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Tumor Microbial Diversity and Compositional Differences among Women in Botswana with High-Grade Cervical Dysplasia and Cervical Cancer

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Abstract

Introduction: We characterized the cervical 16S rDNA microbiome of patients in Botswana with high-grade cervical dysplasia and locally advanced cervical cancer.

Methods: This prospective study included 31 patients: 21 with dysplasia and 10 with cancer. The Shannon diversity index was used to evaluate alpha (intra-sample) diversity, while the UniFrac (weighted and unweighted) and Bray-Curtis distances were employed to evaluate beta (inter-sample) diversity. The relative abundance of microbial taxa was compared among samples using linear discriminant analysis effect size.

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Author Contributions

All authors were involved with subject identification and data collection, interpretation of the statistical analysis, and review and approval of the final manuscript. The study concept was developed by LEC, AK, SG, GWGB, and TTS. GWGB and TTS helped draft the manuscript.

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Conflicts of Interest

The authors report no conflicts of interest, financial or otherwise, related to the subject matter of the article submitted.

Results: Alpha diversity was significantly higher in patients with cervical cancer than in patients with cervical dysplasia ($p<0.05$). Beta diversity also differed significantly (weighted UniFrac Bray-Curtis, $p<0.01$). Neither alpha diversity ($p=0.8$) nor beta diversity ($p=0.19$) varied by HIV status. The results of linear discriminant analysis effect size demonstrated that multiple taxa differed significantly between patients with cervical dysplasia vs. cancer. *Lachnospira* bacteria (in the *Clostridia* class) were particularly enriched among cervical dysplasia patients, while *Proteobacteria* (members of the *Firmicutes* phyla and the *Comamonadaceae* family) were enriched in patients with cervical cancer.

Discussion: The results of our study suggest that differences exist in the diversity and composition of the cervical microbiota between patients with cervical dysplasia and patients with cervical cancer in Botswana. Additional studies are warranted to validate these findings and elucidate their clinical significance among women living in sub-Saharan Africa, as well as other regions of the world.

Keywords

Cervical dysplasia; cervical cancer; gynecologic cancer; cervical microbiota; microbiome; HIV; Botswana; sub-Saharan Africa

INTRODUCTION

Cervical cancer is one of the most common malignancies globally and the most common cause of cancer death among African women[1]. More than half a million new cases of invasive cervical cancer are expected to be diagnosed worldwide in 2020, resulting in over 300,000 deaths[2]. African women have a far higher risk of cervical cancer than women in regions with ready access to preventative health care screening[1]. Fourteen percent of the world's cervical cancer cases and 18% of cervical cancer-related deaths occur in women living in sub-Saharan Africa[1,3]. The incidence of cervical cancer in sub-Saharan Africa—which includes Botswana, Lesotho, Namibia, South Africa, and Swaziland—is expected to increase by 35% come 2030[1].

Persistent exposure to human papilloma virus (HPV) is the well-established antecedent to cervical cancer[4,5]. Women with HIV are at increased risk of HPV infection and thus cervical cancer, despite access to anti-retroviral therapy[6]. The high regional prevalence of HIV in sub-Saharan countries such as Botswana underscores the importance of cervical cancer prevention in these regions[7]. Botswana established one of the first nationwide HIV treatment programs[8] in Africa, with a corresponding decline in HIV-associated mortality; however, the incidence of cervical cancer in Botswana remains among the highest globally (36.6 per 100,000), with nearly two-thirds of cases occurring in HIV-positive women[9].

The microbiome has recently been demonstrated to play a critical role in cancer progression, metastasis, and therapy response[10]. The female cervix is a microbiome-rich environment, but the effect of this microbiome on cancer development and pathogenesis remains limited and poorly understood[11]. Given the rising incidence of cervical cancer, understanding the effect of the cervical flora on cancer progression and response (as well as the converse effect of definitive treatments on said flora), represents an unmet need with high potential benefit

for vulnerable patient populations. Cervical cancer is uniquely positioned for such an investigation, as it allows direct visualization and contact with the primary tumor at the start of treatment. Here we aim to characterize cervical microbiome of patients with cervical dysplasia and cancer living in Botswana.

