The current study uses cervical swab samples obtained from Tanzania, which has among the highest cervical cancer mortality rates by country.

sub-Saharan Africa also has among the highest HIV rates in the world. The association between HIV and cervical cancer has been better studied than any other factor associated with HPV-related cancers. HIV infection has been strongly linked to increased risk of infection with HPV and the severity of HPV pathogenesis (Adler et al., 2014; Salazar et al., 2015; Williamson, 2015). High-risk HPV genotypes are more prevalent in HIV-positive (HIV+) women, suggesting that HIV infection provides an environment where these high-risk HPVs can better establish infection and replicate (McDonald et al., 2014). A likely factor in this is a decrease in T-cell surveillance, which results in an increase in HPV replication with decreasing CD4+ cell count, and other changes in the cervical immune microenvironment as HIV infection progresses. Multiple studies have shown an increase in HPV detection in cervical intraepithelial neoplasms in individuals with less than 200 CD4+ cells per μl of serum (Hanisch et al., 2013; Ezechi et al., 2014; Memiah et al., 2015; Menon et al., 2016). Thus, the cervical immune microenvironment may be a cofactor in suppression of cervical cancer.

Changes in the cervicovaginal bacterial microbiome have been suggested to contribute to the development of precancerous cervical lesions (Guijon et al., 1992; Gillet et al., 2011; Clarke et al., 2012; Guo et al., 2012; Vriend et al., 2015; Mitra et al., 2016; Kyrgiou et al., 2017). Chronic inflammation of the cervix (cervicitis), which is a result of

cervicovaginal pathogens, leads to conditions like pelvic inflammatory disease (PID) and bacterial vaginosis (BV), both of which are associated with persistent HPV infection and cervical cancer (Giraud et al., 1998; Skapinyecz et al., 2003). Both PID and BV are more prevalent in sub-Saharan Africa and in HIV-positive populations (Msuya et al., 2002; Lewis, 2011; Swanepoel et al., 2013). Comparative genomic analyses in women infected with HIV have shown that a shift in microbial diversity as a result of BV is detectable; whether this shift directly affects formation of precancerous cervical lesions is not clear (Spear et al., 2008). Given that cervical cancer rates are expected to rise in sub-Saharan Africa as the HIV-positive population receives life-extending antiretroviral therapy (ART), it is even more important to understand the risk factors associated with the cervical microbiome. There are previous studies that have analyzed how cervical microbiota differ at different stages of cervical cytology or as a function of HIV status (Lee et al., 2013; Borgdorff et al., 2014; Oh et al., 2015; Audirac-Chalifour et al., 2016; Curty et al., 2017). The current study defines bacterial communities associated with cervical lesions and with HIV, which represents a significant advance. Cervical cytology is graded by pap smear screening for nuclear abnormalities according to the Bethesda guidelines.

In this study, we utilized 16S rRNA gene deep sequencing on a set of 144 cervical swab samples from a cohort of Tanzanian women to gain an understanding of the differences in the cervical bacterial community composition as a function of cervical cytology grade and HIV status. The data presented here identify bacterial taxonomies associated with high-grade cervical lesions. In these studies, cervical lesions were

sampled directly by cytobrush, instead of cervicovaginal lavage sampling. The rationale behind this approach was that the sites of the lesions are where tumors form, thus bacteria associated with lesion sites are more likely to be relevant to the process of disease progression than those associated with other regions.

RESULTS

Demographics. Of the 144 patient samples, 41 were HIV positive (HIV+) and 103 were HIV negative (HIV−), with an average patient age of 37 years old. Of these 144 samples, 134 had HPV tests and deep sequencing reads of >1,000. The frequencies of HPV+ and HPV− samples with respect to HIV status are plotted in Figure 2.1A. There were 8 HIV− HPV− samples and 87 HIV− HPV+ samples, but there were no HIV+ HPV− samples and 39 HIV+ HPV+ samples. Among HIV− samples, HPV had a statistically significant effect ($P = 0.02$) on the cervical microbiome (Figure 2.1B and C). Those microbes which were enriched in HPV+ samples were *Bacteriodetes* and *fusobacteria*. Also, there was a decrease in Actinobacteria. Cervical cytology was determined to be negative for intraepithelial lesion or malignancy (NILM) in 23 samples, low-grade squamous intraepithelial lesions (LSIL) in 72 samples, and high-grade squamous intraepithelial lesions (HSIL) in 50 samples. Visual inspection with acetic acid (VIA), the standard for cervical lesion detection in Tanzania, was carried out immediately following sample collection. Twenty-six patients were found to be VIA positive for cervical lesions and 115 were VIA negative. All VIA-positive samples were identified as LSIL or HSIL, while several VIA-negative samples were found to be NILM, LSIL, or HSIL by pap smear. Odds ratios were used to identify risk factors for testing VIA

positive. Testing HIV+, HSIL, having >5 sexual partners, and having been infected with a sexually transmitted infection (STI) were identified as significant risk factors for positive VIA status ($P = 0.0001$, $P = 0.038$, $P = 0.006$, and $P = 0.0008$, respectively).

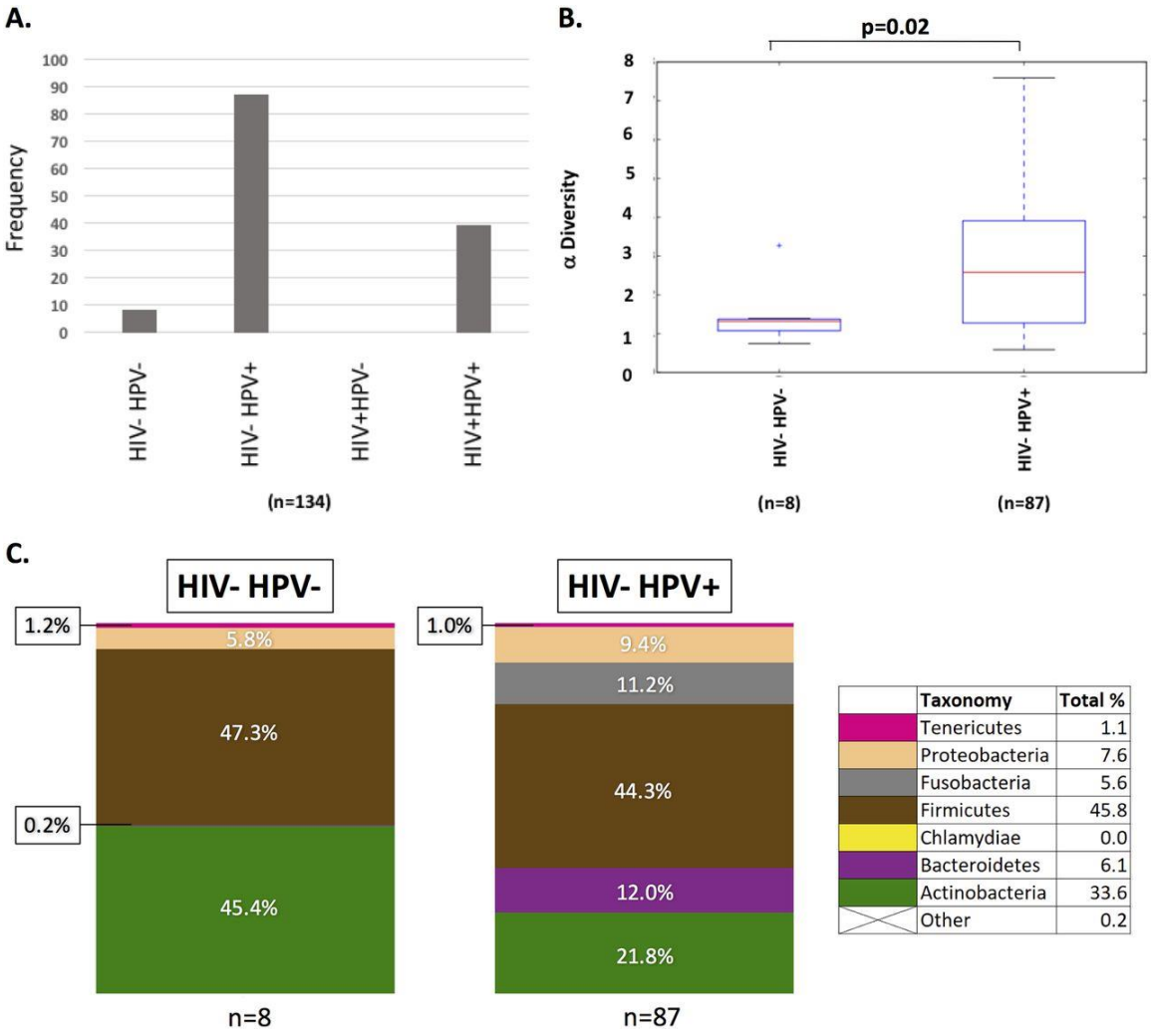

Figure 2.1 Effect of HPV status upon the cervical microbiome diversity. (A) A total of 134 cohort DNA samples were genotyped for HIV and HPV status. The frequency of samples were graphed as the following groups: HIV− HPV− (n = 8), HIV− HPV+ (n = 87), HIV+ HPV− (n = 0), and HIV+ HPV+ (n = 39). Taxonomic groups were determined by analysis of 16S deep sequencing results of bacterial DNAs. (B) Alpha diversity is graphed as a function of HIV− HPV− (n = 8) and HIV− HPV+ (n = 87). A t test showed a significant difference between the HPV− and HPV+ groups (P = 0.02). (C) Bacterial diversity is graphed with each phylum represented as a different color. The color code representing each bacterial phylum is shown in the legend to the right.

Cervical bacteria composition and richness. Samples rarefied to an even depth (1,000 reads) were used to generate 813 operational taxonomic units (OTUs). To assess whether the sampling depth was adequate, rarefaction curves were generated using observed OTUs for HIV status and cervical cytology (see Figure S1 in appendix). Rarefaction curves for both did not converge but showed a diminishing rate of new OTU identification as the number of reads per sample increased, implying that sampling depth was adequate for evaluating dominant members of the cervical bacterial community. Good's coverage test showed that the sequencing depth was able to characterize 99.4% of the bacterial community on average.

The taxonomic analysis of the reads revealed the presence of six main phyla (relative abundance of >1%) in the cervical epithelium, regardless of HIV or cervical cytology status (Figure 2.2). Firmicutes was the predominant phylum across all sampling groups, accounting for 41.3% of total reads. The average relative abundance of Firmicutes decreased slightly in HIV+ samples compared to HIV− samples (44.4% to 40.2%) and varied by cervical cytology, though no obvious trend was apparent. When considering only the HIV+ samples, the relative abundance of Firmicutes appeared to decrease in patients with cervical lesions. Firmicutes reads were primarily from the genus *Lactobacillus*, which accounted for 21.9% of total reads. *Tenericutes* accounted for 1.5% of total reads and showed a clear increase in relative abundance with increasing severity of cervical lesions. In HIV− patients, *Tenericutes* increased from 0.3% of reads in NILM patients to 1.3% in HSIL patients (Figure 2.2C). In HIV+ patients, the shift is larger; the relative abundance of *Tenericutes* increased from 0.2% in NILM patients to 5.0% in

HSIL patients (Figure 2.2D). *Tenericutes* reads were primarily assigned to the *Mycoplasma* and *Ureaplasma* genera, which account for 1.1% and 0.2% of total reads, respectively. Proteobacteria, fusobacteria, Bacteroidetes, and Actinobacteria had smaller or less consistent shifts in relative abundance between HIV and cervical cytology categories. The relative abundance of *Tenericutes* and Bacteroidetes were significantly different between HIV+ and HIV− groups ($P = 0.020$ and $P = 0.017$, respectively). No other phyla reached significance on the basis of HIV status or cervical cytology. Comparison of the relative abundance of bacterial families (Figure 2.3) found that *Mycoplasmataceae* and *Prevotellaceae* were significantly more abundant in HIV+ patients ($P = 0.03$ and $P = 0.07$, respectively). No families were found to be significantly different in abundance on the basis of cervical cytology alone. However, when analyzed among HIV+ patients, *Prevotellaceae* was found to be significantly more abundant in cervical lesions ($P = 0.068$).

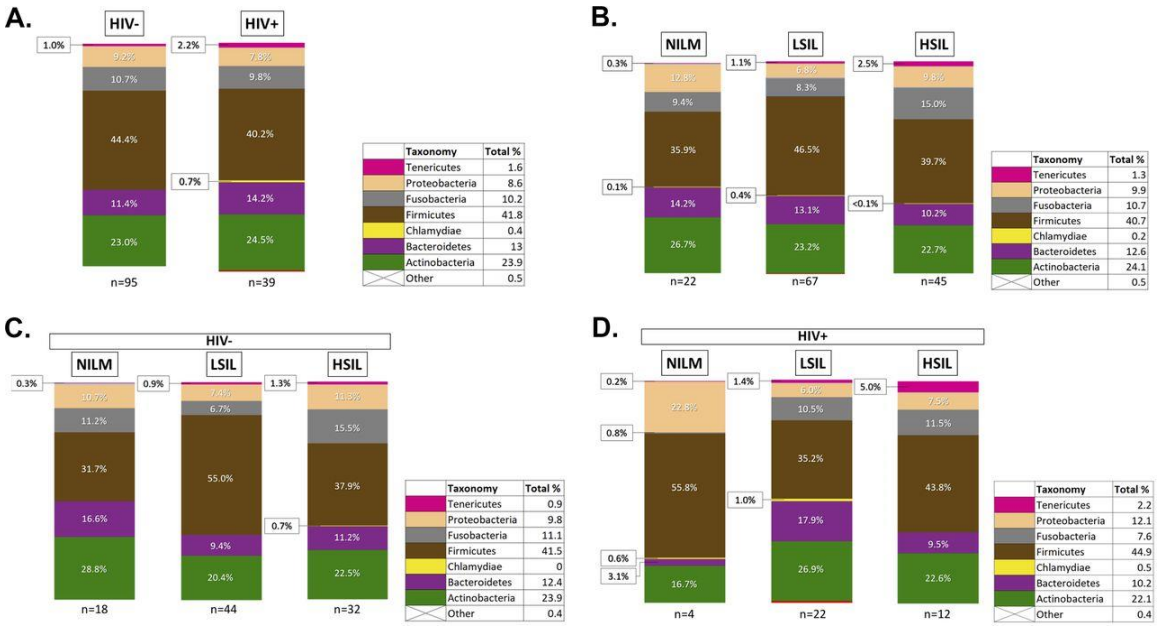

Figure 2.2 Phylum-level taxonomy of the cervical bacterial community composition as a function of HIV status and cervical cytology. (A) Phylum-level bacterial taxonomy of the cohort is displayed by HIV status. (B) Phylum-level bacterial taxonomy of the cohort is displayed as a function of cervical cytology. (C) Phylum-level taxonomy of HIV-negative patients as a function of cervical cytology grade. (D) Phylum-level taxonomy of HIV-positive patients as a function of cervical cytology grade. Each phylum is represented as a percentage of the total.

