THE ASSOCIATION BETWEEN THE PREVALENCE OF HUMAN PAPILLOMAVIRUS, MALE CIRCUMCISION AND THE FORESKIN

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Requirements for the degree of Master of Science in Medicine

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DECLARATION

I, Ewaldé Cutler, declare that the work presented in this dissertation contains both unaided and work performed in collaboration with Prof Bertran Auvert. It is being submitted for the degree of Master of Science in Medicine in the University of Witwatersrand, Johannesburg. This work has not been submitted before for any degree or examination at this or any other university.

_____

Ewaldé Cutler

_____day of _____,2013

DEDICATION

I dedicate this thesis to:

My late husband, Gary Milton Cutler

I know you would be proud.

My two wonderful daughters, Gabrielle and Avigal

Without their unwavering patience, constant support of my academic pursuits, their unyielding love and understanding, the completion of this thesis would not have been possible.

My parents, Flip and Elsabé Cronjé

For the love they have given me throughout my life.

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ABSTRACT

Genital human papillomavirus is one of the most common sexually transmitted infections and a significant cause of morbidity and mortality worldwide, including South Africa. Adult medical male circumcision (MMC) trials in Africa have shown decrease in acquisition of Human Immunodeficiency virus and other sexually transmitted infections.

In this follow-up study, swabs that were collected 21 months post-circumcision were tested for the presence of 13 high risk HPV and 24 low risk HPV, genotypes. Results show that 17.5% and 14.3% participants were infected with at least one low risk or high risk strain of HPV respectively. The most common types of high-risk HPV were type 16 (3.5%) and 18 (3.1%). These results suggest that there is an inverse association between MMC and HPV acquisition.

Participants with at least one high risk strain of HPV were 4.6 times more likely to acquire HIV than participants without high-risk HPV. This was associated exclusively with high-risk HPV infection. HIV acquisition increased dramatically in men with multiple subtypes of HPV.

In a second study, more HPV genotypes were detected on the inner foreskin tissue than on the outer foreskin tissue, highlighting its vulnerability to HPV. Washing of the penis, thus removing possible contaminants reduces the number of detectable HPV genotypes.

This study confirmed that the uncircumcized foreskin, in particular the inner foreskin mucosa, offers a rich environment for HPV infections. This data will allow more insight into the HPV prevalence of young men in South Africa and the multi-factorial mechanisms in play with the association between medical male circumcision and decreased genital HPV prevalence.

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“There are no facts, only interpretations”

(Friedrich Nietzsche 1844–1900)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

%	Percent
µL	Micro litre
AIDS	Acquired immunodeficiency syndrome
AIN	Anal intraepithelial neoplasia
aIRR	Adjusted Incident Rate Ratio
ANRS 1265	Agence Nationale de Recherches sur le SIDA Study 1265
aPMR	Adjusted Poisson mean ratio
aPMRs	Adjusted Poisson mean ratios
aPRR	Adjusted prevalence rate ratio
aPRRs	Adjusted prevalence rate ratios
ASIL	Anal squamous intraepithelial lesions
ASIR	Age standardized incidence rate
AT	As-treated
CD4	CD4 lymphocytes
CI	Confidence interval
CIs	Confidence intervals
DNA	Deoxyribonucleic acid
DOH	Department of Health
ds	Double stranded
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
FDA	Food and Drug Administration
GUD	Genital ulcer disease
GW	Genital wart
GWs	Genital warts
HAART	HIV highly active antiretroviral therapy

HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus type 1
HPV	Human Papillomavirus
HPVs	Human Papillomaviruses
hrHPV	High risk Human Papillomavirus
HRP	Horse-radish peroxidase
HSIL	High grade cervical squamous intraepithelial lesion
HSV	Herpes Simplex Virus
HSV-2	Herpes simplex virus type 2
IARC	International Agency for Research on Cancer
IgG	Immunoglobulin G
IR	Incidence rate
IRR	Incident rate ratio
IRRs	Incident rate ratios
ITT	Intention-to-treat
LA	Linear Array
LCR	Long control region
lrHPV	Low risk Human Papillomavirus
LSIL	Low grade cervical squamous intraepithelial lesion
MC	Male circumcision
mm	Millimetre
MMC	Medical male circumcision
mRNA	Messenger ribonucleic acid
MSM	Men who have sex with men
NA	Not applicable
NCR	National Cancer Registry
NHLS	National Health Laboratory System
NICD	National Institute for Communicable Diseases

ORFs	Open reading frame(s)
P	P-value
Pap	Papanicoloau
PCR	Polymerase chain reaction
PMR	Poisson mean ratio
PRR	Prevalence rate ratio
PRRs	Prevalence rate ratios
PV	Papillomavirus
py	Person-years
qHPV	Quadrivalent HPV
qPCR	Quantitative PCR
Rb	Retinoblastoma protein
RCT	Randomized control trial
RNA	Ribonucleic acid
RR	Rate ratio
rpm	Resolutions per minute
s	Seconds
SCC	Cervical squamous-cell carcinoma
SIL	Squamous intraepithelial lesions
SMDU	Specialized Molecular Unit
ß-globin	Beta-globin
STI	Sexually transmitted infection
STIs	Sexually transmitted infections
TCA	Trichloroacetic acid
UN	United Nations
URR	Upstream regulatory region
USD	United States Dollar
VLP	Virus Like Particle

vs	Versus
WHO	World Health Organization
ZAR	South African Rand

CHAPTER 1: INTRODUCTION

1.1 Human Papillomavirus

Human Papillomavirus belongs to the large *Papillomaviridae* family. These are DNA tumour viruses and more than 100 types of HPV have been identified. Many HPV types do not cause disease whilst some types have varying pathogenic effects (papillomas, verrucae, warts, condylomas, neoplasms and malignant tumours). There are two main HPV genera that infect the ano-genital region: Alpha and Beta papillomaviruses, with 90% of characterized HPVs belonging to these groups. Beta viruses are associated with cutaneous infections, while genital/mucosal HPV types belong to the larger Alpha group of papillomaviruses. The remaining HPVs from the three genera, Gamma, Mu and Nu, cause cutaneous papillomas and verrucae (Figure 1.1) (Bernard, 2005; Bernard, 2006; Doorbar, 2007).

