THE ROLE OF HUMAN PAPILLOMAVIRUS (HPV) VIRAL LOAD IN PENILE HPV INFECTION AND CLEARANCE AMONG YOUNG KENYAN MEN

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Epidemiology.

Chapel Hill
2013

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ABSTRACT
VIRGINIA SENKOMAGO: The role of human papillomavirus (HPV) viral load in penile HPV infection and clearance among young Kenyan men
(Under the direction of Jennifer S. Smith)

Persistent infections with human papillomavirus (HPV) types 16 and 18 are causes of cervical cancer in women and various penile squamous cell carcinomas (SCC) in men. Male circumcision has been found to be protective against penile HPV, but the association between circumcision and HPV viral load remains unclear. Additionally, the role of HPV viral load in HPV persistence and subsequent development of penile SCC is unknown.

An HPV-ancillary study, nested within a randomized controlled trial (RCT) of male circumcision, was conducted in Kisumu, Kenya. Eligible participants were HIV seronegative, uncircumcised and aged 18-24. Penile swabs were collected from glans and shaft sites every 6 months for 24 months. GP5+/6+ PCR was used to identify HPV DNA types. HPV viral load was measured with LightCyler real-time PCR and classified as high (>250copies/scrape) or low (≤250copies/scrape).

Of 2,299 men with HPV baseline results, 1,159 were randomized to immediate circumcision and 1,140 to the control arm and asked to remain uncircumcised until study end. The acquisition of high viral load infections in the glans was lower in the circumcision than control arm for HPV16 [Hazard Ratio(HR)=0.32(0.20-0.49)] and HPV18 [HR=0.34(0.21-0.54)]. For prevalent high viral load infections in the glans at

baseline, risk of persistence to 6 months was lower in the circumcision arm [0.20(0.09-0.34)] than control arm [0.55(0.39-0.68)] for HPV16 and HPV18 [0.17(0.05-0.34) and 0.50(0.25-0.71), respectively].

In uncircumcised men, the hazard of HPV16 clearance at 6 months after first HPV16 detection was found to be lower for high versus low viral load incident infections in the glans [adjusted hazard ratio (aHR) =0.58 (95%confidence interval, 0.36-0.93)]. HPV16 and HPV18 clearance in the shaft was comparable for high and low viral load infections.

Male circumcision reduces the acquisition and possibly enhances the clearance of high viral load HPV16 or HPV18 infections in the glans, and thus could potentially reduce HPV transmission to women. The reduced rate of high versus low viral load HPV16 clearance in uncircumcised men could be associated with increased development of penile SCC, and may also explain the increase in HPV16 transmission in men with high viral load to their female partners.

ACKNOWLEDGEMENTS

I would first like to thank my advisor and dissertation committee chair, Jennifer Smith, for introducing me to HPV research and for her guidance, patience, encouragement and commitment through every step of this project. Her hard work and dedication to HPV research and cervical cancer prevention is truly inspiring. I would also like to sincerely thank Michael Hudgens for his statistical expertise, dedication to this project and frequent accessibility when I needed advice. I am very thankful to Charlie Poole for his guidance in epidemiology methods and for his advice throughout this project. I am also very grateful to Freida Behets and Steve Meshnick for their guidance and helpful comments during my entire dissertation process.

I am thankful to all of my study collaborators including Nicolas Schlecht, Albert Hesselink, Bob Bailey, Stephen Moses, Peter Snijders and Chris Meijer for all of their work on this project. I am very grateful for the guidance and commitment of Danielle Backes who aided me in cleaning the HPV viral load data and provided valuable feedback on my manuscripts. Additionally, I would like to acknowledge the young men who participated in this study and the clinic staff in Kisumu, without whom this research would have been impossible. The main randomized controlled trial was funded by the National Institutes of Health (NIH) AI50440 and the Canadian Institutes of Health Research HCT44180. The human papillomavirus study was funded by NIH R01 CA114773-04.

I am immensely thankful to Michael Schwarz, Andrew Waters and the Tellus Educational Foundation for their encouragement and financial support with the Tellus Foundation Scholarship throughout my doctoral education. I am also very thankful to Louise and Derek Winstanly for the Winstanly scholarship awarded to me in my first year in the doctoral program. I also want to acknowledge the Armand Hammer United World College and Berea College scholarships that supported my previous education and inspired me to pursue a career in global health improvement.

I also want to thank Vangie Foshee, Wendee Wechsberg and Prema Menezes who provided me with research opportunities during my doctoral education and enabled me to further build my research and management skills. I also want to thank Nancy Colvin, Carmen Woody and Bobbi Wallace for their guidance.

Lastly, I want to thank my family, who even from so far away have been my biggest cheerleaders and provided unwavering love and encouragement throughout my education. I am also truly grateful to all my friends at UNC and beyond for the continuous inspiration and support.

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LIST OF ABBREVIATIONS

CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
ELISA	Enzyme-linked immunosorbent assay
DAG	Directed Acyclic Graph
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV-2	Herpes simplex virus type 2
HR	Hazard Ratio
ICC	Invasive cervical cancer
PCR	Polymerase chain reaction
PR	Prevalence Ratio
RCT	Randomized controlled trial
RLB	Reverse line blot
SCC	Squamous Cell Carcinoma
STI	Sexually transmitted infection
UNIM	Universities of Nairobi, Illinois and Manitoba

CHAPTER 1
STATEMENT OF SPECIFIC AIMS

Persistent infections with human papillomavirus (HPV) high-risk types 16 and 18 are causes of approximately 70% of cervical cancer cases (1). Higher HPV16 or HPV18 viral loads in men are believed to be associated with increased HPV transmission female partners compared to lower viral loads (2). Male circumcision has been found to be protective against HIV and penile HPV incidence, however, the effect of circumcision on HPV viral load of incident and prevalent HPV infections remains unclear (3-8).

In men, persistence of HPV16 and HPV18 infections is believed to cause penile squamous cell carcinomas (SCC), particularly basaloid and warty SCC types (9, 10). Higher HPV16 or HPV18 viral loads have been associated with decreased HPV clearance and increased progress to high grade cervical intraepithelial neoplasia (CIN) in women (11-17). However, the association between HPV viral load and HPV clearance has not yet been explored in penile HPV infections in men.

An HPV-ancillary study was nested within a randomized controlled trial (RCT) that began in Kisumu, Kenya in February 2002 with the main aim of determining the effect of male circumcision on human immunodeficiency virus (HIV) incidence (5, 18). Men from the RCT who consented to the collection of penile exfoliated cells every 6 months and their shipment overseas for HPV DNA and viral load testing were enrolled in the ancillary HPV study. The GP5+/6+ PCR assay was used to identify a wide array of HPV DNA types (19, 20). HPV viral load testing was conducted using a LightCyler

real-time PCR assay; viral load was classified as high or low (>250 versus ≤250 copies/scrape) (21). HPV Incidence was defined as the first HPV16 or HPV18-positive result in the glans or shaft. HPV clearance was defined as an HPV-negative result in participants who were positive for that given HPV type in the same anatomical site (glans or shaft) at their last visit.

With the data collected from this study, the following research aims were addressed:

Aim 1: To examine the association between male circumcision and HPV16 or HPV18 viral load in Kenyan men aged 18-24.

1.1. To examine the association between circumcision and the incidence of high viral load infections for HPV types 16 and 18 in the glans or shaft.

1.2. To examine the association between circumcision and the prevalence of high versus low HPV viral load infections among men with incident HPV 16 or HPV18 infections in the glans or shaft.

1.3. To examine the association between circumcision and clearance of prevalent high and low viral load infections for HPV types 16 and 18 in the glans or shaft

Hypotheses:

Circumcision reduces the incidence of high viral load HPV18 and HPV16 infections. Among HPV-positive men, circumcision is associated with a lower prevalence of high versus low viral load HPV16 and HPV18 infections. Circumcision increases the clearance of prevalent high viral load HPV16 and HPV18 infections.

Rationale: Circumcision and lower HPV viral load in men are believed to be associated with reduced HPV transmission to women (2). The effect of circumcision on HPV viral load, however, remains unclear. Only one study, an RCT of male circumcision in Uganda, has examined the association between male circumcision and penile HPV viral load (22).This study did not have longitudinal data on HPV viral load and was therefore unable to examine the association between circumcision and the incidence of high viral load HPV infections, or the association between circumcision and the clearance of high and low viral load prevalent HPV infections. Higher HPV viral loads in men are possibly associated with greater HPV transmission to their female partners (2); thus, circumcision may reduce the transmission of HPV infections to women by reducing incidence and enhancing clearance of high viral load HPV infections in their male partners.

Aim 2: **To examine the association between viral load at HPV detection and HPV clearance in uncircumcised Kenyan men aged 18-24.**

2.1. To examine the association between HPV viral load (high versus low) at detection of prevalent HPV 16 and 18 infections and HPV clearance in the glans or shaft

2.2. To examine the association between HPV viral load (high versus low) at detection of incident HPV 16 and 18 infections and HPV clearance in the glans or shaft

Hypothesis

High viral load at HPV detection is associated with a lower rate of HPV clearance for prevalent and incident HPV infections compared to low HPV viral load.

Rationale: High viral load of high-risk HPV types is suggested to be associated with increased HPV persistence in women; however the association between HPV viral load and HPV clearance has not been examined among penile HPV infections in men (11-17). Higher HPV16 or HPV18 viral loads could possibly be associated with lower HPV clearance and subsequently increased progression to penile SCC.

Additionally, higher HPV viral load in men is suggested to be associated with increased HPV transmission to their female partners (2). It is believed that lower HPV clearance of high versus low viral load HPV infections in men may enhance HPV transmission to their female partners; however the association between higher HPV viral load and lower HPV clearance in penile HPV infections is yet to be established.

CHAPTER 2
BACKGROUND AND SIGNIFICANCE

Significance of HPV infections in males

HPV is one of the most common sexually transmitted infections (STIs) worldwide, the overall global prevalence of HPV in men ranges from 1% to 84% among low-risk and from 2% to 93% among high-risk male populations (23). In Africa, HPV prevalence in males has been found to be as high as 58% (23). The overall HPV prevalence at baseline in the men who participated in this study was 51%; the type-specific prevalences for HPV 16 and HPV 18 were 9.8% and 4.3% respectively (24). HPV infection in men is associated with penile, oral and anal cancers as well as HPV transmission to females and cervical cancer incidence (25). HPV infection in men has also been shown to be associated with an increased risk of HIV acquisition; data from men in this study found the risk of HIV acquisition in HPV-positive men to be 1.8 times that of HPV-negative men (26)

Determinants of HPV infection in males

Cross-sectional studies examining factors associated with HPV penile cells in males have found prevalence to vary by anatomical site; a higher HPV prevalence has been detected in the glans/coronal sulcus than in the penile shaft (24). HPV prevalence has also been associated with male sexual characteristics such as a greater number of

lifetime sexual partners and no condom use. The presence of other sexually transmitted infections, particularly *N. gonorrhea*, *C. trachomatis* and herpes virus simplex virus type 2 (HSV-2) has also been associated with a higher prevalence of HPV in males (26). Personal characteristics of males including less than a secondary school education and less than daily bathing have also been associated with a greater HPV prevalence (24, 27, 28). Longitudinal studies examining factors associated with acquisition of HPV in males have found a greater number of lifetime sexual partners and infection with multiple HPV types to be associated with a greater risk of HPV acquisition (29, 30).

Determinants of HPV clearance in males

The average duration of clearance for overall HPV infections in males is reported to range from 5.9 - 7.52 months (31). Infection with HIV and multiple HPV types was found to be associated with decreased HPV clearance (29, 32). The results on the association of age with HPV clearance remain unclear; HPV clearance has to be found to increase in older men in one study (30), but was found to have no effect on clearance in another study (31).

HPV infection and Development of Penile Carcinomas

Persistence of HPV16 and HPV18 infections is a risk factor for the development of penile squamous cell carcinomas (SCC), particularly basaloid and warty SCC types (9, 10). Approximately half (45% - 47.9%) of penile carcinomas have detectable HPV DNA and the most common HPV types include HPV16 (30.8%) and HPV18 (6.6%) (33). Although invasive penile SCC is rare in comparison to other cancers, incidence in developing countries (4.4 and 4.2 per 100,000 in Uganda and Paraguay, respectively) is

over 5 times higher than in developed countries and is associated with high morbidity and mortality (33, 34).

The role of Male HPV infections in female HPV and cervical cancer

Even before the association between HPV and cervical cancer was understood, male sexual behavior was found to be associated to cervical cancer in female partners. Earlier studies found an association between a greater number of male sexual partners and cervical cancer even after adjustment for age of first intercourse and number of female sexual partners (35). This association generated the hypothesis of an infectious cause of cervical cancer.

Studies on concordance of HPV infections in heterosexual couples further highlight the role of men in transmitting HPV to their female partners. A recent meta-analysis of HPV concordance studies worldwide including 33 populations found that of HPV-positive couples, 63% were infected with one or more of the same HPV types (36). Concordance of HPV infection was found to be sex-dependent; 36% of male partners of HPV-positive females were found to have the same HPV type, whereas 55% of female partners of HPV-positive men were infected with the same HPV type. These findings further support higher rates of infection in women when their partners are HPV-positive. Concordance of HPV types was found to be significantly higher for types 16, 18 and 11 that are high-risk types for cervical cancer in women.

Male Circumcision and HPV prevalence, incidence, clearance and transmission

The effect of male circumcision on HPV infections has been investigated by several studies. Two global meta-analyses including results from 2 RCTs and several longitudinal studies have examined the association between male circumcision and HPV prevalence, incidence and clearance (37, 38). Circumcision was found to greatly reduce the prevalence of overall penile HPV in both meta-analyses [Odds Ratio (OR) = 0.57 (0.45 -0.71) and OR= 0.57(0.42-0.77) respectively], particularly in the glans/sulcus [OR =0.47 (0.37-0.60)].

The two meta-analyses found different results on the association between male circumcision and acquisition of HPV infections; one meta-analysis found that circumcision decreases HPV incidence [Rate Ratio (RR) = 0.75 (0.57-0.99)] and the other found no effect of circumcision on HPV incidence [OR = 1.01 (0.66-1.53)] (37, 38). However, results from RCTs in Rakai and Kisumu also found that circumcision decreases HPV incidence [RR = 0.67 (0.51-0.89) and HR = 0.61 (0.50-0.70) respectively] (7, 8). Similarly, both meta-analyses found no effect of male circumcision on HPV clearance [RR= 1.33 (0.65, 1.33) and HR =1.57 (0.51 - 4.89), respectively](37, 38). However, results from the two RCTs in Rakai and Kisumu found that circumcision increases HPV clearance of existing HPV infections [RR= 0.67 (0.51-0.89) and HR =1.47 (1.31 -1.64)](7, 8). Due to the heterogeneity of studies included in the meta-analyses, more studies are needed to understand the association between male circumcision and HPV incidence and clearance. Results from two RCTs suggest that male circumcision decreases HPV incidence and enhances clearance of existing HPV infections.

The RCT in Rakai also examined the effect of male circumcision on HPV infection in female partners. In HIV-negative men, circumcision was found to decrease the prevalence and incidence of HPV in their female partners [PRR =0.72 (0.62 -0.85)] (39). However, in HIV-positive men, circumcision was found to have no effect on transmission of HPV to female partners (40).

The Role of HPV Viral Load in the Natural History of HPV infections

Determinants of HPV Viral load in males

The studies done on HPV viral load in men so far have been cross-sectional and focused on the distribution and factors associated with viral load. Flores et al found that the penile shaft specimens had the highest viral load values than any another penile site, suggesting that the penile shaft is probably the preferred site for viral replication. Xi et al found that higher viral load values for types 16 and 18 were associated with current smoking status at baseline (41). Other factors such as number of lifetime partners, young age, STI infections, and condom use are known risk factors for HPV infection in men, but their association with HPV viral load remains unclear (24, 27, 28).

HPV Viral Load and HPV Transmission from men to women

The current understanding of the role of HPV viral load in transmission of HPV is based on a study of 238 heterosexual couples examining concordance and viral load (2). This study longitudinally examined HPV type concordance in females based on the viral load of their male partners. Men with detectable viral load levels (>250 copies/scrape) were more likely to have the same HPV type as their female partners than men with viral loads below the detection level (<250 copies/scrape). When

stratified by HPV type, the association between viral load in men and concordance was observed for HPV 16 but not for HPV 18 and 31. This study showed that men with higher viral loads, particularly for HPV type 16, are more likely to transmit infection to their partners than men with lower viral loads.

