HPV-16 viral load in association with cervical neoplasia and cancer in Senegal

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Abstract

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Background: The importance of certain characteristics of human papillomavirus (HPV) infection and human immunodeficiency virus (HIV) infection in the pathogenesis of cervical cancer has not yet been established. Although infection with HPV is a necessary cause of cervical cancer and its precursor lesions, cervical intraepithelial neoplasia (CIN), the exact role of HPV type-specific infection and HPV DNA concentration (viral load) in cervical disease development remains unclear. Furthermore, data are lacking regarding the association between HPV infection, human immunodeficiency virus (HIV) infection, and HIV-induced immunosuppression. This dissertation examines the role of HPV-16 viral load in the development of CIN and cervical cancer among women from Senegal, West Africa. In addition, the relationship between overall and type-specific HPV detection, HIV infection and immunosuppression is investigated.

Methods: Three major strategies are used to investigate these research questions: 1) A cross-sectional analysis of HPV type 16 viral load in relation to severity of cervical disease, 2) A longitudinal analysis of baseline HPV type 16 viral load in relation to future clearance of infection and development of CIN or cancer, and 3) An historical cross-sectional analysis of HPV DNA detection among HIV-positive and HIV-negative women, by immune status.

Results: Compared to women with no cervical disease, HPV-16 viral loads were increased in women with CIN and invasive cancer. Longitudinally, HPV-16 viral load was inversely associated with clearance of HPV-16 infection and marginally associated with development of CIN or worse. The prevalence of HPV DNA was greater among HIV-positive compared to HIV-negative women. This trend was also seen for HPV types 16 and 18, which are responsible for the majority of cervical cancer. In addition, HIV-infected women with severe immunodepression were at increased risk of overall and type-specific HPV infection.

Conclusions: HPV-16 viral loads may be useful correlates of existing disease severity and of development of adverse HPV infection outcomes, including viral persistence and CIN. Measures to prevent initial HPV infection and subsequent development of cervical cancer should be emphasized in high risk populations, especially among HIV-positive women. Further studies are needed to clarify the role of HPV viral load in the natural history of HPV infection.

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Chapter 1

HPV-16 viral load associated with cervical dysplasia and invasive cancer (cross-sectional)

Introduction

Cervical cancer is the third most common cancer among women worldwide[1]. The role of human papillomavirus (HPV) as a central and causal agent in the development of invasive cervical carcinoma has been widely acknowledged [2-6]. Although HPV is the most common sexually transmitted infection worldwide, most of these infections are transient and are cleared within 1 to 2 years after exposure [7-13]. After the establishment of infection, a variety of host and viral cofactors are thought to act upon the immune system and the tissue microenvironment in the cervix to induce development of cervical lesions. A minority of these lesions, however, will ultimately progress to cervical cancer. Persistence of high-risk types of HPV, particularly HPV type 16, is recognized as a crucial event in the development of cervical cancer precursor lesions and squamous cervical cancer [14,15]. Once viral persistence has been established, a series of disruptions to the normal cervical epithelial cell cycle can promote the future development of neoplastic lesions and malignant disease. Cervical precursor lesions can be classified cytologically as high-grade squamous intraepithelial lesions (HSIL) or histologically as cervical intraepithelial neoplasia grades 2 and 3 (CIN2-3).

It has been suggested that high concentrations of HPV DNA in cervical specimens, or viral load, could be etiologically associated with cervical disease development. Indeed, correlation between increasing viral load and disease severity has been documented with other infections including chronic hepatitis B virus, herpes simplex virus, and human immunodeficiency virus, thus indicating a biologically feasible role for HPV viral load in cervical disease outcomes[16-19]. While some studies have found a positive association between HPV viral load and cervical disease [20-23], others have not, thus contributing uncertainty to the etiologic link between HPV viral load and disease development [24-28]. However, a portion of the previous studies evaluating HPV viral load have used only semiquantitative methods of measurement, limiting the ability to draw firm conclusions about HPV viral load and cervical disease [20,21,28-34]. Additionally, it has been cautioned that lower HPV viral load does not entirely exclude the possibility of progression to disease [7].

Women with HIV infection, compared to those who are uninfected, are at greater risk for acquiring HPV infection and are more likely to experience persistence of their infection, thus leaving them at increased risk for cervical dysplasia and subsequent invasive cancer [35-40]. HIV-infected women are also more likely

to harbor a greater diversity of HPV genotypes, including most oncogenic types [41-43]. Among oncogenic types, HPV type 16 has been found to be the most common genotype in both HIV-positive as well as HIV-negative women [2,44-46]. Since a great majority of HIV-positive women are infected with HPV and are therefore at risk of developing persistent infection, understanding the mechanism for HPV persistence would be particularly valuable in this population. Several recent studies have provided data to support a possible association between HPV-16 viral load in HIV-positive women and development of disease[47-50], although similar studies among HIV-negative women have not yielded equally convincing results [27,48,49,51-53]. Of the two known studies that have investigated HPV viral load differences specifically between HIV-positive and HIV-negative women, one reported a higher HPV-16 viral load among HIV-infected women [47], and the other was inconclusive[54].

The role of HPV viral load in the development of cervical cancer is still uncertain. We undertook the current study to more clearly characterize the association between HPV-16 viral load and cervical neoplasia using a cross-sectional sample of HIV-positive and HIV-negative women in Senegal. The long-term goal of our study is to better understand the importance of cervical HPV-16 viral load in the pathogenesis of cervical cancer, as well as its possible association with immunosuppression caused by infection with HIV. Studies such as the present one may also help to understand the utility of HPV-16 viral load as a tool for disease risk prediction, especially among high-risk populations.

Materials and Methods

Women included in the present study were originally recruited from several outpatient clinics in Senegal, West Africa between 1991 and 2009, to participate in one of a series of cross-sectional, case-control, and cohort studies investigating the epidemiology of human papillomavirus (HPV) and cervical cancer [35,55,56]. The primary research goals of these studies included the investigation of molecular biomarkers for cancer prevention, HIV-associated hypermethylation in cervical cancer, and the natural history of cervical neoplasia and HIV infection in Senegal. Participants had previously provided informed consent upon study enrollment, as per the procedures of the Human Subjects Committee of the University of Washington and the University of Dakar. All subjects were all older than 15 years of age, were not pregnant and had an intact cervix. Upon enrollment, demographic, medical, and sexual history information was recorded for all participants. General medical and gynecologic exams were performed

at this time, and blood samples were collected in order to determine each participant's HIV status. Cervical swab samples were also obtained for cytological and HPV screening. All participants donated a cervical swab at their screening visit, and the majority (84.3%) was selected to participate in a subsequent study enrollment visit where an additional cervical swab was taken. Screening and enrollment visits occurred on the same day among 43% of participants.

Sample collection

Participants were included in the present study if they had HPV-16 DNA detected from cervical samples originally collected either at study screening or enrollment visits (see Figure 1). Screening samples were preferentially chosen for analyses unless they were not locatable or lacking sufficient volume for testing, in which case enrollment samples were used. For a small number of participants (n=42), when a study screening or enrollment cervical sample was not available, a cervical sample from a visit subsequent to the enrollment visit was used. At the time of original recruitment and enrollment, cytopathological diagnoses were performed on cervical samples from all of the aforementioned studies. In addition, histological diagnoses were performed in a portion of the studies, as determined by the original study protocol. For the present study, we used the participants' stored HPV-16 positive cervical swab specimens to quantify the presence of HPV-16 DNA.

Information from the original studies, including data on HPV typing, cervical cytologic and histologic findings, and other demographic and reproductive characteristics, were linked to the HPV-16 DNA load values obtained for the present study. Of the original 750 HPV-16 positive women whose samples were identified to be evaluated for HPV-16 DNA load, 244 (33.3%) of these samples were not located and 6 (0.8%) did not have sufficient sample volume remaining for viral load testing. Women with missing or insufficient samples were therefore not included in the present study. Of the remaining 506 study participants, 9 had an unsatisfactory or missing cytological diagnosis for the visit from which the HPV DNA load was evaluated. However, a cytological diagnosis of "normal" was assigned for 6 of these participants with no history of cytological abnormality but who had a normal cytological diagnosis at a visit less than one year from the time of viral load analysis. One woman with a consistent diagnosis of "CIN II/III" at four consecutive visits prior to and after viral load measurement was assigned a value of CIN II/III. The two other women did not have a consistent cytological history from which to assign an

appropriate diagnosis, and were therefore excluded from all analyses. For purposes of evaluation, we used the worst of either histological or cytological diagnosis in order to categorize participants' disease outcomes. However, of the women included in this study, 216 (43.4%) did not have a histological diagnosis available from a previous study, and their cytological diagnosis was therefore used for all disease outcomes.

Laboratory Assays

Serologic testing

Serologic assays for HIV-1 and HIV-2 were performed on patients' baseline blood samples, collected in ethylenediamine tetraacetic acide (EDTA) tubes. A two-test sequence, as previously described, was used to determine HIV status [55,57]. First, serum samples were tested for the presence of either HIV-1 or HIV-2 antibodies (HIV- 1/2 EIA: Sanofi Diagnostics Pasteur). If samples were positive by this test, a second immunoassay was then applied to distinguish HIV-1 and HIV-2 antibodies (Multispot: Genetic Systems). Blood samples were also used to calculate lymphocyte data for HIV-positive participants, including CD4, CD8, and CD3 cell counts per microliter of blood. Cell counts were performed using the fluorescence activated cell sorter (FACS) Count analyser (Becton-Dickinson Biosciences, San Jose, CA, USA). Cervical cellular samples were collected as previously described [55].

HPV detection

Polymerase chain reaction (PCR) assays were used to originally detect HPV DNA in participants' samples. For patients enrolled prior to 1998, assays were performed with HPV L1 consensus primers and HPV type-specific oligonucleotide probes specific for high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, or 56. Consensus primers MY09 and MY11, which are specific to a highly conserved region in the L1 open-reading frame, were used, as previously described [58]. However, beginning in 1998, new probes became available, and a PCR-based reverse-line strip test method (Roche Molecular Systems, Alameda, CA) was used with probes for high-risk HPV types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, 73, 82, and 83, as previously described [59].

HPV-16 DNA load quantification

The number of HPV-16 E7 DNA copies as well as the amount of cellular DNA in each stored cervical swab sample was measured using real-time PCR, as described previously [60]. The assay was set up in a reaction volume of 24µl using the TaqMan Universal PCR Master Mix kit (Applied Biosystems, Foster City, CA). The amplification was carried out on an Applied Biosystems 7900 HT Sequence Detection System. Two log-phase 5-point standard curves were implemented in each PCR run, one for HPV-16 E7 DNA and another for β -actin. The viral load for each sample was measured in triplicate and a mean value of the three measurements for each sample was used. The E7 copy number for each sample was normalized according to the input amount of cellular DNA (β-actin), and values were expressed as copies per 1000 cells (copies/10^3 cell). In total, 6 samples were negative for β-actin, implying that an insufficient amount of DNA was obtained from the original samples for testing, and these participants were excluded from further analyses. HPV-16 DNA was undetectable in 31 samples that were originally identified as being positive for HPV-16. Since these samples were previously identified as having contained HPV-16 DNA, it was assumed that their HPV-16 viral load that was below the assay's lower limit of detection and a value of 1 copy/10^3cell was therefore assigned to them.

Statistical Analysis

The mean value of the triplicate measurement of HPV-16 DNA load was used for analyses. After normalization, viral DNA loads were $\log_{10}$ transformed for analysis and analyzed as a continuous variable. Potential confounding factors included age at enrollment, infection with multiple types, current cigarette smoking, commercial sex worker status, date (year) of original study participation, and clinic where the participant was seen. Age was added as a continuous variable to the model, and all other variables were added as categorical variables. Unless otherwise indicated, all analyses with cervical outcomes were evaluated using the worst of participants' cytological or histological diagnoses, when available. For the purposes of this analysis, cervical disease was classified either as normal (including atypical), cervical intraepithelial neoplasia grade I (CIN1), cervical intraepithelial neoplasia grades 2 and 3 or carcinoma in situ (CIN 2-3+), and invasive cancer. For the analyses involving HIV infection, persons with HIV-1, HIV-2, or dual HIV infections were classified as HIV-positive. The associations between HPV-16 DNA load, CIN, and HIV status were first assessed using univariable linear regression. Multivariable linear regression models were then used to evaluate the association between

HPV-16 DNA load and cervical lesion severity, adjusting for the aforementioned potential confounders. Multinomial logistic regression was used to evaluate the association between HPV-16 viral load and CIN or cancer. Finally, Cohen's weighted kappa coefficient was calculated to assess the agreement between cytology and histology among women with both types of diagnoses available.

Results

Of the women who were originally identified as being HPV-16 positive, 498 had a valid histological or cytological diagnosis and a positive β -globin DNA cervical swab sample, and were therefore included in the study. HPV-16 DNA was quantified in 467 (93.8%) of the 498 women, and a value of 1 copy/10^3cell was assigned to the 31 women from whom no HPV-16 DNA was quantified. The HPV-16 $\log_{10}$ DNA load for women in this study population ranged from 1 to 8.39 copies/10^3 cell, with a mean of 3.50 copies/10^3cell. All of the 498 women included had a cytological diagnosis, and 282 (56.6%) had a histological diagnosis as well.