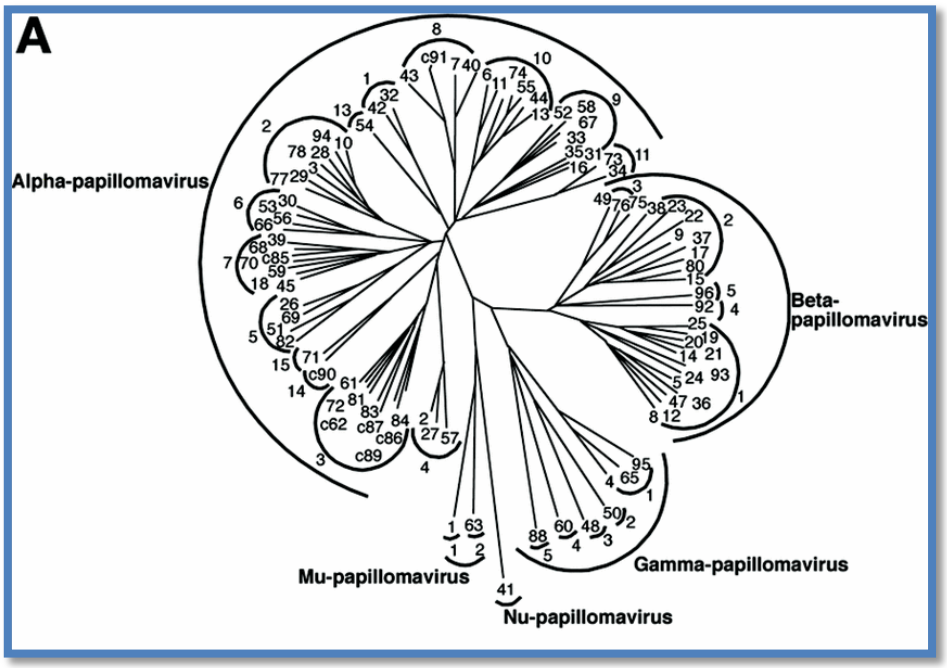

Figure 1.1 HPVs are limited within five evolutionary groups. HPV types that infect the ano-genital region and mucosa come from the Alpha group which contains over 60 members. HPV types in the Beta, Gamma, Mu and Nu groups mainly infect cutaneous sites (Doorbar, 2006)

HPVs infect mucous epithelia in a variety of animals, including humans, causing latent and site-specific sub-clinical and clinical infections. High risk HPV infections generally regress, without causing any serious clinical problems in the host. Approximately one third of all HPV types infect the genital tract and the oral mucosa. There is a subgroup of more than 40 HPV genotypes that preferentially infect mucosal surfaces and are found in the anogenital tract. Fifteen to eighteen of these genotypes are considered oncogenic (Munoz *et al*., 2006) (See Table 1.1). HPV types belonging to this mucosal subgroup are divided into either 'high risk' (hrHPV) or 'low risk' (lrHPV) according to their carcinogenic potential (Calleja-Macias *et al*., 2005; Prado *et al*., 2005). Persistence of high risk HPV causes progression to malignancy. This tumour progression and transformation to malignancy are rare events. Low risk HPV generally progresses to genital warts.

Table 1.1 Risk classification of HPV infections (Munoz *et al*., 2003)

Risk category	**HPV types**
Low risk (lrHPV)	6, 11, 40, 42, 44, 54, 61, 70, 72, 81, CP6108
High risk (hrHPV)	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82
Probable high risk	26, 53, 66
Undetermined risk	34,57,83

HPV is one of the most common sexually transmitted infections and is associated with invasive cancer of sites such as the cervix, anus, penis, vulva, conjunctiva and oesophagus. The individual virus genotypes are defined by DNA sequence and the phylogenetic trees consist of genotypes grouped according to specific pathologies (Figure 1.2) (Burns and Maitland, 2005).