HPV Viral Load and HPV Persistence or Clearance in women

The current published studies on HPV viral load and persistence or clearance of infection have been done in females; to my knowledge, our study would be the first to examine this association in men. Several studies in females have found that higher HPV viral loads for high risk HPV types are associated with persistence of infection (33-36). In these studies, viral load in women was been examined mainly in 2 ways: (binary as less than or greater than 100pg/ml) or as a continuous variable. In the studies that examined viral load dichotomously, the effect of high baseline viral loads on persistence was found to range from OR of 1.3 – 5.7 (13, 42, 43). One study that examined the effect of tertiles of viral load on persistence found that the rate of clearance was higher for patients with lower viral load tertiles than higher ones [HR =2.8 (1.0-8.1)](12). A study examining HPV viral load continuously found that women with ≥ 10^7 HPV 16 copies were infected for longer periods of time than women with lower viral load (11).

More recently, some studies have focused on the dynamics of viral load rather than single time point measurements. Marks et al examined viral load in 50 women and found that whereas the baseline viral load was not associated with clearance, a >2 log decline in viral load over 6 months (2 study visits) was strongly associated with clearance [OR =5.5 (1.4 -21.3)] (44).

Even though the studies on viral load and persistence or clearance in women differ with regards to HPV types examined, viral load tests used, categorization of viral load and definitions of persistence or clearance, they suggest that lower viral loads are associated with clearance of infections in women, particularly for HPV 16.

HPV Viral load and development of CIN in women

Several studies that have examined the association between viral load and CIN have found greater viral load levels to be associated with greater severity of disease. Studies that examined types 16,18, and 31 found a strong association between high viral load (33rd percentile) was found to be strongly associated with ≥ CIN 2 (17, 45). A dose response-relationship has also been found for this association; the RR for association between viral load and CIN3 was found to be 1.9 and 4.5 for 1-10 copies/ cell and >1000copies/cell of viral load respectively (14). A few studies have examined the association between the dynamics of viral load and CIN, these studies found that women who developed CIN2/3 had consistently high or increasing viral load values, whereas those who didn't develop disease had decreasing viral load values over time (46, 47).

Biological Plausibility for Proposed Hypotheses

Aim 1: Male Circumcision reduces acquisition and enhances clearance of high viral load HPV infections

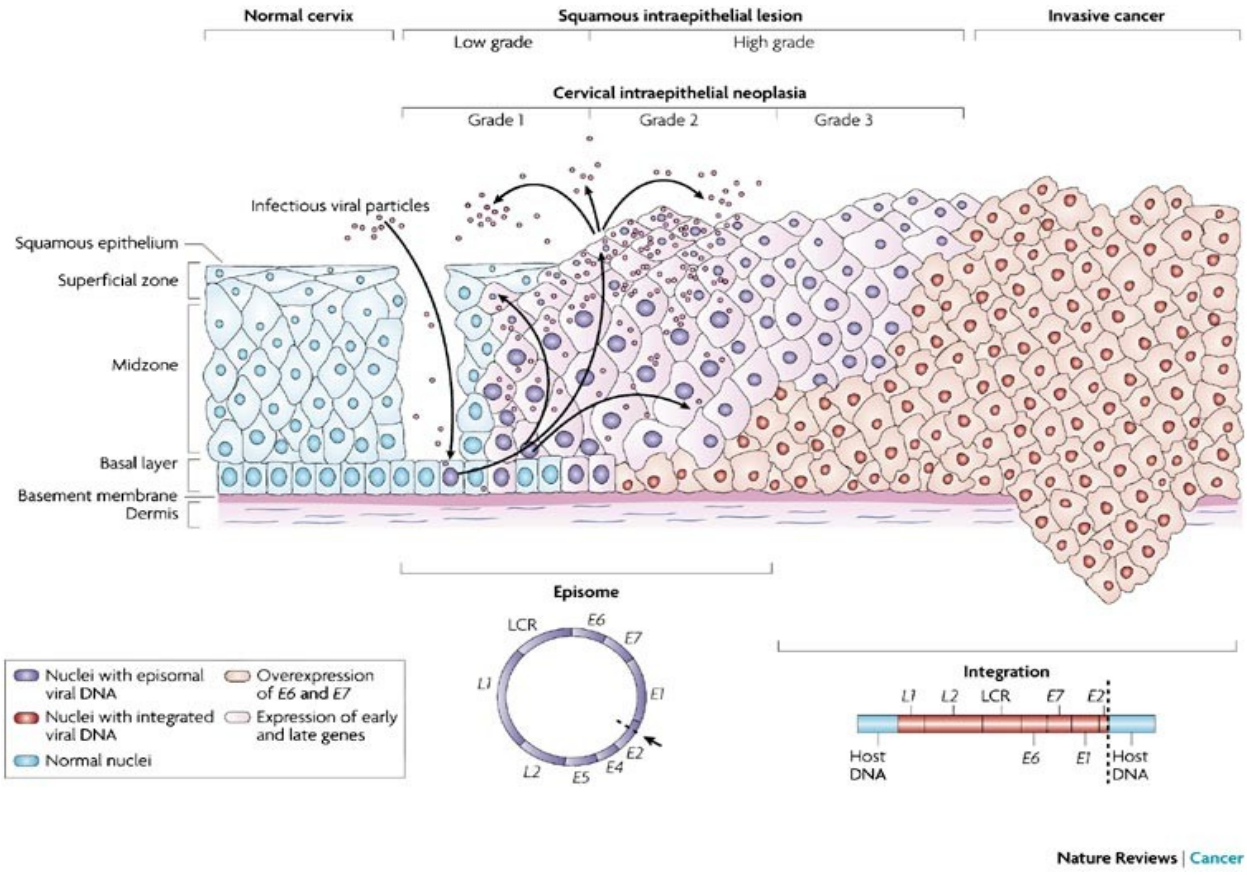
The biological mechanism by which circumcision may reduce HPV viral load is unclear, but it is believed that the keratinized skin surface and scar tissue in circumcised men reduces the entry and subsequent HPV viral replication in the basal epidermal cells

of the penis (39). Male circumcision is believed to enhance clearance by eliminating the moist subpreputial cavity that promotes HPV virus survival and subsequently reduces HPV clearance (39).

Aim 2: High HPV viral load is associated with lower HPV clearance

The process of HPV infection is described in Figure 2.1 (48). Briefly, HPV viral DNA enters basal cells of the dermis and replicates in human cells progressing to the epithelium. In the upper layers of the epithelium, replicated virions are shed and can initiate new infections. The viral load values obtained are a measurement of the copies of a 100ng target DNA region of the HPV viral DNA obtained from samples of exfoliated skin cells (21). It is plausible that a greater number of virus-infected cells in high viral load infections may lead to a lower rate of HPV clearance by the immune system in comparison to low viral HPV viral load (49).

Figure 2.1: Natural History of HPV Infections showing HPV viral replication process (48)



CHAPTER 3
METHODS

These analyses use data from two studies: an RCT of the effectiveness of male circumcision in reducing HIV incidence, and an ancillary HPV study conducted in men in Kisumu, Kenya from 2002-2007(5, 18). Penile exfoliated cells were collected from the men every 6 months for 2 years. Presence of human DNA was evaluated by beta-globin specific PCR, followed by agrose gel electrophoresis (19). The GP5+/6+ PCR assay was used to identify a wide array of HPV DNA types (19, 20). HPV viral load testing was conducted using a LightCyler real-time PCR assay; viral load was classified as high or low (>250 versus ≤250 copies/scrape) (21). The 250 copies/scrape cut-point was chosen because high HPV viral load (>250 copies/scrape) in men has been associated with increased HPV type concordance in female partners (2).

RCT of Male Circumcision for HIV Prevention: Bailey et al, 2007

The RCT was conducted in Kisumu, Kenya, to determine the effect of male circumcision on HIV incidence beginning in February 2002 (5). Inclusion criteria for the study included being residents of Kisumu, uncircumcised, aged 18-24, HIV negative, sexually active within the past 12 months, blood hemoglobin ≥ 90 g/l, not planning to move in the next 2 years and being willing and able to give consent. Exclusion criteria included hemophilia or other bleeding disorder, not being completely uncircumcised,

high prothrombin time index, absolute indication for circumcision and other medical conditions contraindicating surgery.

Screening Visit

Participants were referred to the RCT by private and public clinics, and peer outreach workers recruited participants from local youth organizations. At the screening visits:

- Potential participants were interviewed to determine age, residence and sexual activity history
- Blood test for hemoglobin (to determine if ≥ 90g/l) were done
- HIV testing and counseling was provided
- A physical examination was also done to determine circumcision status.

All eligible individuals were invited to participate in the study and were given a consent form in their preferred language (English, Dholuo or Kiswahili) to take with them and read in detail.

Baseline Visit

Trained study personnel reviewed the consent form and consenting adults were enrolled in the study. At the baseline or randomization visit:

- An interview was conducted to obtain medical history, socio-demographic and health information
- Blood was drawn for HIV and HSV-2 testing. Serum specimens were tested for HIV antibody using 2 rapid tests (Determine, Abbott Diagnostic Division, Hoofddorp, the Netherlands; Trinity Biotech, Wicklow, Ireland) and confirmed bydouble ELISA (Adaltis Inc, Montreal, Canada; Trinity Biotech, Wicklow, Ireland) at the University of

Nairobi. HSV-2 antibodies were tested for in sera using an enzyme-linked immunosorbent assay (ELISA) (Kalon).

- Urine samples were obtained and tested for *N. gonorrhea*, *T. vaginalis* and *C. trachomatis* by PCR (Roche Diagnostics)
- Participants were assigned to the circumcision (intervention) or delayed circumcision (control) groups. A total of 2784 participants were enrolled in the RCT, 1391 participants were assigned to the circumcised group and 1393 were assigned to the intervention group. Circumcision was performed on the same day or within a few days of randomization; participants were checked for complications 3, 8 and 30 days after circumcision.
- Counseling for HIV and STI prevention was done

Follow-up visits

Participants were followed every 6 months at approximately 6, 12, 18 and 24 months after the baseline visit. At the follow-up visits:

- An interview was conducted to obtain socio-demographic and health information
- Counseling for HIV and STI prevention was done
- STI testing (as done at baseline) was done and treatment was provided

Design of HPV Nested Study

Participants enrolling in the parent study of male circumcision and HIV were eligible to participate in the HPV study if they were willing and able to give informed

consent for the collection of penile exfoliated cell specimens at each visit. Of the 2784 participants enrolled in the parent RCT, 2299 (83%) consented to participate and were enrolled in the ancillary HPV study.

Collection of penile exfoliated cell specimens

Penile exfoliated cell specimens were collected by a trained physician or clinical officer working as part of the main RCT at the randomization visit prior to circumcision (i.e. baseline) and every 6 months during follow-up visits. Samples were collected from two anatomical sites: the penile shaft and external foreskin (will be referred to as shaft specimen); and the glans, coronal sulcus, and the internal tissue of the foreskin (will be referred to as glans specimen). Two pre-wetted type 3 Dacron swabs were used to collect exfoliated cells from the two separate anatomical sites: one for the shaft specimen and the other for the glans specimen. Shaft samples were collected by: (a) rubbing the four sides of the external shaft tissue from the proximal to distal penile shaft with sufficient pressure; and (b) rubbing the external surface of the foreskin for uncircumcised men. Glans samples were collected by: (a) swabbing the tip of the urethral opening; completely circling around the urethral orifice 2-3 times; (b) sampling the glans by rubbing back and forth from the top to the bottom in a circular motion; and (c) sampling the coronal sulcus by rotating the swab three times around its circumference. In uncircumcised men, the glans swab included a sample from the inner foreskin tissue.

Each swab was placed in a sterile 15 ml centrifuge tubes with 2-mL of 0.01 mol/L Tris-HCl 7.4 pH buffer with the participant's ID number, the visit number and type of specimen (glans or shaft) (18).

Processing and shipping of collected specimens

Each sample tube (2 per participant, glans and shaft) was placed in a previously labeled centrifuge tubes processed and centrifuged at high speed for 10 minutes in the clinic laboratory on the same day as sample was collected. After centrifuging, the supernatant was discarded using a Pasteur pipette, and the pellet was resuspended in the same volume of 0.01mol/L of Tris-HCl buffer and vortexed. Diluted cell pellets were frozen and stored at -75°C. All samples were transported via Fedex in a dry shipper (with permission from the Kenyan Ministry of Health) from Kisumu to the Department of Pathology at the VU Medical Center in Amsterdam, the Netherlands for HPV DNA testing (5, 18).

HPV DNA testing

DNA was isolated from penile exfoliated cell samples using NucleoSpin 96 Tissue kit (Macherey-Nagel, Germany) and a Microlab Star robotic system (Hamilton, Germany) according to manufacturers' instructions. Presence of human DNA was evaluated by β-globin specific PCR, followed by agrose gel electrophoresis (43). Laboratory testing for HPV positivity was performed using a highly sensitive GP5+/6+ PCR assay followed by hybridization of PCR products using an enzyme immunoassay (EIA) readout with two HPV oligoprobe cocktail probes that detect the following 44 HPV

types: HPV6, 11, 16, 18, 26, 30, 31, 32, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56,57, 58, 59, 61, 64, 66, 67, 68, 69, 70, 71 (equivalent to CP8061), 72, 73, 81 (equivalent to CP8304), 82 (IS39 and MM4subtypes), 83 (equivalent to MM7), 84 (equivalent to MM8), cand85, 86, cand89 (equivalent to CP6108) and JC9710(19). HPV genotyping was subsequently performed on GP5+/6+ PCR positive PCR products by reverse line blot (RLB) hybridization (19, 20). For all analyses, HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 were considered high-risk types. HPV types detected by EIA but not by RLB genotyping were designated as HPVX, indicating a type, sub-type or variant not detectable with probes used for reverse line blot hybridization. Low-risk types included all other HPV types.

HPV16/18 DNA viral load testing

HPV DNA viral load testing was performed on penile exfoliated cell samples that contained HPV16 or 18 using a real time PCR assay and a LightCycler instrument (Roche, Mannheim, Germany) (21). DNA extraction and purification were conducted according manufacturer instructions using 100 µl of the remaining cell suspensions. All samples were run in duplicate in the same run and resulting values were averaged. Dilutions of cloned pHPV DNA were used to determine the standard curve for the HPV target. In order to calculate the number of copies per sample, the amount of HPV DNA in one femtogram was multiplied by the dilution factor and divided by the weight of the cloned pHPV viral genome.

Validity of viral load measures

The LightCycler real time PCR assay used have previously been compared to another real time PCR method (Taq Man assay) and the GP5+/6+ enzyme immunoassay (EIA) to assess validity of measurements obtained (21). The results from the validity study showed that the LightCycler method viral load results were strongly correlated to those from the TaqMan assay (spearman ρ = 0.87), but less correlated to those from the EIA assay (spearman ρ = 0.77). These results showed that viral load results obtained with LightCylcer are comparable to those from other real time PCR methods; these real time PCR methods are more accurate in measuring viral load than EIA methods.

Results from the duplicate runs done per sample were obtained for baseline samples and assessed for agreement. The spearman coefficients for HPV16 and HPV18 were found to be 0.97 and 0.96 respectively (*unpublished data*). These results were limited in assessing for variability since they were done in the same run and do not account for technical variability, but they show very high agreement in viral load measures produced by the LightCycler.

Statistical Analyses

Aim 1: Effect of male circumcision on incidence and clearance of high and low viral load HPV16 and HPV18 infections in Kenyan men

All analyses were performed for each HPV type (HPV16 or HPV18) and anatomical site (glans or shaft) separately. Intent-to-treat analyses were performed to examine the incidence of high viral load infections in circumcision versus control arms.

