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High-risk human papillomavirus in HIV-infected women undergoing cervical cancer screening in Lilongwe, Malawi: A pilot study

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Abstract

Introduction—Rates of abnormal visual inspection with acetic acid (VIA) and prevalence of high-risk human papillomavirus (HPV) subtypes have not been well characterized in HIV-infected women in Malawi.

Methods—We performed a prospective cohort study of VIA (N=440) in HIV-infected women ages 25-59, with a nested study of HPV subtypes in first 300 women enrolled. Wilcoxon's Rank-Sum Test was used to compare continuous variables and Fisher's exact test was used to compare categorical variables between women with normal versus abnormal VIA.

Results: Of 440 women screened, 9.5% (N=42) had abnormal VIA with 69.0% (N=29) having advanced disease not amenable to cryotherapy. Of 294 women with HPV results, 39% (N=114) of women were positive for high-risk HPV infection. Only lower CD4 count (287 cells/mm^3 vs. 339 cells/mm^3, p=0.03) and high-risk HPV (66.7% versus 35.6%, p<0.01) were associated with abnormal VIA. The most common high-risk HPV subtypes in women with abnormal VIA were 35 (33.3%), 16 (26.7%), and 58 (23.3%).

Conclusion—Low CD4 cell count was associated with abnormal VIA and raises the importance of early ART and expanded availability of VIA. HPV vaccines targeting additional non-16/18 high-risk HPV subtypes may have greater protective advantages in countries such as Malawi.

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Keywords

HIV/AIDS; cervical cancer; visual inspection with acetic acid (VIA); human papillomavirus (HPV)

Introduction

In Malawi, cervical cancer is the most common cancer among women, accounting for approximately 45% of all female cancers and resulting in over 1600 deaths per year.[1, 2] The incidence of cervical intraepithelial neoplasia (CIN) is 4-5 times higher in HIV-infected compared to HIV-uninfected women[3, 4] and the risk of invasive cervical cancer is 5-8 times higher.[5-7] Women in Sub-Saharan Africa often do not have access to cervical cancer screening given lack of infrastructure and financial resources for Pap smears, biopsies, and colposcopy.[8, 9] Visual inspection with acetic acid (VIA) has emerged as a more feasible strategy for screening compared to Pap smears, but has been associated with high rates of loss to follow-up when women have been asked to return at a later date for cryotherapy and/or other interventions.[10] As a result of these challenges, many programs in resource-limited settings have implemented a "see and treat" approach using VIA and immediate cryotherapy for abnormalities. Screening programs using VIA in Africa have been found to be feasible, safe, and acceptable for both health care providers and for patients.[11-14]

Cervical cancer is known to be associated with infection with high-risk oncogenic Human Papillomavirus (HPV) subtypes and while at least one study has shown HPV testing followed by cryotherapy to be more efficacious at reducing CIN grade 2 and above (CIN2+) than VIA[15], in many resource-limited settings, including Malawi, there is no availability for HPV testing and the introduction of direct HPV tests for screening purposes may be prohibitive due to lack of infrastructure and high cost. The use of VIA has been piloted in several centers in Malawi since 2005 and has proven to be implementable[16], but has not been widely integrated into HIV care due to limitations in trained personnel, availability of supplies and equipment (such as for cryotherapy), and cost. While there has been discussion of HPV vaccination in Malawi, an HPV vaccine is currently not widely available. We implemented VIA in a large HIV clinic in Lilongwe and sought to determine the prevalence of cervical abnormalities seen with VIA, characterize HPV subtypes, and evaluate the prevalence of high-risk HPV-infection in HIV-infected women with and without abnormalities on VIA.