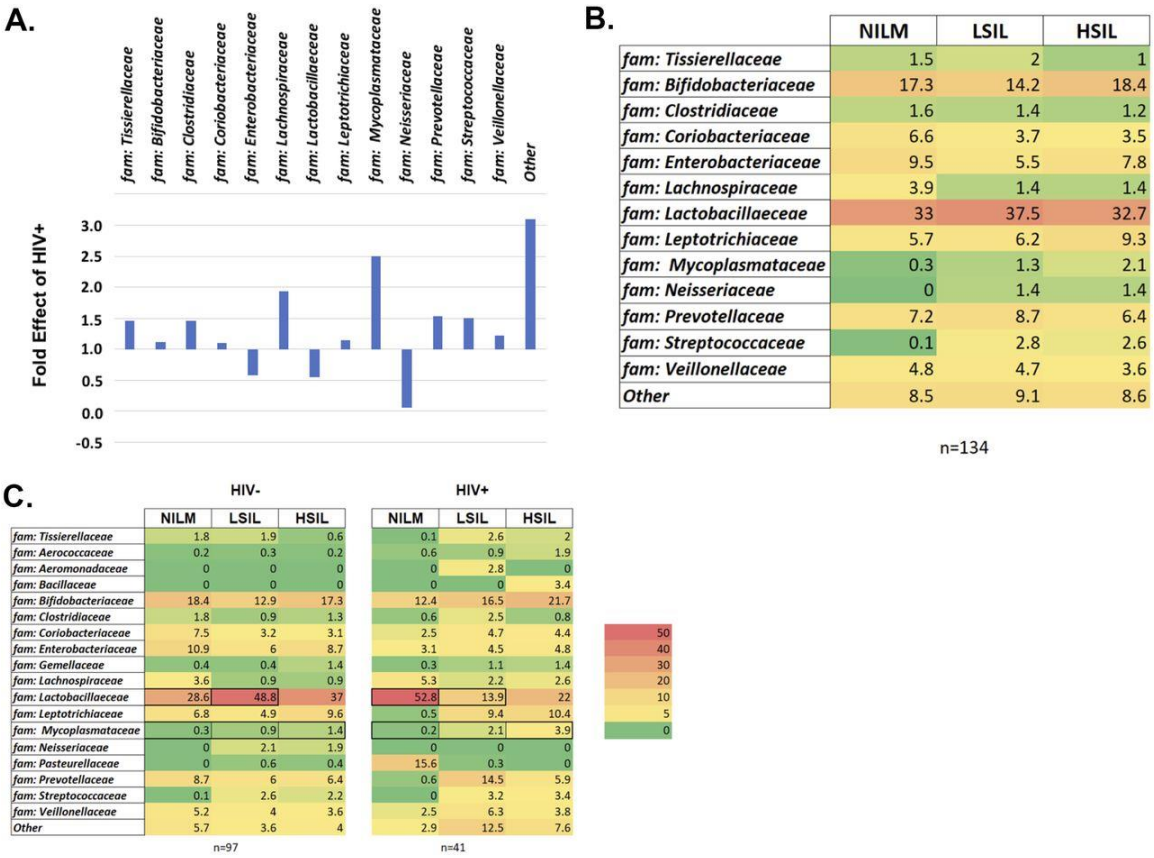

Figure 2.3 Relative abundance heatmap of family-level taxonomy of the cervical bacterial community composition as a function of HIV status and cervical cytology. (A) Fold effect of HIV+ on the family-level bacterial taxonomy within the cohort (normalized to 1). (B) Relative abundance heatmap of the family-level taxonomy of cohort versus cervical cytology. (C) Relative abundance heatmap of the family-level taxonomy of the cohort by cervical cytology, separated by HIV status. The data are presented as percentages of the total. The scale is shown to the right of the heatmap.

Cervical bacterial diversity estimates. Alpha diversity metrics, Chao1, observed OTUs, and PD Whole Tree, displayed higher ($P = 0.009$) bacterial richness in HIV+ patients than in HIV− patients (Figure 2.4). A subset of these samples was matched such that the HIV− and HIV+ groups consisted of the same number of samples, with the same average age, and the same contribution of each cervical cytology to help to control for effects of these confounding variables and to ensure that differences in diversity estimates are not due to differences in sample size. In this matched subset, estimates also displayed higher ($P = 0.003$) bacterial richness in HIV+ patients.

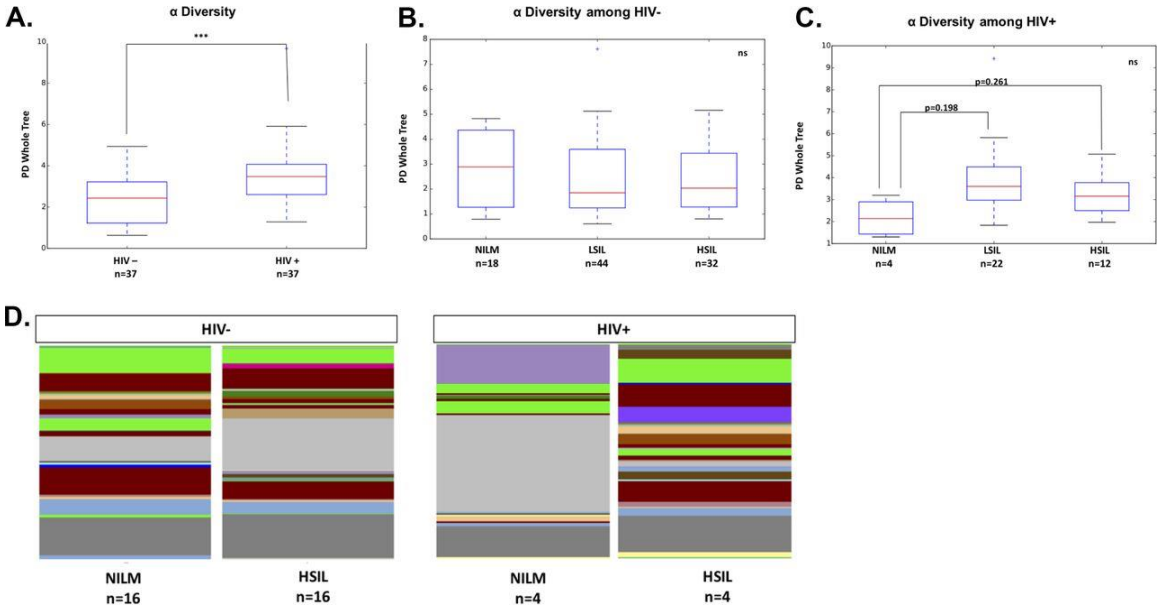

Figure 2.4 Alpha diversity measurements of cohort subgroups. (A) Relationship between HIV status and alpha diversity of cervical bacteria. (B) Relationship between cervical cytology and bacterial alpha diversity in HIV− individuals. (C) Relative abundance of genus-level reads differentiated by cervical cytology in HIV+ and HIV− individuals. Statistical significance is indicated as follows: ns, not significant; *, $P < 0.1$; **, $P < 0.05$; ***, $P < 0.01$. (D) Relative bacterial diversity of cervical microbiota graphed as a function of HIV status. Each color represents a different taxonomic family as defined by deep sequencing of the 16S gene.

Alpha diversity metrics were similar ($P > 0.50$) for the samples from patients at different cervical cytology grades (NILM, LSIL, or HSIL) in both matched and unmatched sets. When alpha diversity metrics were compared between cervical cytology groups separately for HIV+ samples, LSIL and HSIL trended toward a higher diversity compared to NILM ($P = 0.198$ and $P = 0.261$, respectively). Analysis of age-matched, HIV+ NILM/HSIL pairs maintained this trend ($P = 0.264$; Chao1 $P = 0.13$). Comparison of the relative abundance of genus-level reads between these groups showed a noticeably more diverse profile for HSIL samples, which lack the dominance of *Lactobacillus* and *Haemophilus* seen in NILM samples.

Beta diversity analysis showed that bacterial communities were quite varied between samples (Figure 2.5); no discrete communities characterized a large number of samples. On average, the cervical bacterial communities of HIV-positive patients were shown to be significantly different from the communities of HIV-negative patients ($P = 0.001$). Similarly, patients who tested positive for HPV tended to have different bacterial communities from those who tested negative for HPV ($P = 0.008$). Bacterial communities were also shown to differ significantly depending on cervical cytology among HIV-positive patients ($P = 0.05$).

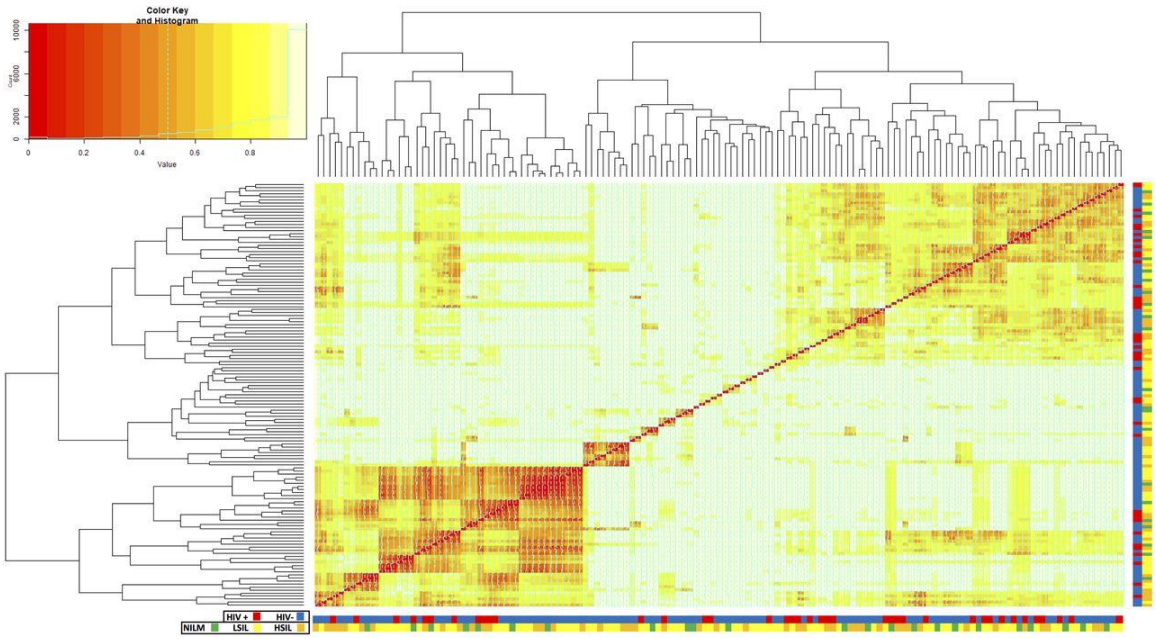

Figure 2.5 Heatmap of the Bray-Curtis distances between each sample (beta diversity). Samples are grouped into a similarity tree based on the abundance of each OTU. Lower values (red) indicate more similarity. HIV status and cervical cytology of each sample are indicated by color beneath each column and beside each row (HIV+ [red], HIV− [blue], NILM [green], LSIL [yellow], HSIL [orange]).

Bacteria associated with cervical cytology states and/or HIV status. Linear discriminant analysis effect size (LEfSe) was used to identify bacterial taxonomies which differentiate cervical microbiota in normal individuals (NILM) from microbiota in patients with precancerous lesions (HSIL). The sum of reads at each taxonomic rank was considered. *Gammaproteobacteria, s24_7,* Paraprevotellaceae (nonverified taxonomy), and *Finegoldia* associated with NILM cervices, while *Pseudomoriadaceae, Staphylococcus, and Mycoplasmatales* associated with precancerous lesions. *Mycoplasmatales* were dominant among *Tenericutes,* resulting in the significant association seen between the phylum and cervical lesions. A distance-based redundancy analysis (db-RDA) analysis of bacterial communities as a function of HIV and/or cervical cytology is summarized in Figure S2. LEfSe was then used to compare HIV+, age-matched pairs of NILM and HSIL patients to determine which bacteria may influence the development of lesions in high-risk, HIV+ populations. *Mycoplasmatales* were most strongly associated with cervical lesions in HIV+ patients, followed by *Parvimonas* and Streptococcus. In NILM patients, an abundance of *Lactobacillus,* especially *Lactobacillus iners* was found, and somewhat less significantly *Finegoldia.* LEfSe analysis of samples by HIV found several bacteria to be associated with being HIV+ (Figure 2.6C). An abundance of non-*Lactobacillus* bacilli was the most significant differentiating taxonomy between HIV-positive and -negative samples. *Mycoplasma* was also associated with HIV+ individuals, supporting the significant difference in relative abundance between HIV-positive and -negative groups shown previously using a direct Kruskal-Wallis comparison. Interestingly, *Ureaplasma* (a member of *Mycoplasmatales*) and *Lactobacillus reuteri* were associated with HIV− patients, while other members of

their respective families were associated with HIV+ patients. This suggests the existence of metabolic niches in the cervical microbiome which may be populated by pathogenic or nonpathogenic bacteria.

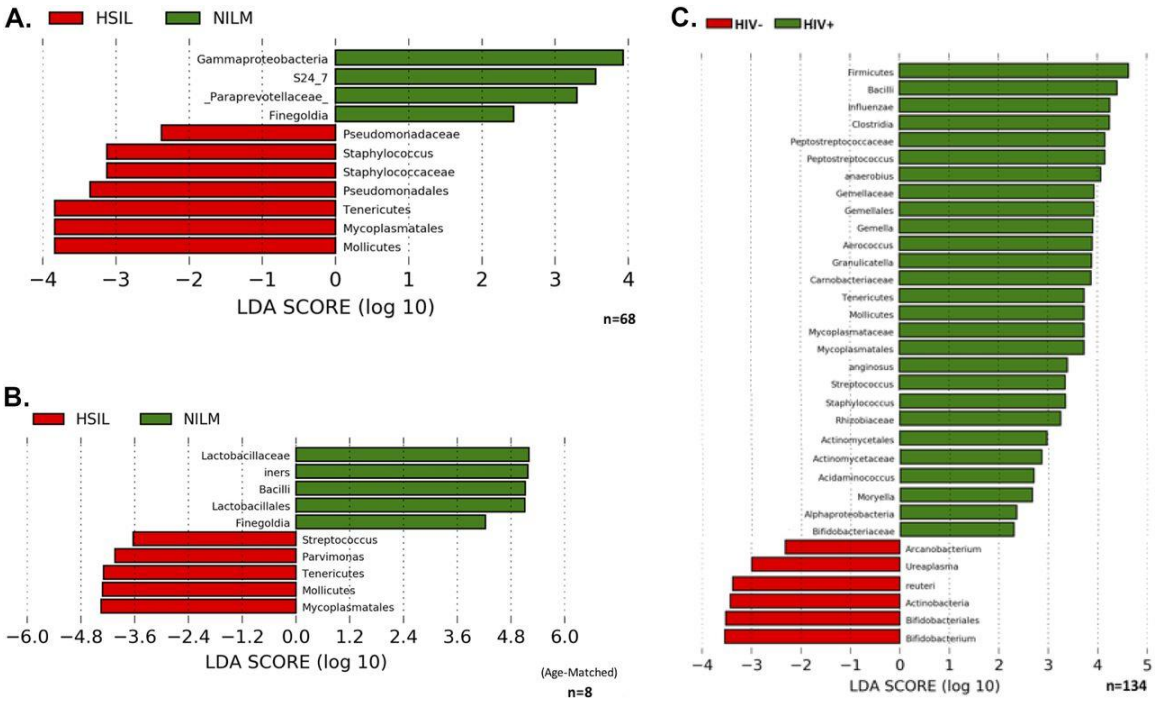

Figure 2.6 LEfSe linear discriminant analysis (LDA) scores. Microbes associated with cervical cytology status and/or HIV status are displayed. (A) Taxonomies differentiating bacterial microbiota in cytologically normal versus HSIL cervices. (B) Taxonomies differentiating bacterial microbiota in cytologically normal versus HSIL cervices in age-matched HIV+ patients. (C) Taxonomies differentiating bacterial microbiota in HIV− versus HIV+ cervices.

DISCUSSION

We found that HPV was in high abundance in the cohort (Figure 2.1A). All of the patients who were HIV positive were positive for one or more HPVs (Figure 2.1A). Among the HIV− samples, HPV was associated with at least a 10-fold increase in Bacteriodetes and fusobacteria as well as a decrease in Actinobacteria (Figure 2.1B and C). Previous studies support the conclusion that HPV affects the microbiome (Lee et al., 2013).

Certain members of the cervicovaginal microbiome are known to protect against infection and pathogenesis. The primary defense mechanisms of the cervicovaginal mucosa are antimicrobial peptides, a pH of less than 4.5, and a microbiome dominated by lactobacilli. An imbalance in these defenses can result in physiochemical changes that produce alterations of the vaginal mucosa and cervical epithelium (Audirac-Chalifour et al., 2016). In particular, an abundance of *Lactobacillus crispatus* shows an inverse relationship with detectable or symptomatic HIV, HPV, or herpesvirus infection (Borgdorff et al., 2014). This suggests that other cervicovaginal microbes may be important in preventing or enhancing the acquisition and pathogenesis of such infections. Microbes that are associated with enhanced pathogenesis have largely gone unidentified or unstudied, especially in the population most at risk, HIV-positive women in sub-Saharan Africa.