Men who were randomized to the circumcision arm but were not circumcised (N=23) and those randomized to the control arm but were circumcised (N=14) were included in the analysis in the group to which they were randomly assigned. Incident infections were assumed to occur at the mid-point between the last HPV-negative and first subsequent HPV-positive result. Men who tested HPV-negative at each visit were censored at their last observed visit. Only the first incident infections for each HPV type (HPV16 or HPV18) at each anatomical site (glans or shaft) were included in incidence analyses. The Kaplan-Meier method was used to estimate the 24-month cumulative risk of HPV16 and HPV18 in the glans or shaft for the circumcision versus control arms. Hazard ratios (HRs) comparing the rate of acquisition of high viral load infections in circumcision versus control arms were estimated by fitting Cox proportional hazards models.

An additional analysis was conducted among only participants with incident HPV16 or HPV18 infections to examine associations between circumcision and the prevalence of high versus low HPV viral load at time of HPV detection. Potential confounders of the association between circumcision and HPV viral load were identified from literature, and analyzed using a directed acyclic graph (DAG) (50). Confounders identified included: HIV infection, condom use, number of sex partners, bathing frequency, infection with multiple HPV types and infection with other STIs (HSV-2, *Trichomonas vaginalis*, *Chlamydia trachomatis* and *Neisseria gonorrhoe*ae). Log binomial models were used to derive prevalence ratios (PRs) comparing the prevalence of high versus low viral load at the time of HPV detection for incident infections observed in the circumcision versus control arms.

Clearance of prevalent infections detected at enrollment was assumed to occur at the mid-point between the last HPV-positive result and the first subsequent HPV-negative result. Men were censored at their last observed visit if they tested positive at each consecutive visit for the same HPV type at the same anatomical site. The Kaplan-Meier method was used to estimate the cumulative risk of clearance for high and low viral load prevalent HPV infections. Risk estimates for clearance of prevalent infections in the circumcision versus control arms were compared with a Z test at 6 and 12 months after baseline. HPV persistence was defined as the detection of the same HPV type at the same anatomical site at 6 and 12 months after baseline in participants who were positive for HPV16 or HPV18 at baseline. For all the analyses of incidence and clearance, HR estimates accounting for interval censoring were obtained by fitting models assuming Weibull distributions for incidence times; results obtained were similar to those from the Cox models (data not shown). Sensitivity analyses were performed by restricting analyses to samples that tested positive for the presence of human DNA with the beta-globin test.

Aim 2: Association between HPV Viral Load and HPV clearance in uncircumcised Kenyan men

The distribution of participant characteristics by HPV16 or HPV18 viral load (high or low) was examined for infections prevalent at baseline. For these analyses, HPV viral load results from the glans and shaft samples were combined; high viral load was defined as >250 copies/scrape in the glans or shaft, and low viral load was defined as $\leq$ 250 copies/scrape in both the glans and shaft. Baseline characteristics examined included age, education, employment, marital status, bathing frequency, number of sexual partners and condom use in the previous 6 months.

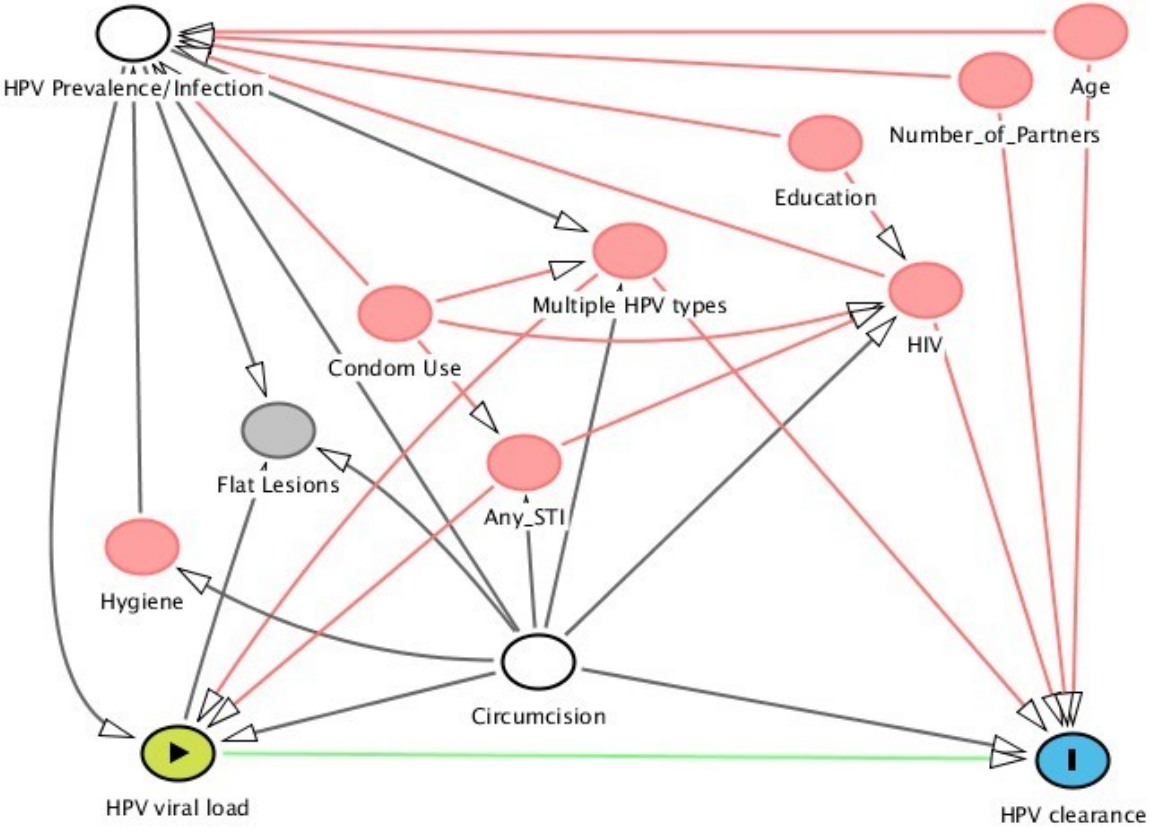
HPV clearance analyses were performed separately for incident and prevalent HPV16 or HPV18 infections. We also examined HPV clearance separately for glans and shaft infections since HPV clearance has been shown to vary by anatomical site (8) . For each participant, only the first detected HPV16 or HPV18 infection (incident or prevalent) at each anatomical site (glans or shaft) was included in clearance analyses. Time-to-clearance since HPV detection was calculated starting at the visit date of first HPV detection; for prevalent infections the start date was baseline, and for incident infections it was the visit date of first HPV detection for each HPV type at each anatomical site. Clearance was assumed to occur at the mid-point between the last HPV-positive result and the first subsequent HPV-negative result. Men were censored at their last observed visit if they remained HPV positive for the same HPV type at the same anatomical site for each observed consecutive visit.

HPV viral load measurements at first detection of HPV16 or HPV18 prevalent or incident infections were used to classify the infections as high (>250 copies/scrape) or low (≤ 250 copies/scrape) viral load infections. For all the clearance analyses, HPV viral load was analyzed as a time-fixed exposure; few HPV16 or HPV18 infections (8.4% overall) were observed to change viral load groups during follow-up. The actual circumcision status of eligible men at each visit was reviewed to verify that they were uncircumcised during HPV detection and clearance; 12 men assigned to the control arm were circumcised later in the study, however, in each case circumcision was performed after clearance of HPV infections so these men were included in clearance analyses.

The Kaplan-Meier method was used to estimate the cumulative incidence for clearance of high versus low viral load HPV infections at 6 months (6-month risk) and

12 months (12-month risk) after HPV detection. Risk ratio estimates were calculated to compare 6-month and 12-month clearance risk estimates of high versus low viral load infections (51). Potential confounders of the association between HPV viral load and HPV clearance were identified from the literature, and analyzed using a DAG (50). Confounders identified and included in Cox regression models were: HIV infection, condom use, number of sex partners, bathing frequency, infection with multiple HPV types, educational attainment, and infection with other STIs (HSV-2, *Trichomonas vaginalis*, *Chlamydia trachomatis* and *Neisseria gonorrhoea*e). Adjusted hazard ratios (aHRs) comparing the clearance hazard at 6 months for high versus low viral load HPV infections were estimated for each HPV type and anatomical site. HRs at 6-months after HPV detection could not be obtained in cases where all high viral load or low viral load infections cleared by 6 months after HPV detection. An interaction term between the HPV viral load group and time-to-clearance was included in all the Cox regression models to account for non-proportional hazards in clearance of high and low viral load HPV infections. Sensitivity analyses for clearance of prevalent and incident infections were performed by restricting analyses to samples that tested positive for the presence of human DNA with the beta-globin test.

Figure 3.1: DAG to Assess Potential Confounders for associations between Circumcision and HPV Viral load, and HPV Viral load and HPV Clearance.



CHAPTER 4
THE ASSOCIATION BETWEEN MALE CIRCUMCISION AND PENILE HPV VIRAL LOAD IN AN RCT IN KISUMU, KENYA

Overview

Persistent infections with human papillomavirus (HPV) types 16 and 18 are causes of approximately 70% of cervical cancer cases. Circumcision and lower HPV viral load in men are associated with reduced HPV transmission to women. The association between circumcision and HPV viral load, however, remains unclear. Penile swabs from glans and shaft sites were collected from men in a circumcision trial in Kisumu, Kenya. GP5+/6+ PCR was used to identify HPV DNA types. HPV viral load was measured with LightCyler real-time PCR and classified as high (>250copies/scrape) or low (≤250copies/scrape). Of 2,299 men with HPV baseline results, 1,159 were randomized to immediate circumcision and 1,140 to the control arm and asked to remain uncircumcised until the study ended. The rate of acquisition of high viral load infections in the glans was lower in the circumcision than control arm for HPV16 [Hazard Ratio(HR)=0.32(95%confidence interval, 0.20-0.49)] and HPV18 [HR=0.34(0.21-0.54)]. Among men with incident infections in the glans, prevalence of high versus low viral load at HPV detection was lower in the circumcision than control arm for HPV16 [Prevalence Ratio(PR)=0.70(0.50-0.99)] and HPV18 [PR=0.59(0.43-0.82)]. For prevalent high viral load infections in the glans at baseline, risk of persistence to 6 months was lower in the circumcision arm [0.20(0.09-0.34)] than control arm [0.55(0.39-

0.68)] for HPV16 and HPV18 [0.17(0.05-0.34) and 0.50(0.25-0.71), respectively]. Results from shaft samples were similar to those from glans samples. Circumcision reduces acquisition and enhances clearance of high viral load HPV infections in the glans, and thus could potentially reduce HPV transmission to women.

Introduction

Persistent infections with human papillomavirus (HPV) high-risk types 16 and 18 are causes of approximately 70% of cervical cancer cases in women (1). Women with higher HPV16 or HPV18 viral loads are more likely to have persistent infections and to progress to high grade cervical intraepithelial neoplasia (CIN) than those with lower viral loads (11-17). Men with higher HPV16 or HPV18 viral loads have a higher prevalence of flat penile lesions and greater HPV type concordance with their female partners than those with lower viral loads (2, 52, 53). As a result, higher HPV viral load in men is suggested to be associated with increased HPV transmission to their female partners (2).

Male circumcision has been found to be protective against HIV and penile HPV infections (3-8, 37). Three randomized control trials (RCTs) in Kisumu, Rakai and Orange Farm, as well as several longitudinal studies have shown that high-risk HPV prevalence is lower in circumcised than uncircumcised men (6-8). RCTs in Kisumu and Rakai also found that male circumcision reduces the acquisition of high-risk HPV infections and enhances clearance of HPV infections over 24 months (7, 8). Prevalence of flat penile lesions after 24 months was lower in circumcised than uncircumcised men in Kisumu (52). Among HIV-negative men in Rakai, lower HPV prevalence was observed in female partners of circumcised men than in female partners of uncircumcised men (40). Circumcision and lower HPV viral load in men are suggested to be associated with reduced HPV

transmission to women, but the association between circumcision and HPV viral load remains unclear (2).

To our knowledge, only the RCT in Rakai, Uganda, has examined the effect of male circumcision on penile HPV viral load (22). This study, however, did not have longitudinal data on HPV viral load and was therefore unable to examine the association between circumcision and incident high versus low viral load HPV infections. The association between circumcision and the clearance of high and low viral load HPV infections present at baseline (prevalent infections) was also not explored.

We present the results of an RCT examining the associations between male circumcision and the incidence and clearance of high and low viral load infections for HPV types16 and 18 in men in Kisumu, Kenya. We have previously shown that male circumcision reduces HPV incidence and increases HPV clearance in this RCT (8); here we expand on those findings to examine the association between male circumcision and HPV viral load.

Methods

Study population and enrollment

Uncircumcised men were screened between February 4, 2002 and September 6, 2005 in Kisumu, Kenya to participate in an RCT of male circumcision (5). The main aim of the trial was to determine the effectiveness of male circumcision in reducing HIV incidence. Study participants were recruited from sexually transmitted infection (STI) clinics, workplaces and community organizations. Study inclusion criteria included being uncircumcised, aged 18-24, HIV negative, sexually active within the past 12 months and having blood hemoglobin ≥ 90 g/l. Participants who met the study criteria were randomly

assigned to either the immediate circumcision arm or to the control arm and asked to remain uncircumcised until the end of their 24 months of study participation. This study protocol was approved by Institutional Review Boards of the Universities of Illinois at Chicago, Manitoba, Nairobi and North Carolina; RTI International and VU University Medical Center.

This analysis includes men from the RCT who consented to the collection of penile exfoliated cells and their shipment overseas for HPV DNA and HPV viral load testing. Of the 2784 men enrolled in the RCT, 2299 (83%) consented to provide penile swab samples and had baseline HPV data; 1159 men were in the circumcision and 1140 in the control arm (Figure 4.I). Eligibility for inclusion in incidence and clearance analyses was determined for each HPV type (HPV16 or 18) and anatomical site (glans or shaft) separately. Participants who were: 1) positive for HPV16 or HPV18 at baseline; 2) had no HPV follow-up data; or 3) had missing HPV viral load data were excluded from incidence analyses for that specific HPV type and anatomical site. Similar proportions of samples in the circumcision and control arms were included in incidence analyses for HPV16 [87% versus 89% in glans, 90% versus 93% in shaft, respectively] and HPV18 [91% versus 93% in glans, 93% versus 95% in shaft, respectively]. Clearance of prevalent high and low viral load infections was examined in participants who were: 1) positive for HPV16 or HPV18 at baseline; 2) had follow-up HPV data; and 3) had viral load data for that HPV type and anatomical site [HPV16 glans=144(82.2%), HPV16shaft =67(77.9%), HPV18 glans=60 (75.9%) and HPV18 shaft =24 (80%)].

Follow-up and specimen collection

Participant follow-up visits were scheduled every 6 months for a period of 24 months. A standardized questionnaire on sociodemographic characteristics and sexual behavior was administered by a trained male interviewer at each visit (5). Penile exfoliated cell specimens were collected by a trained physician or clinical officer at each study visit (18). Samples were collected from two anatomical sites: (i) penile shaft and external foreskin (shaft specimen); and (ii) glans, coronal sulcus, and the internal tissue of the foreskin (glans specimen). Sampling of the foreskin was conducted only in uncircumcised men. Two pre-wetted type 3 Dacron swabs were used to collect penile exfoliated cells from the two separate anatomical sites. Each swab was placed in a sterile 15 ml centrifuge tubes with 2-mL of 0.01 mol/L Tris-HCl 7.4 pH buffer with the participant's ID number, the visit number, and anatomical site of specimen (glans or shaft). Each sample was centrifuged at high speed for 10 minutes in the clinic laboratory on the same day it was collected. Excess Tris-HCl buffer was discarded using a Pasteur pipette, and the pellet was resuspended in the same volume of 0.01mol/L of Tris-HCl buffer and vortexed. Diluted cell pellets were frozen and stored at -75°C. All samples were transported via Fedex in a liquid nitrogen dry shipper from Kisumu to the Department of Pathology at VU Medical Center in Amsterdam, Netherlands, for HPV DNA testing.

HPV DNA, HIV and STI Testing

DNA was isolated from penile exfoliated cell samples using NucleoSpin 96 Tissue kit (Macherey-Nagel, Germany) and a Microlab Star robotic system (Hamilton, Germany) according to manufacturers' instructions. Presence of human DNA was evaluated by beta-globin specific PCR, followed by agrose gel electrophoresis (19). HPV positivity was

assessed by GP5+/6+ PCR followed by hybridization of PCR products using an enzyme immunoassay readout with two HPV oligoprobe cocktail probes that detect 44 HPV types. Subsequent HPV genotyping was performed by reverse line blot (RLB) hybridization of PCR products (19, 20).