Methods

We performed a prospective cohort study of VIA with a nested study of HPV subtypes. The study was performed at Partners in Hope Medical Center in Lilongwe, Malawi, a free HIV/AIDS clinic providing care to approximately 5,000 patients, in a new screen-and-treat VIA program in HIV-infected women. The study took place over a 6-month period from November 2011 to April 2012. All women coming to the clinic for routine antiretroviral therapy (ART) or pre-ART care were offered VIA and given the opportunity to participate in the study. Women were eligible for the study if they were HIV-positive, 25-59 years of age, able to provide informed consent, and had a history of vaginal intercourse. Women

were excluded if they were pregnant, less than 6 weeks postpartum, had a history of hysterectomy, or had prior cryotherapy or cervical cancer. The most recent CD4 cell count prior to VIA was abstracted from the patient chart. The study was approved by the Malawi National Health Sciences Research Committee (#893) and by the University of California Los Angeles Institutional Review Board (#10-001710).

Research Procedures

Participants were administered a baseline questionnaire to assess risk factors for cervical dysplasia and HPV infection. Women underwent a speculum examination with VIA which was performed using 3-5% acetic acid applied to the cervix, waiting one minute, and then examining the cervix to identify suspicious lesions. VIA lesions were classified as per the International Agency for Research on Cancer Guidelines (IARC/WHO).[17] The first 300 participants underwent HPV testing. HPV samples were collected prior to VIA using standard cytobrush technique and placed in ThinPrep preservative (Hologic, Marlborough, MA). The number of women HPV tested was determined by available funding for this nested aim. HPV specimens were shipped to the University of California San Francisco in San Francisco, California for low and high-risk HPV DNA analysis by polymerase chain reaction. A crude DNA preparation was made by pelleting 1.5 ml of the Hologic cell suspension. After the pellet was allowed to dry, it was suspended in 100 μl Qiagen Specimen Transport Media with proteinase K (Invitrogen Life Technologies) at a concentration of 200 μg/ml and incubated at 56°C for 1 hour, then the proteinase K was heat inactivated. PCR was performed using a modified pool of MY09/MY11 consensus HPV L1 primers as well as primers for amplification of the human beta-globin as an indicator of specimen adequacy as described previously.[18] After 40 amplification cycles, specimens were probed with a biotin-labeled HPV L1 consensus probe mixture. A separate membrane was probed with a biotin-labeled probe to the human beta-globin gene. Specimens were also typed by hybridizing to 38 different HPV types.[19]

Women with abnormal lesions seen on VIA that met criteria (raised and thickened white plaques or acetowhite epithelium near the squamocolumnar junction) were offered same day treatment with a standard cryotherapy procedure (two, three-minute freezing periods separated by five minutes). Women who reported increased vaginal discharge, lower pelvic pain, or were noted on exam to have abnormal discharge were give treatment for presumed STD and asked to return on a later date for cryotherapy. Individuals with lesions extending to the vaginal wall, lesions involving more than 75% of the cervix or > 2mm beyond the cryoprobe (including the tip of probe), and women suspected of having invasive cancer were referred to the central hospital in Lilong we for further evaluation. VIA and cryotherapy were performed by three experienced nurses who had completed a five-day training approved by the Ministry of Health. Quality control officials from the Ministry of Health visited eight times over six months to monitor and evaluate the nurse's clinical assessments of cervical lesions.

Statistical Analysis

This study was designed as a pilot to acquire preliminary data over a limited time period (6 months). Summary statistics were generated for basic socio-demographic information and

for baseline clinical information. Wilcoxon's Rank-Sum Test was used to compare continuous variables and Fisher's exact test was used to compare categorical variables between the women who did versus those who did not undergo HPV screening, between the women with normal and abnormal VIA, and between the women with and without one or more high-risk HPV subtypes. A frequency table was also constructed to present the distribution of the various HPV subtypes in the population. All tests are two sided and all analyses were carried out using SAS v9.3 (Cary, North Carolina).