Characteristics of the study population

The median age of those contributing HPV-16 positive specimens was 39 years (range 17-83 years), and 126 (25.3%) of study participants were HIV-positive (Table 1). Of the HIV-positive women, 93 (73.8%) were HIV-1 positive, 21 (16.7%) were HIV-2 positive, and 12 (9.5%) were dually infected. Compared to HIV- women, HIV+ women were younger, had a higher lifetime number of sex partners, and were more likely to be single or divorced. The majority of the study population was nonsmokers (90.7%) and was married (62.0%). Of the study participants, 78 (15.7%) were commercial sex workers. A total of 244 (50.2%) of the study participants were documented as having multiple HPV infections, with 81.8% of HIV-infected women and 39.7% of HIV-uninfected women having more than one type of HPV detected (in addition to HPV-16) at study enrollment.

Association of cervical disease, HIV status, and HPV-16 viral load

A histological or cytological diagnosis (worst of either) of CIN1 was documented in 33 (6.6%) of women, CIN2-3 or CIS in 43 (8.6%), and cancer in 217 (43.6%) of all participants. We observed the mean (sd) $\log_{10}$-transformed values of HPV-16 viral load increasing with increasing lesion severity from 2.65 (1.68)

copies/10^3 cell in women with normal histological or cytological diagnoses to 3.29 (1.78) copies/10^3 cell in women with CIN1, and 4.38 (1.56) copies/10^3 cell in women with CIN2-3, according to worst of either histological or cytological diagnosis. The mean $\log_{10}$ HPV-16 viral load among women with invasive cancer was 4.15 (1.42) copies/10^3cell. After adjustment for HIV status, age, clinic site, infection with multiple types, sample storage time (years), smoking, and commercial sex worker status, women with a diagnosis of CIN 1 exhibited HPV-16 viral load levels that were 4.4 times higher (i.e., $10^{0.64}$) compared to women with a normal cytological outcome (Table 2). Women with CIN 2-3 or CIS had HPV-16 viral load levels that were 93.3 times higher (i.e., $10^{1.97}$), and women with invasive cervical cancer had levels that were 50.1 times higher (i.e., $10^{1.70}$), than women with a normal cytological outcome.

Compared to women with HPV-16 infection only, infection with multiple (>1) types was associated with a 64.5% lower (i.e., $10^{-0.45}$) average HPV-16 viral load, even after adjustment for HIV status and severity of cervical lesion (OR_a 0.35, 95% CI 0.17-0.72). Smoking was also evaluated as a risk factor for HPV-16 viral load among women in the study population. While reported smokers were found to exhibit a lower average HPV-16 viral load than nonsmokers in a univariate model, this difference did not remain after adjustment for potential confounding factors in the adjusted model (OR_a 1.02, 95% CI 0.30-3.55). When compared to HIV-uninfected women in the unadjusted model, women infected with HIV had HPV-16 viral load levels that were on average 1.3 times higher (OR 1.26, 95% CI 0.56-2.82), although this association was not statistically significant. The difference in HPV-16 average viral load between HIV-infected and HIV-uninfected women became even more apparent (3.5 times higher) after additionally adjusting for other variables including severity of cervical lesion and multiple HPV types (OR_a 3.47, 95% CI 1.48-8.32).

Figure 1 gives an illustration of the $\log_{10}$-transformed viral load by disease outcome and HIV status. Since the number of women with HIV type 2 infection and HIV type 1 and 2 dual infection was small in this sample (n=21 and n=12), HIV-1, -2, and dual infections were grouped for all analyses. A general trend of higher HPV-16 viral load associated with HIV infection among study participants can be seen. A more detailed graphical review reveals that increasing HPV-16 viral loads are observed in HIV-positive versus HIV-negative subjects with normal or CIN-grade cytology or histology. However, the average differences in HPV-16 viral load between HIV-positive and HIV-negative subjects among those with

documented CIN2-3 lesions or cancer are less apparent. Among women with HIV type 1 infection, the effect of CD4 count was also evaluated among women who were infected with HIV in this study, and is shown in Table 3. The mean (sd) CD4 cell count for HIV-1 infected, HIV-2 infected, and HIV-1/2 dually infected women in this study were 346 (283) cells/μL, 341 (254) cells/μL, and 259 (144) cells/μL, respectively. Compared to those HIV-infected women classified as having CD4 cell counts of 500 cells per μL or greater, women with CD4 cell counts between 200 and 499 cells per μL (OR_a 3.31, 95% CI 0.33-32.36) and those with CD4 counts of less than 200 cells per μL (OR_a 3.16, 95% CI 0.21-47.86) were at increased risk of a higher HPV-16 viral load in an adjusted model, although these differences did not achieve statistical significance.

A multinomial logistic regression was used to assess the association between increases in HPV-16 viral load and likelihood of cervical disease, as measured by worst of either cytological or histological diagnosis (Table 4). After adjusting for potential confounders, and comparing against women with normal cytology, the likelihood of CIN1 in this sample increased by 25% for each 1-unit $\log_{10}$ increase in HPV-16 viral load (OR_a: 1.25, 95% CI 0.95-1.64), although this association was not statistically significant. Similarly, the risk of CIN2-3 (OR_a 2.35, 95% CI 1.69-3.28) and cancer (OR_a: 2.11, 95% CI 1.50-2.97), compared to women with normal cytology, more than doubled for each 1-unit $\log_{10}$ increase in HPV-16 viral load.

The worst of either cytological or histological outcome was used for all previously-mentioned analysis. However, among women who had both cytological and histological diagnoses available, a sensitivity analysis was also performed to assess the accuracy of classifying disease status by histology compared to cytology. A weighted Cohen's Kappa statistic was calculated based on diagnostic categories of disease in order to compensate for classification that may be due to chance. It was found that the overall agreement between cytological and histological classification of disease was 83.8%, which is considered to be excellent agreement [61,62]. Statistical analyses for all subjects were also performed using histology as the gold standard when available (or, if not available, cytology) as well as using only cytological diagnosis. Results from a univariate analysis of HPV-16 viral load were found to be similar for analyses using histology when available and cytology only compared to those using the worst of either cytological or histological classification, as seen in Table 5.

Discussion

Our analyses demonstrated that HPV-16 viral load increased with increasing grade of cervical disease, and that HIV-infected women were more likely than HIV-uninfected women to have higher HPV-16 DNA loads. Few studies to date have examined the relationship between HPV-16 viral load and cervical disease among both HIV-infected and HIV-uninfected women, and even fewer have assessed this potential relationship using quantitative measures of HPV-16 viral load such as RT-PCR which is proven to be a more accurate measure of HPV type-specific viral load than non-type specific assays including Hybrid Capture[48,49,51,54,63,64].

Our findings that HIV-infection is positively associated with HPV viral load, even after accounting for cervical disease status, is supported by several other recent investigations. Womack et al similarly reported a more than 7-fold higher median overall HPV relative light unit value (as measured by Hybrid Capture II) among HIV-positive women with a final histological diagnosis of normal, compared to normal HIV-negative women [34]. In a study by Lefevre et al, HPV-16 viral load measured by PCR among HIV-positive women who were normal by colposcopy was sevenfold-greater than among normal women who were HIV-negative [48]. Among women who were classified as having a normal histology or cytology in our study, HIV-positive women had statistically significantly increased HPV-16 viral loads compared to those who were HIV-negative. It has been hypothesized that HIV-induced immunosuppression may enable a high level of HPV replication, ultimately causing an increase in HPV viral load, even among women with no documented cervical abnormality. These findings suggest that a strong association between increased HPV16 DNA load and HIV-infection exists, which may highlight a need for increased cervical cancer screening measures among this high-risk group of women.

This theory is further supported by the trend towards increased HPV-16 viral loads observed with decreasing immune status among HIV-infected women, as indicated by CD4 cell count for 200-499 cells/µl and for <200 cells/µl, compared to ≥500 cells/µl), although these differences did not attain statistical significance. Participants were not receiving antiretroviral therapy at the time of study participation, and thus these results are not confounded by variations in immune therapy. Several

studies in addition to ours have also demonstrated an effect of HIV-induced immune suppression, as measured by CD4 cell count, on HPV-16 viral load levels [48,49,64]. However, another study conducted by Fontaine et al. has shown no such association between CD4 cell count and HPV-16 viral load. It has been theorized that the impaired immunity caused by HIV infection leads to less efficient elimination of HPV-infected keratinocytes, which could lead to a higher HPV viral load among women with lower CD4 count [65,66]. However, it has also been theorized that HPV type 16 viral load, compared to other HR-HPV types such as HPV type 18, can more easily evade detection and recognition by the immune system. In this case, we would not expect HPV-16 to be as highly associated with immune status as other HPV types, thereby obscuring the association between CD4 count and HPV-16 viral load in our study [54,67].

We observed a strong association between infection with multiple types of HPV and lower HPV-16 viral load, as found by several others [68-71]. This association remained among both HIV-positive (OR_a 0.30, 95%CI 0.05-0.59) and HIV-negative women (OR_a 0.36, 95%CI 0.17-0.79), possibly indicating that there exist biological interactions between infection with HPV types and HPV type-specific viral replication independent of immune status. The exact mechanism for this interaction is relatively unknown, but it has been suggested that replication of HPV-16 DNA may be altered in the presence of other types of HPV through cross-reactivity of cellular immune response [71]. Xi et al have also suggested that cellular responses induced by natural infection with other types might help with eliminating the infected cells or with restraining viral replication, effectively causing a reduction in viral load [71].

There are several limitations in our study that merit mention. Since the information for this analysis of HPV-16 DNA load was collected in a cross-sectional manner, no conclusion can be drawn concerning the temporal relationship between HPV-16 viral load and development of cervical lesions among these study participants. Therefore, within this study context it is impossible to distinguish between HPV-16 viral load values that are due to prevalent neoplasia or cancer or those that could be indicative of a new neoplastic event. Additionally, as our viral load analyses were limited to HPV type 16, it is possible that a portion of the cervical lesions seen in this population of women could be attributed to infection with another HPV type, thereby obscuring the possible association between HPV-16 viral load and HPV-16 related neoplasia. An additional limitation is that among HIV-infected individuals included in this study, measurements of CD4 count were not available for a portion (35.0%) of the sample of HIV-positive

participants. We do not expect that the subjects with missing CD4 count data will be different than those who have CD4 count information available, however, as the principal reason for missing data is related to protocol differences by site and enrollment procedures between studies. Finally, because we were not able to include HIV-1 and HIV-2 plasma RNA measurements among HIV-infected participants in this study, it is possible that the effect of immune status due to HIV infection has not been fully considered in the analyses presented. Further, because of the small number of HIV-2 and dually HIV-1 and HIV-2 infected participants, we were unable to assess differences in associations by HIV type.

Cervical cytologies were performed on women from all studies included in the present HPV-16 viral load analysis. Additional histological diagnoses, along with cytological diagnoses, were available for a portion (56.6%) of the samples included for viral load testing. Therefore, this study gives a unique opportunity to evaluate the compatibility of cytological and histological diagnostic criteria in relation to HPV-16 viral load and its related risk factors. Sensitivity analyses conducted between cytological and histological classification of disease showed an excellent correlation between cervical and histological diagnoses of women who had both type of diagnoses available. Additional analyses conducted using only cytological diagnostic criteria, as well as analyses using only histological criteria, showed results that were similar to analyses using the worst of either cytological or histological criteria.

During specimen processing, a portion of the samples (91) that were tested for HPV-16 viral load had very low levels of HPV-16 detected in the whole cell negative controls corresponding to their sampling sets, which could indicate laboratory contamination. Of these samples, 52 had sufficient sample remaining for retest, and these new values were used for all analyses. The remaining 39 samples did not have sufficient volume to be retested. However, among samples that did have a retested viral load value, viral load values prior to retesting were highly correlated with retested values (r^2=.995). It was therefore concluded that this possible contamination did not substantially alter viral load values that were not retested. The original HPV-16 viral load values for these samples were included in analyses. Finally, due to the large calendar range over which the samples used for HPV-16 viral load were stored and collected (1991-2009), we considered the possibility that variation in storage time could affect the integrity of DNA samples. Therefore, storage time (in years) of the samples used was added to all multivariate models.