HIV testing was performed at each visit using two HIV antibody rapid tests (Determine, Abbott Diagnostic Division, Hoofddorp, Netherlands; and Unigold, Trinity Biotech, Wicklow, Ireland), and confirmed by double ELISA (Adaltis, Montreal, Canada; Trinity Biotech, Wicklow, Ireland) at the University of Nairobi. Urine samples at each visit were tested for *Trichomonas vaginalis*, *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infections by PCR-based methods (Roche Diagnostics), and serum was tested for Herpes simplex virus (HSV)-2 antibodies by a type-specific enzyme-linked immunosorbent assay (ELISA) (Kalon).

HPV Viral Load Testing

HPV DNA viral load testing was performed on penile exfoliated cell samples that contained HPV16 or HPV18 using a real time PCR assay and a LightCycler instrument (Roche, Mannheim, Germany) (21). DNA extraction and purification were conducted according manufacturer instructions using 100 µl of the remaining cell suspensions. All samples were run in duplicate in the same run and resulting values were averaged. Dilutions of cloned HPV DNA were used to determine the standard curve for the HPV target. To calculate the number of copies per sample, the amount of HPV DNA in one femtogram was multiplied by the dilution factor and divided by the weight of the cloned pHPV viral genome. HPV viral load was categorized as low (≤250 copies/scrape) or high (>250 copies/scrape). The 250 copies/scrape cut-point for high versus low viral load was

chosen because high HPV viral load (>250 copies/scrape) in men has been associated with increased HPV type concordance in female partners (2).

Statistical Analyses

All analyses were performed for each HPV type (HPV16 or HPV18) and anatomical site (glans or shaft) separately. Intent-to-treat analyses were performed to examine the incidence of high viral load infections in circumcision versus control arms. Men who were randomized to the circumcision arm but were not circumcised (N=23) and those randomized to the control arm but were circumcised (N=14) were included in the analysis in the group to which they were randomly assigned. Incident infections were assumed to occur at the mid-point between the last HPV-negative and first subsequent HPV-positive result. Men who tested HPV-negative at each visit were censored at their last observed visit. Only the first incident infections for each HPV type (HPV16 or HPV18) at each anatomical site (glans or shaft) were included in incidence analyses. The Kaplan-Meier method was used to estimate the 24-month cumulative risk of HPV16 and HPV18 in the glans or shaft for the circumcision versus control arms. Hazard ratios (HRs) comparing the rate of acquisition of high viral load infections in circumcision versus control arms were estimated by fitting Cox proportional hazards models.

An additional analysis was conducted among only participants with incident HPV16 or HPV18 infections to examine associations between circumcision and the prevalence of high versus low HPV viral load at time of HPV detection. Potential confounders of the association between circumcision and HPV viral load were identified from literature, and analyzed using a DAG (Figure 3.1) (50). Confounders identified included: HIV infection,

condom use, number of sex partners, bathing frequency, infection with multiple HPV types and infection with other STIs (HSV-2, *Trichomonas vaginalis*, *Chlamydia trachomatis* and *Neisseria gonorrhoe*ae). Log binomial models were used to derive prevalence ratios (PRs) comparing the prevalence of high versus low viral load at the time of HPV detection for incident infections observed in the circumcision versus control arms.

Clearance of prevalent infections detected at enrollment was assumed to occur at the mid-point between the last HPV-positive result and the first subsequent HPV-negative result. Men were censored at their last observed visit if they tested positive at each consecutive visit for the same HPV type at the same anatomical site. The Kaplan-Meier method was used to estimate the cumulative risk of clearance for high and low viral load prevalent HPV infections. Risk estimates for clearance of prevalent infections in the circumcision versus control arms were compared with a Z test at 6 and 12 months after baseline. HPV persistence was defined as the detection of the same HPV type at the same anatomical site at 6 and 12 months after baseline in participants who were positive for HPV16 or HPV18 at baseline. For all the analyses of incidence and clearance, HR estimates accounting for interval censoring were obtained by fitting models assuming Weibull distributions for incidence times; results obtained were similar to those from the Cox models (data not shown). Sensitivity analyses were performed by restricting analyses to samples that tested positive for the presence of human DNA with the beta-globin test.

Results

Characteristics of men enrolled in this RCT have been previously described (5, 8, 18). Briefly, the median age of eligible study participants was 20 (range 18-24 years). Most men were unemployed (65%) and unmarried (93%). At baseline, 44% of participants

reported having more than 2 sex partners and 22% reported always using a condom in the previous 6 months. The prevalence of other STIs at baseline was low (2% for *Trichomonas vaginalis*, 2% for *Neisseria gonorrhoeae* and 5% for *Chlamydia trachomatis)*, with the exception of HSV-2 (27%). There were no appreciable differences in sociodemographic, sexual history, or STI laboratory results at baseline between eligible participants randomized to the circumcision and control arms (Table 4.1).

Incidence of high viral load HPV infections

The 24-month cumulative risk of high viral load infections in the glans was lower in the circumcision than control arm for HPV 16 (2.7% versus 9.5%) and HPV18 (2.2% versus 6.8%, respectively) (Figure 4.2). In the shaft, the estimated 24-month cumulative risk of high viral load infections was comparable in the circumcision and control arms for HPV16 (2.0% versus 2.6%) and HPV18 (2.0% versus 2.6%, respectively).

Of incident HPV infections observed, a total of 151 HPV16 (39.7%) and 135 HPV18 (69.2%) high viral load infections occurred in either the glans or shaft (Table 4.2). The rate of acquisition of high viral load infections in the glans was lower in the circumcision than control arm for both HPV16 [HR=0.32 (0.20-0.49)] and HPV18 [HR= 0.34 (0.21- 0.54)] (Table I). Similar trends were observed in the shaft for both HPV types [HPV16 HR= 0.79 (0.42 -1.45) and HPV18 HR= 0.87 (0.48-1.54)]. Sensitivity analysis by restricting to beta-globin-positive samples yielded similar results (Table 4.2).

Prevalence of high versus low viral load at detection of incident HPV infections

Of the incident HPV infections observed in the glans, most infections in control arm had high viral loads [53.6% (N=83) for HPV16; 60.5% (N=66) for HPV18] whereas most of those in the circumcision arm had low viral loads [62.3% (N=43) for HPV16; 52.2% (N=24)

for HPV18]. In the shaft, most HPV16 incident infections had low viral loads [74.3% (N=52) in circumcision arm; 72.4 %(N=62) in control arm] whereas most HPV18 incident infections had high viral loads [77.8% (N=21) in circumcised arm; 65.0% (N=26) in the control arm]. Few men had incident infections in both the glans and shaft (19% for HPV16 and 25% for HPV18), and in all these cases the viral load category (high or low viral load) was the same in both anatomical sites.

Among men with incident HPV infections in the glans, the prevalence of high versus low viral load at the time of HPV detection was lower in the circumcision than control arm for HPV16 [PR=0.70(0.50-0.99)] and HPV18 [PR=0.59(0.43-0.82)]. However, this association was not observed in the shaft for HPV 16 [PR = 0.93 (0.55-1.57)] or HPV 18 [PR = 1.20 (0.88-1.62)] (Table 4.3). PRs obtained after adjustment for HIV infection, condom use, number of sex partners, bathing frequency, infection with STIs and multiple HPV type infection were similar to the reported unadjusted HRs (results not shown). Sensitivity analyses restricted to beta-globin-positive samples showed similar results as the main analysis (Table 4.3).

Clearance of high and low viral load infections prevalent at baseline

For both HPV types, HPV prevalence at baseline was higher in the glans (HPV16 =6.6%; HPV18 =3.1%) than in the shaft (HPV16 =2.8%; HPV18 =1.1%). Prevalent infections in the glans at baseline were more likely to have high HPV viral loads [HPV16= 54.9% (79); HPV18= 66.7% (N=40) than prevalent infections at baseline in the shaft [HPV16= 11.9% (N=8); HPV18= 41.7% (N=10)] (Table 4.4). Similar proportions of men with

prevalent HPV16 and HPV18 infections at baseline were assigned to the circumcision and control arms (Figure 4. I).

Nearly all prevalent infections at baseline cleared during follow-up, with the exception of 5 (6.6%) HPV16 glans infections in the control arm (4 high viral load infections censored at 18 months and 1 low viral load infection censored at 12 months after baseline). For both HPV types, most prevalent infections in the glans or shaft cleared within 6 months (Figures 4.3 and 4.4). At both 6 and 12 months after baseline, the highest risk of persistence for HPV16 and HPV18 infections was among men in the control arm with high HPV viral load. The estimated risk of persistence to 6 months for prevalent infections in the glans was lower in the circumcision arm [0.20(0.09-0.34)] than control arm [0.55(0.39-0.68)] for HPV 16 and HPV18 [0.17(0.05-0.34) and 0.50(0.25-0.71), respectively] (Table 4.4). For prevalent HPV16 infections in the glans, the estimated risk of persistence to 6 months for high viral load infections in the circumcision arm [0.20 (0.09 – 0.34)] was similar to that of low viral load infections in the control arm [0.13 (0.04 -0.26)] (Table 4.4). A similar trend was observed for prevalent HPV16 infections in the shaft and prevalent HPV18 infections in the glans and shaft.

HR estimates for clearance of prevalent infections were imprecise, but suggest that clearance of high viral load infections was greater in the circumcision than control arm for HPV16 [HR =1.46 (0.91-2.32) in glans; HR=4.34 (0.67-84.9) in shaft] and HPV18 [HR= 2.02 (1.02-4.08) in glans; HR = 4.33 (0.78-33.3) in shaft] (Table 4.4). Results for clearance of low viral load infections were also imprecise, but suggest that clearance in the glans was greater in the circumcision versus control arm [HR=1.31(0.68 -1.89)for HPV16; HR=1.79

(0.70-5.15)for HPV18]. Sensitivity analyses of clearance restricted to prevalent infections that were beta-globin-positive found similar results to the main analysis (data not shown).

Discussion

The two-year cumulative incidence of high viral load HPV16 and HPV18 infections in the glans was lower in the circumcision arm than control arm in young men from Kisumu, Kenya. Circumcision reduced the rate of acquisition of high viral load HPV16 and HPV18 infections in the glans over 24 months. Among men with incident HPV16 or HPV 18 infections, circumcised men were more likely to have low versus high viral load infections in the glans as compared to uncircumcised men. We also found that for prevalent high viral load infections detected at baseline, the risk of HPV persistence to 6 months was lower in the circumcision arm than control arm for both HPV types 16 and 18 in the glans. Similar, but less precise results were observed for the estimated effect of circumcision on the incidence of high viral load HPV16 and HPV18 infections in the shaft samples.

To our knowledge, only one study, in Rakai, Uganda, has examined the effect of circumcision on HPV viral load in men (22). Also an RCT of male circumcision, the study found the combined HPV viral load for HPV types 16, 18, 31, 33, 35 and 52 in the glans was lower in the circumcision arm than control arm at 24 months after randomization. Their estimated protective effect of circumcision on prevalence of high versus low HPV viral load infections in the glans [PR =0.54 (0.21-1.42)] was similar to the PR estimates we obtained for HPV16 [0.70 (0.50 -0.99)] and HPV18 [0.59 (0.43 -0.82)] in the glans.

Our study examined HPV viral load in both the glans and shaft anatomical sites. Estimated effects of circumcision in reducing incidence and enhancing clearance of high

viral load infections in the shaft were similar, but less precise than estimated effects found in the glans. This may be due to fewer HPV infections and a lower proportion of high viral load infections detected in the shaft as compared with the glans. Stronger effects of circumcision on HPV infection in the glans than more distal anatomical sites to the foreskin have been documented in other studies (37).

The biological mechanism by which circumcision reduces HPV viral load is unclear, but it is believed that the keratinized skin surface and scar tissue in circumcised men reduces the entry and subsequent HPV viral replication in the basal epidermal cells of the penis (39). In uncircumcised men, it has been hypothesized that the moist subpreputial cavity promotes HPV virus survival and subsequently reduces HPV clearance (39, 54). Male circumcision has been previously shown to reduce the prevalence of flat penile lesions; our findings are consistent with the current understanding that circumcision reduces the prevalence of flat penile lesions by reducing HPV viral load in men (2, 52, 53). Furthermore, the effect of circumcision in reducing HPV viral load may explain why fewer HPV infections and lower HPV viral loads are observed in female partners of circumcised than uncircumcised men (40, 54). It is possible that circumcision reduces the transmission of HPV infections to women by reducing incidence and enhancing clearance of high viral load HPV infections in their male partners.

Main strengths of this study include the extensive longitudinal data on HPV viral load and quantitative HPV viral load measurements by real time PCR. Our study is also the first to examine type-specific HPV viral load in both the glans and the shaft anatomical sites. Our main study limitation is that HPV viral load measurements were not normalized to account for number of cells in each swab sample (viral load copies/cell). However, the

copies/scrape units have been previously used to show that high HPV viral load (>250 copies /cell) in men is associated with greater HPV type concordance in their female partners (2). We also observed fairly low beta-globin positivity in our samples (61.5% in glans and 43.5% in shaft samples). A possible explanation is that penile cells, particularly in shaft samples, are more keratinized and anucleated than those in the cervix, and therefore may contain less human DNA (24). Lower beta-globin positivity in penile swab samples has also been documented in a similar study (7). Due to our low beta-globin positivity, it is possible that HPV viral load measurements in this study may be underestimated. However, all the incidence and clearance sensitivity analyses restricted to beta-globin positive samples found similar results as the main analyses.

This study adds to the current understanding of HPV viral load in men in general, and the natural history of HPV infections in circumcised and uncircumcised men. Like previous studies examining HPV viral load in men, the interpretation of our results is limited by the inability to distinguish between integrated and episomal DNA (48). Integration is followed by a decrease in HPV viral load, but the level of integrated HPV is believed to increase with progression of HPV infections (48). Future research distinguishing between episomal and integrated DNA is needed to further understand the effect of circumcision on HPV viral load.

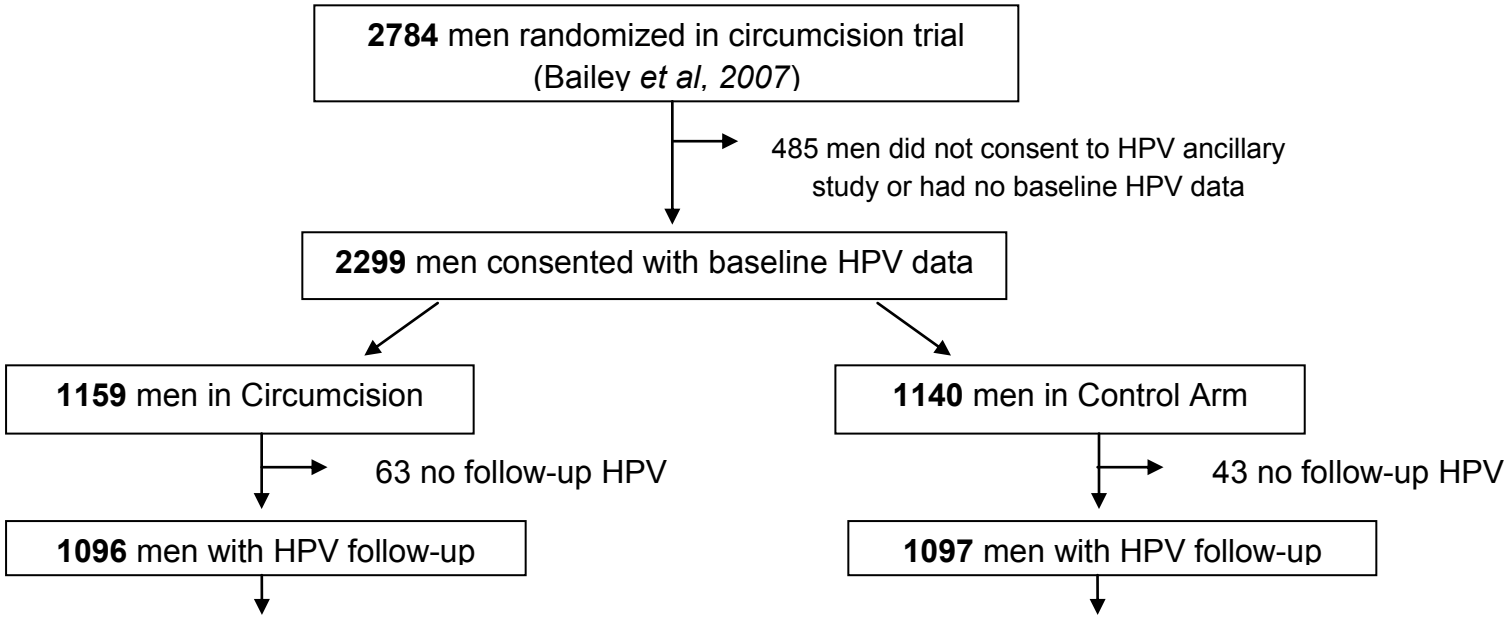
Interestingly, we found that the risk of persistence to 6 and 12 months for prevalent infections detected at baseline was similar in circumcised men with high HPV viral load and uncircumcised men with low HPV viral load, for both HPV types and anatomical sites. This suggests that the viral load of prevalent HPV infections may predict HPV clearance

independently of circumcision, although this needs to be confirmed in a larger study taking into account other factors associated with HPV clearance including treatment of other STIs.