Results

Between November 2011 and April 2012 a total of 446 women were screened for the study. All women from this group were enrolled in the prospective cohort study of VIA, with the first 300 women also enrolled into the nested substudy of HPV infection. Six women were excluded from the analysis because of missing data, leaving 440 women in the cohort and 294 women with HPV results. The median age for all participants was 35.0 years (IQR 31, 42), median CD4 count was 337 cells/mm^3 (IQR 215, 491), 81.8% were on antiretroviral therapy (ART), and 89.5% were undergoing VIA for the first time. Participants who were tested for HPV had a lower CD4 nadir (188 cells/mm^3 vs. 232 cells/mm^3, p=0.04), and were more likely to be on ART (84.7% vs. 76.0%, p= 0.04) compared to women who did not have HPV screening. Table 1 describes baseline characteristics of all women as well as comparisons between those who did versus did not receive HPV testing.

Ten percent of women (N=42) had abnormal VIA. Of these, 16.7% (N=7) received same day cryotherapy, 14.3% (N=6) returned on a later date for cryotherapy, either because they required syndromic sexually transmitted infection (STI) treatment prior to cryotherapy or desired permission from a spouse before undergoing the procedure, and 69.0% (N=29) were referred to KCH. Reasons for referral to KCH included clinically suspected cervical cancer (N=4), lesions extending to the vaginal wall (N=4), lesions involving more than 75% of the cervix (N=2), and lesions more than 2mm beyond the cryoprobe (N=19) (Figure). Lower median CD4 count (287 cells/mm^3 vs. 339 cells/mm^3, p=0.03) and infection with one or more high-risk HPV (66.7% versus 35.6%, p<0.01) were associated with abnormal VIA. Age, duration of HIV infection, ART status and duration, median number of lifetime sex partners, and number of sex partners within the last month were not associated with abnormal VIA.

A total of 57.8% of HPV screened women (N=170) had HPV detected, with 38.8% (N=114) having at least one high-risk subtype. HPV 58 was the most frequent high-risk HPV subtype (9.5%), followed by HPV 35 (8.5%) (Table 2). Thirty women with abnormal VIA were among the subgroup of women tested for HPV infection. Of these, 66.7% (N= 20) had high-risk HPV infection with the most common subtypes being 35 (33.3%), 16 (26.7%), and 58 (23.3%). Among the 264 women with normal VIA who underwent HPV testing, 35.6% (N=94) tested positive for high-risk HPV with the most common subtypes 58 (8.0%), 35 (5.7%), and 16 (5.7%) (Table 2). Twelve percent (N=36) of women were infected with either HPV 16 or 18; however, one of these subtypes was present in 36.7% of women with abnormal VIA. Among those with abnormal VIA, the median number of high-risk HPV

subtypes per woman was 1 (range 0-3) compared to 0 (range 0-1) in women with normal VIA (p<0.01).

Women with and without high-risk HPV were compared (Table 3). Shorter median duration of ART (2 years versus 3 years, p <0.01) and shorter duration since HIV diagnosis (3 years versus 4 years, p < 0.01) were associated with having at least one high-risk HPV type. While younger age was associated with infection with high-risk HPV, the age difference between the two groups was minimal (35 versus 36 years, p= 0.02).

Discussion

Abnormal VIA in HIV-infected women in the ART-era in Malawi

Studies of HIV-infected women in Africa have shown an abnormal VIA rate between 6.5 to 55%.[14, 20-22] Ten percent of women in our study had abnormal VIA, with the majority (69%) having extensive disease not amenable to cryotherapy. The high rate of those with advanced disease is likely attributable to the fact that VIA was not widely available to this population prior to this study, with the majority of women (89.5%) undergoing first-time screening. Similar data was reported by Jhpiego during the implementation of VIA in Guyana, Cote D'Ivoire, and Tanzania, with high prevalence of advanced disease in HIV-infected women screened for the first time.[23]