To our knowledge, no published studies have specifically explored the cervical dysplasia vs tumor microbiome among women in sub-Saharan Africa. As cervical microbial differences can affect cancer risk and treatment response through several pathways, we characterized the 16S rDNA cervical microbiome of women with dysplasia and locally advanced cervical cancer in Botswana. We hypothesized that the cervical microbiome of cancer patients is distinct from that of dysplasia patients. Furthermore, the longitudinal identification of bacterial strains associated with the cervical microbiome will allow further study of the organisms that stably colonize cervical cancers. The detection of bacterial strains associated with treatment response will then lay groundwork for interventions that alter the tumor microbiota to improve cancer outcomes.

PATIENTS AND METHODS

Participants and Clinical Data

Patients with newly diagnosed, biopsy-proven cervical dysplasia or locally advanced, non-metastatic cervical carcinoma presenting at Princess Marina Hospital were prospectively identified between July 2018 and February 2019. Ineligibility criteria included any history of non-cervical primary cancer and active pregnancy. Comprehensive medical history, including current medication, was assessed via interview with clinical provider or trained study staff. Patient medical records were reviewed to obtain demographic and clinico-pathologic data. Tissue samples were collected prior to initiation of definitive chemoradiation entailing external beam radiation therapy and brachytherapy with concurrent cisplatin for invasive cancer.

The study protocol, including subject recruitment and tissue sampling, was approved by the Institutional Review Boards (IRBs) at the University of Botswana (UBR/RES/IRB/BIO/045), the University of Pennsylvania (830039), and The University of Texas MD Anderson Cancer Center (MDACC 2014–0543). Informed consent was mandatory for study participation and documented by patients in writing.

Sample Collection and DNA Extraction

Cervical samples were collected using a matrix-designed quick-release Isohelix swab. The swabs were placed in 20 μL of protease K and 400 μL of lysis buffer (Isohelix) and stored at −80°C within 1 hour of sample collection. Bacterial genomic DNA was extracted using a MO BIO PowerSoil DNA Isolation Kit (MO BIO Laboratories). Samples were shipped to the US for downstream applications including DNA processing and sequencing.

16S rRNA Gene Sequencing and Sequence Data Processing

16S rRNA gene sequencing of the cervical swabs was performed at the Alkek Center for Metagenomics and Microbiome Research at Baylor College of Medicine (Houston, Texas),

using methods adapted from those used for the Human Microbiome Project.[12] The 16S rDNA V4 region was amplified by PCR using primers that contained sequencing adapters and single-end barcodes, allowing the pooling and direct sequencing of PCR products. Amplicons were sequenced on the MiSeq platform (Illumina) using the 2×250-bp paired-end protocol, yielding paired-end reads that overlapped almost completely. The sequence reads were de-multiplexed, quality filtered, and subsequently merged using USEARCH version 7.0.1090 (4). 16S rRNA gene sequences were clustered into OTUs at a similarity cut-off value of 97% using the UPARSE algorithm.[13] To generate taxonomies, we mapped OTUs to an optimized version of the SILVA rRNA database containing the 16S v4 region. A custom script was used to construct an OTU table from the output files generated, as described above, for downstream analyses of alpha diversity, beta diversity, and phylogenetic trends. Principal coordinates analysis was performed by institution and sample set to ensure that no batch effects were present.

Statistical Analyses

For the microbiome analysis, the rarefaction depth was set at 3561 reads. Alpha (within sample) diversity was examined using the Shannon diversity index, and beta (between sample) diversity was examined using UniFrac (weighted and unweighted) and Bray-Curtis distances. The relative abundance of microbial taxa and genera were compared between samples; we then determined differentially abundant bacterial genera by case status using linear discriminant analysis effect size,[14] applying the 1-against-all strategy with a threshold of 4 on the logarithmic linear discriminant analysis score for discriminative features and an α of 0.05 for the factorial Kruskal-Wallis test among classes. linear discriminant analysis effect size was restricted to bacteria that were present in 20% or more of the study population. Observed differences were subjected to paired analysis using two sample Z test for proportions, or Student t test where appropriate.

RESULTS

The 16S rDNA cervical microbiome was characterized among 31 patients: 21 with cervical dysplasia and 10 with cancer. Clinico-pathologic data are summarized in Table 1. Cervical dysplasia patients were classified according to histologic grade of cervical intraepithelial neoplasia ([CIN]1–3). Approximately 58% of study patients (18 of 31) had CIN 3, while 32% (10 of 31) had moderate- or poorly-differentiated squamous cell cancer of the cervix. HPV status was unknown at the time of cervical sampling.