In conclusion, this study showed that circumcision reduces acquisition and enhances clearance of high viral load HPV infections in the glans in Kenyan men. High HPV viral load in men is suggested to be associated with increased HPV transmission to female partners (2). Therefore, male circumcision could potentially reduce HPV transmission to women by reducing acquisition and enhancing clearance of high HPV viral load infections in men. These findings support the effectiveness of male circumcision as an intervention to reduce the incidence of and accelerate the clearance of high viral load infections in men, and potentially reduce HPV incidence in women.

Tables and Figures

Figure 4.1: Study flowchart of HPV samples included in analyses of the effect of male circumcision on penile HPV viral load



HPV Types	HPV 16		HPV 18	
Anatomic Sites	Glans	Shaft	Glans	Shaft
Other Exclusion Criteria : Missing HPV viral load data	19	11	2	1
HPV (+) at baseline **Total samples in Clearance Analyses**	68	39	37	12
HPV (-) at baseline **Total Samples in Incidence Analyses**	1009	1046	1057	1083

HPV 16		HPV 18	
Glans	Shaft	Glans	Shaft
7	6	10	2
76	28	23	12
1014	1063	1064	1083

Figure 4.2: Estimated Cumulative Incidence of High Viral Load infections over 24 months in Kenyan Men

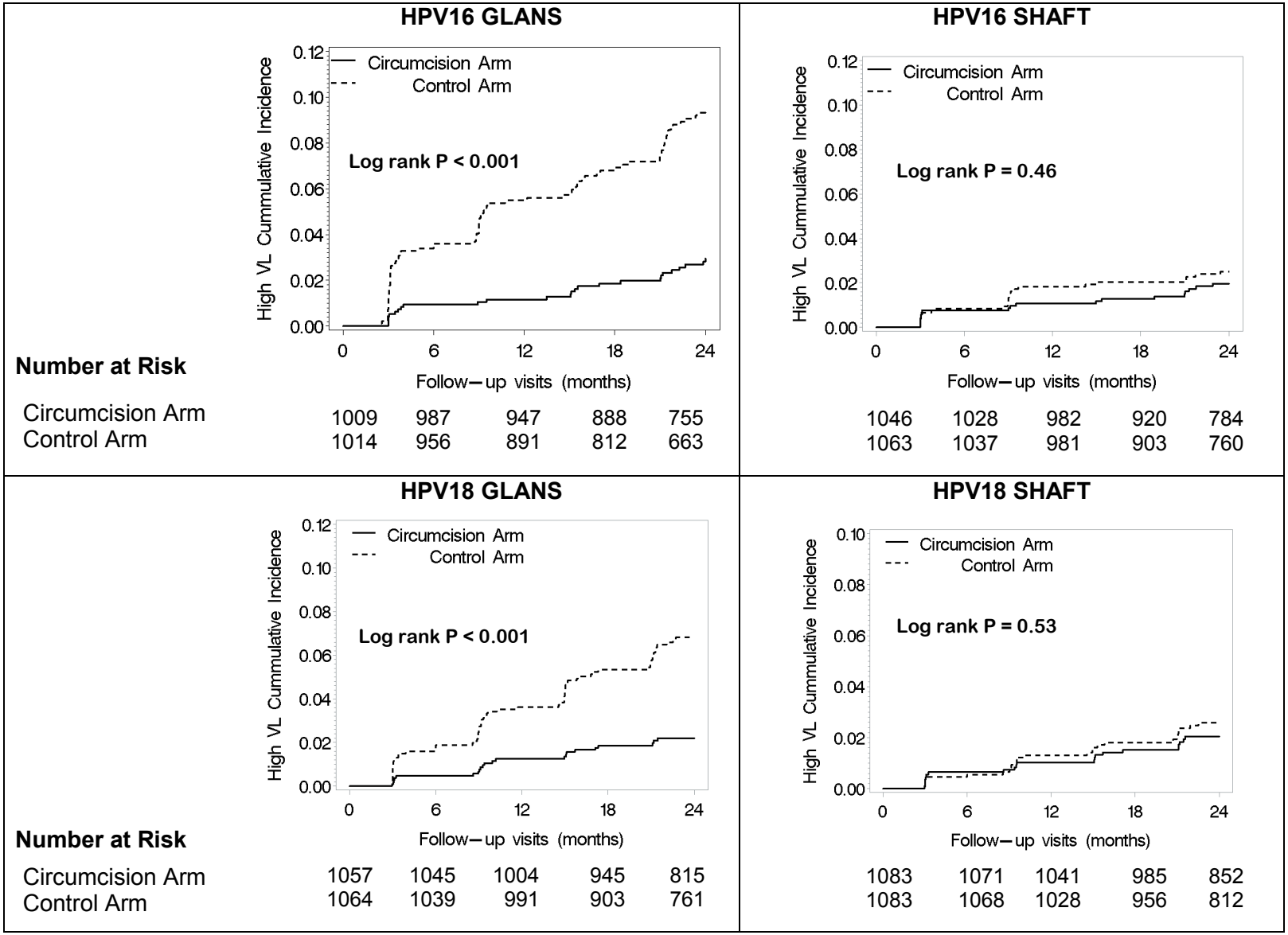


Figure 4.3: Estimated Clearance of Prevalent High Viral Load HPV Infections by circumcision status in Kenyan Men

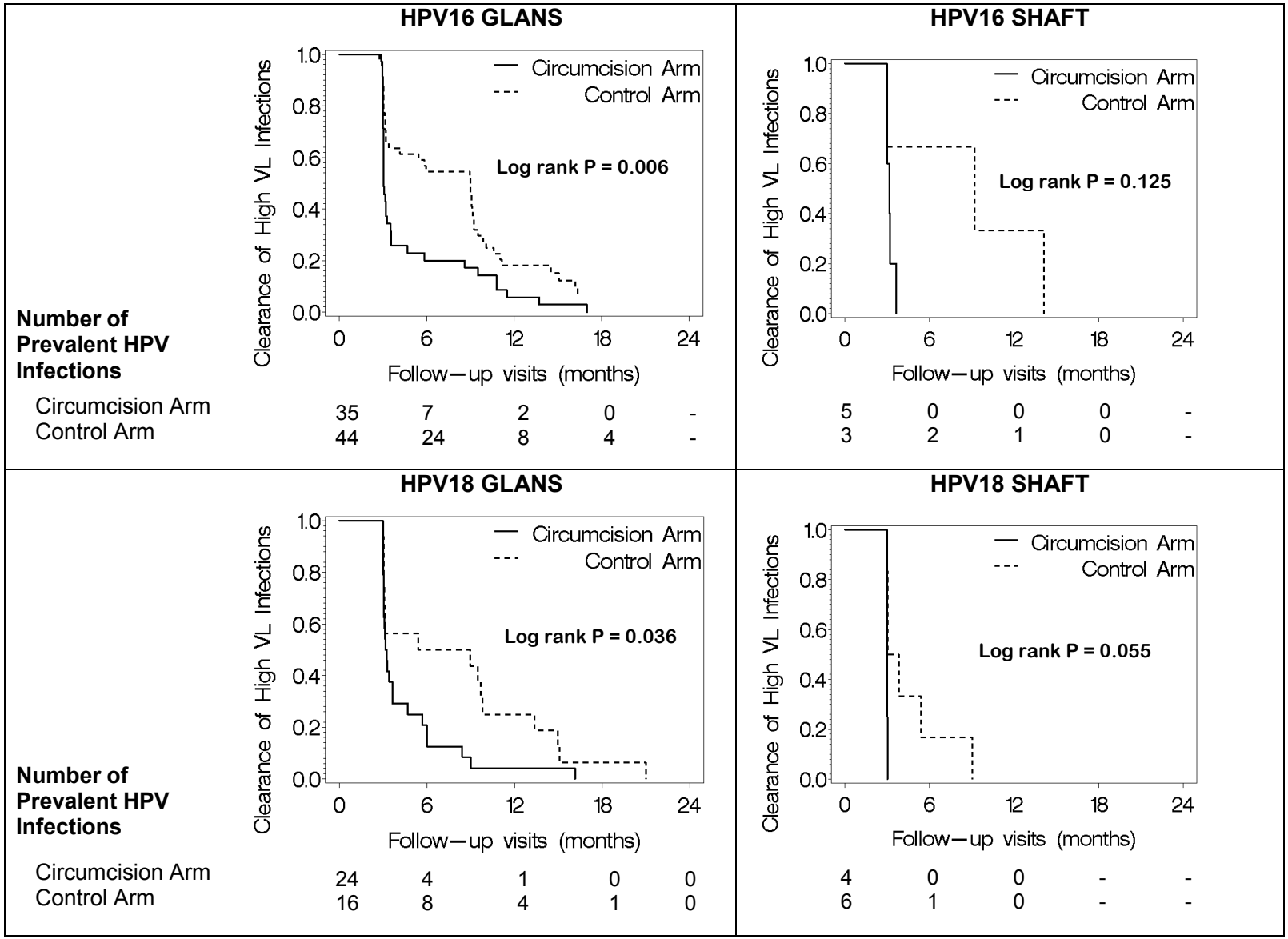


Figure 4.4: Estimated Clearance of Low Viral Load Prevalent HPV Infections by circumcision status in Kenyan Men

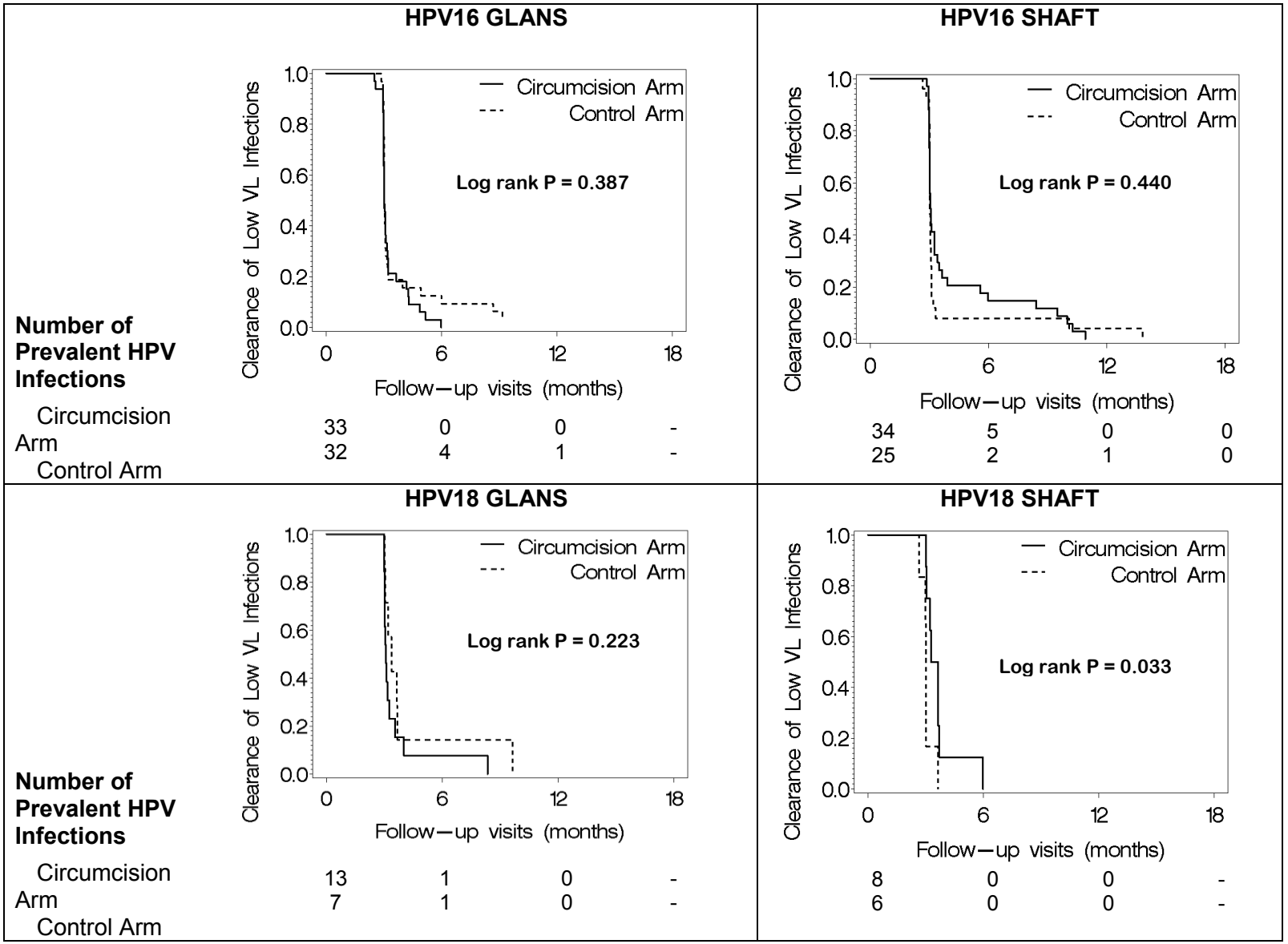


Table 4.1: Baseline Characteristics by Intervention Treatment Arm

Participant Characteristics †	**Circumcision Arm (N= 1096*)**	**Control Arm (N= 1097*)**
Age (years)	20(19-22; 18-28; 1096)	20(19-22; 17-24; 1097)
Education level		
Less than secondary	372 (33.9)	391 (35.6)
Any secondary or above	724 (66.1)	706 (64.4)
Employment status		
Employed and receiving a salary	94 (8.6)	98 (8.9)
Self-employed	303 (27.7)	294 (26.8)
Unemployed	699 (63.8)	705 (64.3)
Marital status		
Not married (no live-in partner)	1024 (93.8)	1018 (93.2)
Not married (with live-in partner)	7 (0.6)	7 (0.6)
Married (not living with wife)	5 (0.5)	15 (1.4)
Married (living with wife)	56 (5.1)	52 (4.8)
Number of partners over lifetime	4 (3-7; 1-120; 1004)	4 (3-7; 1-86; 1016)
Number of sexual partners in previous 6 months		
0	137 (12.5)	136 (12.4)
1	472 (43.2)	472 (44.1)
2+	484 (44.3)	475 (43.5)
Used a condom with intercourse in previous 6 months		
Always	210 (21.9)	208 (21.7)
Inconsistent	511 (53.3)	500 (52.1)
Never	238 (24.8)	251 (26.2)
Herpes simplex virus 2		
Positive	287 (27.3)	278 (26.5)
Negative	764 (72.7)	771 (73.5)
Trichomonas vaginalis		
Positive	21 (1.9)	24 (2.2)
Negative	1064 (98.1)	1059 (97.8)
Neisseria gonorrhoeae		
Positive	30 (2.8)	17 (1.6)
Negative	1053 (97.2)	1066 (98.4)
Chlamydia trachomatis		
Positive	57 (5.3)	42 (3.9)
Negative	1025 (94.7)	1041 (96.1)
Bathing frequency		
Less than daily	23 (2.1)	22 (2.0)
Daily	1063 (97.9)	1062 (98.0)

*This table examines baseline characteristics in men with HPV follow-up data randomized to the circumcision and control arms
† Data are median (IQR; range; n) for continuous data, or n (%) for categorical data. Numbers and percentages may not sum up to totals due to missing values and rounding.