In our cohort, aside from high-risk HPV, only lower CD4 cell count was associated with abnormal VIA, with every 100 cell/mm^3 gain associated with a 19% decrease in the odds of having abnormal VIA (p = 0.03). Several other studies have reported this association, as well as an association between low CD4 cell count and high-risk HPV infection and squamous intraepithelial lesions.[24-26] Higher HIV viral load has also been associated with cervical dysplasia, as well as the number of lifetime sexual partners and >2 sexual partners in the last year.[24, 27, 28] We did not have viral load values available to test this association in our cohort and did not find an association with the number of sex partners. Identifying features strongly associated with cervical abnormalities can help prioritize VIA and other screening strategies, particularly in settings like Malawi, where capacity for routine screening of all women remains extremely limited. Several authors included on this study participate in a large President's Emergency Plan for AIDS Relief (PEPFAR) care and treatment program in Malawi. Among sites supported in this program, VIA is offered at only 9.1% of ART sites (5/55) and is largely limited to hospitals and urban clinics. Increased efforts towards VIA implementation would have tremendous benefit, but are challenging due to limited financial and human resources.

High-risk HPV in HIV-infected women in the ART-era in Malawi

Our study had a similar rate of overall HPV infection (57.8%) compared to a large South African study (61%)[20], but we found lower rates of high-risk HPV (39%) compared to most other studies from Africa, which have reported rates of 46%-90%.[15, 24, 26, 27, 29] The only prior published data on cervical lesions and HPV from Malawi was done in the pre-ART era and utilized Pap smear and cervical lavage in 124 HIV-infected women. This study reported squamous intraepithelial lesions in 15 % of HIV-infected women and found high-risk HPV

DNA in 60% of those with CD4 counts < 300 cells/mm^3; however, the specific distribution of HPV subtypes were not described in this study.[30] It is possible that ART reduces cervical abnormalities and HPV infection rates and this could explain the difference in our results compared to the prior study from the pre-ART era in Malawi. Published data about ART and HPV infection is conflicting,[31] with one study showing ART to be associated with decreased prevalence and incidence of high-risk HPV[32] and another demonstrating ART to be associated with increased HPV clearance in women with CIN, but not in women with normal cervical cytology.[33] Eighty-two percent of participants in our study were on ART and this large number limited power to test associations between ART, cervical abnormalities, and high-risk HPV.

Normal VIA and the presence of high-risk HPV: Implications for screening strategies

In our study, 36% of women with normal VIA had at least one high-risk HPV subtype. HIV-infected women with high-risk HPV infection and normal VIA will be at higher risk for HPV persistence and development of cervical dysplasia in the future.[34, 35] Several studies from Africa have explored direct tests for HPV as a screening method.[15, 20] A recent study from South Africa compared Pap smear, HPV testing, and VIA and found that, as a stand-alone test for CIN2+, HPV was the most sensitive test (92% sensitivity) followed by Pap smear (76%) and VIA (65.5%); however, HPV had the lowest specificity of the three options (51%) with specificity further reduced at CD4 counts   200 cells/mm^3.[20] In the Malawian setting, HPV screening would require a large infusion of funds to establish the necessary infrastructure and would need to be weighed against the lower specificity of this method (51.4% compared to 68% for VIA). A more feasible approach may be forthcoming with the Care-HPV (QIAGEN/PATH)[36] which can be done more easily in resource-limited settings, but implementation would need to be accompanied by expanded availability of VIA and interventions such as cryotherapy.

High-risk HPV subtypes: Implications for an HPV vaccination program

HPV 58 and 35 were the most prevalent high-risk HPV types seen in our study population, accounting for almost 20% of infections. A study done in Zambia found that 98% of HIV-infected women harbored at least one high-risk HPV subtype with a median of four subtypes per person. In this study, HPV 52 was the common (37%), followed by 16 (17%) and 18 (13%)[25], while a study from Botswana showed HPV 58 to be most common (26%) followed by 39and 16 (20% each).[27] Several South African studies have shown high prevalence of HPV 16 and 18.[22, 37] Common themes from these studies include high rates of current HPV infection (68%-98%) and multiple HPV infection, with a median of 2-4 subtypes per woman.