Patient microbiota were initially analyzed with respect to HIV status. Neither α diversity ($p=0.8$) nor β diversity ($p=0.19$) varied by HIV status (Figure 1A,B). Figure 1C shows the top 15 most abundant genera for both HIV positive and HIV negative patients.

Subsequent analysis was conducted to characterize variations in the cervical microbiome among dysplasia vs. cervical cancer. Clinical and demographic characteristics are displayed in Table 2. Mean age and BMI were similar among cervical dysplasia and cancer patients (mean age, 41.8 vs 50.7 years [$p=0.1$]; and mean BMI, 26.3 vs. 30.0 kg/m^2 [$p=0.19$], respectively). Significantly higher α diversity, as measured by SDI ($p<0.05$), was observed in cervical dysplasia patients relative to cervical cancer patients (Figure 2A). Patients with

CIN 3 tended to have higher α diversity than those with CIN 2 (Figure 2B). As with α diversity, overall β diversity differed significantly by cancer status (weighted Bray-Curtis Unifrac; $p<0.01$) (Figure 2C,D). Figure 2E shows the top 15 most abundant genera in cervical dysplasia samples and cervical cancer samples, showing overall higher diversity in the cancer group. The percentage of subjects with a cervical microbiome dominated by *Lactobacillus* or *Gardnerella* appears lower in the cervical cancer cohort (1 of 10 patients).

Linear discriminant analysis effect size was used to identify bacterial genera that were differentially enriched among our patient cohort ($p<0.05$). The genera *Ersipelotrichia*, *Erysipelotrichales*, *Erysipelotrichaceae*, and *Ruminiclostridium* were enriched in HIV-positive patients, while only *Filifactor* had higher abundance in HIV-negative patients (Figure 1D,E). Interestingly, the genus *Lachnospira* (in the *Clostridia* class of bacteria) was significantly enriched among cervical dysplasia patients; while several *Proteobacteria* taxa (*Betaproteobacteria*, *Gammaproteobacteria*, and *Burkholderiaceae*) and members of the *Firmicutes* phyla (*Erysiopelotrichaceae* and *Synergistaceae*) and the *Comamonadaceae* family had higher abundance in cervical cancer patients ($p<0.05$) (Figure 2F,G).

DISCUSSION

In this novel prospective study, we characterized the cervical microbiome of women with cervical dysplasia and cervical cancer living in Botswana, with the hypothesis that the cervical microbiome would be distinct between these two patient groups. Accordingly, significant differences in cervical α and β diversity were observed among these groups of patients, as well as compositional differences. Interestingly, the overall α and β diversity analysis results did not indicate a difference with regard to HIV status.

Prior microbiome cancer research has focused on exploring the relative abundance of bacteria in the vaginal epithelium, with the assignment of community-state types based on the richness of *Lactobacilli* species[13–15]. The presence and abundance of specific *Lactobacilli* species—for example *L. crispatus*, *L. gasseri*, or *L. jensenii*—is associated with a predisposition towards bacterial vaginosis and other pro-inflammatory states consequently leading to DNA cell damage and potentially carcinogenic changes[15,18–20].

However, despite the comparative wealth of data focused on the vaginal microbiome[21], the ectocervical microbiome has yet to be well described. Pertinent studies have thus far concentrated on the setting of pregnancy or pelvic inflammatory disease. Previous work with 16S rDNA sequencing in pregnancy suggests that cervical microbiota diversity differs by race[22] and that the presence of non-*Lactobacillus* community state types is associated with a robust cervical inflammatory response in the setting of pre-term, premature membrane rupture[23,24]. In patients with pelvic inflammatory disease, Wang et al. demonstrated a dominance by *Lactobacillus* and *Gardnerella* in the cervical microbiota, suggesting that the abundance of different taxa is associated with both acute and chronic inflammatory states[25]. In turn, states of polybacterial dysbiosis and chronic local inflammation are thought to encourage the perseverance of HPV, thus ultimately promoting the development of cervical dysplasia and carcinogenesis through persistent HPV exposure[15,17,22–25].

Persistent HPV infections are thought to trigger an innate immune response, resulting in the suppression of infected cervicovaginal mucosal cells[18,29,30]. An altered mucosal microenvironment leads to the growth of anaerobic organisms at the expense of *Lactobacillus* growth, creating cervicovaginal dysbiosis[31]. Linear discriminant analysis effect size was designed to detect bacterial taxa that are associated with a specific state[32]. In our study, linear discriminant analysis effect size identified *Clostridia*, *Firmicutes*, and *Lachnospira* as taxa negatively associated with cervical cancer, while several *Proteobacteria* were identified as taxa positively-associated with cervical cancer (as compared with dysplasia).