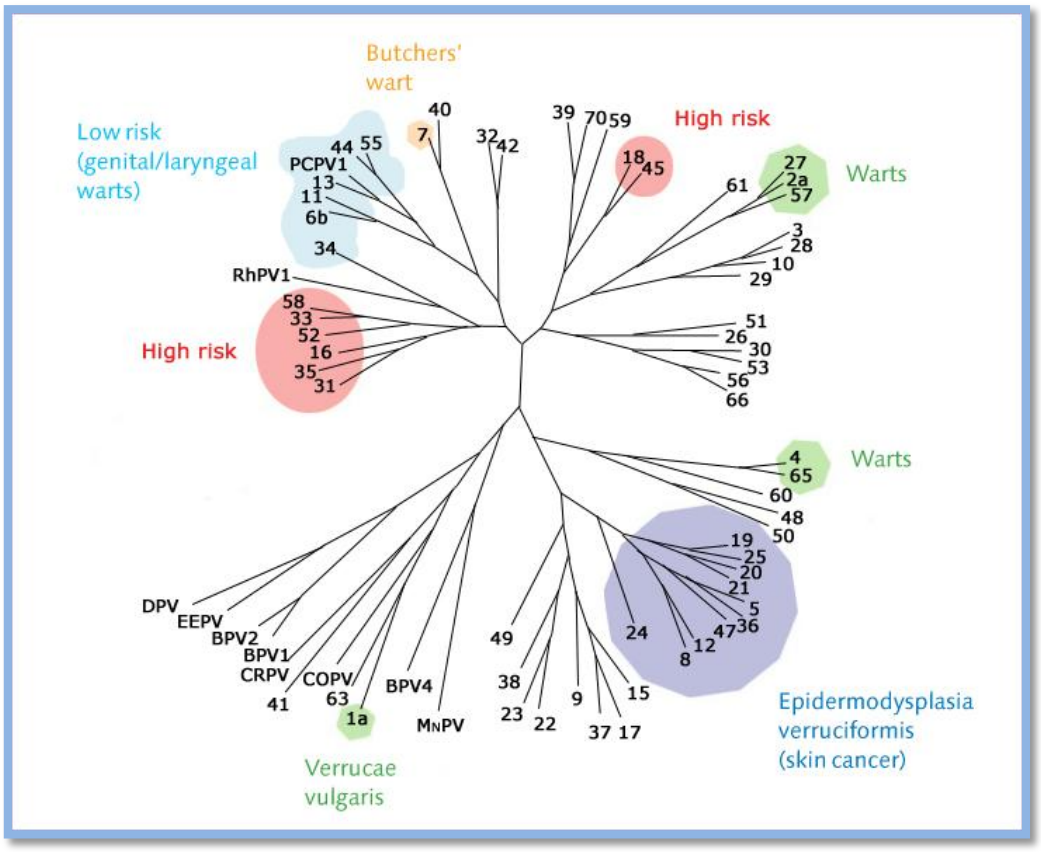

Figure 1.2 Human papillomavirus family tree according to clinical manifestation (adapted from: Burns and Maitland, 2005)

The human papillomavirus consists of a double-stranded (ds) circular deoxyribonucleic acid (Monsonego et al.) genome of about 8000 base pairs, contained within a symmetric icosahedral protein coat. The capsid consists of an assembly of 72 pentamer rosettes of the major capsid protein L1, and L2 minor capsid proteins (Figure 1.3) (Stanley, Lowy, and Frazer, 2006). Papillomaviruses have non-enveloped isometric virions 55nm in diameter. All HPVs have a similar and conserved genomic organization, despite their heterogeneity. The genome is divided into a non-coding region containing regulatory sequences (necessary for replication and transcription), the long control region (LCR), an early region (E region or E genes) involved in transformation and responsible for the pathogenicity of the virus, and a late region (L region/L genes) coding for capsid (structural) proteins (Campo, 2006; IARC, 2005; Kalantari and Bernard, 2006).

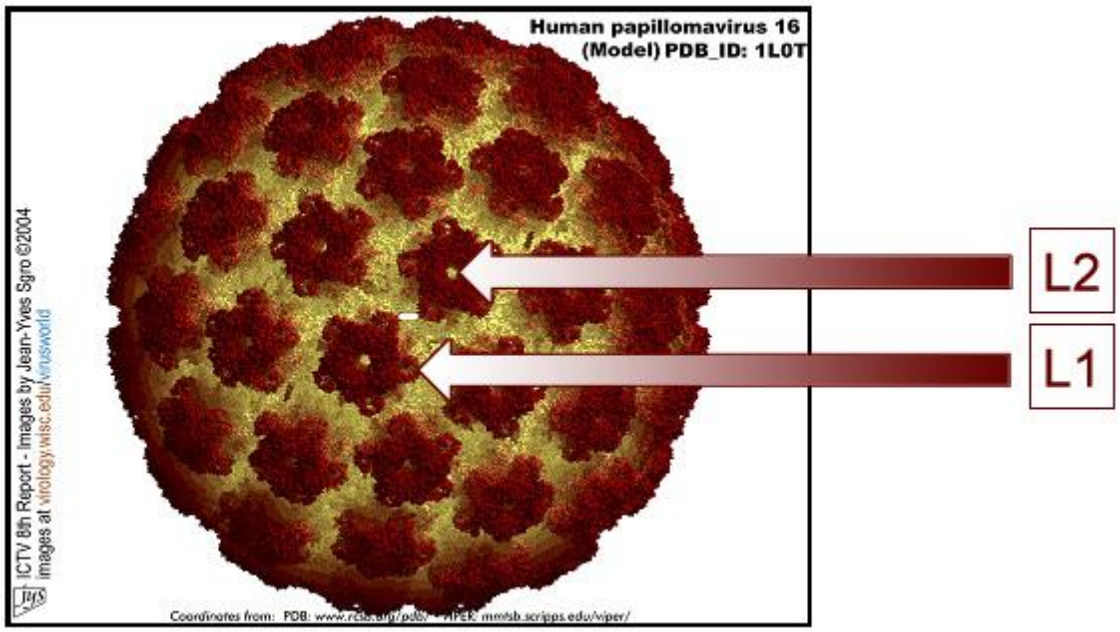

Figure 1.3 Human papillomavirus structure. The rosette-like surface structures (arrowed) are pentamers each consisting of five molecules of L1; one molecule of L2 fits into the central dimple of each pentamer. (Stanley, Lowy, and Frazer, 2006) http://www.ictvonline.org

The virus capsid assists in various critical ways with the establishment of viral infection. The proteinaceous coat protects viral nucleic acid from degradation by external host defences. The virus particle interacts with the target cell surface, mediates inclusion and internalization by the host cell (Day and Schiller, 2006).