Taken together, studies from HIV-infected women in Africa suggest important geographic differences in HPV infection, with a tendency toward non-16 and 18 subtypes to be most prevalent. The quadrivalent HPV vaccine (Gardisil) protects against 6/11/16/18, but is thought to offer some cross protection against subtype 31. The bivalent vaccine (Cervarix) protects against 16/18 and may offer cross protection against subtypes 31/33/45/52/58.[38] Vaccines directly targeting additional HPV subtypes may have even greater protective advantages in countries such as Malawi, due to high prevalence of non-16/18 subtypes

reported from these settings. Randomized clinical trials are currently examining the efficacy of a nonavalent HPV vaccine (types 6/11/16/18/31/33/45/52/58), with preliminary modeling data suggesting high potential to further reduce precancerous lesions and cervical cancer.[39] In early 2013, the GAVI Alliance announced support for eight countries, including Malawi, to receive support to provide HPV vaccination to girls ages 9-13.[40] The funding has been provided in order to allow countries to test their ability to develop the systems that would be needed to roll out the HPV vaccines nationally, and to inform future policy decisions. Sustainable financing for such a vaccination program in Malawi remains a major barrier to date.[41]

Study Limitations

Although we had a quality control officer visiting our site once every two weeks, we did not use photographs or more advanced imaging techniques for confirming VIA findings, and it is possible our study underestimates the rate of abnormal VIA. Given the large number of women on ART (82%), we had limited power to determine associations of ART with abnormal VIA and/or high-risk HPV. Participants who were tested for HPV had a lower CD4 nadir and were more likely to be on ART suggesting that the subset with HPV data may not have been representative of the entire clinical cohort and data may not be generalizable. Finally, we studied HPV among women with abnormal VIA and cannot comment specifically on subtypes most contributory to invasive cervical cancer.

Conclusions

We found high rates of advanced disease at time of VIA among this group of women undergoing screening. Low CD4 cell count was associated with abnormal VIA and raises the importance of early initiation of ART for those eligible, and expanded availability of VIA within HIV care and treatment programs in Malawi. The expansion of ART in Malawi may lead to better overall outcomes via improved clearance of HPV and reduction in CIN, although the role of ART in HPV infection remains unclear and is an important area for further research. Sustainable resources for HPV vaccination programs will be of great benefit in preventing CIN and invasive cervical cancer in Malawi and other areas of Sub-Saharan Africa.

Acknowledgments

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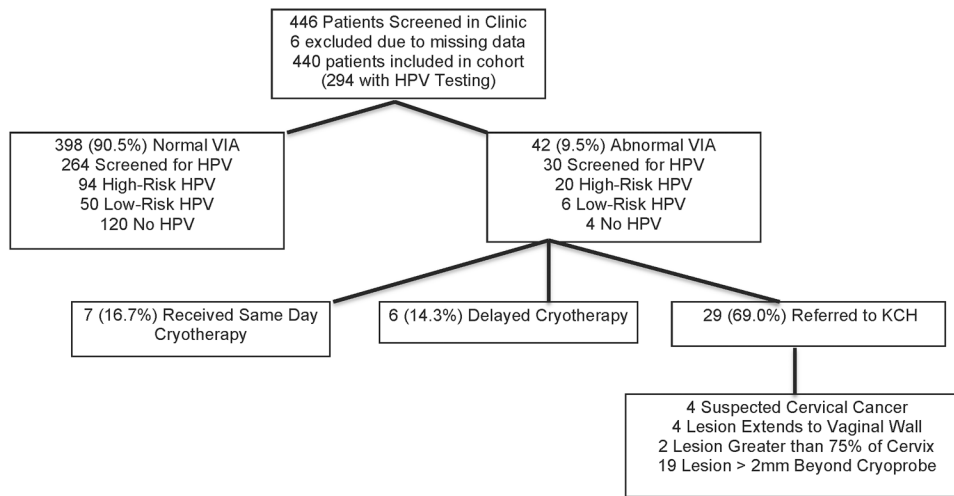41. Financing HPV vaccination in developing countries. The Lancet. 2011; 377:1544.