Table 4.2: Effect of circumcision on incident high viral load HPV16 and HPV18 infections over 24 months in Kenyan Men

HPV Type	Site	Treatment Arm	All Samples			Beta-globin Positive Samples		
			Incident High VL Infections*	Person Years	HR (95% CI) †	Incident High VL Infections*	Person Years	HR (95% CI) †
HPV 16	Glans	Circumcision	26	1386.7	0.32 (0.20-0.49)	19	875.7	0.31 (0.18 – 0.50)
		Control	83	1366.1	1	72	976.0	1
	Shaft	Circumcision	18	1443.8	0.79 (0.42 -1.45)	9	689.4	0.76 (0.31 -1.76)
		Control	24	1481.1	1	13	703.4	1
HPV 18	Glans	Circumcision	22	1473.0	0.34 (0.21 – 0.54)	15	902.6	0.40 (0.21 -0.70)
		Control	66	1486.8	1	45	1055.4	1
	Shaft	Circumcision	21	1521.8	0.87 (0.48 – 1.54)	11	725.1	0.67 (0.31 -1.42)
		Control	26	1539.9	1	17	719.7	1

* Incidence was defined as the first type-specific HPV-positive result in men who were negative for that HPV type at the same anatomical site at baseline. High viral load (VL) was defined as >250 copies/scrape for the given HPV type.
† Hazard Ratio (HR) comparing the risk of incident high viral load infections in circumcision versus control arm.

Table 4.3: Association between circumcision and prevalence of high versus low viral load at detection of incident HPV infections

HPV Type	Site	Treatment Arm	All Samples		Beta-globin Positive Samples	
			Incident HPV High VL/ Low VL*	PR (95% CI) †	Incident HPV High VL/ Low VL*	PR (95% CI) †
HPV 16	Glans	Circumcision	26 /43	0.70 (0.50 - 0.99)	19 /26	0.72 (0.53 - 0.98)
		Control	83 / 72	1	72 /51	1
	Shaft	Circumcision	18 / 52	0.93 (0.55 - 1.57)	9 / 22	0.92 (0.45 – 1.86)
		Control	24 / 62	1	13 /28	1
HPV 18	Glans	Circumcision	22 /24	0.59 (0.43 – 0.82)	15/16	0.59 (0.40 -0.87)
		Control	66 /16	1	45/10	1
	Shaft	Circumcision	21 / 6	1.20 (0.88 – 1.62)	11 / 3	1.25 (0.84 -1.86)
		Control	26 /14	1	17/10	1

* Incidence was defined as the first type-specific HPV-positive result in men who were negative for that HPV type at the same anatomical site at baseline. High viral load (VL) was defined as >250 copies/scrape; low viral load was defined as ≤ 250 copies/scrape for the given HPV type.

† Prevalence Ratio (PR) comparing the prevalence of high versus low viral load at HPV detection in men with incident HPV infections in the circumcision versus control arm.

Table 4.4: Clearance of baseline prevalent infections by circumcision status and HPV viral load in Kenyan Men

Site	Viral Load†	Treatment Arm	Cleared infections n/N*	Person Years	HR for Clearance (95% CI) ‡	Estimated Risk of HPV Persistence (95% CI) ††	
						6 months	12 months
HPV16 Infections							
Glans	High	Circumcision	35/ 35	14.4	1.46 (0.91 -2.32)	0.20 (0.09 -0.34)**	0.06 (0.01-0.17)
		Control	40/44	23.8	1	0.55 (0.39 -0.68)**	0.18 (0.09-0.31)
	Low	Circumcision	33/33	9.2	1.31 (0.68 -1.89)	0	0
		Control	31/32	9.3	1	0.13 (0.04 -0.26)	0.03 (0.00 -0.14)
Shaft	High	Circumcision	5/5	1.3	4.34 (0.67 -84.9)	0	0
		Control	3/3	2.2	1	0.67 (0.05 -0.94)	0.33 (0.01 -0.77)
	Low	Circumcision	34/34	12.1	0.82 (0.49 -1.42)	0.15 (0.05 -0.28)	0
		Control	25/25	7.8	1	0.08 (0.01 -0.22)	0.04 (0.00 - 0.17)
HPV18 Infections							
Glans	High	Circumcision	24/24	9.1	2.02 (1.02- 4.08)	0.17 (0.05 -0.34)**	0.04 (0.00 -0.18)
		Control	16/16	10.8	1	0.50 (0.35 -0.71)**	0.25 (0.08 -0.47)
	Low	Circumcision	13/13	3.9	1.79 (0.70 -5.15)	0.08 (0.00 -0.29)	0
		Control	7/7	2.5	1	0.14 (0.01 -0.46)	0
Shaft	High	Circumcision	4/4	1.0	4.33 (0.78 -33.3)	0	0
		Control	6/6	2.3	1	0.15 (0.01 -0.52)	0
	Low	Circumcision	8/8	2.5	0.34 (0.10 -1.12)	0	0
		Control	6/6	1.5	1	0	0

† High viral load was defined as >250 copies/scrape; low viral load was defined as ≤ 250 copies/scrape for the given HPV type.
*Clearance was defined as an HPV-negative result in participants who were positive for that given HPV type at baseline; n= number of cleared HPV infections; N=total number of HPV infections present at baseline.
‡ Hazard Ratio (HR) comparing clearance of prevalent HPV infections at baseline in circumcision versus control arm over 24 months.
††Persistence was defined as an HPV-positive result in participants who were positive for that given HPV type at the same anatomical site in the previous visit. Estimates for risks of persistence and duration since baseline visit were obtained from Kaplan Meier graphs. **P-value <0.05 for z-score test comparing estimated risk of HPV persistence in circumcision versus control arm.

CHAPTER 5
ASSOCIATION BETWEEN PENILE HPV VIRAL LOAD AND HPV CLEARANCE IN UNCIRCUMCISED KENYAN MEN

Overview

Higher viral loads of high-risk HPV infections in women are associated with lower HPV clearance in comparison to lower viral loads; however, the association between HPV viral load and HPV clearance has not been explored among penile HPV infections. Penile swabs from glans and shaft sites were collected from men enrolled in the control arm of a randomized controlled trial (RCT) of male circumcision in Kisumu, Kenya. GP5+/6+ PCR was used to identify HPV DNA types. HPV viral load was measured with LightCyler real-time PCR and classified as high (>250 copies/scrape) or low (≤250 copies/scrape). Most high and low viral load HPV16 or HPV18 infections cleared within 6 months of HPV detection. The hazard of HPV16 clearance at 6 months after first HPV16 detection was found to be lower for high versus low viral load incident infections in the glans [adjusted hazard ratio (aHR) =0.58 (95%confidence interval, 0.36-0.93)]. The 6-month risk of HPV16 persistence was greater for high versus low viral load infections in the glans for both incident infections [Risk Ratio (RR) = 3.28 (1.29-8.36)] and prevalent infections detected at baseline [RR =4.36 (1.68-11.35)]. HPV16 and HPV18 clearance in the shaft was comparable for high and low viral load infections. The lower HPV16 clearance rate of high versus low viral load infections in the glans could possibly be associated with an increased risk of penile SCC development, and may

explain the higher incidence of penile carcinomas observed in the glans than any other penile site. The lower HPV16 clearance in men with high viral load infections may also explain the reported increase in HPV16 transmission to their female partners in comparison to men with low viral load.

Introduction

Persistent infections with human papillomavirus (HPV) high-risk types 16 and 18 are causes of approximately 70% of cervical cancer in women and approximately half of penile squamous cell carcinomas (SCC) in men, particularly basaloid and warty SCC types (1, 9, 10, 33). Women with higher HPV16 or HPV18 viral loads are more likely to have persistent infections and to progress to high grade cervical intraepithelial neoplasia (CIN) than those with lower viral loads (11-17). The association between HPV viral load and HPV clearance has not yet been examined in penile HPV infections in men; higher HPV16 or HPV18 viral loads could possibly be associated with lower HPV clearance and increased progression to penile SCC.

Higher HPV16 or HPV18 viral loads in men are associated with an increased prevalence of flat penile lesions and greater HPV type concordance with female partners in comparison to lower viral loads (2, 52, 53). As a result, higher HPV viral load in men is suggested to be associated with increased HPV transmission to their female partners in comparison to lower HPV viral load (2). It is believed that lower HPV clearance of high versus low viral load HPV infections in men may enhance HPV transmission to their female partners, however, the association between higher HPV viral load and lower HPV clearance in penile HPV infections is yet to be established.

We present the results of a longitudinal study examining the association between viral load and the clearance of penile HPV infection in uncircumcised men in Kisumu, Kenya. We examine penile HPV viral load measured at first detection of HPV16 or HPV18 infections in the glans and shaft sites and the subsequent rate of HPV clearance over a study period of 24 months. The association between HPV viral load and HPV clearance is examined separately for incident infections and infections detected at baseline (prevalent infections). We have previously examined the effect of male circumcision on HPV viral load, which included men from this study in the control group (55); here we examine the association between penile HPV viral load and the clearance of penile HPV infections among these uncircumcised men.

Methods

Study population and enrollment

Uncircumcised men were screened between February 4, 2002 and September 6, 2005 in Kisumu, Kenya to participate in an RCT of male circumcision (5). The main aim of the trial was to determine the effectiveness of male circumcision in reducing HIV incidence. Baseline study inclusion criteria included being uncircumcised, aged 18-24, HIV negative, sexually active within the past 12 months and having blood hemoglobin ≥ 90 g/l. Study participants were recruited from STI clinics, workplaces and community organizations. The study protocol was approved by Institutional Review Boards of the Universities of Illinois at Chicago, Manitoba, Nairobi and North Carolina; RTI International and VU University Medical Center.

This analysis includes men from the control arm of the RCT who consented to the collection of penile exfoliated cells and their shipment overseas for HPV DNA and HPV viral load testing. Of the 2784 uncircumcised men enrolled in the RCT, 2299 (83%) consented to provide penile swab samples and had baseline HPV data; 1159 men were randomized to immediate circumcision and 1140 to the control arm. Eligibility for inclusion in clearance analyses was determined for each HPV type (HPV16 or 18) and anatomical site (glans or shaft) separately. Participants assigned to the control arm who: i) had no HPV follow-up data; ii) were HPV16 or HPV18-negative throughout follow-up; iii) had their first HPV16 or HPV18-positive result at the last study visit; or iv) had missing HPV viral load data were excluded from clearance analyses for that specific HPV type and anatomical site (Figure 5.1.).

Follow-up and specimen collection

Participant follow-up visits were scheduled at 6 month intervals for a period of 24 months. A standardized questionnaire on sociodemographic characteristics and sexual behavior was administered by a trained male interviewer at each visit (5). Penile exfoliated cell specimens were collected by a trained physician or clinical officer at each study visit (18). Samples were collected from two anatomical sites: (i) penile shaft and external foreskin (shaft specimen); and (ii) glans, coronal sulcus, and the internal tissue of the foreskin (glans specimen). Two pre-wetted type 3 Dacron swabs were used to collect penile exfoliated cells from the two separate anatomical sites. Each swab was placed in a sterile 15 ml centrifuge tubes with 2-mL of 0.01 mol/L Tris-HCl 7.4 pH buffer with the participant's identification (ID) number, the visit number, and anatomical site of specimen (glans or shaft). Each sample was centrifuged at high speed for 10 minutes in the clinic

laboratory on the same day of collection. Excess Tris-HCl buffer was discarded using a Pasteur pipette, and the pellet was resuspended in the same volume of 0.01mol/L of Tris-HCl buffer and vortexed. Diluted cell pellets were frozen and stored at -75°C. All samples were transported via Fedex in a liquid nitrogen dry shipper from Kisumu to the Department of Pathology at VU Medical Center in Amsterdam, Netherlands, for HPV DNA testing.

HPV DNA, HIV and STI Testing

DNA was isolated from penile exfoliated cell samples using NucleoSpin 96 Tissue kit (Macherey-Nagel, Germany) and a Microlab Star robotic system (Hamilton, Germany) according to manufacturers' instructions. Presence of human DNA was evaluated by beta-globin specific PCR, followed by agrose gel electrophoresis (19, 20).HPV positivity was assessed by GP5+/6+ PCR followed by hybridization of PCR products using an enzyme immunoassay readout with two HPV oligoprobe cocktail probes that detect 44 HPV types (high-risk: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68; low-risk: HPV6, 11, 26, 30, 32, 34, 40, 42, 43, 44, 53, 54, 55, 57, 61, 64, 67, 69, 70, 71, 72, 73, 81, 82, 83, 84,cand85, 86, cand89, JC9710). Subsequent HPV genotyping was performed by reverse line blot (RLB) hybridization of PCR products (19, 20).

HIV testing was performed at each visit using two HIV antibody rapid tests (Determine, Abbott Diagnostic Division, Hoofddorp, Netherlands; and Unigold, Trinity Biotech, Wicklow, Ireland), and confirmed by double ELISA (Adaltis, Montreal, Canada; Trinity Biotech, Wicklow, Ireland) at the University of Nairobi. A physician or clinical officer conducted a genital exam during which a swab (if symptomatic) or urine sample were

collected for polymerase chain reaction (PCR) detection of *C. trachomatis* and *N. gonorrhea* (Roche Diagnostics). Blood was collected for herpes simplex virus type 2 (HSV-2; enzyme-linked immunosorbent assay (ELISA) [Kalon]).

HPV Viral Load Testing

HPV DNA viral load testing was performed on penile exfoliated cell samples that contained HPV16 or HPV18 using a real time PCR assay and a LightCycler instrument (Roche, Mannheim, Germany) (21). DNA extraction and purification were conducted according manufacturer instructions using 100 µl of the remaining cell suspensions. All samples were run in duplicate in the same run and resulting viral load values were averaged. Dilutions of cloned HPV DNA were used to determine the standard curve for the HPV target. To calculate the number of copies per sample, the amount of HPV DNA in one femtogram was multiplied by the dilution factor and divided by the weight of the cloned pHPV viral genome. HPV viral load was categorized as low (≤250 copies/scrape) or high (>250 copies/scrape) for each HPV16 and HPV18 infection at each anatomical site. The 250 copies/scrape cut-point for high versus low viral load was chosen because of its significance in previous HPV viral load studies; men with high viral load values (>250 copies/scrape) have been found to have a higher prevalence of flat penile lesions and increased HPV type concordance with female partners (2, 53).

Statistical Analyses

The distribution of participant characteristics by HPV16 or HPV18 viral load (high or low) was examined for infections prevalent at baseline. For these analyses, HPV viral load results from the glans and shaft samples were combined; high viral load was defined as

>250 copies/scrape in the glans or shaft, and low viral load was defined as ≤ 250 copies/scrape in both the glans and shaft. Baseline characteristics examined included age, education, employment, marital status, bathing frequency, number of sexual partners and condom use in the previous 6 months.

HPV clearance analyses were performed separately for incident and prevalent HPV16 or HPV18 infections. We also examined HPV clearance separately for glans and shaft infections since HPV clearance has been shown to vary by anatomical site (8) . For each participant, only the first detected HPV16 or HPV18 infection (incident or prevalent) at each anatomical site (glans or shaft) was included in clearance analyses. Time-to-clearance since HPV detection was calculated starting at the visit date of first HPV detection; for prevalent infections the start date was baseline, and for incident infections it was the visit date of first HPV detection for each HPV type at each anatomical site. Clearance was assumed to occur at the mid-point between the last HPV-positive result and the first subsequent HPV-negative result. Men were censored at their last observed visit if they remained HPV positive for the same HPV type at the same anatomical site for each observed consecutive visit.