In this study, HIV was shown to have a significant effect on the cervical microbiome, increasing bacterial richness and decreasing beta diversity. These results are similar to what has been reported for the cervicovaginal microbiome and suggest that changes in the cervical epithelium microenvironment brought on by HIV exert some selective pressure on cervical bacterial communities (Lee et al., 2013; Borgdorff et al., 2014; Oh et al., 2015; Audirac-Chalifour et al., 2016; Curty et al., 2017). *Mycoplasma* was significantly more abundant in HIV-positive patients and was found to be one of the main categories of bacteria that differentiate the cervical microbiota of HIV-positive and HIV-negative individuals (Figure 2.3). Interestingly, bacteria of the order *Bacilli,* of which *Lactobacillus* is a member, were strongly associated with HIV-positive patients. The absence of *Bacilli* reads classified as *Lactobacillus* among the significant factors of HIV-positive cervical microbiota suggests that this may be due to a shift from protective to nonprotective *Bacilli* in HIV+ individuals. When the cohort was analyzed without taking HIV status into account, cervical cytology did not appear to have a statistically significant association with differences in the cervical microbiome (Figure 2.4B). However, when HIV was controlled for by separating analysis by groups of HIV-positive or HIV-negative patients only, differences in cervical bacterial communities that varied on the basis of cervical lesion status began to reach statistical significance (Figure 2.4C). This suggests that development of precancerous cervical lesions is associated with a certain microbiota. Among these microbiota, *Mycoplasmatales* stood out as the most significant differentiator between the cervical microbiota of a cervix with precancerous lesions from a cervix without precancerous lesions (Figure 2.6). Bacteria belonging to the order *Mycoplasmatales* also showed the clearest linear increase in abundance with

development of more severe lesions in both HIV-positive and HIV-negative populations. The most common *Mycoplasmatales* to infect the urogenital tract of women are *Mycoplasma genitalium* and *Mycoplasma hominis*. *M. genitalium* and *M. hominis* are noncommensal bacteria commonly associated with cervicitis, BV, PID, and HIV infection, though *M. genitalium* has been much better studied (Irwin et al., 2000; Mavedzenge and Weiss, 2009; Soni et al., 2010). It is not well understood whether HIV promotes *Mycoplasma* infection or persistence of an otherwise transient infection in an HIV-negative individual. One study found that HIV-positive women cleared *M. genitalium* infections more slowly than HIV-negative women did, and the infection recurred in 39% of the patients after clearance (Vandepitte et al., 2013). The role of *M. genitalium* infection in influencing initial infection of HIV also remains unclear; however, a strong association between the severity of *M. genitalium* infection and HIV shedding from the cervix has been shown (Manhart et al., 2008). What is clear is that *M. genitalium* infects the epithelia, disrupting tight junctions, and inducing a chronic inflammatory response. The potential for *M. genitalium* to influence replication of HIV suggests that host innate responses to *M. genitalium* infection may influence pathogenesis of other sexually transmitted infections. Induction of HPV in this way is particularly interesting based on the association between *Mycoplasma* and cervical lesions. Infection with *M. genitalium* increases the rate of infection with an HPV genotype associated with a high risk of developing cervical cancer (Ye et al., 2018). Recent work has shown that *Mycoplasma* also increases the risk of development of cervical lesions, supporting the association we report in this study (Ye et al., 2018). *Mycoplasma* can establish persistent, intracellular infections in epithelial cells, which may lead to bacterial vaginosis and/or

cervicitis. *M. genitalium* has been established as an independent, causal microbe responsible for cervicitis (Taylor-Robinson and Jensen, 2011). This suggests that *Mycoplasma* may act as both an intracellular and extracellular stressor, particularly if coinfection with HPV has taken place. This interaction would most likely involve inflammatory cytokines induced by *Mycoplasma* infection. Further study is needed to determine whether the inflammatory cytokines induced by *Mycoplasma* infection include cytokines that are associated with precancerous cervical lesions.

Mycoplasma is a low-abundance microbe that has been shown to cause cervicitis. However, the lack of significant associations in previous metagenomic studies is largely due to a lack of optimization of statistical analyses for the presence of low-abundance microbes. In our study, *Mycoplasma* was a prominent result, likely due to the large HIV-positive proportion of the cohort, wherein immunosuppression allowed higher abundance of the bacteria to accumulate. There was a linear increase in the abundance of *Mycoplasmatales* from NILM to HSIL seen in both HIV-positive and -negative groups.

In this study, we took great effort to control for variation in the cervical microbiome so as to reduce confounding effects that might obscure the bacterial communities that were associated with HPV pathogenesis. The HIV-positive population is of particular interest, since they appear to show changed cervical microbiota associated with HPV pathogenesis (Fig. 2.2, 2.3, 2.4, and 2.6). Future studies, recruiting a cohort of all HIV-positive women with and without cervical lesions would be desirable in order to

better characterize HIV-associated microbiota which promote HPV infection and progression to cervical cancer. Currently, few cervical microbiome cohort studies have been conducted in HIV-positive populations. It is clear that variables such as diet, genetic background, antibiotics or ART, can dramatically affect the microbiota and thus should be carefully controlled at the point of recruitment to the study.

Longitudinal studies of the cervical microbiome are needed to understand how microbe populations change over time, particularly in individuals with HSI*L*. Long-term longitudinal studies will allow determination of early changes in the cervical microbiota that may help predict the development of precancerous lesions. Because progression of HPV infection to cervical cancer is a process that takes decades, and in many individuals never reaches cancer at all, such a study would need to be large. Studies of the cervical microbiome can be further improved using metagenomic sequencing, rather than 16S or other targeted sequencing techniques that lack depth. 16S amplification ignores microbes that lack a gene to match the primers, for example, viruses, archaea, and eukaryotes are not accounted for. Because only a portion of one gene is being sequenced, the microbes present may be estimated only to the genus level or to a higher taxonomic level. Since the majority of medium- or large-scale cervicovaginal microbiome studies have used this method, the role of nonbacterial components of cervicovaginal microbiome in HPV infection and disease has not been characterized.

As the world's HIV-positive population grows, cervical cancer is expected to become an even more significant problem, despite increasing coverage of antiretroviral treatment (ART). Compared to the risk reduction after ART seen in other AIDS-defining cancers like Kaposi's sarcoma and non-Hodgkin's lymphoma, the risk of cervical cancer is not significantly affected, and recurrence rates remain high with or without treatment (Foulot et al., 2008; Mungo et al., 2013; Russomano et al., 2013; Cobucci et al., 2015). Understanding microbes that influence this environment will help identify cervical microbiota associated with low- and high-grade cervical lesions. This may allow certain cervical microbiota to be used as diagnostic markers for those at high risk of developing cervical cancer and for the development of preventative probiotic or antibiotic treatments that could control the cervical microbiome by promoting bacterial colonization with a microbiota associated with healthy cervical cytology. Our studies have identified a unique microbiota associated with HSI*L*. Data derived from our precise sampling of cervical lesions lead us to propose that *Mycoplasma* contributes to a cervical microbiome status that promotes HPV-related cervical lesions. These results suggest a greater influence of the bacterial microbiota on the outcome of HPV infection than previously thought.

MATERIALS AND METHODS

Participants and ethical precautions. This study reports findings derived from a larger cross-sectional cohort study analyzing demographics of HPV and cervical cancer in HIV-positive and -negative women from rural and urban Tanzania.

The cervical microbiome study participants were part of a larger ongoing study to follow HIV- and HPV-associated cervical dysplasia in women at Ocean Road Cancer Institute (ORCI), the only cancer treatment hospital in Tanzania. Between March 2015 and February 2016, female patients undergoing cervical cancer screening were approached for enrollment in the study. Those who were pregnant, menstruating, under 18, reported being sick in the past 30 days, or had a preexisting, non-HIV, immunologic defect were excluded from the study. Disease histories as well as physical examinations were carried out to rule out any clinical symptoms or visible signs for these conditions. Samples were collected at three sites in Tanzania: ORCI in Dar es Salaam and rural clinics in Chalinze and Bagamoyo. A total of 144 cervical cytobrush samples obtained from these women were sequenced, of which 134 samples produced at least 1,000 reads and complete demographic data was available for the women. Of these, 132 had complete HIV data and cervical cytology reads.

Demographic data collection. All study participants gave informed consent and were evaluated by study clinicians. A set of pretested, standardized questionnaires was used to gather demographic data. All personal identifiers were removed from samples to ensure patient confidentiality. With the permission of the patients, medical history was retrospectively retrieved from hospital medical records. More than 30 variables were identified and assessed in the questionnaire. The current study uses only data collected regarding age and laboratory test results (pap smears, visual inspection with acetic acid [VIA], CD4 count, genotyping of HPV, results of serological testing for HIV-1).

Specimen collection, HIV, CD4, and pap tests. Blood samples were collected via venipuncture into acid-citrate-dextrose tubes and processed using centrifugation at the on-site study laboratory within 6 h of being drawn. The separated plasma was tested at the ORCI, as part of standard of care, using Standard Diagnostics HIV-1/2 3.0 detection kit and BD products CD4 FITC, CD8 PE, and CD3 Per CP antibodies to test the CD4 counts using a BD Accuri C6 Plus. Cervical cytobrush samples and pap smears were collected from all patients. Pap smears were examined by at least three trained cytologists and classified according to the pap classification protocol: negative for intraepithelial lesion or malignancy (NILM); atypical squamous cells of undetermined significance (ASC-US); low-grade squamous intraepithelial lesions (LSIL); atypical squamous cells but cannot exclude high-grade lesions (ASC-H); high-grade squamous intraepithelial lesions (HSIL). Cervical cytobrush specimens were placed in lysis buffer and then shipped to the Nebraska Center for Virology at the University of Nebraska-Lincoln (UNL) for processing.

DNA isolation, 16S rRNA library preparation, and sequencing of the V4 region. Cervical cytobrush samples were vortexed and separated from the brush with lysis buffer. DNA was extracted from the lysis buffer using the Qiagen Tissue extraction kit (Dneasy) according to the manufacturer's protocol. The DNA concentration was determined by UV spectrophotometer at 260/280 n*M*.

DNA was then used for tag sequencing of the V4 hypervariable region of the 16S rRNA gene. A 250-bp section of the V4 region was amplified using universal primers described in (Kozich et al., 2013). The PCRs were performed in 25 µl. The cycling conditions were as follows: an initial denaturation of 98°C for 3 min, followed by 25 cycles, with 1 cycle consisting of denaturation at 98°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 45 s, and then a final elongation of 68°C for 4 min. Following amplification, PCR products were analyzed on a 2% agarose gel to confirm correct product size. Normalized amplicons (1 to 2 ng/µl) from 144 samples were pooled together using an epMotion M5073 liquid handler (Eppendorf AG, Hamburg, Germany). Pooled libraries were sequenced using the Illumina MiSeq platform using the dual-index sequencing strategy outlined by (Kozich et al., 2013).

HPV genotyping. To determine HPV status, DNA samples were subjected to HPV redundant primer using the GP5+/GP6+ primer set, which detect up to 40 different mucosal HPVs (Clifford et al., 2005; Ng'andwe et al., 2007; Chisanga et al., 2015). Samples found to be HPV positive were genotyped for HR-HPVs (types 16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66) and LR-HPVs (types 6 and 11) using a low-cost multiplex PCR assay (Samwel et al., 2019).

Data processing and bacterial community analysis. The sequencing data obtained from the sequencer was subsequently analyzed using the Illumina MiSeq data analysis pipeline developed by the Fernando lab (described in detail at

https://github.com/FernandoLab). Briefly, initial quality filtering was carried out to remove sequences that had ambiguous bases, incorrect lengths, and inaccurate assemblies. Subsequently, the quality-filtered reads were run through the UPARSE pipeline (http://www.drive5.com/uparse/) and subjected to chimera filtering and OTU clustering (at a similarity threshold of 97%), followed by the generation of an OTU table. Taxonomy was assigned to the OTUs using the assign_taxonomy.py command available in QIIME using the latest version of the Greengenes database (May 2013).

Statistical analyses. The OTU table was rarefied across samples to the lowest sample depth (1,000 reads) using QIIME based on the Mersenne Twister pseudorandom number generator. All statistical analyses were performed with samples at an even depth. Bar charts summarizing average taxonomic makeup of samples by HIV status and cervical cytology were constructed from the rarefied OTU table in QIIME. Heatmaps showing the relative abundance of bacterial taxonomic families were constructed using the "plot_ts_heatmap" command using the mctoolsR package for R. Differences in bacterial families by HIV status or cervical cytology were evaluated using the "taxa_summary_by_sample_type" command in mctoolsR using Kruskal-Wallis. Families with less than 1% abundance were excluded in this analysis. Alpha diversity estimators Chao1, observed OTUs, and PD whole tree and rarefaction curves were calculated for the overall bacterial community using QIIME. Good's coverage test was performed to evaluate whether adequate sampling depth was achieved. Mean alpha diversity estimates for HIV-positive, HIV-negative, NILM, LSIL, and HSIL groups were compared using nonparametric two-sample t tests using Monte Carlo permutations in QIIME. The

weighted and unweighted UniFrac distance matrix for bacterial communities were calculated using QIIME. Even depth across samples avoided biases that could be encountered when using the UniFrac metric (Lozupone et al., 2011). Bacterial community composition differences were evaluated using the unweighted UniFrac distance matrix as an input for a distance-based redundancy analysis (db-RDA) in QIIME, where HIV status, cervical cytology, and HPV status were used as main effects. A heatmap was generated using the heatmap.2 command in the "ggplots" package for "R" using the Bray-Curtis distance matrix to visualize relationships between samples. Significance was declared at $P \leq 0.1$ throughout this study. The linear discriminant analysis effect size (LEfSe) was used to identify specific OTUs that differed HIV status and cervical cytology (Segata et al., 2011). LEfSe uses a nonparametric factorial Kruskal-Wallis rank sum test followed by a linear discriminant analysis to identify both statistically significant and biologically relevant features. The relative abundances of the OTUs were used as input for LEfSe. Demographic data were examined using odds ratio and an associated P value to test for factors associated with HIV status and/or a positive VIA status. All P values are reported as FDR-corrected P values.

Ethics statement. All human subject protocols were approved by safety committees at the Ocean Road Cancer Institute (ORCI) and UNL in accordance with the Helsinki Declaration. Participation by patients was entirely voluntary, and written patient consent was required for inclusion in the study.

CHAPTER 3

MYCOPLASMA CO-INFECTION IS ASSOCIATED WITH CERVICAL CANCER RISK

ABSTRACT

Tanzania faces one of the highest cervical cancer burdens in the world. Recent work has suggested that the bacterial family *Mycoplasmataceae* is associated with higher levels of HPV, HIV, and pre-cancerous cervical lesions. *Mycoplasmataceae* infection in Tanzania is not well understood, especially when considering the differences between sexually transmitted species. To establish the prevalence of common *Mycoplasmataceae* cervical infections and evaluate their relationship with risk factors for cervical cancer, 1160 Tanzanian women responded to an epidemiological questionnaire and were tested for HIV, HPV, cervical lesions, *Mycoplasma genitalium*, *Mycoplasma hominis*, *Ureaplasma spp.*, and *Lactobacillus iners*. A subset of 134 women were used for 16s metagenomic sequencing of cervical DNA to establish the relative abundance of *Mycoplasmataceae* and *Lactobacillus* present. PCR detection of bacteria at the cervix found *Ureaplasma spp.* in 51.4% of women, *M. hominis* in 34%, *M. genitalium* in 2.3%, and *L. iners* in 75.6%. *M. hominis* and *M. genitalium* infection were significantly more prevalent among women with HPV and HIV. *M. hominis* prevalence was similar despite severity of cervical lesions, however abundance of *M. hominis* increased significantly in women with cervical lesions. These results emphasize the importance of understanding the relationship between *M. hominis* and HPV-related cervical pathogenesis.

INTRODUCTION

Cervical cancer mortality is higher in Eastern Africa than in any other region of the world (Formana et al., 2012). In Tanzania, cervical cancer is the most prevalent cancer in females (Human Papillomavirus and Related Diseases Report WORLD). Tanzania faces many issues which contribute to the burden of cervical cancer, including high HPV prevalence, high HIV prevalence, low condom use, irregular preventative screening, and lack of full implementation of the pap smear. In Europe and the U.S. preventative screening for cervical cancer is usually done by HPV testing or checking for lesions in the cervical epithelium via a pap smear. In Tanzania however, cervical screening is mainly visual inspection with acetic acid (VIA), which is markedly less sensitive for early detection of cervical lesions than the pap smear and does not grade lesions by severity. Cervical lesions detected during screening are usually associated with HPV infection, however recent studies have proposed that the cervical microbiome may be an important co-factor for the development of pre-cancerous and cancerous lesions (Mitra et al., 2016). Currently, it is not understood how, or which cervical microbiota contribute to cervical lesions, though in a previous study we found that the bacterial family *Mycoplasmataceae* was the most significant differential cervical bacteria between women with normal cervical cytology and those with pre-cancerous lesions in Tanzania (Klein et al., 2019). *Mycoplasmataceae* are the smallest known bacteria, in both physical, and genomic size. During infection of the cervicovaginal epithelium, *Mycoplasmataceae* establish a persistent, intracellular infection which can lead to inflammatory cytokine mediated tissue injury. Although it is currently unknown if there is a mechanistic relationship between HPV and *Mycoplasmataceae*, the nature of *Mycoplasma* infection

allows for direct interaction with HPV during co-infection of a single cell, and indirect interaction through cytokine responses.