Our study is unique in the large number of women with a diagnosis of invasive cervical cancer that was included, which few other studies have been able to evaluate [27,29,73]. As limited data is available regarding the association between HPV-16 viral load among women with histologically confirmed cancer, this study provided valuable information concerning viral levels of HPV-16 in well-developed cases of cervical disease. Specifically, our study found a wide range in $\log_{10}$ viral load values among cases of invasive cervical cancer (zero to 8.22 c/10^3 c). It was also noted in our study that average HPV-16 DNA load levels were somewhat lower among cancer specimens (4.15 c/10^3 c) than for CIN2/3 specimens (4.38 c/10^3 c). This finding of lower HPV-16 viral load for cancers compared to CIN 2/3 is in agreement with findings by Cricca et al using cervical swab specimens, although a similar study by Saunier et al. reported very high HPV-16 viral load levels for cancer specimens [73,74]. However, Saunier et al measured viral load using paraffin-embedded cancer specimens, whose higher proportion of cancer cells compared to smears may account for the overall higher viral load found. A study by Moberg et al similarly found a wide range of HPV-16 DNA values for cancer specimens using archival smears, with very low HPV-16 DNA levels noted among cancer cases [29]. These values might be attributed to the fact that cancer specimens may contain normal cells in addition to cancer cells, which could effectively lower the average HPV DNA load found [69]. However, as the women with invasive cervical cancer in our study tend to present with larger and later stage cancer, we expect the contribution of normal cells to HPV DNA load values to be negligible in our measurements. Sherman and colleagues have also studied the effect of low grade mucosal lesions surrounding high-grade cervical lesions, and concluded that the viral load of the surrounding tissue severely limits the clinical interpretation of viral load as a determinant for disease risk [69]. It is also possible that, while high HPV-16 viral loads have been reliably documented among women with high grade lesions, the development of more severe cervical disease is not necessarily limited to those with high viral load. This could indicate that other factors might be responsible for HPV viral DNA quantities in cervical specimens, such as the integration status of the HPV virus into the host's genome, which is known to be a key step in the progression of cervical disease [24,73]. It is also likely that cancer cases included in this study represent a select group of women who were well enough to participate in study enrollment, and therefore likely had less severe cancer.

In summary, our findings support an association between HPV-16 viral loads, HIV status, and increasing severity of cervical disease. We found an association between HPV-16 viral load and increasing severity of cervical disease, including invasive cancer. Although the strength of the association has varied, the majority of studies conducted support the existence of a correlation between HPV-16 viral loads and increasing cervical lesion severity [23,24,73,75-77]. However, even as HPV-16 viral loads were observed to increase with increasing grade of cervical lesion in our study, there was a substantial overlap in the range of viral loads across categories of increasing cervical disease severity, suggesting a possible limitation of the clinical utility of HPV-16 viral load as a definitive marker of disease [27,30,49,78]. Several unknowns remain in order to more completely understand the etiologic role of HPV-16 viral load in the development of cervical disease, including the role of HPV viral DNA persistence and longitudinal fluctuations in HPV-16 DNA load.

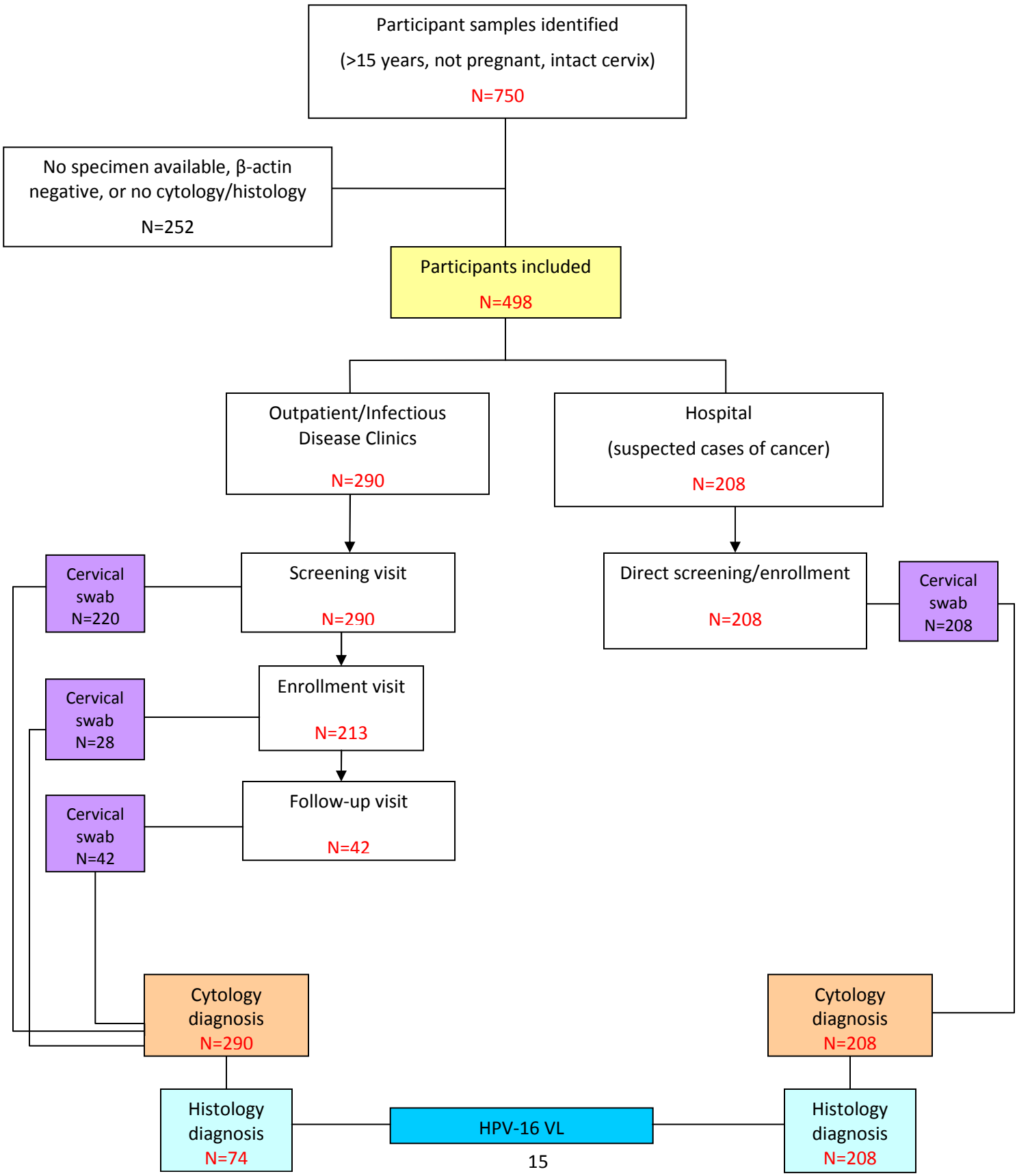


Figure 1. Cervical sample and enrollment tree

Table 1. Demographic characteristics of HPV16-infected Senegalese women, by HIV status, N=498

	HIV negative N (%)	HIV positive N (%)
Total	372	126
Age, years		
<35	106 (28)	61 (48)
35-39	60 (16)	32 (25)
40-44	39 (10)	13 (10)
≥45	167 (45)	20 (16)
Marital status		
Single	33 (9)	24 (19)
Married - monogamous	102 (28)	30 (24)
Married - polygamous	127 (35)	23 (19)
Separated/Divorced	55 (15)	30 (24)
Widow	46 (13)	17 (14)
Cigarette smoker	28 (8)	18 (14)
Alcohol use	16 (4)	20 (16)
Commercial sex worker	49 (13)	29 (23)
Lifetime sexual partners		
1	172 (58)	32 (33)
2-5	86 (29)	45 (46)
>5	38 (13)	21 (21)
Age at first sex		
<=15	130 (47)	39 (37)
16-20	128 (46)	53 (50)
>=21	20 (7)	13 (12)
Lack of contraception use	270 (73)	88 (70)
Children		
None	23 (6)	8 (6)
1-3	108 (29)	73 (58)
≥4	236 (64)	45 (36)
CD4 count		
Mean ± SD, cells/µl	----	336.34±267.27
Subjects with		
>=500 cells/µl	----	20 (22)
200-499 cells/µl	----	41 (46)
<200 cells/µl	----	29 (32)

Table 2. HPV-16 viral loads in relation to cervical disease status, HIV infection, and infection with multiple HPV types.

	N (%)	Log_{10} viral mean copies/10^3 cell (±SD)	Crude β* (95% CI)	Adjusted β† (95% CI)
Cervical disease status§				
Within normal limits(inc. atypical)	205 (41.2)	2.65 (±1.68)	ref	ref
CIN1	33 (6.6)	3.29 (±1.78)	0.65 (0.07-1.23)	0.64 (0.03-1.24)
CIN2-3/CIS	43 (8.6)	4.38 (±1.56)	1.73 (1.21-2.25)	1.97 (1.38-2.56)
Cancer	217 (43.6)	4.15 (±1.42)	1.51 (1.21- 1.81)	1.70 (1.05-2.35)
Multiple HPV types				
No	242 (49.8)	3.80 (±1.51)	ref	ref
Yes	244 (50.2)	3.21 (±1.89)	-0.59 (-0.89 - -0.28)	-0.45 (-0.76 - -0.14)
Smoking				
No	449 (90.7)	3.55 (±1.71)	ref	ref
Yes	46 (9.3)	2.94 (±1.89)	-0.61 (-1.14--0.09)	0.01 (-0.53-0.55)
HIV status				
HIV-negative	372 (74.7)	3.47 (±1.78)	ref	ref
HIV-positive	126 (25.3)	3.57 (±1.60)	0.10 (-0.25 - 0.45)	0.54 (0.17 - 0.92)

* Interpretation: HPV-16 viral load level (c/10^3 cell) among women with CIN1 is $10^{(0.65)}$= 4.5 times the viral load level of women with normal cytology/histology

§ Evaluated using the worst of either cytological or histological diagnosis

†Adjusted for age, infection with multiple (>1) types, cigarette smoking, clinic site, commercial sex work, hiv status, and sample storage time (y)

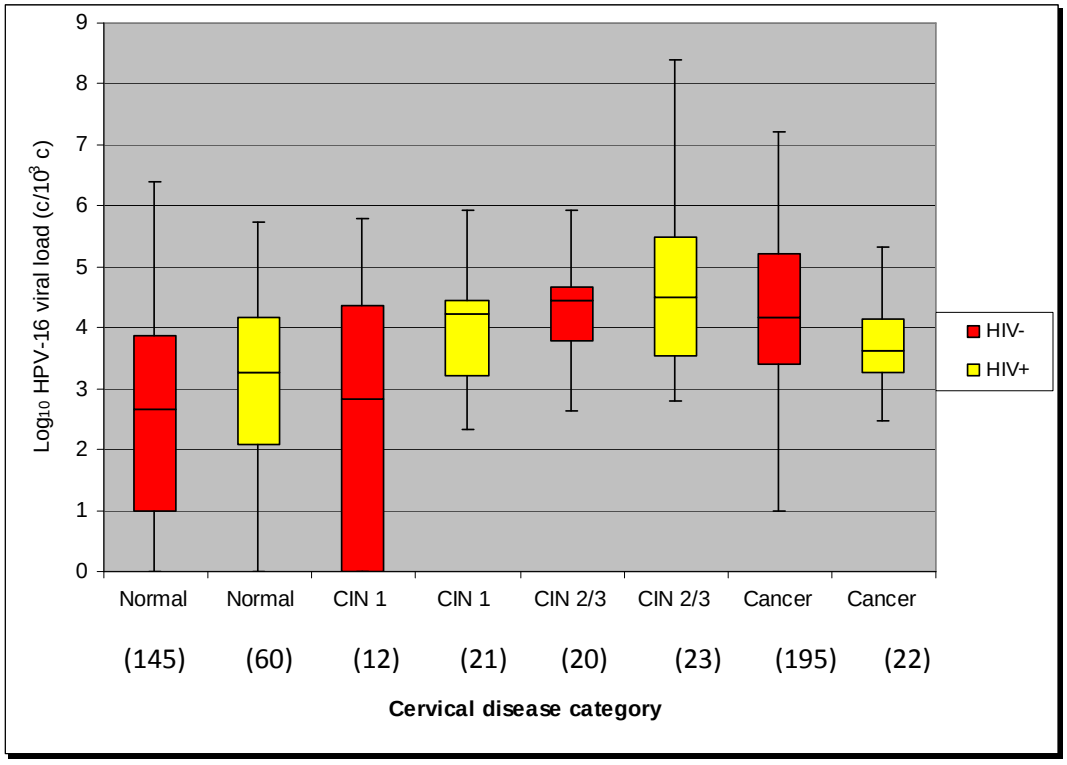


Figure 2. Box and whiskers plot of log_{10} HPV-16 viral load, by cervical disease status and HIV infection status.

Table 3. Among HIV positive women, HPV-16 viral loads by CD4 cell counts.

	Log_{10} viral mean copies/10^3 cell (±SD)	Crude β* (95% CI)	Adjusted β† (95% CI)
CD4+ cell count			
>=500 cells/ml	2.79 (±1.73)	ref	ref
200-499 cells/ml	3.65 (±1.82)	0.86 (-0.07-1.79)	0.52 (-0.48-1.51)
<200 cells/ml	3.86 (±1.56)	1.07 (0.08-2.07)	0.50 (-0.67-1.68)

* Interpretation: HPV-16 viral load among HIV-infected women with CD4 cell count 200-499 cells/µl is $10^{(0.86)}$=7.2 times the viral load level of women with CD4 cell count >=500 cells/µl.
†Adjusted for age, infection with multiple (>1) types, cigarette smoking, clinic site, commercial sex work, and sample storage time (y)

Table 4. Multinomial logistic regression analysis of the risk of cervical neoplasia and cancer with increasing HPV-16 viral load

	Crude OR* (95% CI)	Adjusted OR† (95% CI)
Cervical disease status[§]		
Within normal limits(inc. atypical)	ref	ref
CIN1	1.26 (1.00-1.58)	1.25 (0.95-1.64)
CIN2-3/CIS	2.06 (1.61-2.64)	2.35 (1.69-3.28)
Cancer	1.84 (1.59-2.13)	2.11 (1.50-2.97)

* Odds of disease per 1-unit increase in $\log_{10}$ HPV-16 viral load
†Adjusted for age, infection with multiple (>1) types, cigarette smoking, clinic site, commercial sex work, hiv status, and sample storage time (y)
§ Evaluated using the worst of either cytological or histological diagnosis

Table 5. Univariate linear regression analysis of the association between increasing HPV-16 viral load and risk of cervical intraepithelial neoplasia (CIN) and cancer, by method of disease diagnosis.