HPV viral load measurements at first detection of HPV16 or HPV18 prevalent or incident infections were used to classify the infections as high (>250 copies/scrape) or low (≤ 250 copies/scrape) viral load infections. For all the clearance analyses, HPV viral load was analyzed as a time-fixed exposure; few HPV16 or HPV18 infections (8.4% overall) were observed to change viral load groups during follow-up. The actual circumcision status of eligible men at each visit was reviewed to verify that they were uncircumcised during HPV detection and clearance; 12 men assigned to the control arm

were circumcised later in the study, however, in each case circumcision was performed after clearance of HPV infections so these men were included in clearance analyses.

The Kaplan-Meier method was used to estimate the cumulative incidence for clearance of high versus low viral load HPV infections at 6 months (6-month risk) after HPV detection. Risk ratio estimates were calculated to compare 6-month and 12-month clearance risk estimates of high versus low viral load infections (51). Potential confounders of the association between HPV viral load and HPV clearance were identified from the literature, and analyzed using a DAG (50). Confounders identified and included in Cox regression models were: HIV infection, condom use, number of sex partners, bathing frequency, infection with multiple HPV types, educational attainment, and infection with other STIs (HSV-2, *Trichomonas vaginalis*, *Chlamydia trachomatis* and *Neisseria gonorrhoea*e). Adjusted hazard ratios (aHRs) comparing the clearance hazard at 6 months for high versus low viral load HPV infections were estimated for each HPV type and anatomical site. HRs at 6-months after HPV detection could not be obtained in cases where all high viral load or low viral load infections cleared by 6 months after HPV detection. An interaction term between the HPV viral load group and time-to-clearance was included in all the Cox regression models to account for non-proportional hazards in clearance of high and low viral load HPV infections. Sensitivity analyses for clearance of prevalent and incident infections were performed by restricting analyses to samples that tested positive for the presence of human DNA with the beta-globin test.

Results

Of the 1140 men randomized to the control arm of the main RCT, 1097 (96.2%) men had follow-up HPV data (Figure 5.I). Eligibility for inclusion in clearance analyses was determined separately for each HPV type and for each anatomical site. Of the samples with HPV-positive results, similar proportions were excluded for missing HPV16 or HPV18 viral HPV load results [HPV16 glans= 7(3.1%), HPV16 shaft =6(5.3%), HPV18 glans=8 (7.4%) and HPV18 shaft =2(3.9%)]. Also, similar proportions of incident HPV infections were observed at the 24-month visit (last study visit) and thus excluded from clearance analyses for each HPV type and anatomical site [HPV16 glans =37(23.9%), HPV16 shaft =17(19.8%), HPV18 glans= 17 (20.2%) and HPV18 shaft = 11(27.5%)] (Figure 5.I).

Distribution of Participant characteristics by HPV viral load at baseline

Of the 2299 uncircumcised men enrolled in the RCT with baseline HPV results, 239 (10%) men were HPV16-positive and 103 (5%) were HPV18-positive in either the glans or shaft sites and had HPV viral load data at baseline (Table 5.1). Few men had infections in both the glans and shaft [25(11%) for HPV16; 8 (8%) for HPV18], and in each case the HPV viral load category was the same for both anatomical sites. Infection with multiple HPV types at baseline was associated with high versus low HPV viral load for both HPV16 and HPV18 prevalent infections. For HPV18 prevalent infections, the presence of sexually transmitted infections (HSV-2, *Trichomonas vaginalis*, *Chlamydia trachomatis* or *Neisseria gonorrhoeae*) was associated with high versus low viral load, however this association was not observed for HPV16 prevalent infections. Men with high versus low viral HPV16 or HPV18 viral load appeared to have similar distributions

of age, employment status, educational attainment, number of sexual partners and condom use in previous 6 months, marital status and bathing frequency at baseline.

HPV Viral load and Clearance of Penile HPV Infections Prevalent at Baseline

At baseline, a greater proportion of high viral load HPV infections was detected in the glans for both HPV16 [57.9% (N=44)] and HPV18 [69.6% (N=16)], than in the shaft [HPV16 =10.7% (N=28); HPV18 =50.0% (N=6)] (Table 5.2). All HPV18 infections and most HPV16 (93.4%) infections detected at the baseline visit in the glans or shaft cleared during follow-up (Figure 5.2). In the glans, the 6-month risk of HPV persistence was greater in high versus low viral load infections for HPV16 [RR =4.36 (1.68-11.35)] (Table 5.2).Less precise results were obtained for the 6-month risk of HPV persistence for high and low viral load HPV16 infections in the shaft [RR = 3.50 (0.53 - 22.92)]. Results suggest that the hazard of HPV16 clearance at 6 months after baseline [aHR = 0.61 (0.34-1.09)] was lower for high versus low HPV16 viral load infections in the glans after adjustment for possible confounders. Sensitivity analyses restricted to HPV 16 prevalent infections in the glans that were β-globin positive (52.6%) found weaker and less precise results [aHR = 0.88 (0.34-2.36)] for clearance of high versus low viral load HPV16 prevalent infections.

HPV Viral Load and Clearance of Incident Penile HPV Infections

Of the incident HPV infections observed, a greater proportion of high viral load infections was detected in the glans for both HPV16 [55.1% (N=65)] and HPV18 [80.1% (N=54)] than in the shaft [HPV16 =29.0% (N=20); HPV18 =65.5% (N=19)] (Table 5.2). Most of the incident HPV16 (89.1%) or HPV 18 (85.4%) infections in the glans or shaft

cleared during follow-up (Figure 5.3). The 6-month risk of HPV16 persistence was greater for high versus low HPV16 viral load infections in the glans [6-month RR = 3.28 (1.29 - 8.36)] (Table 5.2). The hazard of HPV16 clearance at 6 months after HPV detection [aHR = 0.58 (0.36-0.93)] was lower for high versus low viral load HPV16 infections in the glans after adjustment for possible confounders. In the shaft, the hazard of HPV16 clearance at 6 months after HPV detection was similar for high and low viral load HPV16 infections [aHR = 0.83 (0.34 -1.65)]. For HPV 18 incident infections, the 6-month risk of HPV persistence was similar for high and low viral load infections in the glans [6-month risk = 1.78 (0.21 -14.90)] (Table 5.2). Sensitivity analyses restricted to HPV 16 incident infections that were β-globin positive in the glans (59.3%) or shaft (50.7%) found less precise results as the main analyses [aHR glans = 0.52 (0.22- 1.15); aHR shaft = 0.32 (0.04- 1.69)] for the clearance of high versus low viral load infections.

Discussion

Among uncircumcised Kenyan men, the clearance rate of HPV16 incident infections in the glans was found to be lower for high versus low HPV viral load infections at 6 months after HPV16 detection. For both prevalent and incident infections, the 6-month risk of HPV16 persistence was greater for higher than lower HPV viral load infections in the glans. A greater proportion of high viral load infections was observed in the glans than the shaft for both prevalent and incident HPV16 or HPV 18 infections. In the shaft, the risk of HPV16 or HPV18 persistence to 6 months after detection was similar for high and low viral load infections.

This is the first study, to our knowledge, to examine the association between penile HPV viral load and subsequent HPV clearance. Direct comparison of our results to those from HPV viral load studies in women is complicated by the differences in detected HPV types, viral load categories and the different cervical cytology profiles (normal versus abnormal) across studies. One study in French women with normal cytology found that those with higher viral loads of high-risk HPV types (≥10pg/ml) at baseline had lower HPV clearance than women with lower viral loads [risk ratio= 0.65 (0.44-0.94)] (43). Another study in Danish women with abnormal cytology found that those with the 50% lowest viral load had an increased risk of HPV16 clearance [OR =5.0 (1.7 -15)] (56). A study in Korean women who underwent conization treatment for CIN found that higher pre-cone HPV viral load (≥ 100 RLU/PC) was the only risk factor for HPV persistence after conization [OR =5.75 (1.08 -30.52)] (42).Two studies found conflicting results in examining a dose-response relationship between HPV viral load and HPV clearance in women with normal cytology; one in Colombian women found no dose-response relationship whereas the other in Canadian women found that HPV clearance was greater in women with lower tertiles as compared to higher tertiles of HPV16 or HPV 18 viral load [HR =2.4 (1.8-6.2)] (12, 57). Thus, results on HPV viral load and HPV clearance in women are inconclusive, but results from several studies suggesting that high HPV viral load is associated with lower HPV clearance, are similar to our findings in Kenyan men (12, 42, 43, 56, 57).

The lower HPV clearance observed in cases of high HPV viral load infections may be explained by the greater number of virus-infected cells the immune system may have to eliminate as compared to low viral HPV viral load infections (49). Alternatively,

lower clearance of high viral load infections may reflect a weaker immune response to the HPV virus and virus-infected cells (49). Our baseline risk factor analyses found that high HPV16 or HPV18 viral load is associated with a greater presence of STIs or infections with multiple HPV types; this suggests that high HPV viral loads are possibly more likely to occur in men with weaker immune systems in comparison to low HPV viral loads.

We also found a greater proportion of high viral load infections in glans than the shaft for both HPV16 and HPV18 incident and prevalent infections. These findings are different from results of a cross-sectional study of men in the United States (Arizona and Florida) that found higher HPV viral load in the shaft than any other penile site (58). This difference is likely due to the fact that 86% of men in the U.S. cross-sectional study were circumcised, whereas all the men in our study were uncircumcised during detection of HPV infection and HPV clearance. In uncircumcised men, the moist subpreputial cavity that surrounds the glans is believed to promote HPV survival and HPV viral replication (39).

Main strengths of this study include the extensive longitudinal data on HPV viral load, quantitative HPV viral load measurements by real time PCR, and the separate evaluation of samples from the glans and the shaft anatomical sites. Our main study limitation is that HPV viral load measurements were not normalized to account for number of cells in each swab sample (viral load copies/cell). However, the copies/scrape units have been previously used to show the associations between high HPV viral load (>250 copies/scrape) and higher prevalence of flat penile lesions as well as increased HPV type concordance with female partners (2, 53). We also observed fairly low beta-globin positivity

in our samples (56.7% in glans and 48.4% in shaft samples). It is possible that HPV viral load measurements in this study may be underestimated, although similar prevalences of beta-globin positivity have been observed among men (7). Sensitivity analyses restricted to beta-globin positive samples generated less precise results, however estimates suggested that high versus low viral load HPV infections were associated with lower HPV clearance as observed in the main analyses. Our analyses also examined HPV viral load measurements every 6 months; it is possible that different time intervals between visits could produce different results for the association between HPV viral load and clearance. The challenge of defining HPV clearance and persistence based on study visits is still an unresolved issue in HPV research, however, the 6–month time interval used in this study is comparable to the reported average durations of penile HPV infection in men (range 5.9 -7.5 months) (31, 48). Lastly, the interpretation of our results is limited by the inability to distinguish between integrated and episomal HPV DNA; studies in women have shown that the physical state of viral DNA is important in understanding the association between HPV viral load, HPV clearance and progression to CIN (59-61). Future research distinguishing integrated and episomal HPV DNA by examining the ratio of expressed E2 and E6 HPV genes is needed to further understand the association between detected HPV viral load and HPV clearance.

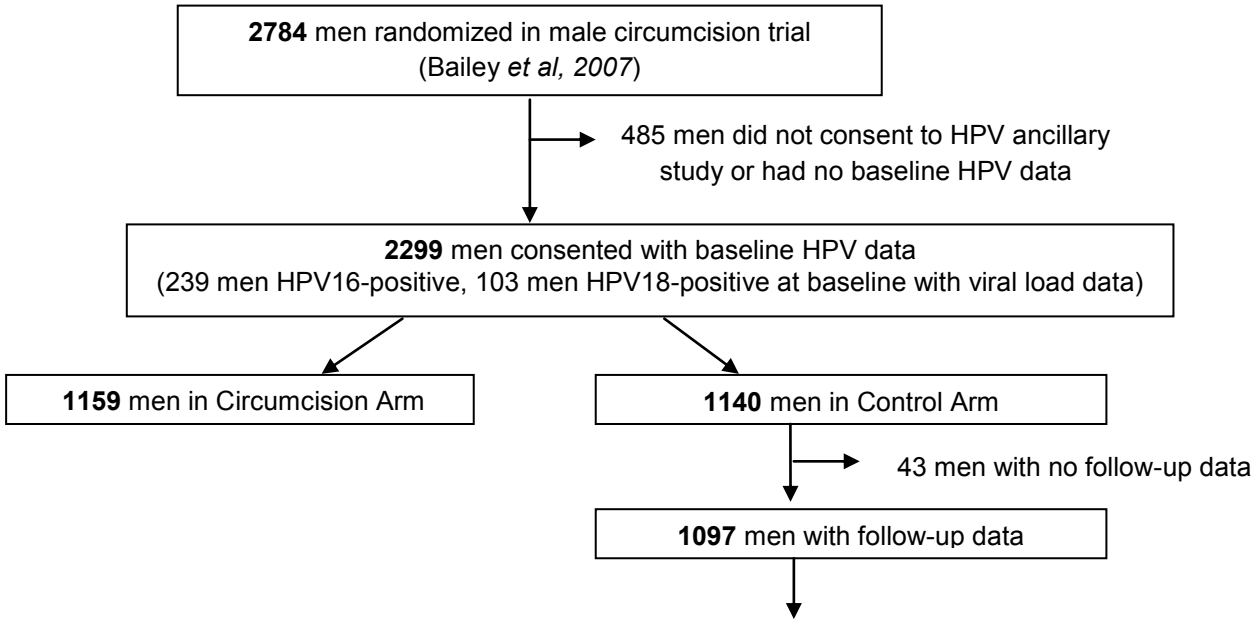
High HPV16 viral load in the glans is associated with lower HPV16 clearance and thus, could possibly be associated with an increased risk of penile squamous cell carcinoma development in the glans in comparison to low HPV16 viral load. The majority of anatomically classified penile carcinomas are reported to occur in the glans (62%) than any other penile site; this may be in-part due to the lower clearance of high versus low viral load HPV infections observed in the glans but not in the shaft (62). Our

results may also explain why men with higher HPV16 viral loads may have greater HPV16 transmission to their female partners than those with lower viral loads; the longer duration of infection for high versus low viral load infections in men could increase the possibility of HPV transmission to their female partners (2). The generalizability of our results is limited to uncircumcised men since circumcision has been found to independently enhance HPV clearance; future research is needed to examine if the association between HPV viral load and HPV clearance exists in circumcised men (7, 8).

In conclusion, this study found that most HPV16 or HPV18 infections in the glans or shaft cleared within 6 months of first detection in young uncircumcised men. For both prevalent and incident infections, the 6-month risk of HPV16 persistence was greater for high viral load than low viral load infections in the glans. High viral load of HPV16 incident infections in the glans was associated with lower HPV clearance at 6 months after HPV detection in comparison to low HPV16 viral load, and thus, could possibly be associated with an increased risk of penile SCC development in uncircumcised men. Our results also provide further support for the suggested association between high HPV viral load in men and increased HPV transmission to their female partners.