Figure. Schematic showing distribution of normal versus abnormal VIA, HPV, and outcome (cryotherapy versus referral)

Table 1

Baseline characteristics of women in the cohort and comparisons of women who did versus did not receive HPV testing

Variable	All N = 440	No HPV Testing N = 146	HPV Testing N = 294	P Value[*]
Median age$_{(IQR)}$	35 (31, 42)	34.5 (31, 41)	36 (30, 43)	0.32
Median months on ART$_{(IQR)}$	36 (12, 48)	36 (24, 60)	24 (12, 48)	0.06
Median months since HIV diagnosis$_{(IQR)}$	36 (24, 60)	48 (24, 60)	36 (24, 60)	0.72
^Median CD4 Count (cells/mm³)$_{(IQR)}$	337 (215, 491)	358.5 (257, 493)	329.5 (200, 488)	0.14
%Median CD4 Nadir (cells/mm³)$_{(IQR)}$	203 (117, 314)	232 (127, 333.5)	188 (111, 275)	0.04
Median number of lifetime sex partners$_{(IQR)}$	3 (2, 4)	3 (2, 4)	3 (2, 4)	0.44
Median number of sex partners in last month$_{(IQR)}$	1 (0, 1)	1 (0, 1)	1 (0, 1)	0.87
Median age at first intercourse$_{(IQR)}$	18 (16, 20)	18 (16, 20)	18 (16, 20)	0.20
Education N, %				0.52
No schooling	37 (8.4)	10 (6.9)	27 (9.2)	
Primary school completed	251 (57.1)	79 (54.1)	172 (58.5)	
Secondary school completed	141 (32.1)	53 (36.3)	88 (29.9)	
University completed	11 (2.5)	4 (2.7)	7 (2.4)	
On ART N, %				0.04
Yes	360 (81.8)	111 (76.0)	249 (84.7)	
No	80 (18.2)	35 (24.0)	45 (15.3)	
Number of pregnancies N, %				0.71
0	10 (2.3)	3 (2.1)	7 (2.4)	
1	32 (7.3)	12 (8.2)	20 (6.8)	
2	47 (10.7)	12 (8.2)	35 (11.9)	
3-5	232 (52.7)	82 (56.2)	150 (51.0)	
6-10	112 (25.5)	36 (24.7)	76 (25.9)	
More than 10	7 (1.6)	1 (0.7)	6 (2.0)	
Bleeding after sex N, %				0.10
Yes	45 (10.2)	20 (13.7)	25 (8.5)	
No	395 (89.8)	126 (86.3)	269 (91.5)	
History of sexually transmitted infection N, %				0.40
Yes	127 (28.9)	48 (32.9)	79 (26.9)	
No	307 (69.8)	96 (65.8)	211 (71.8)	
Not sure	6 (1.4)	2 (1.4)	4 (1.4)	
Primary sex partner circumcised N, %				0.53
Yes	103 (31.2)	38 (33.6)	65 (30.0)	
No	227 (68.8)	75 (66.4)	152 (70.1)	

[*] P-value for HPV tested versus not tested. For numeric variables, the Wilcoxon Rank Sum test was used. For categorical variables, Fisher's Exact Test was used

^ Median CD4 cell count was available for 420 of 440 participants (95.5%) and was collected a median of 14.6 months (IQR: 8.4, 18.0) prior to VIA

*%*Median nadir CD4 cell count was available for 417 of 440 participants (94.8%)

Table 2

Distribution of high-risk HPV subtypes overall and by VIA status (N = 294)