1.2 HPV lifecycle and pathogenesis

HPV is an ubiquitous virus and infection with subsequent virion synthesis requires differentiation of the basal epithelial host cell. Initial infection by HPVs is thought to occur through micro-abrasions or micro trauma of the epithelial tissue, this allowing entry of the HPV particle into the cells of the basal layer (Lehoux, D'Abramo, and Archambault, 2009).

HPV demonstrates tropism towards the keratinocytes and therefore the lifecycle of HPV is strictly linked to the differentiation program of the host keratinocytes. Thus, the assembly of mature virions is restricted to terminally differentiated suprabasal cells. Basal layer cells consist mainly of stem cells and transit-amplifying cells and it is epithelial stem cells that

must be infected for a lesion to be maintained. These cells continuously divide and replenish cells that are lost due to desquamation (Doorbar, 2007).

HPV infects the squamous epithelial cells in the cervix, glans of the penis, penile shaft, the scrotum and anus by interaction with the host cell surface receptors such as heparin sulfate proteoglycans and alpha 6 integrins. The virus enters the cell via clathrin-mediated and caveoline-mediated endocytosis depending on the HPV type. Following penetration of the target cell, a controlled process of un-coating occurs to make the viral genome accessible to the cellular transcription and replication processes (Day and Schiller, 2006).

An uncoated virus delivers its genome of eight genes to the host cell nucleus, but details are lacking regarding the delivery of the viral genome into the nucleus. Viral DNA is replicated and then separated into progeny cells using the host cell apparatus. A daughter cell remains in the basal layer to serve as a reservoir for the replication of further viral DNA. Other daughter cells migrate suprabasally, where HPV inhibits the cell from its normal exit from the cell cycle. Viral proteins are produced at defined times and levels as the infected cell moves towards the epithelial surface. The lifecycle of HPV depends on continuous replication of the host cell; the virus uses the infected cell for replication of its genetic material and expression of oncoproteins (Figure 1.4). Differentiation of infected cells is necessary for productive infection of the host cells and is coupled with expression of the late viral capsid genes for the completion of the viral lifecycle (Conway and Meyers, 2009).

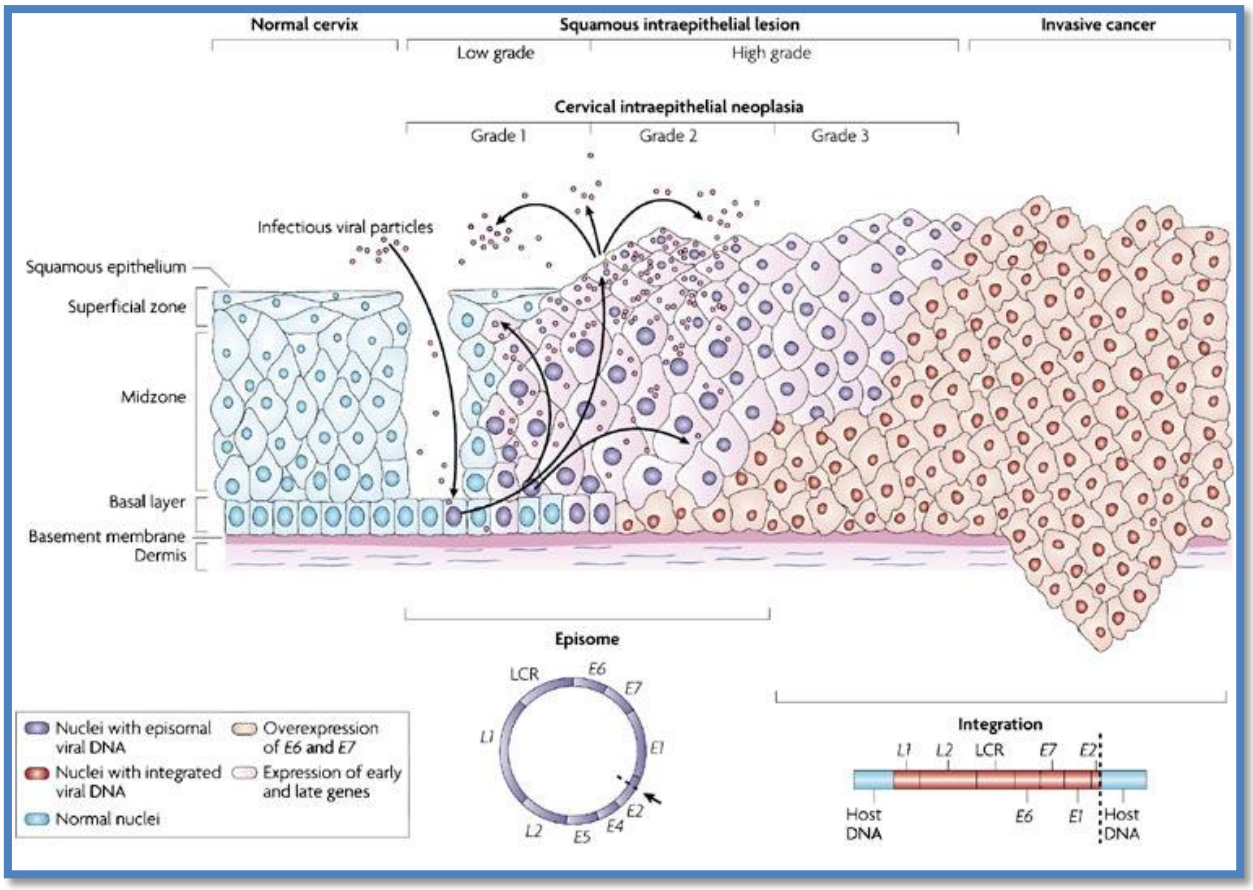

Figure 1.4 Schematic diagram to show the disease continuum of cancer development following human papillomavirus infection and role of early and late HPV genes (Woodman, Collins, and Young, 2007).