Mycoplasmataceae is comprised of the genera *Mycoplasma* and *Ureaplasma*, which include several sexually transmitted species with global prevalence. Most notably, *M. hominis, M. genitalium*, and U. urealyticum are relatively common sexually transmitted infections (STIs) associated with cervical inflammation (Byers et al., 2009; Dehon et al., 2016; Onywera et al., 2019). Among *Mycoplasma*, only *M. genitalium* is sometimes included in regular STI screening, although *M. hominis* is believed to have similar pathogenesis. As a result, *M. hominis* has received significantly less study, and its relationship with HPV, HIV, and cervical lesions remains unclear. The prevalence of *M. genitalium, M. hominis*, and U. urealyticum in Tanzania has not previously been established in a large and diverse cohort, nor has it been considered alongside established risk factors for cervical dysplasia.

It has been suggested that high levels of cervicovaginal dysbiosis and transmission of *Mycoplasma* and other STIs in Eastern Africa is in part due to the commensal cervicovaginal bacteria in the region. Specifically, *L. iners* is the most prevalent cervicovaginal *Lactobacillus* in Eastern Africa, especially in HIV+ women, but has been shown to be less protective against cervicovaginal infection than other *Lactobacillus* (Jespers et al., 2015b). Whether a cervical microbiome dominated by *L. iners* is conducive to infection and proliferation of *Mycoplasmataceae*, and the

relationship between the bacteria and HPV pathogenesis, remains unclear. This study aims to establish the prevalence of common *Mycoplasmataceae* species in Tanzania and evaluate their relationship with *L. iners* and risk factors for cervical cancer, including HPV, HIV, and lifestyle factors.

RESULTS

Cohort demographics. DNA was successfully isolated from the cervical cytobrush samples of 1060 women. Complete data of cervical cytobrush DNA, pap-smear, VIA, HIV status, and epidemiological questionnaire response was available for 1002 women. Women with incomplete data were included in analyses where the missing data was not relevant. The cohort averaged 38.3 years old, ranging from 18 to 73. A large majority (92.3%) of the women screened reported at least one previous pregnancy, and 84.1% were sexually active within the 3 months preceding sampling. 67.4% of the cohort reported the use of at least one type of birth control, although it is unclear if they had recently used birth control at time of sampling. 17.6% of the cohort had tested positive for HIV and was on antiretroviral therapy at the time of sampling. Using a multiplex HPV genotyping PCR, we found that 46.1% of the cohort tested positive for at least one HPV genotype, and 38.2% of HPV positive women were coinfected with at least two genotypes.

There was a significant difference between the identification of cervical lesions between VIA and pap smear. Only 17% of women with pap smears graded HSIL had

lesions identified by VIA. Additionally, although 88.9% of the cohort tested negative for cervical lesions by VIA, only 20.1% of the cohort was graded NILM by pap smear. The majority of women had pap smears exhibiting low-grade cervical dysplasia: ASCUS (24.8%) and LSIL (30.8%). More severe cervical dysplasia was apparent in 24.4% of women (14.9% ASC-H, 9.5% HSIL).

***Mycoplasma* Screen.** Table 3.1 shows the breakdown of all data collected in this study and the variation of cervical cancer risk factors and *Mycoplasmataceae* prevalence within each group. PCR detection of *Mycoplasmataceae* at the cervix found a high prevalence of the bacterial family among Tanzanian women, 66% of whom tested positive for at least one *Mycoplasmataceae*. *Ureaplasma spp.* was the most prevalent *Mycoplasmataceae*, detectable in 51.4% of the cohort, followed by *Mycoplasma hominis* in 34%, and *Mycoplasma genitalium* in only 2.3% of women. *Lactobacillus iners* was more prevalent than *Mycoplasmataceae*, detectable in 75.6% of women. Detection of any *Mycoplasmataceae* significantly increased the likelihood of detection of other *Mycoplasmataceae* species in that individual (Supplemental 1). Women with *L. iners* also had higher prevalence of *Ureaplasma spp.* and *M. hominis* than woman without *L. iners.* Both *M. hominis* and *M. genitalium* were more common in women who reported previously having been diagnosed with an STI, though it is unclear if the STI was *Mycoplasma* related (Supplemental 1.3 and 1.4). *Mycoplasma* was prevalent amongst all age groups.

Table 3.1 Prevalence of *Mycoplasma*, HPV, HIV, and epidemiological factors. Values are listed as percentage of women positive for the condition labeled in each column. The cohort is broken down into sub-groups in each row, depending on results from testing or survey. A one-proportion Z-test was used to identify prevalence in subgroups that differ significantly from the cohort average. Values were considered significant when $p < 0.05$ and are labeled with a `*`. The column 'LSIL+' includes LSIL and ASCUS pap smear results for ease of interpretation. Similarly, the column 'HSIL+' includes HSIL and ASC-H pap smear results.

	n	Urea-plasma *spp.*	*M. hominis*	*M. genitalium*	*L. iners*	HPV+	HIV+	NILM	LSIL*	HSIL*	Age
Total	1060	51.4	34	2.3	75.6	46.1	17.6	20.1	55.5	24.4	38.3
HPV											
HPV+	489	53.6	**42.9***	2.2	**82.4***	--	**24.8***	17.3	52.6	**30.1***	36.9
HPV-	571	49.6	**26.3***	2.3	**69.7***	--	**11.4***	22.4	58.1	**19.5***	39.5
1 HPV	302	55.3	38.1	**1***	79.8	--	19.4	20.7	51.7	27.6	37.2
2+ HPV	187	50.8	**50.8***	4.3	**86.6***	--	**33.5***	**11.9***	54	**34.1***	36.5
HIV											
HIV+	181	**60.8***	**64.6***	**6.1***	**81.8***	**65.2***	--	16.9	60.1	23	39.2
HIV-	847	49.5	**27.6***	1.5	74.3	**42.3***	--	20.8	54.6	24.6	38.1
Cytology											
NILM	208	48.6	32.7	2.4	75.5	39.9	14.9	--	--	--	38.4
ASCUS	257	53.3	36.6	1.2	77.8	**34.6***	19.5	--	--	--	37.6
LSIL	319	48.9	32.3	2.8	74.9	**35.7***	18.8	--	--	--	38.8
ASC-H	155	55.5	35.5	1.3	75.5	41.3	14.8	--	--	--	37.6
HSIL	98	55.1	33.7	2	66.3	50	19.8	--	--	--	39.5
VIA											
0	873	51.1	33.7	2.3	75.6	44.3	**14.7***	21	55.7	23.3	38
1	109	49.5	35.8	2.8	79.8	**63.3***	**33***	15.7	55.6	28.7	37.1

Age											
18-29	209	54.5	33.5	2.4	**83.7***	52.6	**10.6***	21.4	53.4	25.2	25.8
30-39	374	50.3	37.7	2.9	76.7	50	20.9	18.1	55.5	26.4	34.5
40-49	321	51.1	33.6	2.2	74.8	41.7	19.2	21.6	58.1	20.3	44.1
50+	133	51.9	27.1	0.8	**59.4***	**36.1***	16	21.4	53.4	25.2	54.8
Last Sex											
< 3 months	876	52.9	35.3	2.5	77.4	47.1	15.7	19.6	56.7	23.7	37.4
4-12 months	81	48.1	32.1	1.2	65.4	38.3	**29.1***	17.5	50	32.5	39.6
> 12 months	85	42.4	**23.5***	1.2	**64.7***	44.7	26.2	28.2	50.6	21.2	46.6
Sex Partners											
1-2	455	47.9	**25.5***	1.5	**71.4***	**38.5***	**8.9***	20	59.1	20.9	38.6
3-5	465	54.8	**40.2***	2.8	77.4	**52.9***	**21.4***	19.2	54.3	26.5	37.7
> 5	102	52	**44.1***	2.9	82.4	50	**36.6***	26.2	47.5	26.3	39.3
Pregnancies											
0	80	47.5	32.5	2.5	76.3	52.5	**8.9***	20.3	58.2	21.5	32.5
1-2	319	53.9	36.4	2.8	**82.8***	50.5	19.2	21	57.1	21.9	32.9
3-5	492	51	33.3	2	72.8	45.3	19.3	21.1	55.7	23.2	39.8
> 5	151	51	32.5	2	68.2	**37.1***	13.2	15.4	51	**33.6***	47.7
Birth Control											
No	340	49.7	33.8	3.2	73.2	47.1	21.4	20.7	56.8	22.5	38.3
Yes	702	52.6	34.2	1.9	76.5	45.9	15.8	19.9	55.1	25	38.3
Birth Control Type											
Pills	370	51.6	34.6	1.4	75.1	42.7	17	19	56.6	24.4	40.7
Injection	416	54.6	35.8	2.6	78.1	48.8	15.9	20	**50***	**30***	37.4
Condom	44	43.2	40.9	4.5	81.8	**61.4***	**47.6***	27.3	50	22.7	35.9
Implant	124	57.3	37.9	1.6	78.2	42.7	12.9	19.1	**64.2***	**16.7***	33.9
Loop	70	47.1	24.3	2.9	67.1	42.9	**2.9***	20.6	47.1	32.3	43.8
Natural	12	41.7	33.3	0	**91.7***	41.7	16.7	25	58.3	16.6	40.1
STI Self-Report											
No recent	939	51.8	33.2	1.9	75.5	45.4	**15.1***	19.7	57	23.3	38.2
Yes recent	73	53.4	38.4	4.1	75.3	50.7	**37.5***	27.4	**42.5***	30.1	39

PCR Detection												
Ureaplasma spp.	545	--		38	2.9	**80.6***	48.1	20.8	18.9	54.9	26.2	38.1
M. hominis	360	**57.5***	--		3.9	**83.6***	**58.3***	**33.3***	19.3	55.8	24.9	37.7
M. genitalium	24	66.7	**58.3***	--		83.3	45.8	**45.8***	23.8	57.1	19.1	35.5
L. iners	801	54.8	**37.6***	2.5	--		**50.3***	19	20.2	56.4	23.4	37.4

Effects of HIV Infection. Being HIV+ increased odds of detection of all *Mycoplasmataceae* and *L. iners*. *Mycoplasma hominis* and *genitalium* infections were especially prevalent among HIV+ women when compared to HIV- (odds ratio (OR) 4.8 and 4.2 respectively), while *Ureaplasma spp.* and *L. iners* were only slightly more common (OR 1.6 for both). This data supports previous research suggesting the HIV+ population acts as a reservoir for *M. hominis* infection (Djigma et al., 2011). *Mycoplasma* was still quite prevalent among HIV- women (49.5%, 27.6%, 1.5% prevalence respectively for *Ureaplasma spp.*, *M. hominis*, and *M. genitalium* respectively).

Effects of HPV Infection. Women infected with at least one HPV genotype were significantly more likely to have cervical dysplasia, especially high-grade lesions (OR 1.3773 for non-NILM, OR 2.7108 for HSIL). HPV+ women were also more likely to be infected with *M. hominis* (OR 2.1, P<0.0001), while *M. genitalium* and *Ureaplasma* did not have a significant increase in prevalence associated with HPV (Figure 3.1). Commensal bacteria *L. iners*, was more likely to be present in HPV+ women (OR 2.0, P<0.00001). Co-infection with 2 or more different HPV genotypes was associated with higher prevalence of *M. hominis* and *M. genitalium* than women infected by 1 HPV genotype. Multiple HPV infection was much more common amongst HIV+ women, however this increase in *Mycoplasma* prevalence was also apparent in HIV- women with multiple HPV when compared to HIV-, single HPV women.

Effects of Cervical Cytology. *Mycoplasmataceae* were not significantly more or less prevalent among women with cervical dysplasia (Figure 3.1). Multivariate analysis of cervical cytology found that prevalence of HPV, number of pregnancies, use of injection-based birth control, and self-reporting of a previous STI varied significantly between cytology groups (Figure S3.1). Only HPV prevalence had an obvious positive relationship with severity of cervical lesions, while having more than 5 pregnancies or using injection-based birth control were associated with increased odds of high-grade cervical lesions.

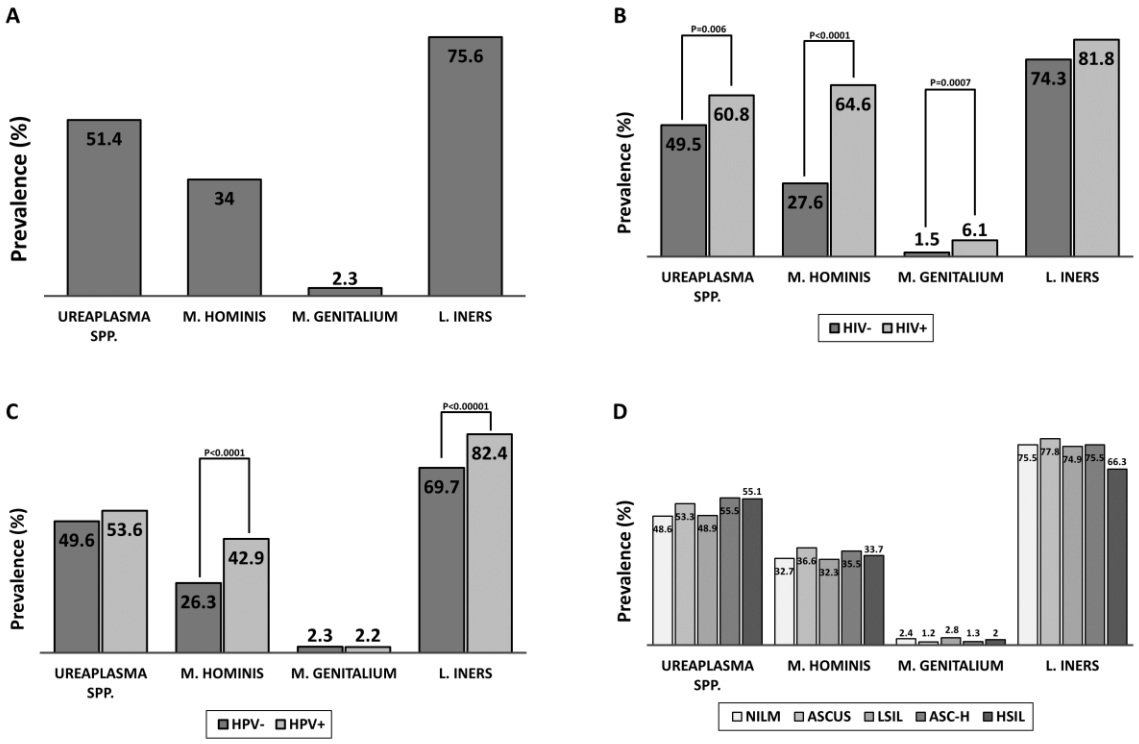

Figure 3.1 Relationship between *Mycoplasmataceae* abundance and cervical cancer risk factors. (a) Overall prevalence of the screened *Mycoplasmataceae* and *Lactobacillus* species in the cohort; (b) Comparison of prevalence between HIV+ and HIV- women; (c) Comparison of prevalence between HPV+ and HPV- women; (d) Comparison of prevalence between women based on cervical cytology.

Effects of Other Factors. Sexual history was an important factor for detection of *Mycoplasmataceae* and *L. iners*. Women with 3 or more unique previous sex partners were significantly more likely to be infected with *M. hominis*, HPV, and HIV and were more likely to test HSI*L*. Prevalence of *M. hominis* and *L. iners* was significantly higher among women who had been sexually active during the 3 months prior to sampling (Figure S3). Self-reported condom use was very low, especially for HIV- women (2.6%), contributing to increased transmission of *Mycoplasma* among sexually active women. Aging was associated with a significant decrease (P=0.0004) in *L. iners* prevalence, decreasing from 83.7% in women 18-29 to 59.4% in women 50+. Age did not appear to be related with a shift in *Mycoplasmataceae* prevalence, though women aged 50+ did have somewhat lower prevalence of *M. hominis* and *M. genitalium*, possibly related to menopause or decreased sexual activity. *L. iners* prevalence also decreased in women with 3 or more previous pregnancies, however this may have been influenced by a higher average age among high gravidity women.