	Cytology only Crude β (95% CI)	Histology (cytology when not available) Crude β (95% CI)	Worst of histology or cytology Crude β (95% CI)
Cervical disease status			
Within normal limits(inc. atypical)	ref	ref	ref
CIN1	0.79 (0.18 - 1.39)	0.85 (0.29 - 1.42)	0.64 (0.70 - 1.23)
CIN2-3/CIS	1.45 (0.97 - 1.94)	1.57 (1.04 - 2.10)	1.73 (1.21 - 2.25)
Cancer	1.27 (0.95 - 1.60)	1.39 (1.08 - 1.70)	1.51 (1.21 - 1.81)

Chapter 2

HPV-16 viral load associated with cervical dysplasia and invasive cancer (longitudinal)

Introduction

It is widely acknowledged that infection with high-risk human papillomavirus (HR-HPV) types invariably precedes the development of cervical cancer. However, compared to the high prevalence of HR-HPV infection among sexually active women, infection with HR-HPV does not always lead to development of cervical disease [3,6]. In fact, among women with newly-acquired HPV infections, some estimates show that roughly half of infections are cleared within 8-10 months of acquisition [7,13,79]. However, among women who do not successfully clear their HR-HPV infection, development of cervical intraepithelial neoplasia (CIN) and cervical cancer is a conceivable outcome. High-grade CIN lesions (grades 2 and 3) are considered precursor events to cervical cancer.

Among high-risk HPV types, HPV-16 is considered to have the highest oncogenic potential, and has been identified in 50% of cervical cancers[3,46,80-84]. Due to the consistent identification of HR-HPV, especially HPV type 16, in cervical precursor lesions and cancer, HPV testing has been considered useful for the detection of cervical intraepithelial neoplasia grade 2 or higher. The ability to reliably amplify HPV in laboratory assays further advocates for use of this highly sensitive assay as a cervical cancer screening tool. However, the detection of HR-HPV at an unknown point during a woman's HPV infection may not necessarily provide an accurate estimate of future disease risk. Since the predictive value of a single HPV test remains low, other methods of better identifying HPV-positive women at risk of CIN development have been considered [85]. Quantitative measurement of HPV DNA (viral load) could add complementary information to a positive HPV test concerning a woman's risk for future neoplasia.

Some cross-sectional studies have found that HPV-16 viral load is elevated among those with cervical lesions [30,40,49,54,68,73,86], although others have provided conflicting results[27,52,87]. In our recent Chapter 1 cross-sectional analysis, the risk of CIN2-3 and of cancer more than doubled per 1 unit increase in $\log_{10}$ HPV-16 viral load when comparing to women with normal cytology. These cross-sectional studies can help to clarify the role ofHPV-16 viral load and its association with current cervical disease. However, the measurement of baseline viral load could also be informative of future events in the natural history of HPV infection, including viral clearance and development of CIN [88].

It is thought that HPV viral persistence, or the inability to clear an existing HPV infection, could greatly predispose an individual to development of future neoplasia and cancer. If it is accepted that HPV persistence is related to the development of cervical neoplasia, risk factors that lead to persistence of infection need to be thoroughly evaluated. Several studies have shown a correlation between increases in the concentration of HPV viral load, persistence of HR-HPV infection, and eventual development of cervical neoplasia. HPV type 16 has been indicated as being most predictive of the development of incident cervical intraepithelial or worse, among high-risk types [29,75,89].

Women with human immunodeficiency virus (HIV) are disposed to both a higher prevalence of HPV infection and a greater risk of cervical squamous intraepithelial lesions [47,48,90]. A study by Ahdieh et al found that HIV-positive women, compared to HIV-negative women, were at 1.8 times higher risk of incident infection with any high-risk HPV type, and 1.5 times higher risk of acquiring HPV type-16 infection [91]. However, of the studies recently conducted to examine the relationship between HPV-16 viral load and cervical neoplasia, few have addressed the association among HIV-positive women[34,48-50,54,92,93]. The impact of HIV-infection on HPV DNA levels, persistence of HPV infection, and subsequent disease risk, therefore, remains to be investigated.

The specific role of HPV viral load in HPV persistence and development of cervical neoplasia, especially among high-risk populations, remains largely unclarified. Therefore, a more extensive study of the role of viral load in viral persistence and cancer progression, as well as factors that affect viral load in individuals with HIV infections, are important research objectives. The objective of the present study was to determine the association between baseline HPV-16 viral load, persistence of HPV-16 infection, and development of cervical neoplasia. In order to evaluate this, we assessed determinants of HPV-16 viral load among women from Senegal, West Africa who had longitudinal follow-up data available.

Materials and Methods

Study population

The study population and sample collection methods for this study have been described in detail elsewhere[35,55]. Briefly, between October 1, 1994 and January 1, 1998, women older than 15 years that presented either to the University of Dakar Infectious Disease Clinic and commercial sex workers attending sexually transmitted disease clinics either in Dakar or M'Bour were enrolled in a study evaluating HSIL development among HIV-positive and HIV-negative women. At the time of enrollment, women underwent serologic testing for HIV-1 and HIV-2 and screening for HPV DNA and cytology. No HIV-infected woman was receiving antiretroviral therapy at the time of this study. Those women with HIV infection or with high-risk HPV infection were recruited to participate in a longitudinal study to assess the risk of developing cervical lesions, with follow-up visits scheduled at 4-month intervals. Participants included in the present study were those who tested positively for HPV type 16 DNA during study enrollment or who had an incident infection identified after study enrollment. All participants had at least one additional documented HPV test performed at a follow-up visit. Written consent was obtained from all women who were enrolled in the original cohort study. The study was conducted according to procedures approved by the institutional review boards of both the University of Washington and the University of Dakar. For the present study, we used the participants' stored cervical swab specimens to quantify the amount of HPV-16 DNA at the participant's baseline visit. Baseline visit was defined as the first visit at which the participant was tested positively for HPV-16 DNA, and for which a cervical sample was available for HPV-16 DNA quantification.

Specimen collection and study procedures

Blood for HIV-1 and HIV-2 testing was collected from each participant at the original screening visit, and cervical samples were also taken for HPV and cervical screening, as described previously [35,55]. Participants received a general physical exam, and also completed a questionnaire that solicited medical history and sexual behavior information. Pap smears were interpreted and classified according to the Bethesda system with atypical results including: atypical squamous cells (ASCUS), low grade squamous intraepithelial lesion (LSIL), high grade squamous intraepithelial lesion (HSIL), and squamous cell carcinoma[94]. Conventional Pap smears were used for all cytological diagnoses. These slides were initially read by a pathologist in Senegal and then sent to Seattle, Washington for a second confirmatory

reading. For study purposes, the confirmatory diagnosis made by the Seattle pathologist was used as the final diagnosis.

HPV DNA Detection

Cervical cellular samples were collected as previously described[55]. Polymerase chain reaction (PCR) assays were used to originally detect HPV DNA in participants' samples, using HPV L1 consensus primers and HPV type-specific oligonucleotide probes specific for high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, or 56. First, a 10-probe mixture using consensus primers MY09 and MY11 tested for the presence of HR-HPV. These MY09 and MY11 primers are specific to a highly conserved region in the L1 open-reading frame. Positive samples were then re-amplified to allow detection of HPV low-risk and high-risk types, with high-risk types including HPV-16, HPV-18, combined HPV 31, 33, 35, and 39; combined HPV 45 and 56, and combined HPV 51 and 52[58].

Quantification of HPV-16 DNA loads

The number of HPV-16 E7 DNA copies as well as the amount of cellular DNA in each stored cervical swab sample was measured using real-time PCR, as described previously [60]. The assay was set up in a reaction volume of 24*u*l using the TaqMan Universal PCR Master Mix kit (Applied Biosystems, Foster City, CA). The amplification was carried out on an Applied Biosystems 7900 HT Sequence Detection System. Two log-phase 5-point standard curves were implemented in each PCR run, one for HPV-16 E7 DNA and another for β -actin. The viral load for each sample was measured in triplicate and a mean value of the three measurements for each sample was used. The E7 copy number for each sample was normalized according to the input amount of cellular DNA (β-actin), and values were expressed as copies per 1000 cells (copies/10^3 cell). None of the specimens assayed were negative for β-actin. HPV-16 DNA was undetectable in 19 samples that were originally identified as being positive for HPV-16 when tested previously. Since these samples were previously identified as containing HPV-16 DNA, it was assumed that their HPV-16 viral load that was below the lower limit of detection and a value of 1 copy/10^3cell (or a $\log_{10}$-transformed value of zero) was therefore assigned to them.

Statistical methods

The associations between baseline HPV-16 viral load and HPV-related outcome variables, including clearance of HPV infection and development of high grade squamous intraepithelial lesions (HSIL) or worse (HSIL+), were evaluated in this analysis. HPV-16 viral load, expressed as copies/10^3cell, was $\log_{10}$ transformed and analyzed as a continuous variable for all analyses. Clearance of HPV-16 infection was defined as having at least two consecutive negative HPV tests after one or more HPV-16 positive tests. If, at the end of the participant's follow-up period, only one HPV-16 negative test was documented, the participant was considered to have cleared her infection. Development of HSIL+ was defined as having a cytological diagnosis of HSIL or worse documented at a follow-up visit occurring after the baseline visit at which HPV-16 viral load was measured. Potential confounding factors in this analysis were selected *a priori* and included age, HIV status, prevalent (versus incident) HPV-16 infection at baseline, and infection with multiple HPV types. Age was added to the model as a continuous variable, and all other variables were added as categorical variables. The Cox proportional hazards model was used to calculate hazard ratios (HR) for $\log_{10}$-transformed values of HPV-16 viral load in relation to clearance of HPV infection and development of HSIL. Time to event for clearance of infection was measured from the baseline visit when HPV-16 viral load was measured to the first visit at which HPV-16 was not detected (only for those with two consecutive HPV-16 negative visits). Time to event for development of HSIL+ was measured from the baseline visit when HPV-16 viral load was measured to the first occurrence of HSIL+ development. Women who remained HPV-16 positive at the last documented study visit were considered censored for the outcome involving clearance of infection. Univariable and multivariable hazard models were used to assess associations. Statistical analyses were performed using STATA (StataCorp LP, College Station, Texas).

Results

Study population

Among the 179 participants who were originally identified as being positive for HPV-16 DNA and having longitudinal follow-up data (≥2 HPV tests), baseline frozen cervical specimens were located and HPV-16 DNA quantification was performed for 121 (67.6%) of these participants. Of these, 8 women were diagnosed as having a high-grade lesion or worse at baseline, and were excluded from further analyses. Of the remaining 113 women included in this analysis, 73 were HIV-negative at enrollment, and 40 of

these women were HIV-positive, including 29 HIV-1 infected women, 6 HIV-2 infected women, and 5 HIV-1/2 dually infected women (Table 6). The median age of the 113 women included in this analysis was 30 (range 17-54). The median age at first sex was 17 (range 11-31), and 31.5% of the study population was married. Multiple type HPV infections at enrollment were documented in 45 (41.3%) of all women. As detailed in Table 7, the median follow-up time for women in this study was 2.0 years (range 3 months to 5.5 years) and the average number of follow-up visits was 7.4.

HPV-16 DNA viral loads

HPV-16 DNA was identified in 94 (83.2%) of the 113 samples that were included in this analysis. The $\log_{10}$ HPV-16 viral load values among all positive samples ranged from one to 6.40 copies per 10^3 cells. A value of 1 copy/10^3 cell (or zero $\log_{10}$ copies/10^3cell) was assigned to the 19 samples from which HPV-16 DNA could not be quantified since it was assumed that their viral load levels were less than the assay's detection threshold of 10 viral copies[71]. Among the 40 HIV-infected women contributing viral load samples to this study, the mean (sd) $\log_{10}$ quantity of HPV-16 DNA at baseline visit was 3.12 (1.61) copies/10^3cell. Of the 73 HIV-negative baseline cervical samples that were tested, the mean $\log_{10}$ HPV-16 DNA amount was 2.67 (1.70) copies/10^3cell.