1.3 HPV genome and replication

The HPV genome contains eight genes or open reading frames (ORFs) with multiple promoters and a number of splice variants. These open frames can be subdivided into early (E) and late (L) genes according to the time of their expression in the HPV lifecycle, as well as a non-coding region called upstream regulatory region (URR) (Figure 1.5). Summary of HPV genes and functions are summarized in Table 1.2. The early genes E1, E2, E4, E5, E6 and E7 encode the non-structural proteins that participate in DNA replication, transcriptional regulation, cell transformation and viral assembly and release. The late ORFs encode the L1 and L2 viral capsid proteins. L1 serves as the major capsid protein, while L2 serves as the minor capsid protein (Pereira, Hitzeroth, and Rybicki, 2009).

Table 1.2 Function of HPV ORF's (Day and Schiller, 2006; Graham, 2006; Stanley, Pett, and Coleman, 2007)

Protein	Function
E6	Binds p53, directs p53 ubiquitin-mediated degradation With E7 immobilizes primary keratinocytes inhibits apoptosis and differentiation
E7	Binds retinoblastoma protein (Rb) Co-operates with E6 to immobilize primary cells Allow cell cycle progression in differentiating virus infected cells
E1	Virus genome replication Helicase, ATPase, ATP-binding protein essential for viral DNA replication
E2	Viral transcription factor, virus genome replication, segregation, encapsidation, regulates cellular gene expression, cell cycle regulation
E4	Interacts with cytoskeletal proteins, allows viral assembly Remodels cytokeratin network
E5	Weak transforming activity, up-regulates growth factor receptors Preserves differentiation, facilitates replication and late gene expression
L1	Major capsid protein, structural
L2	Minor capsid protein, virus assembly

Papillomaviruses have two modes of replication: stable and vegetative. Stable replication exists as a circular episomal genome in the cells of the basal and parabasal layers of lrHPV infected squamous epithelial cells while the vegetative form occurs in highly differentiated cells at the epithelial surface. The hallmark of the malignant transformation by hrHPV types is the integration of the viral genome into the host cell genome (McBride, 2008).

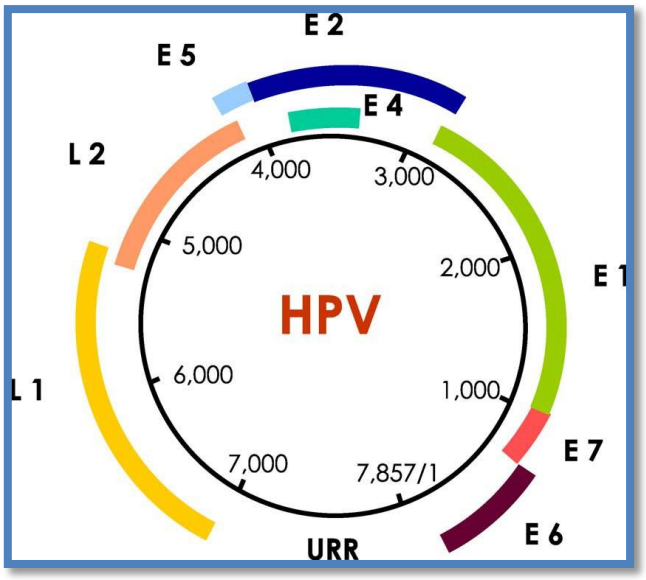

Figure 1.5 Schematic representation of the HPV genome showing the arrangement of the early E or non-structural genes, the capsid genies (L1 and L2) and the upstream regulatory region (URR) (Munoz *et al*., 2006)

The first viral genes to be expressed, E6 and E7, interfere with p53 that regulates normal cell division and the function of the human genes retinoblastoma protein (Rb) (Ganguly and Parihar, 2009). Rb, is a tumour suppressor protein and p53 is responsible for repairing DNA damage in cells and apoptosis if the damage is too severe to be repaired by the cell. In HPV infection, E6 binds with p53 and E7 binds with Rb. This allows the virus to take over the cell processes and reproduce itself. E7 binds to Rb to inactivate the E2F transcription factor and trigger expression of proteins necessary for DNA replication. The consequence is continued cell division of damaged cells with multiple mutations in the host genome (Doorbar, 2007; Lehoux, D'Abramo, and Archambault, 2009). Integration of HPV DNA at specific sites in the host cell genome is an early and critical step in cancer progression (Stanley, 2006; Stanley, Pett, and Coleman, 2007). E6 and E7 are well-known oncoproteins in hrHPV (Ganguly and Parihar, 2009). Persistent HPV infection facilitates constant activity of viral proteins E6 and E7 as well as accumulation of oncogene mutations, and both are prerequisites for cancer development. Progression to cancer results after years of active infection. These oncoproteins are expressed in low levels, but when the virus genome is no longer episomal in the nucleus and has

incorporated in the host genome, these oncoproteins become over-expressed (Graham, 2006; Munoz *et al*., 2006; Stanley, Pett, and Coleman, 2007).