Dysbiosis causes cervicovaginal inflammation and other unfavorable changes in the cervicovaginal mucosal barrier. Worldwide, the most common type of cervicovaginal dysbiosis (defined as a cervicovaginal microbiome not dominated by *Lactobacilli*) is bacterial vaginosis[33]. Bacterial vaginosis is characterized by a persistent decrease in *Lactobacilli* and an increase in fastidious anaerobes[29]. Globally, the prevalence of bacterial vaginosis is highest among women living in sub-Saharan Africa and in women of sub-Saharan African descent[33]. Cervicovaginal dysbiotic states, such as bacterial vaginosis, lead to an altered metabolic profile and reduced cervicovaginal barrier function. This dysbiotic state is not only associated with an increased acquisition of HIV, but also with high-risk HPV, cervical dysplasia, and ultimately cervical cancer[29,34]. The percentage of subjects with their cervical microbiome dominated by *Lactobacillus* was low in our cohort of patients, although the proportion of dysplasia patients with *Lactobacillus*-dominated cervical microbiomes was higher than that of cancer patients. The lack of *Lactobacilli* identified in our cervical dysplasia and cervical cancer patients supports this rationale and suggests that cervicovaginal microbes are important in preventing or enhancing the acquisition and pathogenesis of HPV and HIV. Identifying the microbes that are associated with enhanced pathogenesis and ultimately oncogenesis or tumorigenesis is especially important in susceptible populations such as HIV-positive women in Botswana. Previous studies evaluating the noncancerous cervical microbiome have shown HIV status to be associated with a decrease in microbiome diversity and increase in bacterial richness[11,35,36]. In our study, although neither α nor β diversity varied by HIV status, HIV status was shown to have a significant effect on the cervical microbiome bacterial composition with the genera *Ersipelotrichia, Erysipelotrichales, Erysipelotrichaceae*, and *Ruminiclostridium* being enriched in HIV-positive patients, and only *Filifactor* being enriched in HIV-negative patients. These results support what has been previously reported in other studies investigating the cervicovaginal microbiome and imply that a patients HIV status exerts some selective pressure on the cervical epithelium microenvironment[11,37].

The gut microbiome and its influence on carcinogenesis and prognosis has been well described, most notably in melanoma and colorectal cancer[10,38,39]. Bullman et al. recently identified colonization by *Fusobacterium* (and its associated microbiome *Bacteriodes*, *Selenomas*, and *Prevotella*) at both the primary tumor and distant paired metastatic sites of colorectal cancer. This finding suggests that colonized organisms inhabiting the primary tumor could potentially migrate with primary tumor cells to distant locations and manipulate microbiota diversity at metastatic sites, contributing to poor anti-tumor immunity[40]. Thus, identifying the specific organisms that colonize the tumor microbiota may provide further

insight into mechanisms that modulate immune response and potentiate tumor cell growth[31]. Historically, microbiome cervical cancer research has been limited to mainly Western industrialized populations. We hope that our findings in women in Botswana provide a timely and critical glimpse into this uniquely vulnerable population.

Although the present study yielded intriguing findings, an important limitation is the lack of a control group of “normal” patients without dysplasia or cancer. Additionally, details of menstrual phase, sexual, behavioral and reproductive characteristics, HPV infection and genotypes, antibiotics, synthetic hormones for contraceptive purposes, vaginal douching and presence of other sexually transmitted diseases were not known in our data set. Furthermore our modest sample size remains a potential limitation. Yet despite the relatively small size of this prospective patient cohort, large statistically significant differences were still observed between cervical dysplasia and cancer patients, alluding to the underlying complexity associated with the cervical microbiome in this patient population. Furthermore, this proof-of-concept study demonstrates the feasibility of using 16S rDNA next-generation sequencing to evaluate cervical microbiome differences, even among a unique remote population and within an international collaborative setting.