Clearance of infection

During the course of follow-up, 77 women (68.1%) cleared their initial HPV-16 infection by their last documented follow-up visit, with a median time to clearance of infection, defined as first negative HPV-16 detection event, of approximately 1 year. Univariate hazard ratios for clearance of infection by risk factors are presented in Table 8. Each 1-unit $\log_{10}$ increase in HPV-16 baseline viral load was associated with a decreased likelihood of infection clearance (HR: 0.71, 95% CI 0.61-0.82). Each 1-unit $\log_{10}$ increase in HPV-16 baseline viral load was associated with a decreased likelihood of clearance of infection among women with prevalent infections (HR_a: 0.81, 95% CI 0.66-1.00), but was particularly strong among those with incident infections (HR_a: 0.64, 95% CI 0.48-0.86). Infection with HIV (HR_a: 0.52, 95% CI 0.31-0.86) was associated with a decreased likelihood of infection clearance. In addition, having a prevalent versus incident infection at time of baseline visit (HR_a: 0.71, 95% CI 0.44-1.14), and having multiple HPV types detected at enrollment (HR_a: 0.82, 95% CI 0.52-1.30) appeared to be moderately

associated with decreased likelihood of clearance of infection, although these associations were not statistically significant. Only HPV-16 baseline viral load (HR_a: 0.75, 95% CI 0.64-0.87) achieved statistical significance in the adjusted Cox model, however. Figure 3 depicts the Kaplan-Meier survival curve of participants according to tertiles of HPV-16 baseline viral load.

In order to further investigate the role of HPV-16 baseline viral load in infection clearance, we restricted analyses to women with normal cytology at baseline (Table 10). Of these women, 28 (33.3%) had incidently-detected HPV-16 infection, and 56 (66.7%) had prevalently-detected HPV-16 infection. Clearance of infection was associated with a 1-unit log_{10} increase in HPV-16 baseline viral load among women with normal cytology (HR_a: 0.77, 95% CI 0.65-0.92). Among those women with incident HPV-16 infection, each 1-unit log_{10} increase in HPV-16 baseline viral load was associated with a 38% decreased risk of infection clearance (HR_a: 0.62, 95% CI 0.44-0.88). This association was less marked among women with prevalent HPV-16 infections, however (HR_a: 0.90 per log_{10}, 95% CI 0.71-1.14).

Development of HSIL or worse (HSIL+)

Among this sample of women, 33 developed high grade squamous intraepithelial lesion or worse during study follow-up. In univariate analysis, the association between HPV-16 viral load and development of HSIL+ was associated with a 1-unit log_{10} increase in HPV-16 baseline viral load (HR 1.21, 95% CI 0.95-1.54), although not significantly. Those with prevalent (versus incident) infections (HR: 0.63, 95% CI 0.30-1.33) and those with multiple type infections (HR: 0.80, 95% CI 0.38-1.68) also had lower likelihood of developing HSIL+, although neither association was statistically significant. After controlling for potential confounders, a 1-unit increase in log_{10} HPV-16 viral load was associated with an 18% increased likelihood of developing CIN2+ by the end of the study period (HR_a: 1.18, 95% CI 0.90-1.54), as shown in Table 4. However, this association did not achieve statistical significance. Figure 1 shows trends in HPV-16 viral load, by tertiles, in relation to development of HSIL+.

Additional analyses among women with normal cytology at baseline, and by prevalent versus incident HPV-16 detection, were also performed (Table 10). Among women with normal cytology, no relationship between increasing HPV-16 viral and development of HSIL+ was found (HR_a: 0.93 per log_{10},

95% CI 0.66-1.32). No significant relationship between HPV-16 viral load and HSIL+ development was seen, either, among women with prevalent (HR_a: 1.12 per log_{10}, 95% CI 0.67-1.89) or incident (HR_a: 0.82 per log_{10}, 95% CI 0.84-1.53) HPV-16 detection.

Discussion

In order to investigate the role of HPV-16 viral load in the natural history of HPV infection and lesion development, we measured HPV-16 DNA load in cervical samples collected from HPV-16 infected Senegalese women with longitudinal follow-up data. We then estimated the association of HPV-16 viral load with clearance of HPV-16 infection and subsequent development of HSIL or worse. We found a strong association between increasing first-measured HPV-16 viral load and decreasing likelihood of infection clearance, but only a modest association between baseline viral load and HSIL development.

Cross-sectional associations between HPV viral load and cervical lesions have been reported by others previously, and have additionally been found in Chapter 1 among our cross-sectional sample of women from Senegal [20-23]. Several other longitudinal studies have suggested a predictive relationship between HPV-16 viral load and the development of cervical lesions [7,75,76,95,96]. Constandinou-Williams et al found a substantial increase in risk of acquiring a cervical abnormality per 10-fold increase in HPV-16 copy number (HR: 1.76, 95% CI 1.38-2.25). An analysis of HPV-16 viral load in relation to CIN2+ development after 18 months of follow-up conducted by Hesselink et al found a relative risk of 1.6 (95% CI 1.3-1.9) using survival techniques. In the present study, after adjusting for potential confounders, development of HSIL+ subsequent to HPV-16 viral load baseline measurement showed a modest but not significant association (HR_a: 1.18 per log_{10}, 95% CI 0.90-1.54). Several theories have been offered to explain the lack of a strong association between viral load and future cervical dysplasia. Xi et al have suggested that HPV-16 DNA load is associated with the risk of developing CIN-3, but that these associations varied with cytological findings at the time of viral load measurement [60]. Indeed, when baseline viral loads were analyzed without making the exclusion of no high-grade lesion present at baseline in our sample of women, the apparent association between HPV-16 viral load and development of future lesions was even weaker. However, to further elucidate this hypothesis, we additionally restricted this analysis to women who had a normal cytological finding at baseline. No association with baseline HPV-16 viral load was found among women with normal cytology. Another hypothesis is that RNA levels of HPV-16 are

more representative of disease risk than HPV-16 DNA levels. A recent study by Winer et al evaluated the association between both RNA and DNA HPV-16 levels and risk of developing CIN 2-3[97]. This study found that, while HPV-16 E7 RNA levels were substantially associated with an increase in lesion development (HR: 6.36 per $\log_{10}$, 95% CI 2.00-20.23), HPV-16 E7 DNA levels were not (HR: 1.18 per $\log_{10}$, 95% CI 0.77-1.80), perhaps suggesting that DNA may be a less sensitive marker of disease progression.

In line with our previous cross-sectional study, HPV-16 viral load was increased among HIV-positive women, as compared to HIV-negative women. Few others have evaluated HPV-16 viral load in relation to HIV status in a longitudinal manner [39,93]. As we have shown in our previous parent study, in the present longitudinal substudy, women infected with HIV had a lower likelihood of clearing their infection (HR_a: 0.58, 95% CI 0.33-1.02) and a slightly higher likelihood of developing HSIL+ (HR_a: 1.30, 95% CI 0.58-2.93)[37,55].

Several limitations in our study should be addressed. Among women who exhibit HPV infection, it is often difficult to attribute HPV DNA measured to a newly productive HPV infection rather than that resulting from a pre-existing cervical lesion [88]. In our analysis of the association of HPV-16 baseline viral load with the development of HSIL+, we attempted to address this issue by excluding women who had a recent history of HSIL+ previous to baseline, or who had documented high grade lesions or worse at baseline. Even after these exclusions, we cannot be entirely sure that baseline HPV-16 DNA values measured are not due to underlying pre-existing lesions which were not identified by cytology.

An ideal study of the natural history of HPV viral load and HPV infection would be conducted among women who have only incident infections. In this way, a more accurate measurement of HPV infection duration and its association with HPV DNA load may be achieved. In order to simulate this type of study, we completed an additional analysis restricted to only women with normal cytology having newly-detected (incident) HPV-16 infections in our study. No significant association of HPV-16 DNA with HSIL+ development was found among women with newly-detected infections (HR_a: 0.82, 95% CI 0.44-1.53), although small sample size may be partially responsible for this finding. In our analysis of all women and compared to women with newly-detected (incident) HPV infection, women with prevalent HPV infection

were somewhat less likely to develop HSIL (HR: 0.70, 95% CI 0.25-1.95) or clear HPV infection (HR: 0.75, 95% CI 0.41-1.37), although these findings were not particularly strong. It seems plausible that the natural histories of women with prevalent versus incident HPV infection may each affect the predictive nature of the relationship between HPV infection and lesion development, although it may be important to consider other factors such as age in this interpretation. In this study, it seems feasible that women with prevalently-detected HPV infection represent a mixed background of infection histories, with many of the women harboring a long-time persistent but non-progressive infection.

It is also possible that the disappearance and reappearance of HPV type 16 infections were missed among our group of study participants as HPV testing occurred only at specified intervals during study follow-up (usually every 4 months or longer). In the event of this occurrence, a woman who cleared, then acquired a new HPV-16 infection, would be misclassified as having a persistent infection, and an overestimation of the length of infection persistence would occur. Additionally, among the sample of women included in this longitudinal study, a portion (8) were noted as having transiently-detected HPV-16 DNA based on longitudinal HPV-16 DNA testing, defined as having one or more negative HPV-16 tests both subsequent to and preceding HPV-16 positive detection in the same woman. These longitudinal fluctuations in HPV-positivity could be due to low-level viral shedding, sample variation, or the appearance of new HPV type 16 infections in the same person [13,98,99] This could also carry certain implications for the prediction of HSIL+ and clearance of HPV infection in our study, as the HPV infection characteristics of a woman with transient HPV infection might be unique from a woman with persistent HPV infection. A study by Dalstein et al reported that development of CIN 2-3 was only achieved by women classified as having persistent HR-HPV infection, compared to women with transiently positive HR-HPV or HR-HPV negative infections[7]. Hawes et al found that, compared to women with no HPV DNA detected, women with transient high-risk HPV infection had a 14 times increased risk of HSIL development, but women with persistent high-risk HPV infection had a 47 times increased risk[55]. While it has been suggested that sporadic detection of HPV infections is a common event among women, especially those who have become newly sexually active[13], it is currently unclear how HPV DNA viral load might be related to the sporadic nature of HPV infections.

Our study focused on HPV-16 DNA measurement at baseline and its association with future disease risk. However, it has been suggested by several others that multiple measurements of HPV viral load could be a more useful indicator of future disease development than viral load measured at a single point alone. A study by Monnier-Benoit et al showed that HPV-16 viral loads which increased over time were more likely to be associated with CIN2/3 incidence than those which did not increase over time [100]. Constandinou-Williams et al found that baseline measurement of HPV-16 viral load had only a modest association with risk of acquiring a cytological abnormality. However, a more detailed analysis of their data implied that HPV copy number was prone to waxing and waning during follow-up [95]. Data on multiple HPV-16 viral load measurements over time was also available for a small portion of the participants in our study (Supplementary Figure 5). A closer look at these changes in viral load over time among these participants revealed that, even among women who developed a lower grade cytological abnormality (CIN1), viral load was variable over study follow-up. Longitudinal profiles of women who did develop a cytological abnormality by the end of study follow-up tended to follow a pattern of either increasing viral load over time or relatively constant viral load over time. However, it is interesting to note that in several instances among our longitudinal participants, a spike in HPV-16 viral load was followed closely by a change in cytological diagnosis of the participant from less severe to more severe disease. In addition to cervical lesion development, kinetics of HPV-16 viral load has also been used recently to predict HPV clearance events. A recent study by Marks et al showed that, although HPV 16 viral load measured at a single point in time was not associated with viral clearance, repeated measurements of HPV-16 viral load proved be a more useful predictor of clearance[101].

In conclusion, our study findings indicate that increased baseline HPV-16 viral load is associated with decreased likelihood of clearance of HPV-16 infection. However, the association of viral load with development of cervical lesions remains unclear from our data. Viral load might indeed be important for early events in the establishment of HPV infection among women. However, a single viral load measurement may not be entirely indicative of future risk of disease. Future studies may be useful for investigating the potential usefulness of HPV viral load kinetics in cervical disease development.

Table 6. Baseline demographic and biological characteristics of participants, according to clearance of HPV-16 infection by end of follow-up, N=113

Participant characteristics*	All women †	%	Cleared §	% cleared
Total	113		77	
HIV status				
HIV-negative	73	64.6%	57	78.1%
HIV-1	29	25.7%	15	51.7%
HIV-2	6	5.3%	3	50.0%
HIV-dual	5	4.4%	2	40.0%
CD4 count				
Mean ± SD, cells/µl	381.2 (±210.5)	---	416.3 (±233.6)	---
Subjects with				
>=500 cells/µl	9	23.7%	6	66.7%
200-499 cells/µl	23	60.5%	12	52.2%
<200 cells/µl	6	15.8%	2	33.3%
Age, y				
<25	25	22.1%	17	68.0
25-34	58	51.3%	39	67.2
≥35	30	26.5%	21	70.0
Marital status				
Single	35	31.5%	25	71.4%
Married (monogamous)	24	21.6%	16	66.7%
Married (polygamous)	11	9.9%	5	45.5%
Divorced	37	33.3%	27	73.0%
Widow	4	3.6%	2	50.0%
Smoking				
Yes	31	27.4%	21	67.7%
No	82	72.6%	56	68.3%
Alcohol use				
Yes	18	15.9%	13	72.2%
No	95	84.1%	64	67.4%

Table 6 continued

Participant characteristics*	All women †	%	Cleared §	% cleared
Contraceptive Use				
No	44	39.3%	23	52.3%
Yes	68	60.7%	53	77.9%
Number of children				
0	15	13.5%	11	73.3%
1-3	63	56.8%	45	71.4%
>3	33	29.7%	20	60.6%
Age at first sex, y				
<=15	40	39.2%	28	70.0%
16-20	50	49.0%	34	68.0%
≥21	12	11.8%	8	66.7%
Commercial sex worker				
Yes	62	54.9%	47	75.8%
No	51	45.1%	30	58.8%
Lifetime partners				
1	28	26.2%	19	67.9%
2-5	30	28.0%	14	46.7%
6-10	12	11.2%	10	83.3%
>10	37	34.6%	29	78.4%
Multiple (>1) HPV infections				
Yes	45	41.3%	32	71.1%
No	64	58.7%	44	68.8%
Baseline cytology status				
Normal	84	74.3%	66	78.6%
LSIL	29	25.7%	11	37.9%

*The following variables had missing data: CD4 count (among HIV+) (2), marital status (2), contraceptive use (1), number of children (2), age at first sex (12), lifetime sexual partners (6), multiple HPV infections (5).