Relative Abundance. A subset of 104 cervical samples was analyzed via 16s metagenomic sequencing to establish the relative abundance of *Mycoplasmataceae* and *L. iners* present. Each sample was rarefied to an even depth of 1,000 reads. After rarefication, women with more than 5 reads from *Ureaplasma spp.*, *M. hominis*, *M. genitalium*, or *L. iners* were considered positive for that bacteria. The prevalence of each bacteria was similar to results from PCR screening, though no *M. genitalium* reads were present among the subset of samples tested. By using the number of reads generated we were able to determine the relative abundance of each bacteria in each woman's cervical

microbiome. Using this, we estimated the relative abundance of the screened bacteria within cervical cytology groups by adjusting the prevalence of a bacteria by the average relative abundance of that bacteria in positive samples of each cytology grade. When looking at the relative abundance of *Mycoplasmataceae* in women with cervical dysplasia, it becomes apparent that a significantly larger portion of the cervical microbiota is *M. hominis* (Figure 3.2). *M. hominis* is the only *Mycoplasmataceae* which increases linearly with the development of more severe cervical lesions. *Ureaplasma spp.* were most abundant among HSIL women, however LSIL had a lower abundance than NILM women. *L. iners* was least abundant among HSIL women, but significantly more abundant in LSIL than NILM women. *Lactobacillus crispatus* is considered to be the most protective cervicovaginal microbe. Though we did not PCR screen for *L. crispatus*, no women with *L. crispatus* reads by 16s had *M. hominis*, suggesting *L. crispatus* protects against *Mycoplasma* infection, while *L. iners* does not.

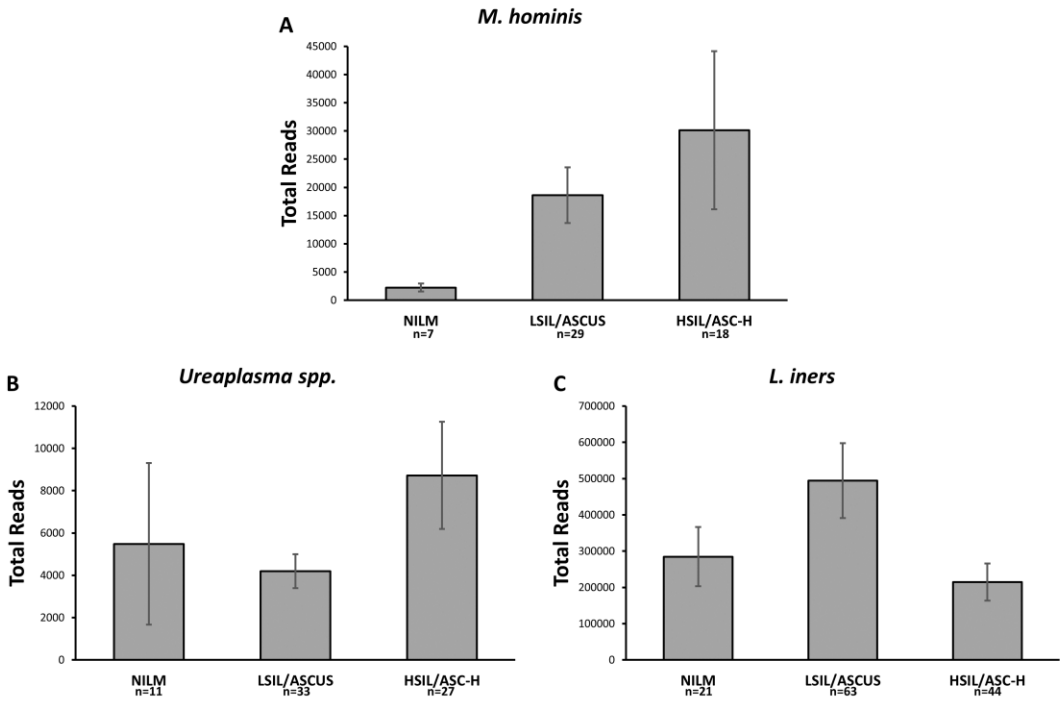

Figure 3.2 Relative abundance of *Mycoplasma* based on cervical cytology. Abundance among infected is the mean of positive 16s samples (n) adjusted by prevalence determined by PCR screen. Error bars represent standard error of the mean. (**a**) Expected number of *M. hominis* 16s DNA reads for 100 Tanzanian women of varying cervical cytology; (**b**) Expected number of *Ureaplasma spp.* 16s DNA reads for 100 Tanzanian women of varying cervical cytology; (**c**) Expected number of *L. iners* 16s DNA reads for 100 Tanzanian women of varying cervical cytology.

DISCUSSION

In this study, we found that cervical *Mycoplasma* infection is prevalent among Tanzanian women. Even though *M. genitalium* is more often screened for as a cervicovaginal infection, we found that *M. hominis* and *Ureaplasma spp.* were significantly more common in Tanzania. Similar results have been found in *M. hominis* and *M. genitalium* screens from other sub-Saharan African countries (You et al., 2002; Agbakoba et al., 2007; Redelinghuys et al., 2013; Kouegnigan Rerambiah et al., 2015b). The primers we used to detect *Ureaplasma spp.* included both U. urealyticum and U. parvu*M.* U. urealyticum is known to be a cervicovaginal pathogen, however U. parvum is sometimes commensal in the uterus. Because we took our samples partially from the endocervix, it is likely U. parvum originating from the internal cervical os may also have been detected. For this reason, we did not consider *Ureaplasma spp.* as a non-commensal infection and focus on the importance of *M. hominis* as a common, poorly understood cervical infection in Tanzania.

Women who reported having had an STI were more likely to have a *M. hominis* or *M. genitalium* infection, however most women with such an infection did not report any history of STI's. This indicates that most *M. hominis* and *M. genitalium* infections are asymptomatic, and thus go untreated. Currently, it is unclear how long a *Mycoplasma* infection of the cervix can persist while untreated. We detected higher prevalence of *M. hominis* among sexually active women, even those with a single long-term partner, suggesting sex may be important for persistence of *M. hominis* infection. Despite similar prevalence, significantly higher abundance of *M. hominis* in the presence of cervical

lesions, especially high-grade cervical lesions, suggests that proliferation of *M. hominis* and development of cervical lesions have some form of mechanistic relationship. It is possible proliferation of *M. hominis* drives the formation of cervical lesions, or that cervical lesions create a microenvironment that favors proliferation of *M. hominis*. Longitudinal sampling of *M. hominis* abundance and cervical cytology would help to clarify this relationship. *M. hominis* may also contribute to HPV-driven cervical lesion formation by increasing persistence of the pathogens during co-infection. We found that prevalence of *M. hominis* was significantly higher among HPV+ women, which could result from prolonged persistence increasing the likelihood of sampling an infection. This idea is supported by previous studies which have identified cervical pathogens, including *Mycoplasma*, as cofactors in the persistence of HPV infection (Gillet et al., 2011; Clarke et al., 2012; Guo et al., 2012; Vriend et al., 2015). The intracellular nature of *Mycoplasma* infection is particularly interesting when considering its relationship with HPV. Intracellular bacterial infections may directly interact with HPV replication in epithelial cells, while also contributing to the epithelium's immune microenvironment by influencing cytokine expression. The data presented here highlights the need for further research into *M. hominis* prevalence and pathogenesis, especially related to HPV, HIV, and cervical cancer.

Our data supports previous research suggesting *L. iners* is an especially common commensal cervical bacteria in sub-Sahara African countries. Increased prevalence of commensal *L. iners* among HIV+, HPV+, and *Mycoplasmataceae*+ women suggests that *L. iners* does not protect the cervix from infection, as other *Lactobacillus* species are

believed to do. This is further evidenced by our 16s data, where co-detection of *M. hominis* and *L. iners* was common, but *M. hominis* and *L. crispatus* were never detected together.

This study highlights the need to account for significant regional differences in cervicovaginal microbiota, especially *Mycoplasma*. The high prevalence of *M. hominis* and its association with risk factors for cervical cancer (HPV, HIV, and cervical lesions) demonstrates the importance of better understanding *M. hominis* pathogenesis. Our results suggest screening for *Mycoplasma* is especially important in Tanzania, particularly among women at high risk for cervical cancer. Establishing a screening and treatment protocol to address the prevalence of asymptomatic *Mycoplasma* infection could reduce transmission of HPV and HIV by reducing susceptibility to infection, and potentially prevent progression of cervical lesions. Long-term, longitudinal studies are needed to clarify whether *Mycoplasma* becomes abundant at the cervix preceding or following the development of lesions, which would help to clarify if *Mycoplasma* is driving formation of cervical lesions or benefitting from the microenvironment associated with lesions.

MATERIAL AND METHODS

Participants and ethical precautions. This study reports findings derived from an ongoing cross-sectional cohort study analyzing demographics of HPV and cervical cancer in HIV-positive and -negative women from rural and urban Tanzania. Between

March 2015 and February 2017, female patients undergoing cervical cancer screening were approached for enrollment in the study. Those who were pregnant, menstruating, under 18, reported being sick in the past 30 days, or had a preexisting, non-HIV, immunologic defect were excluded from the study. Disease histories and physical examinations were used to rule out any clinical symptoms or visible signs for these conditions. Samples were collected at three sites in Tanzania: Ocean Road Cancer Institute (ORCI) in Dar es Salaam and rural clinics in Chalinze and Bagamoyo. After collection of cervical samples and demographic data, samples from 1060 women were screened for *Mycoplasma* species and *Lactobacillus iners*. A subset of 132 women were also used for 16s metagenomic sequencing.

Demographic data collection. This study was approved for human subjects work by the University of Nebraska-Lincoln Institutional Review Board (IRB) under protocol ID: 14709. All study participants gave informed consent and were evaluated by study clinicians. A set of pretested, standardized questionnaires was used to gather demographic data. All personal identifiers were removed from samples to ensure patient confidentiality. With the permission of the patients, medical history was retrospectively retrieved from hospital medical records. More than 30 variables were identified and assessed in the questionnaire, including time since last sexual intercourse, number of sexual partners, number of pregnancies, use and type of birth control, and self-reported history of STI infections.

Specimen collection, HIV and pap tests. Blood samples were collected via venipuncture into acid-citrate-dextrose tubes and processed using centrifugation at the

on-site study laboratory within 6 h of being drawn. The separated plasma was tested at the ORCI, as part of standard of care, using Standard Diagnostics HIV-1/2 3.0 detection kit. Cervical cytobrush samples and pap smears were collected from the cervical transformation zone of all patients. Pap smears were examined by at least three trained cytologists and classified according to the pap classification protocol: negative for intraepithelial lesion or malignancy (NILM); atypical squamous cells of undetermined significance (ASC-US); low-grade squamous intraepithelial lesions (LSIL); atypical squamous cells but cannot exclude high-grade lesions (ASC-H); high-grade squamous intraepithelial lesions (HSIL). Cervical cytobrush specimens were placed in lysis buffer and then shipped to the Nebraska Center for Virology at the University of Nebraska-Lincoln (UNL) for processing.

DNA isolation. Cervical cytobrush samples were vortexed and separated from the brush with lysis buffer. DNA was extracted from the lysis buffer using the Qiagen Tissue extraction kit (Dneasy) according to the manufacturer's protocol. The DNA concentration was determined by UV spectrophotometer at 260/280 n*M*.

HPV genotyping. To determine HPV status, DNA samples were genotyped for HR-HPVs (types 16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66) and LR-HPVs (types 6 and 11) using a low-cost multiplex PCR assay (Samwel et al., 2019).

***Mycoplasmataceae* and *L. iners* Screen.** A multiplex PCR targeting *M. genitalium*, *M. hominis*, and *Ureaplasma spp.* was adapted from (Stellrecht et al., 2004),

with the addition of primers targeting *L. iners* established in (Kusters et al., 2015). Primers were mixed with sample DNA and Qiagen Multiplex PCR Master Mix according to the manufacturer's protocol.

The PCRs were performed in 25 µl. The cycling conditions were as follows: an initial denaturation of 95°C for 15 minutes, followed by 35 cycles, with 1 cycle consisting of denaturation at 94°C for 15 s, and annealing and extension at 60°C for 1 minute, then a final elongation of 72°C for 5 minutes. After amplification, DNA samples were run in 0.5% agarose gels containing Ethidium Bromide at 95 volts for 1 hour. Gels were then imaged using a Bio Rad ChemiDoc MP Imaging System to visualize bands.

Statistical Analyses. Multivariate analysis of variance (MANOVA) using one variable selected as fixed versus the other remaining dependent variables collected (*Ureaplasma spp.*, *M. hominis*, *M. genitalium*, *L. iners*, HPV, HIV, Age, time since last sexual activity, number of sex partners, number of pregnancies, self-reporting of STI infection, use of birth control, and type of birth control used) was used to identify significant differences between women with different cervical cytology, HIV, or HPV status. The birth control types considered were pills, injections, condoms, implants, loop, and natural. Odds ratios were calculated to identify groups with significantly increased odds of HPV, HIV, or *Mycoplasmataceae*. A p value of 0.05 was the maximum considered to be significant throughout the study.

16S rRNA library preparation, and sequencing of the V4 region. DNA samples were used for tag sequencing of the V4 hypervariable region of the 16S rRNA gene. A 250-bp section of the V4 region was amplified using universal primers described in reference 40. The PCRs were performed in 25 μl. The cycling conditions were as follows: an initial denaturation of 98°C for 3 min, followed by 25 cycles, with 1 cycle consisting of denaturation at 98°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 45 s, and then a final elongation of 68°C for 4 min. Following amplification, PCR products were analyzed on a 2% agarose gel to confirm correct product size. Normalized amplicons (1 to 2 ng/μl) from 144 samples were pooled together using an epMotion M5073 liquid handler (Eppendorf AG, Hamburg, Germany). Pooled libraries were sequenced using the Illumina MiSeq platform using the dual-index sequencing strategy outlined by (Kozich et al., 2013).

16S data processing and bacterial community analysis. The sequencing data obtained from the sequencer was subsequently analyzed using the Illumina MiSeq data analysis pipeline developed by the Fernando lab (described in detail at https://github.com/FernandoLab). Briefly, initial quality filtering was carried out to remove sequences that had ambiguous bases, incorrect lengths, and inaccurate assemblies. Subsequently, the quality-filtered reads were run through the UPARSE pipeline (http://www.drive5.com/uparse/) and subjected to chimera filtering and OTU clustering (at a similarity threshold of 97%), followed by the generation of an OTU table. Taxonomy was assigned to the OTUs using the assign_taxonomy.py command available in QIIME using the Greengenes database (May 2013). The OTU table was rarefied across

samples to the lowest sample depth (1,000 reads) using QIIME based on the Mersenne Twister pseudorandom number generator. All statistical analyses were performed with samples at an even depth.

Ethics statement. All human subject protocols were approved by safety committees at the Ocean Road Cancer Institute (ORCI) and UNL in accordance with the Helsinki Declaration. Participation by patients was entirely voluntary, and written patient consent was required for inclusion in the study.

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Understanding the factors which work with HPV infection to be sufficient for development of cervical cancer is a longstanding goal in this field. It is believed that HPV-dependent cervical cancer pathogenesis is affected by factors including HPV prevention and treatment practices, HPV genotype, HIV infection, human genetics, sociocultural factors, and the cervical microbiome. The work presented in this dissertation analyzed the cervical microbiota of 1160 Tanzanian women in relation to these risk factors for cervical cancer. In particular, *Mycoplasma hominis* was identified for its association with cervical cancer risk factors.

Women infected with at least one HPV genotype showed a shift in cervical microbiota which was more pronounced in those infected with multiple HPV genotypes. Though the nature of this relationship is unclear, cervical pathogens, including *Mycoplasma*, can act as cofactors in the persistence of HPV infection through yet-unknown mechanisms. *M. hominis* prevalence was significantly higher among HPV+ women, and higher still among women with multiple HPV genotypes, which could result from prolonged persistence increasing the likelihood of sampling an infection.