In conclusion, our study demonstrated hypothesis-generating differences in the cervical microbial profiles of women in Botswana with cervical cancer compared to those with cervical dysplasia. The cervical microbiome of women with cervical dysplasia and cervical cancer did not differ with respect to HIV status. The lack of *Lactobacilli* in our samples supports the rationale that cervicovaginal dysbiotic states (characterized by a persistent decrease in *Lactobacilli*) are associated with a higher incidence of HIV, cervical dysplasia, and cervical cancer. These findings help improve comprehension of the essential function of the tumor microbiome in cervical cancer. Additional studies are warranted to validate these findings and to elucidate the biological significance of these observed differences among women living in sub-Saharan Africa.

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Highlights:

- This prospective study fills the void of data on cervical microbiome in sub-Saharan African women
- Among patients in Botswana, cervical microbiome diversity was greater for cancer versus dysplasia
- Cervical microbiota of women with cancer are distinct in composition as compared with dysplasia

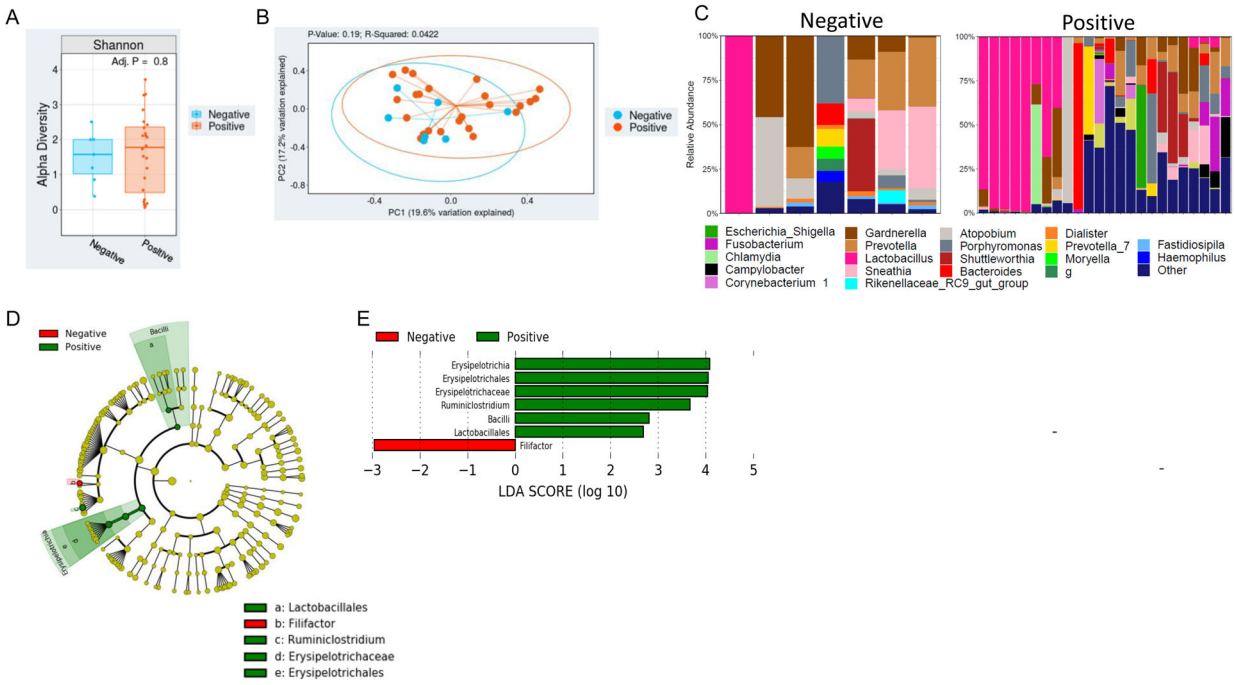

Figure 1. The cervical microbiota of cervical dysplasia and cervical cancer individuals with and without HIV.

A) Overall alpha diversity, as assessed by Shannon diversity in HIV positive cervical dysplasia and cervical cancer patient's (n=24) vs negative patients (n=7). B) Beta diversity, as assessed by Bray-Curtis unweighted UniFrac in HIV positive patients vs HIV negative patients. C) Stacked bar plot of the top 10 most abundant genus-level bacteria in HIV positive vs negative patients. Each bar represents a single participant. D,E) LEfSe analysis identified the most differentially abundant taxa between HIV positive and negative patients. D) Cladogram representation of the significantly different taxa features from phylum (inner circle) to genus (outer circle). E) Histogram showing the LDA scores of genera differentially abundant between the two groups. LEfSe was restricted to $p < 0.05$ for class and subclass analysis and a minimum LDA score of 2.0.