† Column percentages

§ Row percentages

Table 7. Follow-up characteristics of participants with longitudinal data (n=113).

Characteristic	Mean	range
Mean follow-up period, years (range)	2.0	0.25-5.5
Mean number of study visits (range)	7.4	2-22
Follow-up results	**N**	**%**
Clearance of infection	77	68.1%
Development of HSIL or worse	33	29.2%

Table 8. Factors associated with clearance of HPV-16 infection.

Variable	Univariable HR	95% CI	Multivariable HR*	95% CI
HPV-16 VL (per log_{10} VL)	0.71	0.61-0.82	0.75	0.64-0.87
HIV-positive	0.52	0.31-0.86	0.58	0.33-1.02
Prevalent HPV-16	0.71	0.44-1.14	0.75	0.41-1.37
Multiple HPV Types	0.82	0.52-1.30	0.77	0.44-1.37
Age <35	0.92	0.56-1.52	0.92	0.54-1.56

*Adjusted for HIV status, prevalent (versus incident) HPV-16 infection at baseline, infection with multiple HPV types, age, and HPV-16 viral load at baseline.

Table 9. Factors associated with development of high-grade squamous intraepithelial lesions or worse.

Variable	Univariable HR	95% CI	Multivariable HR*	95% CI
HPV-16 VL (per log_{10} VL)	1.21	0.95-1.54	1.18	0.90-1.54
HIV-positive	1.75	0.88-3.50	1.30	0.58-2.93
Prevalent HPV-16	0.63	0.30-1.33	0.70	0.25-1.95
Multiple HPV Types	0.80	0.38-1.68	0.95	0.39-2.30
Age <35	0.97	0.46-2.07	0.97	0.44-2.17

*Adjusted for HIV status, prevalent (versus incident) HPV-16 infection at baseline, infection with multiple HPV types, age, and HPV-16 viral load at baseline.

Table 10. Association of clearance of HPV-16 infection and development of HSIL+ with 1-unit log_{10} HPV-16 viral load among women with normal cytology at baseline, by prevalent and incident baseline HPV-16 infection.

	Participants with normal cytology n=84		Among Prevalent Infections n=56		Among Incident Infections n=28	
	Multivariable HR*	95% CI	Multivariable HR	95% CI	Multivariable HR	95% CI
Clearance of HPV-16 Infection	0.77	0.65-0.92	0.90	0.71-1.14	0.62	0.44-0.88
Development of HSIL+	0.93	0.66-1.32	1.12	0.67-1.89	0.82	0.44-1.53

*Adjusted for HIV status, prevalent (versus incident) HPV-16 infection at baseline, infection with multiple HPV types, and age.

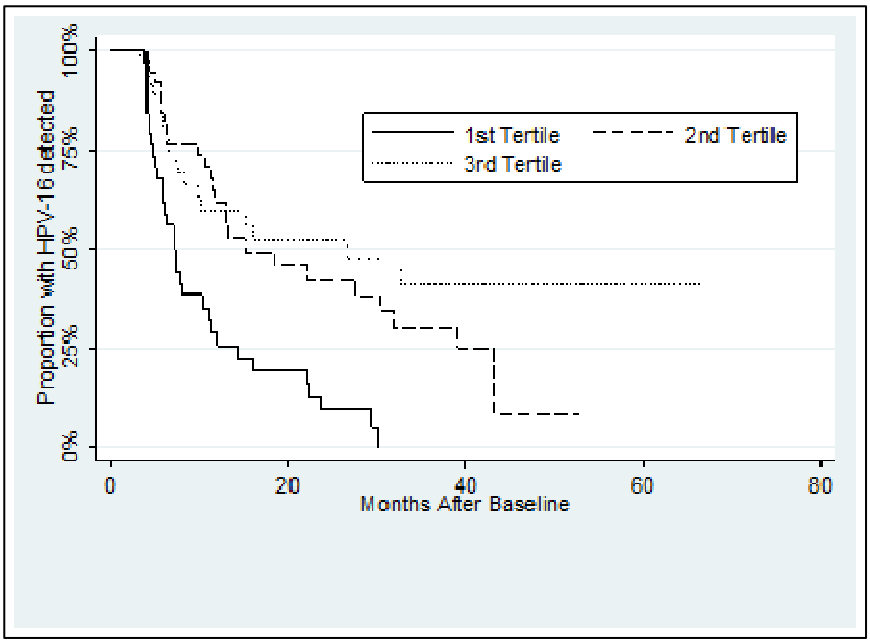


Figure 3. Cumulative incidence of clearance of HPV-16 Infection according to tertiles of HPV-16 viral load

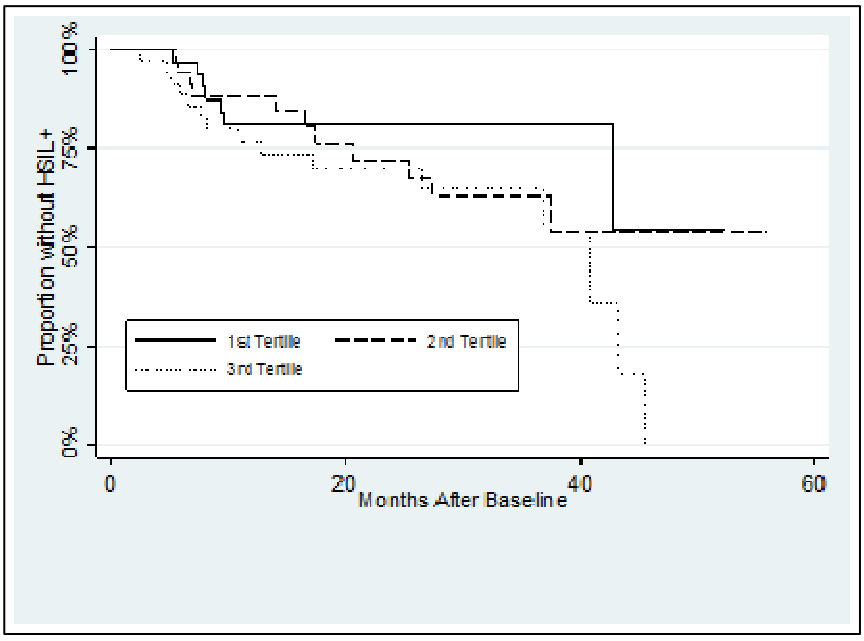
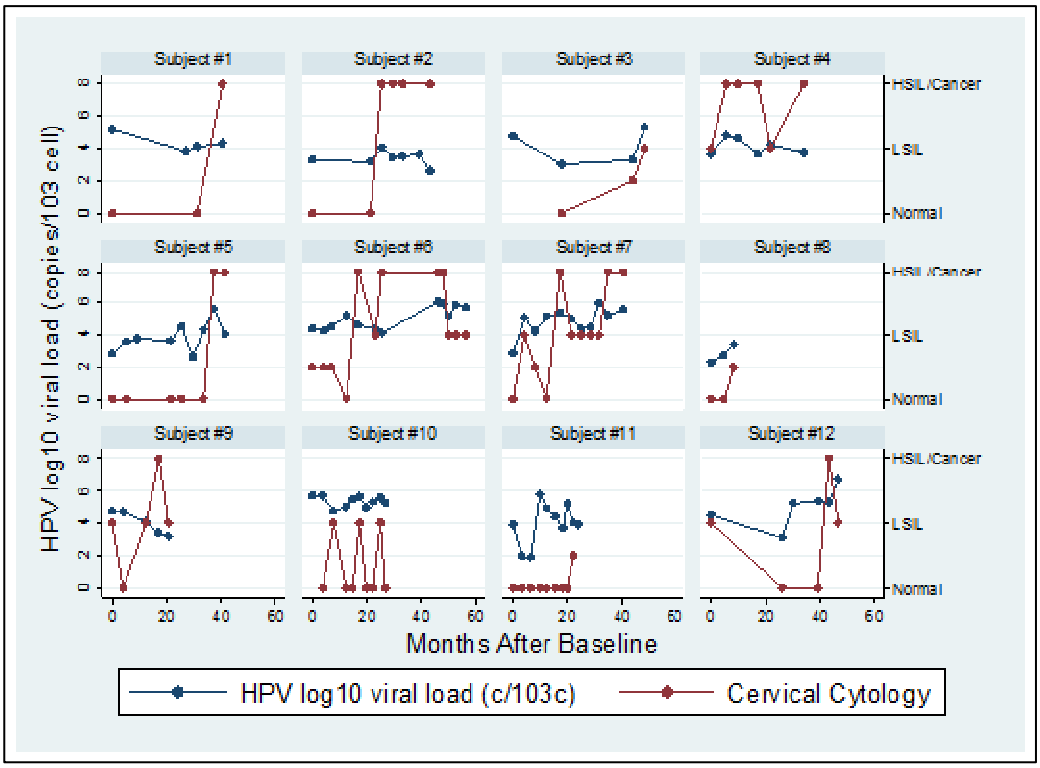


Figure 4. Cumulative incidence of development of high-grade intraepithelial lesion or worse (HSIL+), according to tertiles of HPV-16 viral load

Figure 5. HPV-16 viral load longitudinal kinetics, by worst achieved cervical disease outcome



Chapter 3

HPV detection and HIV infection

Introduction

Infection with human papillomavirus (HPV) is universally recognized as a major risk factor for cervical cancer and for cervical intraepithelial neoplasia, its precursor lesions [6,102,103]. Detection of HPV is considerably more common among women infected with the human immunodeficiency virus (HIV) compared to uninfected women [37,38,104-109]. Furthermore, women infected with HIV are known to be at increased risk of HPV-associated disease, including cervical cancer [110,111]. A likely explanation for this association is that HIV-induced immunosuppression may limit the immune system's ability to effectively eliminate HPV infection, leaving an individual at greater risk of developing cervical neoplasia or cancer [105]. However, the exact etiologic pathway between HIV-induced immunosuppression, HPV infection, and its clinical sequelae has yet to be clearly established.

CD4 lymphocyte count is used to characterize risk of advanced immunosuppression of HIV-infected individuals, generally defined as a CD4 cell count of less than 200 cells per mm^3. It is hypothesized that immunosuppression may increase the risk of HPV detection and potential later development of cervical neoplasia among HIV-infected individuals through the lowering of CD4 T-cells. Limited data exist to confirm the relationship between measurement of CD4 count and HPV detection, however, and the information that does exist has not been entirely corroborative. In addition, little is known concerning the effect of CD4 count on detection of HPV type-specific detection, including the most commonly-detected oncogenic types, HPV-16 and HPV-18.

In order to investigate the relationship between these factors, we conducted a cross-sectional historical analysis of women attending clinics in Senegal. The primary goals of this study were to determine the associations between overall and genotype-specific HPV detection among HIV-positive and HIV-negative women and to explore the trend of increased risk of HPV detection with CD4 count. A secondary objective was to analyze these trends by HIV subtype (HIV-1, HIV-2, and dual HIV-1,-2).

Materials and Methods

Data collection

This study consisted of historical data from women who participated in research studies in Senegal between 2000 and 2010. The aims of these studies were to investigate the epidemiology of HPV and its association with HIV-associated immune responses to human papillomavirus (HPV) and cervical cancer, DNA methylation, and to investigate new approaches to cancer control, as described previously [112,113]. All participants in these studies provided written informed consent upon enrollment, as per the procedures of the Human Subjects Committee of the University of Washington and the University of Dakar. Subjects were all older than 15 years of age, and were excluded from participation if they were pregnant or if they did not have an intact cervix. Upon study enrollment, a structured interview soliciting demographic information and detailed medical history (including reproductive and sexual history information) was given. General medical and gynecologic exams were also carried out at this time, and blood samples were collected and analyzed in order to determine patients' HIV-1 and HIV-2 status using RNA and DNA assays, and for lymphocyte subset analysis. Cervical swab samples were also obtained for HPV detection.