As the infected cells differentiate, the remainder of the early genes become switched on. E1 is the primary replication protein and E2 is a polypeptide often referred to as transactivator. Both are DNA binding proteins that regulate viral transcription and replication as well as the coordinated expression of the other early viral genes. E4 and E5 genes assist in the virus genome production, activating the productive phase of the HPV lifecycle and E5 is involved in transformation and enhancing the rate of the epidermal growth factor (EGF) to maintain replication in the upper epithelial layers (McBride, 2008). As infected supra-basal cells become terminally differentiated, the late genes, L1 and L2 are expressed, allowing assembly and release of viral genome into infectious particles and increased infectivity. Capsid synthesis is dependent on mRNA splicing changes. HPV are non-lytic and increased copy numbers of viral particles are not released from infected cells until it reaches the epithelial surface. Infectious virions in the upper epithelial layers are shed in the dead squames of the host epithelium to infect other cells and hosts (Doorbar, 2007; Stanley, 2006).

1.4 Link between HPV and cervical cancer

Cervical cancer arises in the transformation region of the uterine cervix. This is the area which undergoes physiological metaplasia from glandular to squamous epithelium during the beginning of adolescence. There are two major types of cervical cancers. The most common form, cervical squamous-cell carcinoma (SCC), derives from squamous cells. Squamous cells are scale-like cells constituting the majority of the epithelia of the lower cervix. The other form, cervical adenocarcinomas, arises in cervical glandular cells and occurs in 15% of cases (Cuzick *et al*., 2008).

Sexual intercourse (vaginal and anal) is the primary route for genital HPV transmission. Oral-genital and digital-genital infections do occur. Vertical transmission is associated with development of recurrent laryngeal papillomatosis (Burchell *et al*., 2006). HPV infection is very common in young women after the onset of sexual activity and when it persists, the viral oncoproteins produce disruption of the cell-cycle controls resulting in low grade cervical squamous intraepithelial lesions (LSIL) (Kjaer *et al*., 2001).

Cervical cancer is the first cancer recognized by the World Health Organization (WHO) to be 100% attributable to infection. The causal role of HPV has been well documented. Infection with the human papillomavirus, especially genotypes 16 and 18, has been established as a cause of cervical pre-cancerous and cancerous lesions (Clifford *et al*., 2005b; Clifford *et al*., 2003; Dillner *et al*., 1997; Kjaer *et al*., 2002; Sigstad *et al*., 2002; Wallin *et al*., 1999). Type 16 and 18 together cause about 70% of cervical cancer and types 31 and 45 together cause another 15% (IARC, 2003). Studies demonstrated the DNA of hrHPV types in virtually all cervical cancer biopsies (zur Hausen, 1996; zur Hausen, 2002; Zur Hausen, 2008).

The development of the disease is dependent on persistent infection with HPV. The vast majority of HPV infections will regress spontaneously with no serious sequelae (Bosch *et al*., 2002). Research has shown that new HPV infections median duration is eight months and that approximately 70% clear up within a year and 91% clear within two years (Kjaer *et al*., 2002).

Potential co-factors for development of persistent HPV infections and cancer in women are: use of oral contraceptives, younger age at time of sexual debut, high number of sexual partners, high number of live births (parity linked as a behavioural co-factor), immunologic status, older age, hrHPV types, infection with multiple HPV types and co-infections with other STI pathogens like *Chlamydia trachomatis* and herpes simplex virus and condom use (Castellsague *et al*., 2006; Mayeaux, 2008).

Papanicoloau and Traut published their findings on vaginal cytology as a screening method for uterine cancer in 1943 (Papanicolaou and Traut, 1943). The Pap smear has been the best screening tool introduced for any cancer and has been responsible for a significant reduction of cervical cancer (Runowicz, 2007).

The causal relationship between HPV and cervical cancer was first documented in the 1970s by Harald zur Hausen (zur Hausen, 1976; zur Hausen, 1977). The discovery of hrHPV as causative agents for cervical cancer was clearly a prerequisite for the development of new approaches in the prevention and therapy of the disease (zur Hausen, 2000).

1.5 HPV prevalence and HPV related cancer distribution

1.5.1 Worldwide

HPV infection has been identified as a definite human carcinogen for six types of cancer: cervix, penis, vulva, vagina, anus and oropharynx (including the base of the tongue and tonsils). Cervical cancer is the second most common malignancy in women worldwide and the first for developing countries and women living in Africa, where the majority of deaths occur (Parkin and Bray, 2006). The high burden of cervical cancer in these countries is due both to a high prevalence of HPV infection (Naucler *et al*., 2004; Sahasrabuddhe *et al*., 2007) and ineffective screening programs (Sankaranarayanan, Budukh, and Rajkumar, 2001).

Global cervical cancer statistics show a large diversity in risk and mortality (Parkin, Pisani, and Ferlay, 1999). 493 000 new cases of invasive cervical carcinoma are diagnosed globally, with 78 897 cases occurring in Africa. Although cervical cancer rates in developed countries decrease due to effective screening programs, high levels are still found in developing countries (Boffetta and Parkin, 1994).

Most of the 274 000 deaths per annum caused by cervical cancer occur in developing countries, with a lack of infrastructure and resources to implement routine screening. The worldwide age standardized mortality rate due to cervical cancer is 14.8 per 100 000 in Africa and 9 per 100 000 globally (Parkin *et al*., 2005). Cancer is an under-accentuated disease in Africa, partly due to the devastating burden of other communicable diseases (Parkin *et al*., 2008).