Tables and Figures

Figure 5.1: Study flowchart of samples included in analyses of HPV viral load and HPV clearance



HPV Types	HPV 16		HPV 18	
Anatomic Sites	Glans	Shaft	Glans	Shaft
Exclusion Criteria for Samples:				
1. HPV (-) throughout study	859	977	982	1043
2. First HPV(+) result at 24month (last) visit	37	17	17	11
3. Missing Viral load	7	6	8	2
Total Samples included in Analyses				
Prevalent HPV Infections	76	28	23	12
Incident HPV Infections	118	69	67	29

Figure 5.2*:* Estimated clearance of prevalent HPV infections by HPV Viral Load in uncircumcised Kenyan Men

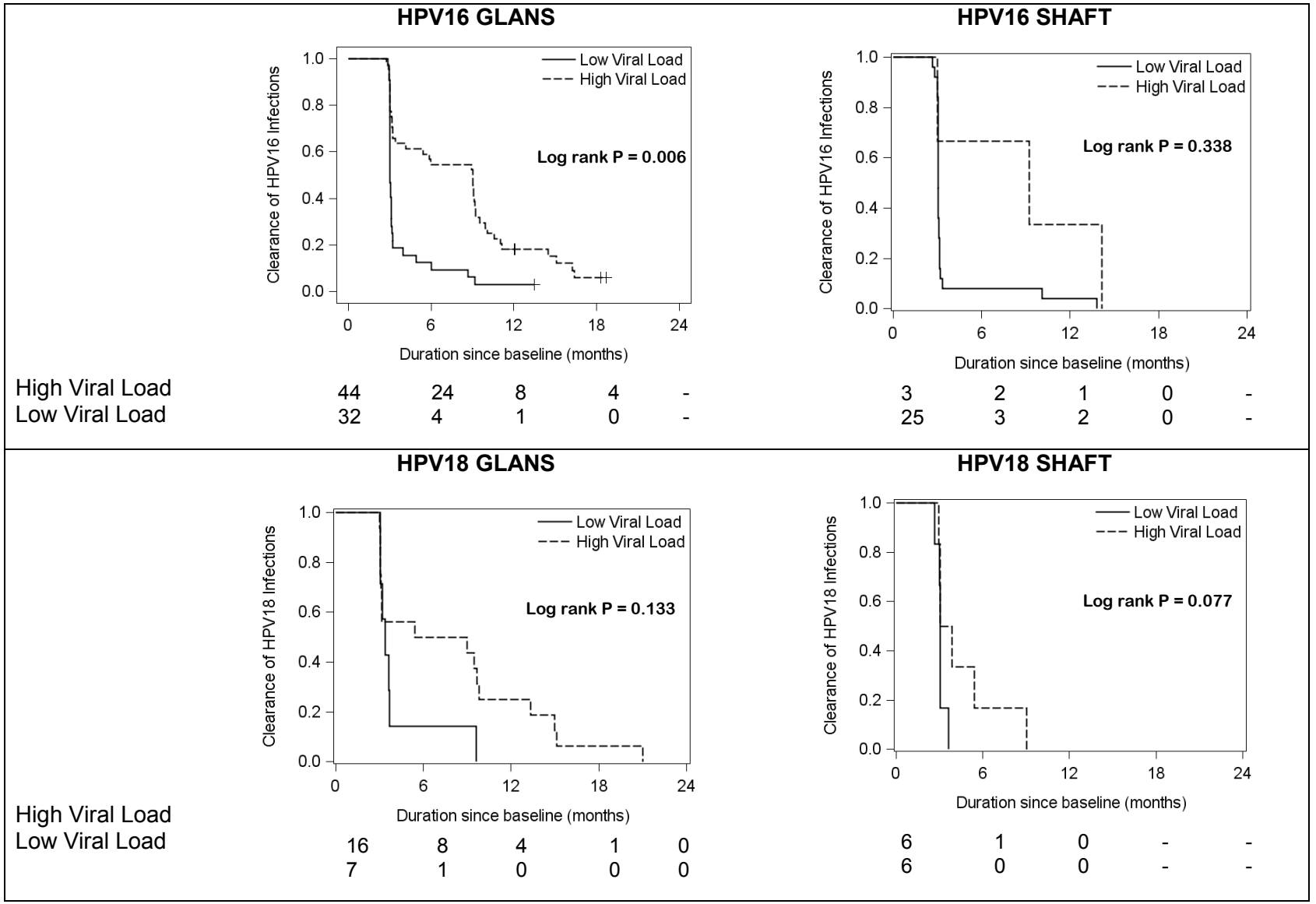
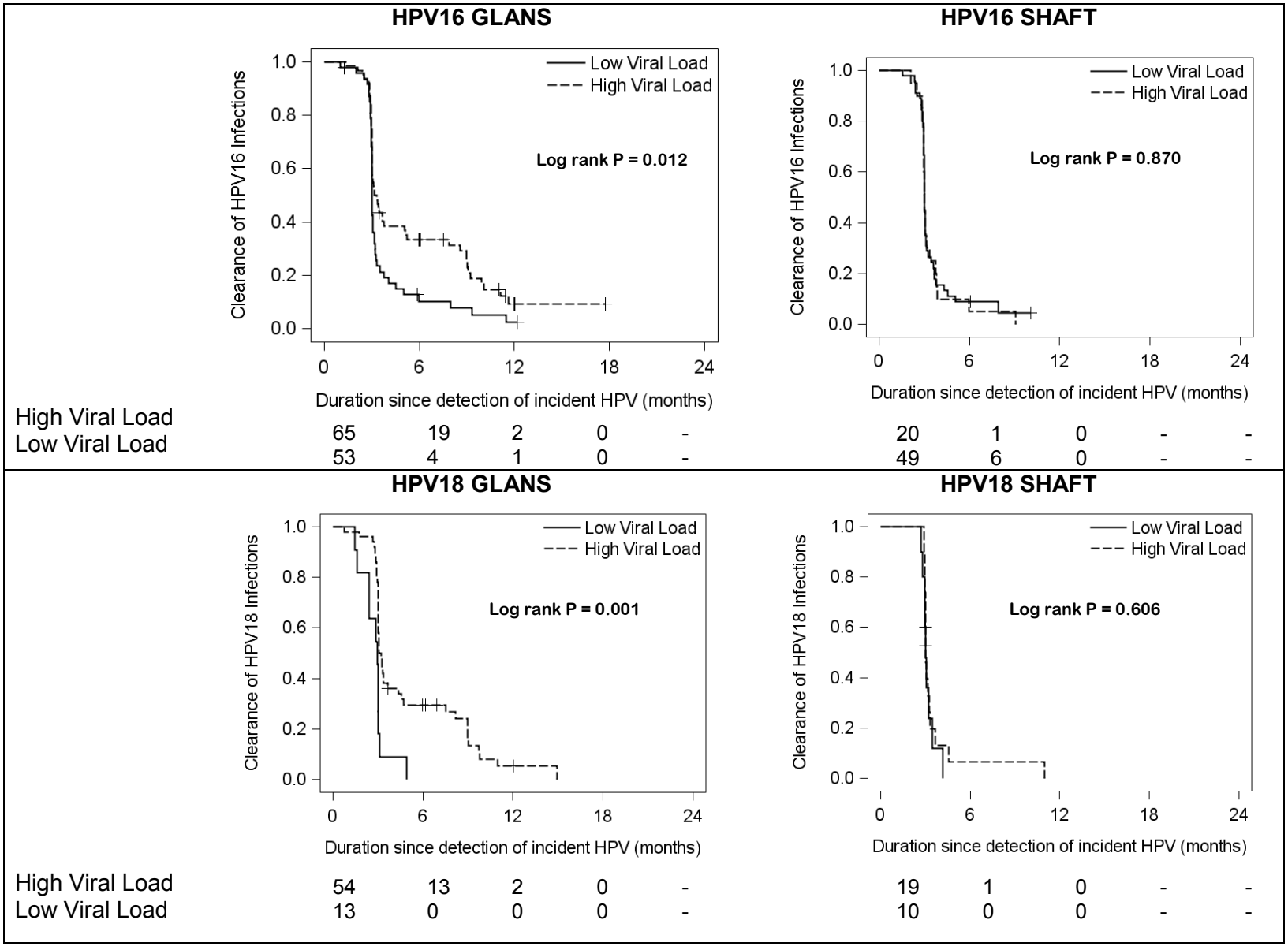


Figure 5.3*:* Estimated clearance of incident HPV infections by HPV Viral Load in uncircumcised Kenyan Men



High Viral Load	65	19	2	0	-	20	1	0	-	-
Low Viral Load	53	4	1	0	-	49	6	0	-	-
High Viral Load	54	13	2	0	-	19	1	0	-	-
Low Viral Load	13	0	0	0	-	10	0	0	-	-

Table 5.1: Participant characteristics, stratified by baseline HPV viral load for HPV types 16 and 18 in men in Kisumu, Kenya

Demographic characteristics†	HPV16 Prevalent Infections (N =239)*		HPV18 Prevalent Infections (N=103)*	
	High HPV16 Viral Load [a] (N =109)	Low HPV16 Viral Load [a] (N =130)	High HPV18 Viral Load [a] (N =65)	Low HPV18 Viral Load [a] (N =38)
Age (years)	20 (19-21;18-24;109)	20 (19-22;18-24;130)	20 (19-22;18-24;65)	20 (20-22;18-24;38)
Education level				
Less than secondary	40 (36.7)	55 (42.3)	23(35.4)	15 (39.5)
Any secondary or above	69 (63.3)	75 (57.7)	42(64.6)	23 (60.5)
Employment status				
Employed and receiving a salary	6 (5.5)	7(5.4)	7 (10.8)	3 (7.9)
Self-employed	38 (34.7)	41 (31.5)	21 (32.3)	12 (31.6)
Unemployed	65 (59.6)	82 (63.1)	37 (56.9)	23 (60.5)
Marital status				
Not married (no live-in partner)	103 (95.4)	125 (96.1)	61 (93.8)	34 (89.5)
Married (not living with wife)	2 (1.8)	0	4 (6.2)	3 (7.9)
Married (living with wife)	3 (2.8)	5 (3.9)	0	0
Infection with Multiple HPV types [‡]				
Yes	74 (67.9)	75 (57.7)	52 (80.0)	23 (60.5)
No	35 (32.1)	55 (42.3)	13 (20.0)	15 (39.5)
Infection with sexually transmitted infections [§]				
Positive	32 (29.3)	45 (34.6)	24 (36.9)	7 (18.4)
Negative	77 (70.7)	85 (65.4)	41 (63.1)	31 (81.6)
Number of partners in previous 6 months				
0	12 (11.1)	12 (9.3)	8 (12.3)	4 (10.5)
1	41 (38.0)	63 (48.8)	27 (41.5)	19 (50.0)
2+	55 (50.9)	54 (41.9)	30 (46.2)	15 (39.5)
Used condom with intercourse in previous 6 months	17 (17.5)	32 (27.1)	11 (18.6)	5 (14.7)
Always	54 (55.7)	59 (50.0)	33 (55.9)	18 (52.9)
Inconsistent	25 (25.7)	27 (22.9)	14 (23.7)	11 (32.4)
Never				
Bathing Frequency				
Less than daily	3 (2.8)	3 (2.3)	2 (3.1)	2 (5.3)
Daily	105 (97.2)	127 (97.7)	63 (96.9)	36 (94.7)

* This table includes only men with detected HPV16 DNA or HPV18 DNA at baseline. High viral load was defined as >250 copies/scrape in the glans or shaft. Low viral load was defined as ≤ 250 copies/scrape in the both the glans and shaft. Numbers and percentages may not sum up to totals due to missing values and rounding.
† Data are median (IQR; range; n) for continuous data, or n (%) for categorical data.
‡ Infection with multiple HPV types was defined as co-infection with at least one other HPV type
§ Positive result for HSV-2, *Trichomonas vaginalis*, *Chlamydia trachomatis* or *Neisseria gonorrhoea* was examined.

Table 5.2: The association of penile HPV viral load and HPV clearance in uncircumcised Kenyan Men

HPV Type	Site	Viral Load†	Cleared infections (n/N*)	Person-Years	Estimated 6-month Risk of HPV Persistence (95% CI) ††	Estimated 6-month RR of HPV Persistence (95% CI) ††	Adjusted HR (aHR) for clearance at 6-months (95% CI) ‡
Prevalent HPV infections							
HPV16	Glans	High	40/44	28.88	0.56 (0.40-0.69)	4.36 (1.68 – 11.35)	0.61 (0.34-1.09)
		Low	31/32	11.61	0.13 (0.01-0.24)	1	1
	Shaft	High	3/3	2.19	0.67 (0.13-0.99)	8.34 (1.76- 39.36)	-- ¶
		Low	25/25	7.81	0.08 (0.01-0.19)	1	
HPV 18	Glans	High	16/16	10.75	0.50 (0.26-0.75)	3.50 (0.53- 22.92)	-- ¶
		Low	7/7	2.47	0.14 (0.03-0.40)	1	
	Shaft	High	6/6	2.28	0.17 (0.00-0.46)	-- ¶	-- ¶
		Low	6/6	1.53	0		
Incident HPV infections							
HPV16	Glans	High	52/65	27.20	0.34 (0.22-0.45)	3.28 (1.29 – 8.36)	0.58 (0.36-0.93)
		Low	45/53	14.96	0.10 (0.01-0.19)	1	1
	Shaft	High	20/20	5.86	0.09 (0.01-0.17)	1.78 (0.21 -14.90)	0.83 (0.34 -1.65)
		Low	42/49	13.11	0.05 (0.00-0.15)	1	1
HPV 18	Glans	High	45/54	19.92	0.30 (0.17-0.42)	-- ¶	-- ¶
		Low	11/13	2.56	0		
	Shaft	High	17/19	5.14	0.06 (0.00-0.18)	-- ¶	-- ¶
		Low	9/10	2.36	0		

†High viral load was defined as >250 copies/scrape; low viral load was defined as ≤ 250 copies/scrape

* Clearance was defined as an HPV-negative result in participants who were positive for that given HPV type at previous visit; n= number of cleared HPV infections; N=total number of HPV infections observed

‡ Adjusted Hazard Ratio (aHR) comparing clearance of high versus low viral load infections at 6 months after infection. HR adjusted for infection with multiple HPV types, number of sex partners, bathing frequency, education, HIV infection, infection with other STIs and condom use.

†† Persistence was defined as an HPV-positive result in participants who were positive for that given HPV type in the previous visit. Estimates for risk and RR of persistent infections at 6 months since infection were derived from Kaplan Meier survival estimates

¶ Risk Ratio (RR) or adjusted hazard ratio (aHR) could not be obtained because all HPV infections in one group had cleared at 6 months after HPV detection

CHAPTER 6
CONCLUSION

The first aim of this dissertation was to examine the association between male circumcision and HPV viral load in men aged 18-24 in Kisumu, Kenya. As hypothesized, we found that male circumcision reduces acquisition of high viral load infections for both HPV16 [HR =0.32(0.20-0.49)] and HPV18 [HR=0.34(0.21-0.54)] in the glans. Among men with incident infections in the glans, prevalence of high versus low viral load at HPV detection was lower in the circumcision than control arm for HPV16 [PR =0.70 (0.50-0.99)] and HPV18 [PR=0.59(0.43-0.82)]. Although overall HR estimates suggested that clearance of baseline prevalent high and low infections was comparable in circumcision and control arm, the risk of persistence to 6 months after baseline for high viral load infections was lower in the circumcision than control arm in the glans for HPV16 and HPV18. Interestingly, we found that the risk of persistence to 6 and 12 months for prevalent infections detected at baseline was similar in circumcised men with high HPV viral load and uncircumcised men with low HPV viral load, for both HPV types and anatomical sites.

High HPV viral load in men is suggested to be associated with increased HPV transmission to female partners (2). Also, fewer HPV infections and lower HPV viral loads have been reported in female partners of circumcised than uncircumcised men (40, 54). Findings from this analysis suggest that male circumcision could potentially

reduce HPV transmission to women by reducing acquisition and enhancing clearance of high HPV viral load infections in men. Voluntary Medical Male Circumcision for HIV prevention is currently being promoted in East and Southern Africa, our findings suggest that male circumcision may also be beneficial in decreasing HPV transmission from men to women (63).

The second aim of this dissertation was to examine the association between viral load at HPV detection and HPV clearance in uncircumcised men. This analysis was conducted only in uncircumcised men since male circumcision has been shown to enhance HPV clearance (7, 8). Analyses showed that the clearance rate of HPV16 incident infections in the glans was lower in high viral load than low viral load infections at 6 months after HPV16 detection [aHR =0.58 (0.36-0.93)]. For both prevalent and incident infections, the risk of HPV16 persistence to 6 after detection was greater for high viral load than low viral load infections in the glans. A greater proportion of high viral load infections was observed in the glans than the shaft for both prevalent and incident HPV16 or HPV 18 infections. The risk of HPV16 or HPV18 persistence to 6 months after detection was similar for high and low viral load infections in the shaft.

To our knowledge this is the first study to examine the association between penile HPV viral load and HPV clearance. High HPV16 viral load in the glans was associated with a reduced HPV clearance, and thus, could possibly be associated with development of penile SCC. The lower clearance rate and longer duration of infection in men with high versus low HPV16 viral load could also explain the suggested increase in HPV16 transmission to their female partners.

HPV viral load measurements in women have been suggested to have possible prognostic value in detecting HPV infections that are more likely to persist and possibly progress to CIN during HPV screening (45, 56). HPV screening in men is not recommended since penile HPV infections are common and likely to clear as shown in our results (64). These analyses add to the current understanding of the role of HPV viral load in penile HPV clearance and possibly penile SCC development in men, as well as transmission of HPV infections to their female partners.

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