HIV Serology and Lymphocyte Subset Analysis

Serologic assays for HIV-1 and HIV-2 were performed on patients' baseline blood samples, collected in ethylenediamine tetraacetic acide (EDTA) tubes. A two-test sequence, as previously described [114], was used to determine HIV status. First, serum samples were tested for the presence of either HIV-1 or HIV-2 antibodies (HIV ½ EIA: Sanofi Diagnostics Pasteur). If samples were positive by this test, a second immunoassay was then applied to distinguish HIV-1 and HIV-2 antibodies (Multispot: Genetic Systems). Blood samples were also used to calculate lymphocyte data for HIV-positive participants, including CD4, CD8, and CD3 cell counts per microliter of blood (cells/µl). Cell counts were performed using the fluorescence activated cell sorter (FACS) Count analyser (Becton-Dickinson Biosciences, San Jose, CA, USA).

HPV DNA Detection

Specimens were tested for HPV DNA with a polymerase-chain-reaction (PCR) assay using L1 consensus primers, and with amplification of the cellular β-globin gene as a control. Of the samples included, DNA isolation was performed on 43 (1.6%) without use of the Qiagen column due to protocol differences between studies contributing historical data. In the case of these samples, digestion was performed with 20 µg/ml proteinase K at 37°C for 1 h, and genomic DNA was ethanol precipitated from 200 µl of the digested samples. For those samples using the Qiagen column, genomic DNA was isolated from 200 µl of the digested samples using QIAamp DNA blood mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA). The presence of HPV DNA was determined by PCR amplification followed by dot blot hybridization for all samples, and positive samples were subsequently genotyped for type-specific HPV. Of the historical data available, 16 (0.6%) of the samples were originally genotyped using the Roche line blot [115] or the Roche Linear array assay [116] with probes for low-risk HPV types 6, 11, 40, 42, 53, 54, 57, 66, and 84 and high-risk HPV types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, 73, 82, and 83. For the remaining samples, positive samples were genotyped using a liquid bead microarray assay (LBMA) [117,118].

Statistical Analysis

The associations between HIV serostatus (positive versus negative) and HPV-related outcomes were evaluated in this analysis using log binomial regression (a generalized linear model with robust variance) The STATA command used for all analyses was "glm outcome predictor(s), family(poisson) robust eform" [119,120]. In addition, among HIV-positive women only, a log binomial regression was used to determine the association between CD4 count and HPV-related outcomes. Prevalence ratios were chosen for all study outcomes since detection of HPV was common among women in this study population. Cervical HPV DNA types were classified according to their oncogenic potential, with high-risk types including HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, and 56 [14]. For some analyses, HPV types were further divided into groups based on their genotypical association with high-risk species types HPV-16 and HPV-18, which are likely to have similar biological and medical properties [121]. Alpha-9 type species related to HPV type 16 included HPV-31, 33, 35, 52, 58, and 67. Alpha-7 type species related to HPV type 18 included HPV-39, 45, 59, 68, and 70.

Potential confounding factors that were deemed to have an association with the exposure and outcomes of interest were chosen *a priori* for these analyses, and included age, age at first sexual intercourse, lifetime number of sexual partners, smoking, and use of Qiagen column for cervical specimen purification[114,122]. Women with values of age at first sexual intercourse of less than 10 years were assigned as missing for this variable. Age, age at first sexual intercourse, and lifetime number of sexual partners were all evaluated as continuous variables. Smoking and use of Qiagen column were added to the log binomial regression model as dichotomous (yes/no) variables. Women who reported undertaking commercial sex work were excluded from this analysis. Both univariate and multivariate models were used to assess the potential relationship between HIV status, CD4 count, and HPV detection. Among women with HIV-infection, the relationship between ARV treatment, when available, and HPV-related outcomes was also evaluated.

Results

The median age of women included in the present analysis was 42.7 years (range 15 to 84). Socio-demographic and health characteristics for women with and without HIV-1 and/or HIV-2 infection are listed in Table 11. Compared to HIV-uninfected women, women with HIV-infection were more likely to be younger, be widowed or separated, initiate sex at a younger age, and have a greater number of lifetime sexual partners.

HIV-infected women were more likely to have any HPV detected (78.2% vs. 27.1%), to have high-risk HPV detected (65.7% vs. 17.6%), and to have multiple types of HPV detected (62.3% vs. 11.6%) than HIV-uninfected women. The most common types of high-risk HPV detected among HIV-positive individuals included types 58 (18.8%), 52 (17.3%), 16 (13.1%), and 18 (10.9%) (Figure 6). Among HIV-negative women, the mostly commonly detected high-risk HPV types also included types 58 (3.0%), 83 (2.7%), 52 (2.3%), and 16 (2.2%). Table 12 describes the relationship between HPV risk factors and their association with HIV-positivity. Compared to HIV-negative women, HIV-positive women were more likely to have any HPV (PR_a: 2.28, 95% CI 2.01-2.58), high-risk HPV (PR_a: 3.02, 95% CI 2.57-3.57), and multiple types of HPV (PR_a: 4.51, 95% CI 3.71-5.49) detected. With respect to the two types most commonly detected in cervical cancer, HIV-positive women had a higher likelihood of having HPV type 16 and HPV type 18 detected than HIV-negative women (HPV-16 PR_a: 4.76, 95% CI 2.62-8.63; HPV-18

PR_a: 5.77, 95% CI 3.47-9.60). In order to more clearly evaluate the relationship between HIV infection and phylogenetically similar HPV types, HIV infection was compared in relation to HPV16 -like types (including HPV-31, 33, 35, 52, 58, and 67) and HPV 18-like types (including HPV-39, 45, 59, 68, and 70). The association between HIV infection and detection of HPV-16 and -18 held equally for phylogenetically-similar groupings. Compared to HIV-negative women and adjusted for confounding factors, HIV-positive women were 3.64 times more likely to have HPV 16 –like DNA detected (95% CI 2.92-4.54) and 3.85 times more likely to have HPV 18-like DNA detected (95% CI 2.87-5.16).

Of the 467 HIV-positive subjects included in the study, 373 (79.9%) were infected with HIV-1, 78 (16.7%) were infected with HIV-2, and 16 (3.4%) were dually infected with HIV types -1 and -2. Of HIV-positive women infected with HIV types -1, -2, and dual infection, the average (SD) CD4 count was 357.6 (291.0), 535.7 (337.9), and 268.4 (267.2) cells/µl, respectively. HIV-1, -2 and dually-infected women were more likely than HIV-negative women to have any HPV, high-risk HPV, and multiple types of HPV detected (Table 3). In addition, HIV-1 infected women (PR_a: 4.60, 95%CI 2.52-8.37), HIV-2 infected women (PR_a: 4.72, 95% CI 2.04-10.92), and HIV dually-infected women (PR_a: 7.41, 95% CI 2.24-24.51) were all at higher risk of having HPV type 16 detected. A similar association was seen for HPV type 18 and infection with HIV-1 (PR_a: 4.16, 95% CI 3.08-5.62) and HIV-dual infection (PR_a: 6.51, 95% CI 4.09-10.38), although this increased risk was more modest for HIV-2 infection (PR_a: 1.33, 95% CI 0.67-2.65).

To further elucidate the relationship between immune factors related to HIV-infection and HPV detection, HIV-positive study participants were divided into 3 subgroups by level of immune function, as measured by CD4 count. A total of 63.2% of all HIV-infected women included in this study had CD4 counts measuring greater than or equal to 500 cells/µl, 19.7% between 200 and 499 cells/µl, and 17.1% less than 200 cells/µl. Compared to HIV-infected individuals having CD4 counts measuring 500 cells/µl or above, severely immunosuppressed individuals with CD4 counts below 200 cells/µl were more likely to have any HPV (PR_a: 1.30, 95% 1.07-1.59), multiple HPV types (>1 HPV types versus ≤1 HPV types) (PR_a: 1.52, 95% CI 1.14-2.01), and high-risk HPV detected (PR_a: 1.67, 95% CI 1.25-2.25). HPV types classified as being related to HPV type 16 as well as those related to HPV type 18 were also more likely to be detected among women with severe immune depression (HPV 16 –like PR_a: 1.79, 95% CI 1.21-2.64; HPV 18 –like PR_a: 1.91, 95% CI 1.09-3.34). Compared to women with CD4 counts ≥500 cells/µl, women who

were mildly immunosuppressed (CD4 counts 200-499 cells/µl) were not at appreciably greater risk of HPV detection, detection of multiple HPV types, or detection of any high-risk HPV. Among the HIV-infected women in this study, 185 (39.6%) had ARV information available. Compared to those who were not taking ARV at the time of study, and controlling for CD4 count and other potential confounders, those taking ARV showed no significant difference in HPV overall, high-risk, multiple, or HPV16/18 type-specific detection (Table 13).

Discussion

HPV infection in HIV-infected versus HIV-uninfected women

We observed associations between infection with HIV and detection of HPV, including HPV high-risk and multiple types. Our estimate of the association with any HPV (PR_a: 2.28) complements prevalence ratio estimates from other large studies comparing HIV-infected and HIV-uninfected individuals, which range from 1.0 to 3.6 in other African settings [123]. Our finding that HIV-positive individuals are more likely to have multiple-type infections is also supported by several recent studies, and may indicate both an increased exposure to inoculations due to high-risk sexual activity among this group, and a greater overall susceptibility to HPV infection [108,116,124].

Among HPV types that have been identified as having the highest oncogenic potential, HPV-16 and 18 are estimated to account for 70% of all cancers [14]. In the present study, HIV-positive women were more likely than HIV-negative women to have HPV-16 detected (PR_a: 4.76, 95%CI 2.62-8.63) as well as HPV-18 (PR_a: 5.77, 95%CI 3.47-9.60). The finding of increased likelihood of HPV-16 infection with HIV infection is consistent with results from two other studies, where a prevalence ratio of 1.87 and an odds ratio of 1.52 were reported, respectively [106,125]. In a study conducted by Ahdieh et al, HIV-infected women were also shown to have a lower likelihood of clearing HPV type 16 infection, especially among those with CD4 counts below 200 cells/µl [105]. An increased risk of the two HPV types most commonly linked to cervical cancer among HIV-positive women could suggest a predisposition to cervical neoplasia or cancer development as well.

Prevalence ratios for any HPV, multiple types, and any high risk type appeared to be similar among women with HIV-1 or HIV-2 compared to HIV-uninfected women. Interestingly, while prevalence ratios for HPV-16 and HPV-16 like types were elevated regardless of HIV type, estimates for HPV-18 and HPV-18 like types among women with HIV-2 infection were somewhat attenuated. These findings are especially noteworthy as HIV-2 infection is known to confer a more mild form of immunosuppression than HIV-1 infection, with comparatively longer duration of disease, suggesting a different mechanism of immune control than HIV-1 [126,127]. If verifiable, a differential risk of HPV infection according to HIV type could allude to a complex interplay between HIV-related immune factors such as clinical latency, immune control, and type-specific high-risk HPV infection [37].

HPV infection among HIV-positive women

Detection of HPV and of high-risk HPV was increased among HIV-positive women with lower CD4 counts in our study, especially among those with CD4 counts less than 200 cells/µl. Among HIV-positive women with the lowest CD4 counts, there was a noted increased likelihood of any HPV detection (PR 1.30), HPV multiple-type infection (PR 1.52), and high-risk HPV infection (PR 1.67). Other studies with CD4 count information have produced similar estimates, supporting an association between markers of severe immune depression and HPV detection [90,106]. In addition, a consistent dose-response relationship was observed with decreasing levels of CD4 count in our study in relation to any, high-risk, and multiple-type HPV detection. A study by Singh et al also found consistent dose-response trends for any, high-risk and multiple-type infection [128]. If supported by further studies, such dose-response relationships with CD4 count could support the hypothesis that immune status influences HPV detection.

An increase in HPV types 16, 18, and 16/18-like detection was noted among women with decreasing CD4 counts in this study, a finding which is supported by findings from other studies [67,90,128]. A study by Firnhaber et al conducted in South Africa found consistent evidence that, among women with negative cytology, there existed a significant association between CD4 count of less than 200 cells/µl and HPV-16 detection [129]. However, a more detailed evaluation of this relationship will need to rely on studies with larger sample sizes to make any definitive conclusions of risk.

The most common high-risk genotypes among HIV-infected women in the present study were 58 (19%), 52 (17%), 16 (13%) and 18 (11%) (Figure 1). This finding is not unlike other similar studies conducted in the African region, where HPV types other than types 16 and 18 were most common among HIV-infected women [106,109,130,131]. Similarly, a meta-analysis by Clifford et al showed that HPV types 52 and 58 accounted for a high proportion of all HR-HPV infections among HIV-infected women from Africa [108]. Among HIV-negative women from our study, HPV types 58 (3.0%), 83 (2.7%), and 52 (2.3%) were found more often than HPV type 16 (2.2%). These finding support a similar study conducted in Senegal among women >35 years of age, where HPV types 52 and 58 were commonly detected among HIV-negative women, regardless of cervical cytology diagnosis [132]. Importantly, Xi found that HPV types 58, 52, and 33 were most commonly detected among women with HSIL or cancer, after HPV type 16. The findng that high-risk types other than HPV types 16 and 18 are commonly detected in this region among HIV-positive and HIV-negative women carries great implications concerning the effectiveness of the currently available HPV vaccine among women in Sub-Saharan Africa. Although some evidence of cross-protection has been noted, the currently available vaccines only cover oncogenic HPV types 16 and 18 [133-135]. Infection with other high-risk HPV types not currently covered by the available vaccines may infer an added risk of cancer development in African settings. However, a comprehensive comparison of HPV type-specific prevalence between studies should account for differences in laboratory measurement of HPV types.