We also showed that HIV has a significant effect on the cervical microbiome, suggesting that changes in the cervical microenvironment brought on by HIV exert some selective pressure on cervical bacterial communities. The HIV+ population is of particular interest due to their greatly increased risk of cervical cancer, and more

pronounced changes in the cervical microbiota in the presence of cervical lesions. A significant increase in *Mycoplasma* prevalence among HIV+ women may contribute to the increased cervical cancer risk seen among this group, though further study is necessary to establish a mechanistic relationship which supports this idea. *M. genitalium* infection's ability to increase HIV viral shedding suggests that host innate responses to *M. genitalium* infection may similarly influence pathogenesis of other sexually transmitted infections. Induction of HPV in this way is particularly interesting based on the association between *Mycoplasma* and cervical lesions shown in this study, and warrants further research.

The majority of the 66% of Tanzanian women that tested positive for at least one *Mycoplasmataceae* were asymptomatic and untreated. This represents a large reservoir of *Mycoplasma* which persistently infects the population for an indeterminant amount of time, influencing the cervical immune microenvironment in both HIV+ and HIV- populations. The intracellular nature of these persistent *Mycoplasmataceae* infections is particularly interesting when considering a relationship with HPV pathogenesis, as it allows the bacteria to act as both an intracellular and extracellular stressor. Further study is needed to determine whether cytokine expression induced by persistent *Mycoplasma* infection in sub-Saharan Africa includes cytokines associated with developing cervical lesions (IL1, IL6, TNFα, IFNγ) at sufficient levels to influence HPV pathogenesis and cervical dysplasia. *Mycoplasma* infection also causes genomic stress on its host cell, which may increase the mutation and integration rate of HPV in a coinfected cell. This

warrants investigation as another mechanism contributing to cervical cancer risk in *Mycoplasma* and HPV coinfected women.

Among sexually transmitted *Mycoplasmataceae*, *M. genitalium* is the most studied and screened infection, however this study shows that *M. genitalium* is relatively uncommon among sub-Saharan African populations in comparison to *M. hominis* and *Ureaplasma spp.* Considering taxonomic differences in prevalence and pathogenesis is particularly important, as this study found different species of *Mycoplasmataceae* were differentially associated with cervical cancer risk factors. In particular, while all *Mycoplasmataceae* had similar prevalence among women with and without cervical lesions, *M. hominis* stood out as the only *Mycoplasmataceae* that greatly increased in relative abundance with more severe cervical lesions. This suggests that proliferation of *M. hominis* and development of cervical lesions have some form of mechanistic relationship. Long-term, longitudinal studies are needed to clarify whether *M. hominis* becomes abundant at the cervix preceding or following the development of lesions, which would help to clarify if *M. hominis* is driving formation of cervical lesions or benefitting from the microenvironment associated with lesions.

The high prevalence of *M. hominis* and its association with risk factors for cervical cancer demonstrates the importance of better understanding *Mycoplasma* pathogenesis and establishing a screening and treatment protocol to address the prevalence of asymptomatic *Mycoplasma* infection. This study emphasizes the need to

account for significant regional differences in cervicovaginal microbiota and suggests that the cervical microbiota could be used as a diagnostic marker for cervical cancer. With sufficient understanding, there is potential for the development of preventative probiotic or antibiotic treatments that could reduce cervical cancer risk by promoting colonization with cervical microbiota associated with healthy cytology. These results suggest a greater influence of the bacterial microbiota on the outcome of HPV infection than previously thought, and highlight *M. hominis* as a common, poorly understood cervical infection in sub-Saharan Africa.

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APPENDIX A (SUPPLEMENTARY FIGURES)

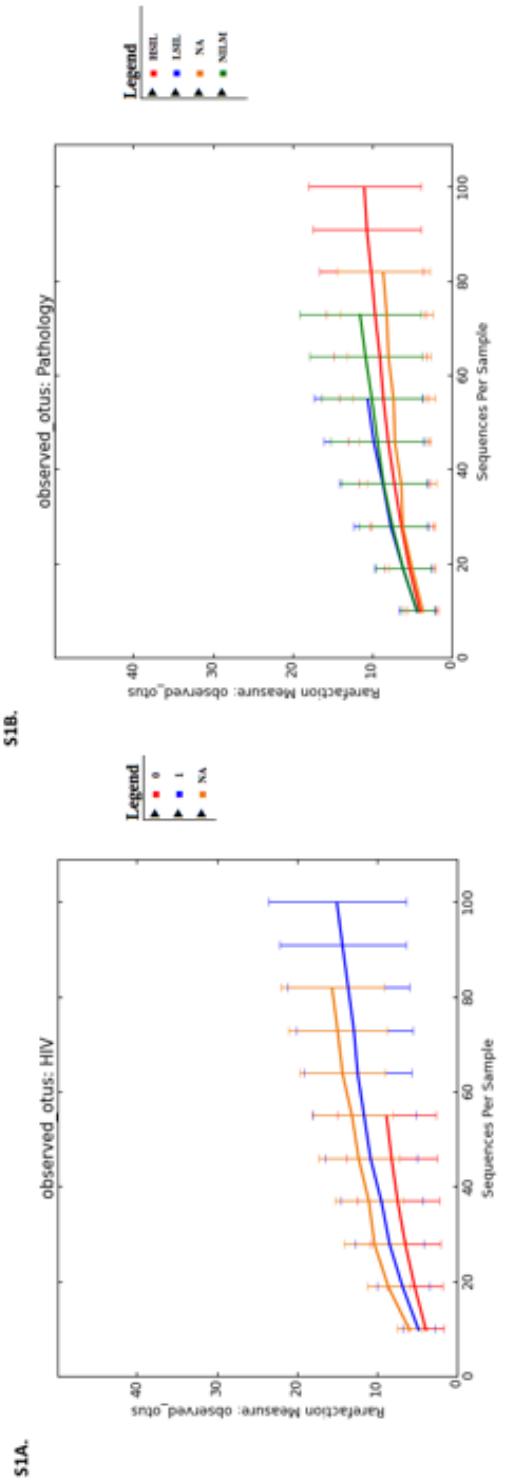

Figure S1 Bacterial 16S deep sequencing data were analyzed with rarefaction curves generated from the OTU data. These rarefactions were then compared with HIV status and cervical cytology. (S1A) The red squares and " 0" represent HIV-negative samples. The black squares and " 1" represent HIV-positive samples. The line indicated as " NA" is the unadjusted control. (S1B) Red squares represent HSIL, and blue squares represents LSI*L*. Green squares represent NIL*M*. The line indicated as " NA" is the unadjusted control.

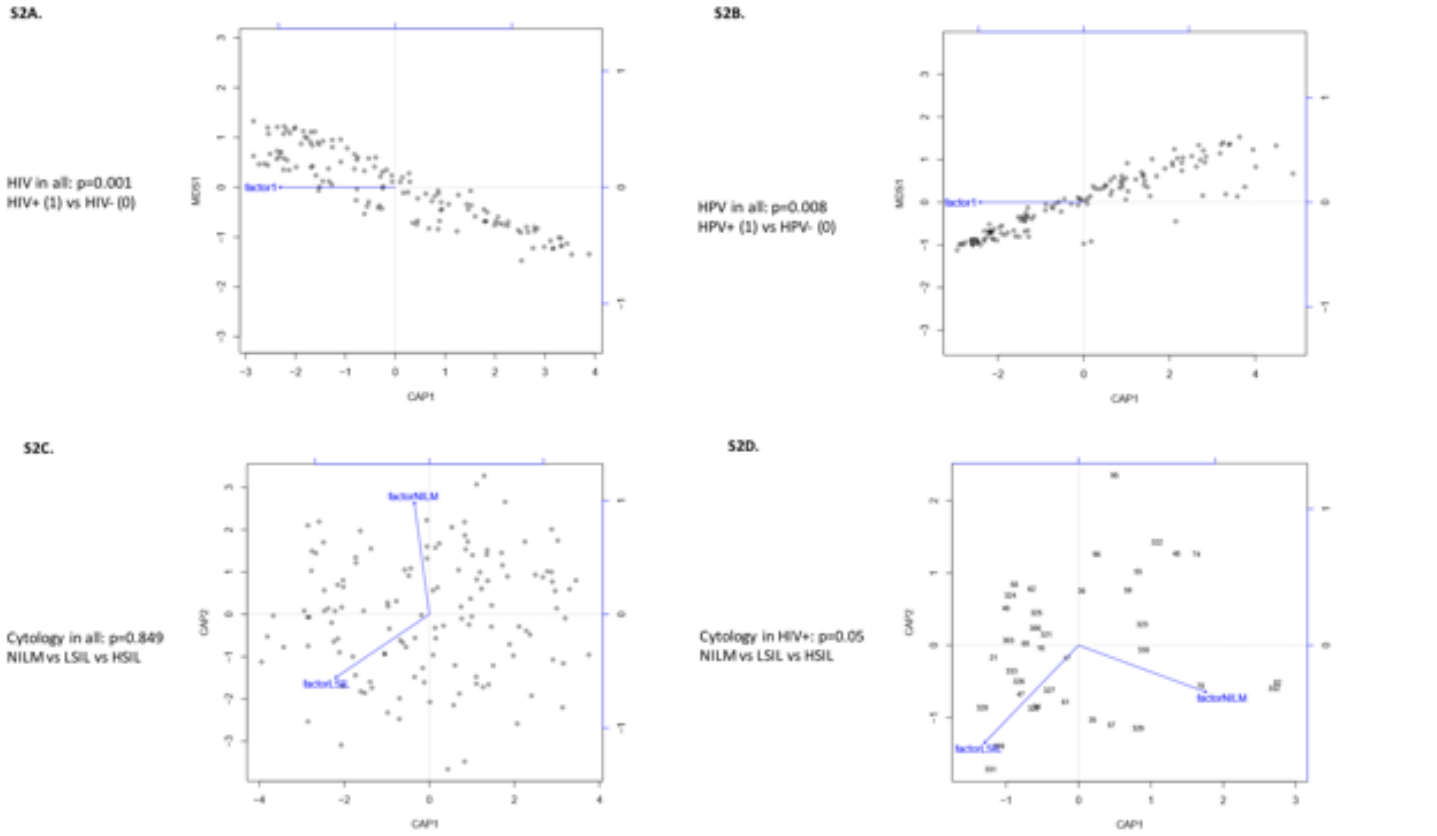

Figure S2 The bacterial community composition differences were analyzed in reference to cervical cytology and HIV status using db-RDA with the unweighted UniFrac distance matrix. (S2A) Db-RDA analysis of the bacterial community as a function of HIV status. (S2B) Db-RDA analysis of the bacterial community as a function of HIV status. (S2C) Db-RDA analysis of the bacterial community as a function of cytology (NILM versus LSIL or HSIL) at P = 0.849. (S2D) Db-RDA analysis of the bacterial community as a function of cytology (NILM versus LSIL or HSIL) at P = 0.05.

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[s]
Corrected Model	Ureaplasma spp.	1.391[a]	4	.348	1.393	.234	.006	5.573	.437
	M. hominis	.251[b]	4	.063	.279	.892	.001	1.116	.112
	M. genitalium	.052[c]	4	.013	.628	.643	.003	2.511	.207
	L. iners	1.063[d]	4	.266	1.416	.226	.006	5.665	.444
	HPV	5.389[e]	4	1.347	5.510	.000	.022	22.042	.977
	HIV	.452[f]	4	.113	.772	.543	.003	3.090	.258
	Age	456.037[g]	4	114.009	1.223	.299	.005	4.891	.386
	Last Sex	1.109[h]	4	.277	.784	.535	.003	3.137	.253
	Sex Partners	139.035[i]	4	34.759	1.947	.101	.008	7.789	.589
	Pregnancies	50.859[j]	4	12.715	2.491	.042	.010	9.963	.712
	BirthC	.979[k]	4	.245	1.113	.349	.004	4.453	.353
	Pills	1.323[l]	4	.331	1.443	.218	.006	5.773	.452
	Inject	3.834[m]	4	.959	4.038	.003	.016	16.151	.913
	Condom	.078[n]	4	.020	.498	.737	.002	1.992	.170
	Implants	.909[o]	4	.227	2.164	.071	.009	8.655	.642
	Loop	.345[p]	4	.086	1.401	.232	.006	5.603	.439
	Natural	.041[q]	4	.010	.874	.479	.003	3.497	.288
	STI	1.692[r]	4	.423	2.489	.042	.010	9.958	.712
Intercept	Ureaplasma spp.	233.363	1	233.363	934.826	.000	.484	934.826	1.000
	M. hominis	99.389	1	99.389	440.991	.000	.307	440.991	1.000
	M. genitalium	.343	1	.343	16.670	.000	.016	16.670	.983
	L. iners	462.929	1	462.929	2467.314	.000	.712	2467.314	1.000
	HPV	201.360	1	201.360	823.631	.000	.452	823.631	1.000
	HIV	26.382	1	26.382	180.247	.000	.153	180.247	1.000
	Age	1251308.347	1	1251308.347	13420.507	.000	.931	13420.507	1.000
	Last Sex	52.767	1	52.767	149.246	.000	.130	149.246	1.000
	Sex Partners	10091.962	1	10091.962	565.376	.000	.362	565.376	1.000
	Pregnancies	9836.266	1	9836.266	1926.934	.000	.659	1926.934	1.000
	BirthC	394.785	1	394.785	1794.763	.000	.643	1794.763	1.000
	Pills	109.767	1	109.767	478.975	.000	.325	478.975	1.000
	Inject	148.956	1	148.956	627.453	.000	.386	627.453	1.000
	Condom	1.459	1	1.459	37.078	.000	.036	37.078	1.000
	Implants	10.231	1	10.231	97.409	.000	.089	97.409	1.000
	Loop	4.408	1	4.408	71.687	.000	.067	71.687	1.000
	Natural	.103	1	.103	8.683	.003	.009	8.683	.837
	STI	17.795	1	17.795	104.705	.000	.095	104.705	1.000
Cytology	Ureaplasma spp.	1.391	4	.348	1.393	.234	.006	5.573	.437
	M. hominis	.251	4	.063	.279	.892	.001	1.116	.112
	M. genitalium	.052	4	.013	.628	.643	.003	2.511	.207
	L. iners	1.063	4	.266	1.416	.226	.006	5.665	.444
	HPV	5.389	4	1.347	5.510	.000	.022	22.042	.977
	HIV	.452	4	.113	.772	.543	.003	3.090	.258
	Age	456.037	4	114.009	1.223	.299	.005	4.891	.386
	Last Sex	1.109	4	.277	.784	.535	.003	3.137	.253
	Sex Partners	139.035	4	34.759	1.947	.101	.008	7.789	.589
	Pregnancies	50.859	4	12.715	2.491	.042	.010	9.963	.712
	BirthC	.979	4	.245	1.113	.349	.004	4.453	.353
	Pills	1.323	4	.331	1.443	.218	.006	5.773	.452
	Inject	3.834	4	.959	4.038	.003	.016	16.151	.913
	Condom	.078	4	.020	.498	.737	.002	1.992	.170
	Implants	.909	4	.227	2.164	.071	.009	8.655	.642
	Loop	.345	4	.086	1.401	.232	.006	5.603	.439
	Natural	.041	4	.010	.874	.479	.003	3.497	.288
	STI	1.692	4	.423	2.489	.042	.010	9.958	.712
Error	Ureaplasma spp.	248.884	997	.250					
	M. hominis	224.700	997	.225					
	M. genitalium	20.508	997	.021					
	L. iners	187.062	997	.188					
	HPV	243.745	997	.244					
	HIV	145.927	997	.146					
	Age	92958.813	997	93.239					
	Last Sex	352.496	997	.354					
	Sex Partners	17796.459	997	17.850					
	Pregnancies	5089.306	997	5.105					
	BirthC	219.305	997	.220					
	Pills	228.482	997	.229					
	Inject	236.686	997	.237					
	Condom	39.244	997	.039					
	Implants	104.720	997	.105					
	Loop	61.308	997	.061					
	Natural	11.815	997	.012					
	STI	169.441	997	.170					
Total	Ureaplasma spp.	516.000	1002						
	M. hominis	341.000	1002						
	M. genitalium	21.000	1002						
	L. iners	751.000	1002						
	HPV	464.000	1002						
	HIV	178.000	1002						
	Age	1564112.000	1002						
	Last Sex	414.000	1002						
	Sex Partners	29263.000	1002						
	Pregnancies	16187.000	1002						
	BirthC	675.000	1002						
	Pills	357.000	1002						
	Inject	401.000	1002						
	Condom	41.000	1002						
	Implants	120.000	1002						
	Loop	66.000	1002						
	Natural	12.000	1002						
	STI	188.000	1002						
Corrected Total	Ureaplasma spp.	250.275	1001						
	M. hominis	224.951	1001						
	M. genitalium	20.560	1001						
	L. iners	188.125	1001						
	HPV	249.134	1001						
	HIV	146.379	1001						
	Age	93414.850	1001						
	Last Sex	353.605	1001						
	Sex Partners	17935.494	1001						
	Pregnancies	5140.165	1001						
	BirthC	220.284	1001						
	Pills	229.805	1001						
	Inject	240.520	1001						
	Condom	39.322	1001						
	Implants	105.629	1001						
	Loop	61.653	1001						
	Natural	11.856	1001						
	STI	171.134	1001						

a. R Squared = .006 (Adjusted R Squared = .002)
b. R Squared = .001 (Adjusted R Squared = -.003)
c. R Squared = .003 (Adjusted R Squared = -.001)
d. R Squared = .006 (Adjusted R Squared = .002)
e. R Squared = .022 (Adjusted R Squared = .018)
f. R Squared = .003 (Adjusted R Squared = -.001)
g. R Squared = .005 (Adjusted R Squared = .001)
h. R Squared = .003 (Adjusted R Squared = -.001)
i. R Squared = .008 (Adjusted R Squared = .004)
j. R Squared = .010 (Adjusted R Squared = .006)
k. R Squared = .004 (Adjusted R Squared = .000)
l. R Squared = .006 (Adjusted R Squared = .002)
m. R Squared = .016 (Adjusted R Squared = .012)
n. R Squared = .002 (Adjusted R Squared = -.002)
o. R Squared = .009 (Adjusted R Squared = .005)
p. R Squared = .006 (Adjusted R Squared = .002)
q. R Squared = .003 (Adjusted R Squared = -.001)
r. R Squared = .010 (Adjusted R Squared = .006)
s. Computed using alpha = .05