The annual incidence of anal cancer is 1 per 100,000 in the heterosexual population in western countries (Denny and Ngan, 2006). The incidence in men who practice anal-receptive intercourse is much higher at 35 per 100,000 (Daling *et al*., 2004). The incidence of anal cancer has increased in recent decades (de Pokomandy *et al*., 2009). The incidence of anal cancer is high among women with cervical dysplasia and cervical cancer, HIV positive individuals, men who have sex with men and transplant recipients (Chin-Hong, 2008; Chin-Hong *et al*., 2009)

Penile carcinoma is an uncommon malignant tumour which accounts for less than 1% of all malignant tumours in males in Europe and North America, but in regions of South America, Africa and Asia the incidence is markedly higher to up to 10% (Miralles-Guri *et al*., 2009). The penile cancer burden is about 26 300 new cases per year and 40% of penile cancers are attributable to HPV infection (Parkin and Bray, 2006). It occurs predominantly in elderly men with an average age of diagnosis at 60 years. The incidence of penile carcinoma varies a great deal among different populations, with the highest incidence in developing countries (Bleeker *et al*., 2009).

The HPV-associated invasive vulvo-vaginal cancers are verrucous carcinoma (lrHPV) and the hrHPV-induced (non-keratinizing), squamous cell carcinoma, the condylomatous carcinoma and the very rare vaginal squamo-transitional carcinoma (Alexander and Giuliano, 2012; Carter and Downs, 2012).

The virus has also been linked in the oncogenesis of non-genital cancers for example head and neck malignancies, specifically oropharyngeal cancers. Worldwide, the incidence and prevalence of HPV-associated oropharyngeal cancer has been increasing over time. This is likely due to an increased risk of exposure to the virus as the persistence of oral HPV infection increases the risk of eventual oropharyngeal cancer (Zandberg et al., 2013)

1.5.2 South Africa

South Africa launched a pathology-based cancer registry in 1986, relying on information reported by 80 private and public laboratories. 16 559 cancers were reported in women, of which 2 897 (17.4%) were new cases of histologically confirmed cervical cancer (Sitas *et al*., 1997; Sitas, Madhoo, and Wessie, 1998). In 1998 and 1999, 84 laboratories countrywide reported 6 061 and 5 203 cervical cancer cases respectively (20% and 17% of all cancers) (Mqoqi *et al*., 2004). Cervical cancer was the leading cancer in women in 1998 and second to breast cancer in 1999. The lifetime risk for a South African woman to develop cervical cancer was 1 in 26 in 1998 and 1 in 31 in 1999 (Mqoqi *et al*., 2004; Sitas, Madhoo, and Wessie, 1998). The age standardized incidence rate (ASIR) of cervical cancer in South Africa is similar to those of other developing countries, 34.4 per 100 000 (1998) and 28.7 per 100 000 (Mqoqi *et al*., 2004). The South African National Cancer Registry (NCR) had not produced subsequent reports.

In South Africa, HIV positive women showed increased risk of cervical pre-cancer, but not for invasive cancer (Moodley *et al*., 2006). This is probably due to women dying of HIV-related causes before presenting with cervical cancer (Denny, 2006). Although high levels of high-risk HPV infections are found in women that are HIV-1 infected, progression to high grade cervical squamous intraepithelial lesion (HSIL) occurred in the minority of cases (Denny *et al*., 2008; Strickler *et al*., 2005).

The penile cancer age standardize incidence, based on histopathology-based registries in Southern Africa, is between 0.4 and 1.8 per 100 000, varying between ethnic groups (WHO/IARC, 2003). The crude incidence rate by registry for the African continent is between 0.0 and 0.7 per 100 000 men (WHO/ICO, 2007).

Less information is available regarding incidence of anal cancer in South Africa. The crude incidence rate for anal cancer by registry for the African continent is between 0.1 and 0.3 per 100 000 men (WHO/ICO, 2007).

1.5.3 HPV prevalence

Global HPV prevalence in women with normal cytology at any given point in time is approximately 10% confirming that HPV is one of the most common sexually transmitted infections. Case control studies and prevalence surveys have unequivocally shown that HPV DNA can be detected in cervical cancer specimens in 90-100% of cases compared with a prevalence of 5-10% in women with normal cytology (Bosch *et al*., 2002).

HPV 16 is constantly the most common type and HPV 18 is the second most detected type worldwide, with some slight regional differences. HPV 52 is the second most prevalent type in Africa followed by HPV 18. HPV 16 is also consistently the most common HPV type contributing to 50-55% of invasive cervical cancers cases (Bosch *et al*., 2008).

The overall HPV and HPV 16 prevalences in sub-Saharan African women are higher than the rest of the world (Clifford *et al*., 2005a). There is a high prevalence of HPV 16 in South African women with cancer of the cervix and cervical intraepithelial neoplasia (Kay *et al*., 2003). In women with squamous cell cancers of the cervix in South Africa, 64% tested positive for HPV types 16 and 18, which is higher than other African (sub-Saharan) countries (Cooper *et al*., 1991). HPV 16 and HPV 18 are accountable for 70% of cervical

cancer worldwide and for 63% in South Africa. HPV 45 and HPV 31 are responsible for 10% of cases (WHO/ICO, 2007).

Prevalence and incidence of HPV appear to be high in men (Dunne *et al*., 2006; Lu *et al*., 2009), with hrHPV present in 100% of anal and penile cancers in immune-deficient individuals and 86% of cancers in immune-competent individuals (Wong *et al*., 2009). Prevalence varies on the basis of adequacy of sampling, anatomic sites and processing methods (Dunne *et al*., 2006). These observed differences in HPV distribution may be due to disparity in tissue tropism of HPV types. The HPV prevalence in Africa is higher than in other continents, with HPV 6 and HPV 52 with the highest prevalences of 5.6% and 5.2%, respectively. HPV 16 and 18 have a prevalence of 4.4%, compared to 3.8% (HPV16) and 2.0% (HPV18) for all continents combined (Vardas *et al*., 2011). In South Africa, HPV prevalence have an adjusted odd ratio of 3.56 (1.50-8.50) and 1.97 (1.03-3.77) for multiple types and any type respectively (Muller, Chirwa, and Lewis, 2009).