This study has several strengths. First, this is one of the largest known reports of HIV-positive and HIV-negative women in Africa with HPV detection and typing data available. This study also contributes CD4 count measurement information among HIV-infected women, and in this way supplements existing data regarding the possible link between CD4 count measurement and HPV detection. In addition, the data from our study is uniquely important because little is currently known concerning the effect of HIV serotypes on the risk of HPV infection, including HIV-2 and HIV type -1 and -2 dual infection [37,136]. Our study suggests that, despite the known comparatively mild effects of immune suppression among HIV-2 infected individuals, this group is still at increased risk of HPV infection. This potential increased susceptibility but overall lack of data concerning risk of HPV-related neoplasia among HIV-2 infected individuals underlines the need to conduct future longitudinal studies among this group.

A number of limitations also exist in this study. First, information on antiretroviral treatment (ARV) was only available for a portion (39.6%) of the study subjects, and the possible associations between the effect of antiretroviral therapy and detection of HPV could therefore not be fully evaluated in this population. This missingness was primarily due to protocol differences between studies contributing data to this historical analysis, with ARV treatment not having been collected for two of the four studies contributing data. However, similar analyses between HPV detection and risk factors for HIV infection were carried out among the subset of the population that did have antiretroviral information available. Among women with known ARV status, and accounting for CD4 count, estimates of HPV detection were not different among those with and without ARV use. Furthermore, several recent studies have suggested that there is limited effect of ARV on HPV viral persistence [123,129,137,138], indicating that it may be possible to interpret associations between HPV and HIV infection outside of the context of ARV. Finally, it was beyond scope of our aims to evaluate the association between cervical cytology and factors related to HIV infection in this study. However, as women with high grade intraepithelial neoplasia are observed to have higher oncogenic HPV prevalence than women with low grade lesions or normal cytology, it would be beneficial to supplement our findings among HIV-positive women with information from other studies regarding the relationship between existing severity of cervical dysplasia, HIV positivity, and markers of immune status [108].

Our most noteworthy findings are that HIV-positive women have a higher likelihood than HIV-negative women of having cervical HPV DNA detected, including the most high-risk types. In addition, among HIV-infected women, there is evidence that increased immunosuppression as measured by CD4 cell count is associated with a higher likelihood of HPV detection. One possible explanation for these findings is that reactivation of latent HPV viral infections is responsible for the greater susceptibility to HPV infection among HIV-positive individuals [129,139]. Future longitudinal studies involving the type-specific associations among HIV-positive individuals and by CD4 count could help to further clarify the complex immunological relationship between HIV and HPV infection in women.

Table 11. Socio-demographic, sexual behavior and disease characteristics of the study population, by Human Immunodeficiency Virus (HIV) status

	HIV-positive N (%)	HIV-negative N (%)
Total	467	2139
Age		
<35	179 (38.3)	411 (19.2)
35-39	82 (17.6)	297 (13.9)
40-44	85 (18.2)	370 (17.3)
≥45	121 (25.9)	1061 (49.6)
Marital status		
Monogamous	139 (30.1)	850 (39.9)
Polygamous	98 (21.2)	925 (43.4)
Single	27 (5.8)	74 (3.5)
Separated/Divorced	74 (16.0)	145 (6.8)
Widow	124 (26.8)	136 (6.4)
Alcohol use	10 (3.3)	21 (1.1)
Smoker	6 (1.9)	14 (0.8)
No contraceptive Use	408 (88.7)	1752 (82.3)
Education		
None	261 (56.4)	1121 (52.8)
Primary	136 (29.4)	643 (30.3)
Secondary or above	66 (14.3)	361 (17.0)
Age at first sex		
<15	157 (33.6)	579 (27.1)
16-20	215 (46.0)	1042 (48.7)
≥21	95 (20.3)	518 (24.2)
Lifetime partners		
1	205 (46.3)	1372 (65.3)
2-5	228 (51.5)	702 (33.4)
>5	10 (2.3)	26 (1.2)
HIV type*		
HIV-1	373 (79.9)	---
HIV-2	78 (16.7)	---
HIV-1/2	16 (3.4)	---
CD4 count		
≥500	79 (29.9)	---
200-499	99 (37.5)	---
<200	86 (32.9)	---

Table 12. Prevalence ratio (PR) estimates for the association of HIV-infection (HIV-positive versus HIV-negative) with the detection of overall and type-specific human papillomavirus (HPV) infection.

HPV risk factor	HIV-positive	HIV-negative	Univariable PR	95% CI	Multivariable PR*	95% CI
HPV presence	365	579	2.89	2.65-3.14	2.28	2.01-2.58
High-risk HPV	307	377	3.73	3.33-4.17	3.02	2.57-3.57
Multiple type HPV	291	248	5.37	4.69-6.16	4.51	3.71-5.49
HPV 16 presence	61	46	6.07	4.20-8.79	4.76	2.62-8.63
HPV16-like(alpha9)†	230	219	4.81	4.12-5.62	3.64	2.92-4.54
HPV 18 presence	51	36	6.49	4.29-9.82	5.77	3.47-9.60
HPV18-like(alpha7) §	137	134	4.68	3.77-5.81	3.85	2.87-5.16

*Adjusted for age (continuous), age at first sex (continuous), lifetime sexual partners (continuous), use of qiagen column for DNA processing, and project.
† Includes HPV types 16, 31, 33, 35, 52, 58, and 67.
§ Includes HPV types 18, 39, 45, 59, 68, and 70.

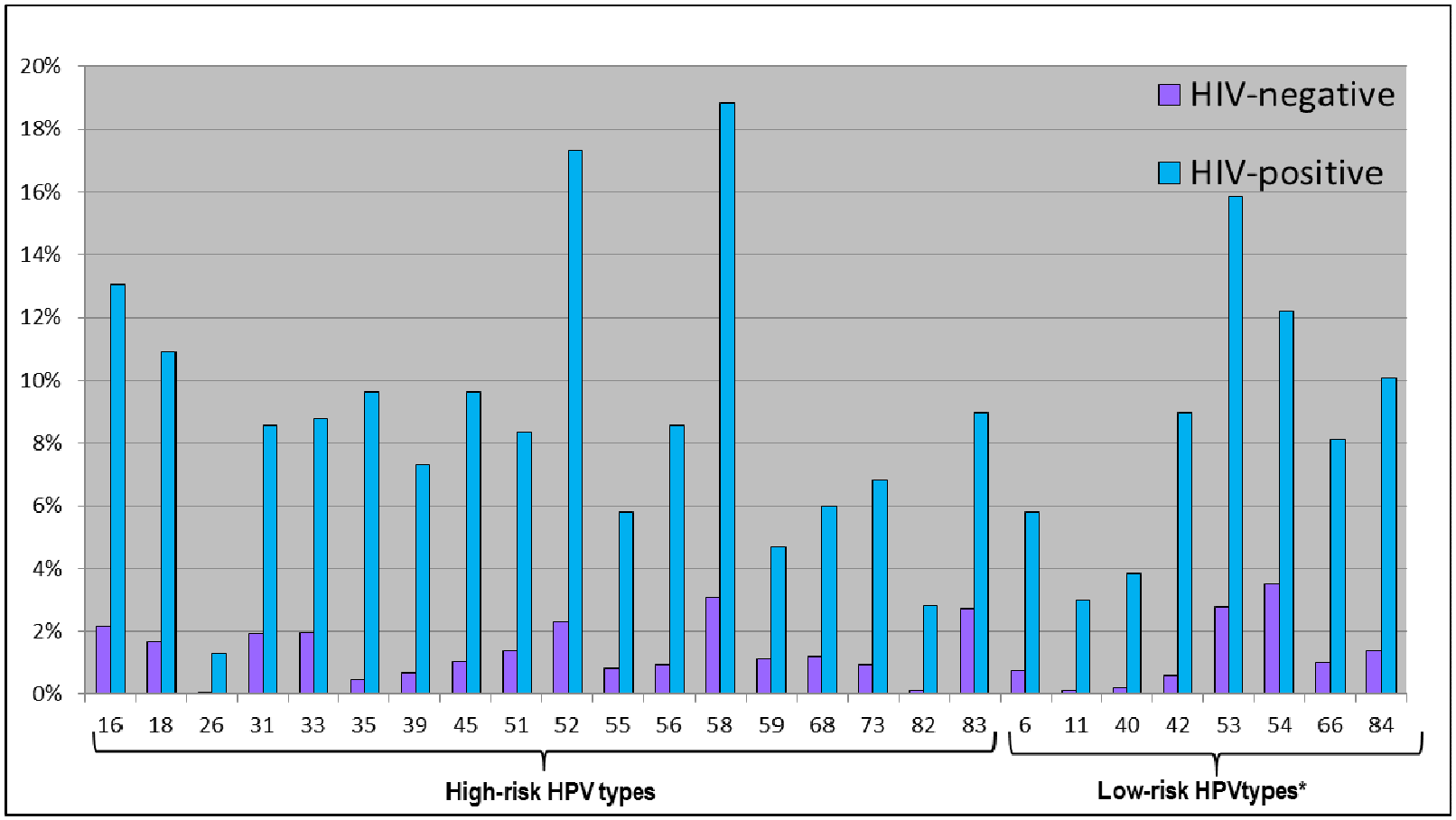


Figure 6. Type-specific prevalence of HPV among HIV-positive and HIV-negative women.

HPV type	16	18	26	31	33	35	39	45	51	52	55	56	58	59	68	73	82	83	6	11	40	42	53	54	66	84
HIV-, N	46	36	1	41	42	10	14	22	29	49	17	20	65	24	25	20	3	58	16	3	4	12	59	75	21	29
HIV+, N	61	51	6	40	41	45	34	45	39	81	27	40	87	22	28	32	13	42	27	14	18	42	73	57	38	47

***No observations for HPV type 57 in the study population**

Table 13. Prevalence ratio estimates for the association of HIV subtype and CD4 count among HIV-positive women with detection of overall and type-specific HPV infection (n=467).

	HPV-positive		HPV multiple infection		High risk HPV		HPV16		HPV16-like†		HPV18		HPV18-like§	
	aPR*	95% CI	aPR	95% CI	aPR	95% CI	aPR	95% CI	aPR	95% CI	aPR	95% CI	aPR	95% CI
HIV type*														
HIV-negative	ref		ref		ref		ref		ref		ref		ref	
HIV-1	2.33	2.05-2.64	4.66	3.81-5.69	3.14	2.66-3.70	4.60	2.52-8.37	3.74	2.99-4.69	6.21	3.70-10.42	4.16	3.08-5.62
HIV-2	1.97	1.62-2.39	3.69	2.76-4.94	2.35	1.78-3.12	4.72	2.04-10.92	2.87	1.97-4.16	2.29	0.70-7.54	1.33	0.67-2.65
Dual HIV-1/2	2.37	1.83-3.06	4.71	3.29-6.75	3.18	2.26-4.46	7.41	2.24-24.51	4.27	2.69-6.80	8.51	2.92-24.83	6.51	4.09-10.38
CD4 count¶**														
≥500	ref		ref		ref		ref		ref		ref		ref	
200-499	1.00	0.80-1.25	1.06	0.78-1.46	1.27	0.93-1.74	0.82	0.11-6.27	1.21	0.80-1.84	0.77	0.27-2.23	1.28	0.71-2.30
<200	1.30	1.07-1.59	1.52	1.14-2.01	1.67	1.25-2.25	9.00	1.66-48.67	1.79	1.21-2.64	1.20	0.45-3.24	1.91	1.09-3.34
ARV treatment€														
No	ref		ref		ref		ref		ref		ref		ref	
Yes	0.97	0.76-1.23	1.09	0.79-1.51	1.10	0.78-1.54	1.10	0.43-2.86	1.07	0.69-1.65	1.26	0.42-3.79	1.51	0.78-2.92

*Adjusted for age (continuous), age at first sex (continuous), lifetime sexual partners (continuous), use of qiagen column for DNA processing, and project.

† Includes HPV types 16, 31, 33, 35, 52, 58, and 67.

§ Includes HPV types 18, 39, 45, 59, 68, and 70.

¶ Adjusted for age (continuous), age at first sex (continuous), lifetime sexual partners (continuous), use of qiagen column for DNA processing, and project.

** Wald test values for CD4 count trend: HPV-positive (p=0.005), HPV multiple infection (p=0.002), High risk HPV (p<0.001), HPV16 (p=0.005), HPV 16-like (p=0.002), HPV18 (p=0.599), HPV18-like (p=0.019)

€ Adjusted for age (continuous), age at first sex (continuous), lifetime sexual partners (continuous), use of qiagen column for DNA processing, project, and CD4 count (continuous). Excludes 282 missing values for ARV treatment.

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