High-risk HPV subtype	Percent of all HPV subtypes[1]	Frequency	Overall Prevalence (%)[2]	Abnormal VIA N = 30	Normal VIA N = 264	P-value*
Type 58	5.7	28	9.5	7 (23.3)	21 (8.0)	0.01
Type 35	5.1	25	8.5	10 (33.3)	15 (5.7)	< 0.01
Type 16	4.7	23	7.8	8 (26.7)	15 (5.7)	< 0.01
Type 33	3.4	17	5.8	3 (10.0)	14 (5.3)	0.40
Type 52	3.4	17	5.8	6 (20.0)	11 (4.2)	0.05
Type 18	2.6	13	4.4	3 (10.0)	10 (3.8)	0.13
Type 51	2.4	12	4.1	2 (6.7)	10 (3.8)	0.35
Type 56	1.8	9	3.1	1 (3.3)	8 (3.0)	1.0
Type 82	1.8	9	3.1	3 (10.0)	6 (2.3)	< 0.01
Type 68	1.4	7	2.4	2 (6.7)	5 (1.9)	0.15
Type 31	1.0	5	1.7	1 (3.3)	4 (1.5)	0.42
Type 39	1.0	5	1.7	1 (3.3)	4 (1.5)	0.42
Type 45	0.6	3	1.0	1 (3.3)	2 (0.8)	0.28
Type 59	0.6	3	1.0	1 (3.3)	2 (0.8)	0.27
Type 73	0.2	1	0.3	1 (3.3)	0(0)	0.10

[1] Frequency divided by the total number of occurrences of all HPV subtypes (Total = 495).

[2] Frequency divided by the number of women with HPV Testing (N = 294)

*Fisher's exact test for abnormal versus normal VIA

Table 3

Comparison of women with and without high-risk HPV

Variable	HPV Tested N = 294	Negative for high-risk HPV N = 180	Positive for ≥1 high-risk HPV subtype N = 114	P Value
Median age$_{(IQR)}$	36 (30, 43)	36 (32, 44)	35 (30, 42)	0.02
Median months on ART$_{(IQR)}$	24 (12, 48)	36 (24, 60)	24 (11, 36)	<.01
Median months since diagnosis$_{(IQR)}$	36 (24, 60)	48 (24, 72)	36 (12, 60)	<.01
Median CD4 count (cells/mm^3)$_{(IQR)}$	329.5 (200, 488)	364 (247, 534)	256 (159, 380)	<.01
Median CD4 nadir (cells/mm^3)$_{(IQR)}$	188 (111, 275)	194 (120, 288)	180 (86, 274)	0.20
Median number of lifetime sex partners$_{(IQR)}$	3 (2, 4)	3 (2, 3)	3 (2, 4)	0.48
Median age at first intercourse$_{(IQR)}$	18 (16, 20)	18 (16, 20)	18 (16, 19)	0.32
Education N, %				0.44
No schooling	27 (9.18)	16 (8.89)	11 (9.65)	
Primary school completed	172 (58.5)	111 (61.67)	61 (53.51)	
Secondary school completed	88 (29.93)	50 (27.78)	38 (33.33)	
University completed	7 (2.38)	3 (1.67)	4 (3.51)	
On ART N, %				0.87
Yes	249 (84.69)	153 (85)	96 (84.21)	
No	45 (15.31)	27 (15)	18 (15.79)	
Bleeding after sex N, %				0.67
Yes	25 (8.5)	14 (7.78)	11 (9.65)	
No	269 (91.5)	166 (92.22)	103 (90.35)	
History of sexually transmitted infection^ N, %				0.92
Yes	79 (26.87)	49 (27.22)	30 (26.32)	
No	211 (71.77)	129 (71.67)	82 (71.93)	
Not sure	4 (1.36)	2 (1.11)	2 (1.75)	
Primary sex partner circumcised N, %				1
Yes	65 (29.95)	39 (29.77)	26 (30.23)	
No	152 (70.05)	92 (70.23)	60 (69.77)	

[*]
For numeric variables, the Wilcoxon Rank Sum test was used. For categorical variables, Fisher's Exact Test was used