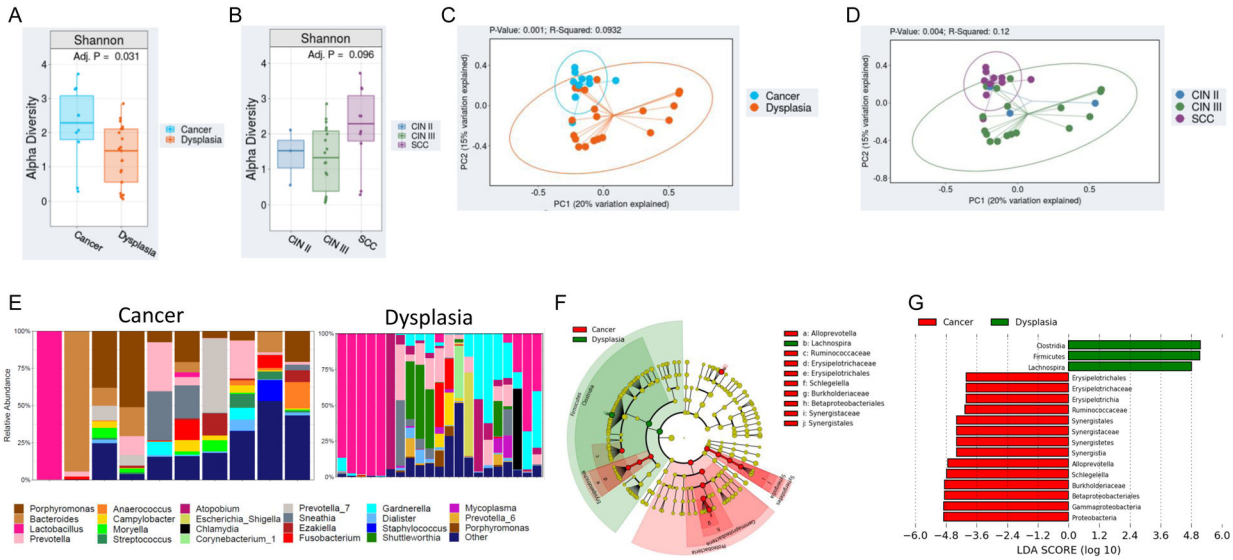

Figure 2. The cervical microbiota in individuals with cervical cancer is significantly different than individuals with cervical dysplasia.

A,B) Overall alpha diversity, as assessed by Shannon diversity in cervical dysplasia (n=21) and cervical cancer patients (n=10). C,D) Beta diversity, as assessed by Bray-Curtis weighted UniFrac in Cervical dysplasia vs cervical cancer patients. E) Stacked bar plot of the top 15 most abundant genus-level bacteria in cervical dysplasia patients vs cervical cancer patients. Each bar represents a single participant. F,G) LEfSe analysis identified the most differentially abundant taxa between cervical dysplasia and cervical cancer patients. F) Cladogram representation of the significantly different taxa features from phylum (inner circle) to genus (outer circle). G) Histogram showing the LDA scores of genera differentially abundant between the two groups. LEfSe was restricted to $p < 0.05$ for class and subclass analysis and a minimum LDA score of 2.0.

Table 1.

Clinico-pathological features of 31 patients in Botswana with cervical dysplasia or cervical cancer

Feature	Result
Type of cervical lesion, (n=31)	
CIN 1	0
CIN 2	3
CIN 3	18
Cervical cancer	10
HIV status, %	
Positive	77
Negative	23
Smoking status, %	
Smoker	6
Non-Smoker	94

CIN, cervical intraepithelial neoplasia.

Table 2.

Selected characteristics of 31 patients in Botswana with cervical dysplasia vs. cervical cancer

Characteristic	Dysplasia (n=21)	Cancer (n=10)	P value*
Mean age (SD), years	41.8 (7.5)	50.7 (12)	0.1
Mean BMI (SD), kg/m^2	26.3 (6.4)	30.0 (7.2)	0.2
HIV status, %			
Positive	17 (81%)	7 (70%)	0.5
Negative	4 (19%)	3 (30%)	0.5
Smoking status, %			
Smoker	2 (10%)	0 (0%)	0.3
Non-Smoker	19 (90%)	10 (100%)	0.3

*P values were based on a t-test (continuous variables) or z-test (proportions). All tests were 2-sided.