Figure S3.1 MANOVA analysis of factors in realtionship cervical cytology

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[s]
Corrected Model	Cytology	2.639[a]	1	2.639	1.775	.183	.002	1.775	.265
	M. hominis	2.004[b]	1	2.004	8.989	.003	.009	8.989	.850
	M. genitalium	.041[c]	1	.041	1.976	.160	.002	1.976	.290
	L. iners	3.190[d]	1	3.190	17.252	.000	.017	17.252	.986
	HPV	.328[e]	1	.328	1.316	.252	.001	1.316	.209
	HIV	1.066[f]	1	1.066	7.337	.007	.007	7.337	.772
	Age	38.116[g]	1	38.116	.408	.523	.000	.408	.098
	Last Sex	1.112[h]	1	1.112	3.155	.076	.003	3.155	.427
	Sex Partners	.147[i]	1	.147	.008	.928	.000	.008	.051
	Pregnancies	.130[j]	1	.130	.025	.874	.000	.025	.053
	BirthC	.077[k]	1	.077	.351	.554	.000	.351	.091
	Pills	.003[l]	1	.003	.012	.911	.000	.012	.051
	Inject	.528[m]	1	.528	2.201	.138	.002	2.201	.317
	Condom	.068[n]	1	.068	1.723	.190	.002	1.723	.259
	Implants	.207[o]	1	.207	1.967	.161	.002	1.967	.289
	Loop	.064[p]	1	.064	1.032	.310	.001	1.032	.174
	Natural	.006[q]	1	.006	.469	.494	.000	.469	.105
	STI	.062[r]	1	.062	.364	.547	.000	.364	.093
Intercept	Cytology	2842.655	1	2842.655	1911.721	.000	.657	1911.721	1.000
	M. hominis	115.034	1	115.034	515.969	.000	.340	515.969	1.000
	M. genitalium	.432	1	.432	21.042	.000	.021	21.042	.996
	L. iners	559.837	1	559.837	3027.221	.000	.752	3027.221	1.000
	HPV	214.172	1	214.172	860.798	.000	.463	860.798	1.000
	HIV	31.246	1	31.246	215.024	.000	.177	215.024	1.000
	Age	1469826.967	1	1469826.967	15740.826	.000	.940	15740.826	1.000
	Last Sex	60.833	1	60.833	172.578	.000	.147	172.578	1.000
	Sex Partners	11314.909	1	11314.909	630.872	.000	.387	630.872	1.000
	Pregnancies	11034.668	1	11034.668	2146.808	.000	.682	2146.808	1.000
	BirthC	453.953	1	453.953	2061.483	.000	.673	2061.483	1.000
	Pills	127.117	1	127.117	553.156	.000	.356	553.156	1.000
	Inject	159.786	1	159.786	665.796	.000	.400	665.796	1.000
	Condom	1.696	1	1.696	43.214	.000	.041	43.214	1.000
	Implants	14.255	1	14.255	135.222	.000	.119	135.222	1.000
	Loop	4.375	1	4.375	71.034	.000	.066	71.034	1.000
	Natural	.145	1	.145	12.259	.000	.012	12.259	.938
	STI	16.913	1	16.913	98.862	.000	.090	98.862	1.000
Ureaplasmaspp	Cytology	2.639	1	2.639	1.775	.183	.002	1.775	.265
	M. hominis	2.004	1	2.004	8.989	.003	.009	8.989	.850
	M. genitalium	.041	1	.041	1.976	.160	.002	1.976	.290
	L. iners	3.190	1	3.190	17.252	.000	.017	17.252	.986
	HPV	.328	1	.328	1.316	.252	.001	1.316	.209
	HIV	1.066	1	1.066	7.337	.007	.007	7.337	.772
	Age	38.116	1	38.116	.408	.523	.000	.408	.098
	Last Sex	1.112	1	1.112	3.155	.076	.003	3.155	.427
	Sex Partners	.147	1	.147	.008	.928	.000	.008	.051
	Pregnancies	.130	1	.130	.025	.874	.000	.025	.053
	BirthC	.077	1	.077	.351	.554	.000	.351	.091
	Pills	.003	1	.003	.012	.911	.000	.012	.051
	Inject	.528	1	.528	2.201	.138	.002	2.201	.317
	Condom	.068	1	.068	1.723	.190	.002	1.723	.259
	Implants	.207	1	.207	1.967	.161	.002	1.967	.289
	Loop	.064	1	.064	1.032	.310	.001	1.032	.174
	Natural	.006	1	.006	.469	.494	.000	.469	.105
	STI	.062	1	.062	.364	.547	.000	.364	.093
Error	Cytology	1486.962	1000	1.487					
	M. hominis	222.947	1000	.223					
	M. genitalium	20.519	1000	.021					
	L. iners	184.934	1000	.185					
	HPV	248.806	1000	.249					
	HIV	145.313	1000	.145					
	Age	93376.734	1000	93.377					
	Last Sex	352.493	1000	.352					
	Sex Partners	17935.347	1000	17.935					
	Pregnancies	5140.035	1000	5.140					
	BirthC	220.207	1000	.220					
	Pills	229.803	1000	.230					
	Inject	239.992	1000	.240					
	Condom	39.255	1000	.039					
	Implants	105.421	1000	.105					
	Loop	61.589	1000	.062					
	Natural	11.851	1000	.012					
	STI	171.072	1000	.171					
Total	Cytology	4340.000	1002						
	M. hominis	341.000	1002						
	M. genitalium	21.000	1002						
	L. iners	751.000	1002						
	HPV	464.000	1002						
	HIV	178.000	1002						
	Age	1564112.000	1002						
	Last Sex	414.000	1002						
	Sex Partners	29263.000	1002						
	Pregnancies	16187.000	1002						
	BirthC	675.000	1002						
	Pills	357.000	1002						
	Inject	401.000	1002						
	Condom	41.000	1002						
	Implants	120.000	1002						
	Loop	66.000	1002						
	Natural	12.000	1002						
	STI	188.000	1002						
Corrected Total	Cytology	1489.601	1001						
	M. hominis	224.951	1001						
	M. genitalium	20.560	1001						
	L. iners	188.125	1001						
	HPV	249.134	1001						
	HIV	146.379	1001						
	Age	93414.850	1001						
	Last Sex	353.605	1001						
	Sex Partners	17935.494	1001						
	Pregnancies	5140.165	1001						
	BirthC	220.284	1001						
	Pills	229.805	1001						
	Inject	240.520	1001						
	Condom	39.322	1001						
	Implants	105.629	1001						
	Loop	61.653	1001						
	Natural	11.856	1001						
	STI	171.134	1001						

a. R Squared = .002 (Adjusted R Squared = .001)
b. R Squared = .009 (Adjusted R Squared = .008)
c. R Squared = .002 (Adjusted R Squared = .001)
d. R Squared = .017 (Adjusted R Squared = .016)
e. R Squared = .001 (Adjusted R Squared = .000)
f. R Squared = .007 (Adjusted R Squared = .006)
g. R Squared = .000 (Adjusted R Squared = -.001)
h. R Squared = .003 (Adjusted R Squared = .002)
i. R Squared = .000 (Adjusted R Squared = -.001)
j. R Squared = .000 (Adjusted R Squared = -.001)
k. R Squared = .000 (Adjusted R Squared = -.001)
l. R Squared = .000 (Adjusted R Squared = -.001)
m. R Squared = .002 (Adjusted R Squared = .001)
n. R Squared = .002 (Adjusted R Squared = .001)
o. R Squared = .002 (Adjusted R Squared = .001)
p. R Squared = .001 (Adjusted R Squared = .000)
q. R Squared = .000 (Adjusted R Squared = -.001)
r. R Squared = .000 (Adjusted R Squared = -.001)
s. Computed using alpha = .05

Figure S3.2 MANOVA analysis of factors in relationship to *Ureaplasma spp.* detection

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[s]
Corrected Model	Cytology	.036[a]	1	.036	.024	.876	.000	.024	.053
	Ureaplasma spp.	2.230[b]	1	2.230	8.989	.003	.009	8.989	.850
	M. genitalium	.105[c]	1	.105	5.119	.024	.005	5.119	.618
	L. iners	3.848[d]	1	3.848	20.880	.000	.020	20.880	.995
	HPV	6.450[e]	1	6.450	26.579	.000	.026	26.579	.999
	HIV	13.167[f]	1	13.167	98.840	.000	.090	98.840	1.000
	Age	165.895[g]	1	165.895	1.779	.183	.002	1.779	.266
	Last Sex	1.396[h]	1	1.396	3.962	.047	.004	3.962	.511
	Sex Partners	99.308[i]	1	99.308	5.568	.018	.006	5.568	.654
	Pregnancies	5.212[j]	1	5.212	1.015	.314	.001	1.015	.172
	BirthC	.002[k]	1	.002	.010	.919	.000	.010	.051
	Pills	.028[l]	1	.028	.121	.727	.000	.121	.064
	Inject	.136[m]	1	.136	.566	.452	.001	.566	.117
	Condom	.019[n]	1	.019	.474	.491	.000	.474	.106
	Implants	.021[o]	1	.021	.197	.657	.000	.197	.073
	Loop	.133[p]	1	.133	2.155	.142	.002	2.155	.311
	Natural	3.124E-5[q]	1	3.124E-5	.003	.959	.000	.003	.050
	STI	.841[r]	1	.841	4.941	.026	.005	4.941	.603
Intercept	Cytology	2565.849	1	2565.849	1722.550	.000	.633	1722.550	1.000
	Ureaplasma spp.	253.583	1	253.583	1022.322	.000	.506	1022.322	1.000
	M. genitalium	.536	1	.536	26.196	.000	.026	26.196	.999
	L. iners	534.027	1	534.027	2897.959	.000	.743	2897.959	1.000
	HPV	216.143	1	216.143	890.637	.000	.471	890.637	1.000
	HIV	42.089	1	42.089	315.953	.000	.240	315.953	1.000
	Age	1311260.933	1	1311260.933	14061.937	.000	.934	14061.937	1.000
	Last Sex	48.821	1	48.821	138.613	.000	.122	138.613	1.000
	Sex Partners	10824.290	1	10824.290	606.872	.000	.378	606.872	1.000
	Pregnancies	9775.440	1	9775.440	1903.706	.000	.656	1903.706	1.000
	BirthC	407.723	1	407.723	1850.912	.000	.649	1850.912	1.000
	Pills	115.365	1	115.365	502.074	.000	.334	502.074	1.000
	Inject	146.954	1	146.954	611.332	.000	.379	611.332	1.000
	Condom	1.615	1	1.615	41.101	.000	.039	41.101	1.000
	Implants	13.238	1	13.238	125.354	.000	.111	125.354	1.000
	Loop	3.458	1	3.458	56.208	.000	.053	56.208	1.000
	Natural	.128	1	.128	10.777	.001	.011	10.777	.907
	STI	17.512	1	17.512	102.836	.000	.093	102.836	1.000
M.hominis	Cytology	.036	1	.036	.024	.876	.000	.024	.053
	Ureaplasma spp.	2.230	1	2.230	8.989	.003	.009	8.989	.850
	M. genitalium	.105	1	.105	5.119	.024	.005	5.119	.618
	L. iners	3.848	1	3.848	20.880	.000	.020	20.880	.995
	HPV	6.450	1	6.450	26.579	.000	.026	26.579	.999
	HIV	13.167	1	13.167	98.840	.000	.090	98.840	1.000
	Age	165.895	1	165.895	1.779	.183	.002	1.779	.266
	Last Sex	1.396	1	1.396	3.962	.047	.004	3.962	.511
	Sex Partners	99.308	1	99.308	5.568	.018	.006	5.568	.654
	Pregnancies	5.212	1	5.212	1.015	.314	.001	1.015	.172
	BirthC	.002	1	.002	.010	.919	.000	.010	.051
	Pills	.028	1	.028	.121	.727	.000	.121	.064
	Inject	.136	1	.136	.566	.452	.001	.566	.117
	Condom	.019	1	.019	.474	.491	.000	.474	.106
	Implants	.021	1	.021	.197	.657	.000	.197	.073
	Loop	.133	1	.133	2.155	.142	.002	2.155	.311
	Natural	3.124E-5	1	3.124E-5	.003	.959	.000	.003	.050
	STI	.841	1	.841	4.941	.026	.005	4.941	.603
Error	Cytology	1489.564	1000	1.490					
	Ureaplasma spp.	248.046	1000	.248					
	M. genitalium	20.455	1000	.020					
	L. iners	184.277	1000	.184					
	HPV	242.684	1000	.243					
	HIV	133.212	1000	.133					
	Age	93248.955	1000	93.249					
	Last Sex	352.209	1000	.352					
	Sex Partners	17836.186	1000	17.836					
	Pregnancies	5134.952	1000	5.135					
	BirthC	220.282	1000	.220					
	Pills	229.777	1000	.230					
	Inject	240.384	1000	.240					
	Condom	39.304	1000	.039					
	Implants	105.608	1000	.106					
	Loop	61.520	1000	.062					
	Natural	11.856	1000	.012					
	STI	170.292	1000	.170					
Total	Cytology	4340.000	1002						
	Ureaplasma spp.	516.000	1002						
	M. genitalium	21.000	1002						
	L. iners	751.000	1002						
	HPV	464.000	1002						
	HIV	178.000	1002						
	Age	1564112.000	1002						
	Last Sex	414.000	1002						
	Sex Partners	29263.000	1002						
	Pregnancies	16187.000	1002						
	BirthC	675.000	1002						
	Pills	357.000	1002						
	Inject	401.000	1002						
	Condom	41.000	1002						
	Implants	120.000	1002						
	Loop	66.000	1002						
	Natural	12.000	1002						
	STI	188.000	1002						
Corrected Total	Cytology	1489.601	1001						
	Ureaplasma spp.	250.275	1001						
	M. genitalium	20.560	1001						
	L. iners	188.125	1001						
	HPV	249.134	1001						
	HIV	146.379	1001						
	Age	93414.850	1001						
	Last Sex	353.605	1001						
	Sex Partners	17935.494	1001						
	Pregnancies	5140.165	1001						
	BirthC	220.284	1001						
	Pills	229.805	1001						
	Inject	240.520	1001						
	Condom	39.322	1001						
	Implants	105.629	1001						
	Loop	61.653	1001						
	Natural	11.856	1001						
	STI	171.134	1001						

a. R Squared = .000 (Adjusted R Squared = -.001)
b. R Squared = .009 (Adjusted R Squared = .008)
c. R Squared = .005 (Adjusted R Squared = .004)
d. R Squared = .020 (Adjusted R Squared = .019)
e. R Squared = .026 (Adjusted R Squared = .025)
f. R Squared = .090 (Adjusted R Squared = .089)
g. R Squared = .002 (Adjusted R Squared = .001)
h. R Squared = .004 (Adjusted R Squared = .003)
i. R Squared = .006 (Adjusted R Squared = .005)
j. R Squared = .001 (Adjusted R Squared = .000)
k. R Squared = .000 (Adjusted R Squared = -.001)
l. R Squared = .000 (Adjusted R Squared = -.001)
m. R Squared = .001 (Adjusted R Squared = .000)
n. R Squared = .000 (Adjusted R Squared = -.001)
o. R Squared = .000 (Adjusted R Squared = -.001)
p. R Squared = .002 (Adjusted R Squared = .001)
q. R Squared = .000 (Adjusted R Squared = -.001)
r. R Squared = .005 (Adjusted R Squared = .004)
s. Computed using alpha = .05

Figure S3.3 MANOVA analysis of factors in relationship to *M. hominis* detection

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[s]
Corrected Model	Cytology	.009[a]	1	.009	.006	.940	.000	.006	.051
	Ureaplasma spp.	.494[b]	1	.494	1.976	.160	.002	1.976	.290
	M. hominis	1.146[c]	1	1.146	5.119	.024	.005	5.119	.618
	L. iners	.077[d]	1	.077	.411	.522	.000	.411	.098
	HPV	.026[e]	1	.026	.103	.749	.000	.103	.062
	HIV	1.912[f]	1	1.912	13.233	.000	.013	13.233	.953
	Age	178.258[g]	1	178.258	1.912	.167	.002	1.912	.282
	Last Sex	.226[h]	1	.226	.640	.424	.001	.640	.126
	Sex Partners	.007[i]	1	.007	.000	.984	.000	.000	.050
	Pregnancies	1.087[j]	1	1.087	.212	.646	.000	.212	.075
	BirthC	.064[k]	1	.064	.290	.590	.000	.290	.084
	Pills	.300[l]	1	.300	1.306	.253	.001	1.306	.208
	Inject	.124[m]	1	.124	.515	.473	.001	.515	.111
	Condom	.063[n]	1	.063	1.612	.204	.002	1.612	.245
	Implants	.013[o]	1	.013	.122	.727	.000	.122	.064
	Loop	.019[p]	1	.019	.300	.584	.000	.300	.085
	Natural	.003[q]	1	.003	.260	.611	.000	.260	.080
	STI	1.915[r]	1	1.915	11.319	.001	.011	11.319	.919
Intercept	Cytology	231.246	1	231.246	155.241	.000	.134	155.241	1.000
	Ureaplasma spp.	28.549	1	28.549	114.298	.000	.103	114.298	1.000
	M. hominis	16.906	1	16.906	75.539	.000	.070	75.539	1.000
	L. iners	49.890	1	49.890	265.303	.000	.210	265.303	1.000
	HPV	16.373	1	16.373	65.726	.000	.062	65.726	1.000
	HIV	8.618	1	8.618	59.656	.000	.056	59.656	1.000
	Age	111983.192	1	111983.192	1201.065	.000	.546	1201.065	1.000
	Last Sex	3.136	1	3.136	8.875	.003	.009	8.875	.845
	Sex Partners	934.770	1	934.770	52.118	.000	.050	52.118	1.000
	Pregnancies	847.514	1	847.514	164.916	.000	.142	164.916	1.000
	BirthC	34.419	1	34.419	156.294	.000	.135	156.294	1.000
	Pills	7.326	1	7.326	31.919	.000	.031	31.919	1.000
	Inject	15.733	1	15.733	65.445	.000	.061	65.445	1.000
	Condom	.375	1	.375	9.543	.002	.009	9.543	.870
	Implants	.955	1	.955	9.042	.003	.009	9.042	.852
	Loop	.529	1	.529	8.591	.003	.009	8.591	.834
	Natural	.003	1	.003	.260	.611	.000	.260	.080
	STI	6.263	1	6.263	37.010	.000	.036	37.010	1.000
M.genitalium	Cytology	.009	1	.009	.006	.940	.000	.006	.051
	Ureaplasma spp.	.494	1	.494	1.976	.160	.002	1.976	.290
	M. hominis	1.146	1	1.146	5.119	.024	.005	5.119	.618
	L. iners	.077	1	.077	.411	.522	.000	.411	.098
	HPV	.026	1	.026	.103	.749	.000	.103	.062
	HIV	1.912	1	1.912	13.233	.000	.013	13.233	.953
	Age	178.258	1	178.258	1.912	.167	.002	1.912	.282
	Last Sex	.226	1	.226	.640	.424	.001	.640	.126
	Sex Partners	.007	1	.007	.000	.984	.000	.000	.050
	Pregnancies	1.087	1	1.087	.212	.646	.000	.212	.075
	BirthC	.064	1	.064	.290	.590	.000	.290	.084
	Pills	.300	1	.300	1.306	.253	.001	1.306	.208
	Inject	.124	1	.124	.515	.473	.001	.515	.111
	Condom	.063	1	.063	1.612	.204	.002	1.612	.245
	Implants	.013	1	.013	.122	.727	.000	.122	.064
	Loop	.019	1	.019	.300	.584	.000	.300	.085
	Natural	.003	1	.003	.260	.611	.000	.260	.080
	STI	1.915	1	1.915	11.319	.001	.011	11.319	.919
Error	Cytology	1489.592	1000	1.490					
	Ureaplasma spp.	249.782	1000	.250					
	M. hominis	223.805	1000	.224					
	L. iners	188.047	1000	.188					
	HPV	249.108	1000	.249					
	HIV	144.467	1000	.144					
	Age	93236.592	1000	93.237					
	Last Sex	353.379	1000	.353					
	Sex Partners	17935.487	1000	17.935					
	Pregnancies	5139.878	1000	5.139					
	BirthC	220.220	1000	.220					
	Pills	229.506	1000	.230					
	Inject	240.396	1000	.240					
	Condom	39.259	1000	.039					
	Implants	105.616	1000	.106					
	Loop	61.634	1000	.062					
	Natural	11.853	1000	.012					
	STI	169.218	1000	.169					
Total	Cytology	4340.000	1002						
	Ureaplasma spp.	516.000	1002						
	M. hominis	341.000	1002						
	L. iners	751.000	1002						
	HPV	464.000	1002						
	HIV	178.000	1002						
	Age	1564112.000	1002						
	Last Sex	414.000	1002						
	Sex Partners	29263.000	1002						
	Pregnancies	16187.000	1002						
	BirthC	675.000	1002						
	Pills	357.000	1002						
	Inject	401.000	1002						
	Condom	41.000	1002						
	Implants	120.000	1002						
	Loop	66.000	1002						
	Natural	12.000	1002						
	STI	188.000	1002						
Corrected Total	Cytology	1489.601	1001						
	Ureaplasma spp.	250.275	1001						
	M. hominis	224.951	1001						
	L. iners	188.125	1001						
	HPV	249.134	1001						
	HIV	146.379	1001						
	Age	93414.850	1001						
	Last Sex	353.605	1001						
	Sex Partners	17935.494	1001						
	Pregnancies	5140.165	1001						
	BirthC	220.284	1001						
	Pills	229.805	1001						
	Inject	240.520	1001						
	Condom	39.322	1001						
	Implants	105.629	1001						
	Loop	61.653	1001						
	Natural	11.856	1001						
	STI	171.134	1001						

a. R Squared = .000 (Adjusted R Squared = -.001)
b. R Squared = .002 (Adjusted R Squared = .001)
c. R Squared = .005 (Adjusted R Squared = .004)
d. R Squared = .000 (Adjusted R Squared = -.001)
e. R Squared = .000 (Adjusted R Squared = -.001)
f. R Squared = .013 (Adjusted R Squared = .012)
g. R Squared = .002 (Adjusted R Squared = .001)
h. R Squared = .001 (Adjusted R Squared = .000)
i. R Squared = .000 (Adjusted R Squared = -.001)
j. R Squared = .000 (Adjusted R Squared = -.001)
k. R Squared = .000 (Adjusted R Squared = -.001)
l. R Squared = .001 (Adjusted R Squared = .000)
m. R Squared = .001 (Adjusted R Squared = .000)
n. R Squared = .002 (Adjusted R Squared = .001)
o. R Squared = .000 (Adjusted R Squared = -.001)
p. R Squared = .000 (Adjusted R Squared = -.001)
q. R Squared = .000 (Adjusted R Squared = -.001)
r. R Squared = .011 (Adjusted R Squared = .010)
s. Computed using alpha = .05

Figure S3.4 MANOVA analysis of factors in relationship to *M. genitalium* detection

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[s]
Corrected Model	Cytology	2.975[a]	1	2.975	2.001	.158	.002	2.001	.293
	M. genitalium	.008[b]	1	.008	.411	.522	.000	.411	.098
	Ureaplasma spp.	4.244[c]	1	4.244	17.252	.000	.017	17.252	.986
	M. hominis	4.601[d]	1	4.601	20.880	.000	.020	20.880	.995
	HPV	5.185[e]	1	5.185	21.254	.000	.021	21.254	.996
	HIV	.714[f]	1	.714	4.901	.027	.005	4.901	.599
	Age	2314.393[g]	1	2314.393	25.405	.000	.025	25.405	.999
	Last Sex	3.423[h]	1	3.423	9.776	.002	.010	9.776	.878
	Sex Partners	38.343[i]	1	38.343	2.142	.144	.002	2.142	.310
	Pregnancies	54.860[j]	1	54.860	10.788	.001	.011	10.788	.907
	BirthC	.348[k]	1	.348	1.581	.209	.002	1.581	.241
	Pills	.001[l]	1	.001	.004	.948	.000	.004	.050
	Inject	.580[m]	1	.580	2.419	.120	.002	2.419	.343
	Condom	.057[n]	1	.057	1.448	.229	.001	1.448	.225
	Implants	.050[o]	1	.050	.471	.493	.000	.471	.105
	Loop	.106[p]	1	.106	1.723	.190	.002	1.723	.259
	Natural	.021[q]	1	.021	1.807	.179	.002	1.807	.269
	STI	.011[r]	1	.011	.064	.800	.000	.064	.057
Intercept	Cytology	2221.023	1	2221.023	1494.002	.000	.599	1494.002	1.000
	M. genitalium	.280	1	.280	13.620	.000	.013	13.620	.959
	Ureaplasma spp.	171.570	1	171.570	697.350	.000	.411	697.350	1.000
	M. hominis	68.313	1	68.313	310.022	.000	.237	310.022	1.000
	HPV	133.788	1	133.788	548.425	.000	.354	548.425	1.000
	HIV	19.816	1	19.816	136.036	.000	.120	136.036	1.000
	Age	1155523.622	1	1155523.622	12684.059	.000	.927	12684.059	1.000
	Last Sex	58.645	1	58.645	167.470	.000	.143	167.470	1.000
	Sex Partners	7946.487	1	7946.487	444.009	.000	.307	444.009	1.000
	Pregnancies	8983.083	1	8983.083	1766.479	.000	.639	1766.479	1.000
	BirthC	330.703	1	330.703	1503.627	.000	.601	1503.627	1.000
	Pills	95.219	1	95.219	414.346	.000	.293	414.346	1.000
	Inject	112.317	1	112.317	468.106	.000	.319	468.106	1.000
	Condom	1.007	1	1.007	25.645	.000	.025	25.645	.999
	Implants	10.074	1	10.074	95.414	.000	.087	95.414	1.000
	Loop	3.879	1	3.879	63.018	.000	.059	63.018	1.000
	Natural	.065	1	.065	5.518	.019	.005	5.518	.651
	STI	13.041	1	13.041	76.208	.000	.071	76.208	1.000
Liners	Cytology	2.975	1	2.975	2.001	.158	.002	2.001	.293
	M. genitalium	.008	1	.008	.411	.522	.000	.411	.098
	Ureaplasma spp.	4.244	1	4.244	17.252	.000	.017	17.252	.986
	M. hominis	4.601	1	4.601	20.880	.000	.020	20.880	.995
	HPV	5.185	1	5.185	21.254	.000	.021	21.254	.996
	HIV	.714	1	.714	4.901	.027	.005	4.901	.599
	Age	2314.393	1	2314.393	25.405	.000	.025	25.405	.999
	Last Sex	3.423	1	3.423	9.776	.002	.010	9.776	.878
	Sex Partners	38.343	1	38.343	2.142	.144	.002	2.142	.310
	Pregnancies	54.860	1	54.860	10.788	.001	.011	10.788	.907
	BirthC	.348	1	.348	1.581	.209	.002	1.581	.241
	Pills	.001	1	.001	.004	.948	.000	.004	.050
	Inject	.580	1	.580	2.419	.120	.002	2.419	.343
	Condom	.057	1	.057	1.448	.229	.001	1.448	.225
	Implants	.050	1	.050	.471	.493	.000	.471	.105
	Loop	.106	1	.106	1.723	.190	.002	1.723	.259
	Natural	.021	1	.021	1.807	.179	.002	1.807	.269
	STI	.011	1	.011	.064	.800	.000	.064	.057
Error	Cytology	1486.626	1000	1.487					
	M. genitalium	20.551	1000	.021					
	Ureaplasma spp.	246.031	1000	.246					
	M. hominis	220.350	1000	.220					
	HPV	243.949	1000	.244					
	HIV	145.665	1000	.146					
	Age	91100.458	1000	91.100					
	Last Sex	350.182	1000	.350					
	Sex Partners	17997.151	1000	17.997					
	Pregnancies	5085.305	1000	5.085					
	BirthC	219.937	1000	.220					
	Pills	229.804	1000	.230					
	Inject	239.939	1000	.240					
	Condom	39.265	1000	.039					
	Implants	105.579	1000	.106					
	Loop	61.547	1000	.062					
	Natural	11.835	1000	.012					
	STI	171.123	1000	.171					
Total	Cytology	4340.000	1002						
	M. genitalium	21.000	1002						
	Ureaplasma spp.	516.000	1002						
	M. hominis	341.000	1002						
	HPV	464.000	1002						
	HIV	178.000	1002						
	Age	1564112.000	1002						
	Last Sex	414.000	1002						
	Sex Partners	29263.000	1002						
	Pregnancies	16197.000	1002						
	BirthC	675.000	1002						
	Pills	357.000	1002						
	Inject	401.000	1002						
	Condom	41.000	1002						
	Implants	120.000	1002						
	Loop	66.000	1002						
	Natural	12.000	1002						
	STI	188.000	1002						
Corrected Total	Cytology	1489.601	1001						
	M. genitalium	20.560	1001						
	Ureaplasma spp.	250.275	1001						
	M. hominis	224.951	1001						
	HPV	249.134	1001						
	HIV	146.379	1001						
	Age	93414.850	1001						
	Last Sex	353.605	1001						
	Sex Partners	17935.494	1001						
	Pregnancies	5140.165	1001						
	BirthC	220.284	1001						
	Pills	229.805	1001						
	Inject	240.520	1001						
	Condom	39.322	1001						
	Implants	105.629	1001						
	Loop	61.653	1001						
	Natural	11.856	1001						
	STI	171.134	1001						

a. R Squared = .002 (Adjusted R Squared = .001)
b. R Squared = .000 (Adjusted R Squared = -.001)
c. R Squared = .017 (Adjusted R Squared = .016)
d. R Squared = .020 (Adjusted R Squared = .019)
e. R Squared = .021 (Adjusted R Squared = .020)
f. R Squared = .005 (Adjusted R Squared = .004)
g. R Squared = .025 (Adjusted R Squared = .024)
h. R Squared = .010 (Adjusted R Squared = .009)
i. R Squared = .002 (Adjusted R Squared = .001)
j. R Squared = .011 (Adjusted R Squared = .010)
k. R Squared = .002 (Adjusted R Squared = .001)
l. R Squared = .000 (Adjusted R Squared = -.001)
m. R Squared = .002 (Adjusted R Squared = .001)
n. R Squared = .001 (Adjusted R Squared = .000)
o. R Squared = .000 (Adjusted R Squared = -.001)
p. R Squared = .002 (Adjusted R Squared = .001)
q. R Squared = .002 (Adjusted R Squared = .001)
r. R Squared = .000 (Adjusted R Squared = -.001)
s. Computed using alpha = .05

Figure S3.5 MANOVA analysis of factors in relationship to *L. iners* detection