1.6 Cancers caused by HPV in men

As in women, there are strong links between HPV and disease in men (Giuliano *et al*., 2008b). HPV contributes to men's burden of disease such as anal intraepithelial neoplasia, (AIN), penile and oropharyngeal cancers and genital warts. HPV infection in men is mostly asymptomatic and sub-clinical. Ano-genital warts are the most common manifestation of HPV in men (Baldwin *et al*., 2003). Anal HPV infection is common in men who have sex with men (MSM) as well as heterosexual men (Nyitray *et al*., 2008).

Anal cancer has similar biological and epidemiological properties to cervical cancer. It presents with the same histological patterns, is associated with the same types of HPV and has a similar natural history (Pereira, Lacerda, and Barros, 2008). Like the cervix, the anal canal has a transformation zone in which the columnar epithelium of the rectum joins the squamous epithelium of the anus. As in the cervical transformation zone, the ano-

rectal junction is a common site of anal HPV infection and development of AIN, a potential precursor of anal cancer (Pereira, Lacerda, and Barros, 2008).

HPV has been shown to cause intraepithelial neoplasia that can develop into low grade to high-grade to invasive anal cancer (Denny and Ngan, 2006). HPV is strongly associated with anal and some penile cancers (Parkin and Bray, 2006; Rubin *et al*., 2001). HPV is found in 80% of anal cancers in MSM, with HPV 16 accounting for the majority of the cases, 60% of anal cancer in heterosexual men have HPV positivity (Partridge and Koutsky, 2006).

Penile carcinoma is a potentially mutilating disease with a multi-factorial aetiology. It is a disease with high morbidity and mortality (Bleeker *et al*., 2009). Several risk factors have been identified for example: phimosis (unretractable foreskin), poor hygiene, smegma retention, smoking, history of genital warts and other sexually transmitted diseases, multiple sexual partners (Bleeker *et al*., 2009; Castellsague *et al*., 2002). HPV plays an important role in the pathogenesis of penile carcinomas. The prevalence of HPV in penile cancers is estimated to be between 15% and 71% (Micali *et al*., 2004; Micali *et al*., 2006). A systematic review found that 40% of penile cancers are HPV-related and that HPV 16 and HPV 18 are the most common genotypes at 63% (Madsen *et al*., 2008; Miralles-Guri *et al*., 2009; Parkin and Bray, 2006). Penile carcinomas with basaloid and warty characteristics have shown the strongest association with HPV infection (Parkin and Bray, 2006).

1.7 The male role in cervical cancer

Following infection, HPV can adopt three different states in the male genital epithelium: a latent, subclinical (flat lesions) and clinical infection with classical genital warts (Hippelainen *et al*., 1994).

Male HPV disease notably contributes to infection and subsequent cervical disease in women. Women who have many sexual partners or who have sex with men who have many other partners, have a greater risk of cervical cancer. Male sexual behaviour affects rates of HPV prevalence, cervical dysplasia and invasive cervical cancer in female partners, a greater understanding of HPV infection in men is an essential component of cervical cancer prevention (Giuliano *et al*., 2009). Case control studies in women with cervical cancer and their partners have demonstrated that men's sexual behaviour is linked to increased cervical cancer (Bosch *et al*., 1996) and HPV prevalence among men (Vardas *et al*., 2011). Acting as carriers and vectors, male partners evidently contribute significantly to HPV disease in women (Castellsague, Bosch, and Munoz, 2003). Genital HPV infection is widespread and multifocal in young men, with a higher occurrence as what is reported for similar cohorts of young women. There is a growing interest in understanding HPV infection and related disease in men. Several studies investigated the factors associated with male HPV detection. In these studies, sexual behavior (OR = 3.8, 95% CI: 1.8–8.0), circumcision status (OR = 0.2, 95% CI: 0.1–0.4), condom use (OR = 0.4, 95% CI: 0.1–1.0) and smoking (OR = 2.3, 95% CI: 1.1–4.6) were identified with increased risk for HPV infections (Castellsague et al., 2002; Daling et al., 2004; Hernandez et al., 2008b; Lajous et al., 2005; Nielson et al., 2007b; Svare et al., 2002; Vaccarella et al., 2006).

Studies of HPV in men are necessary to improve understanding HPV transmission and carcinogenesis to prevent disease in men and women. There is a need for characterization and understanding of HPV in men in terms of type distribution across countries and age specific prevalence estimates (Baldwin *et al*., 2003). This will also elucidate the impact of vaccines in men and implementing sex neutral vaccine policies (Giuliano *et al*., 2008a).

1.8 Sampling techniques and genital sites for the detection of HPV in men

One of the main challenges for studying HPV in heterosexual men is specimen collection. Recent studies have gathered data on regarding the optimal genital sites for sampling and how specimens should be collected (Fife *et al*., 2003; Giuliano *et al*., 2007; Hernandez *et al*., 2006; Nielson *et al*., 2007b; Nielson *et al*., 2009; Ogilvie *et al*., 2009).

Various studies evaluated multiple anatomical sites and type of specimens (Baldwin et al., 2003; Fife et al., 2003; Flores et al., 2008a; Giovannelli et al., 2007; Giuliano et al., 2007; Hernandez et al., 2006; Lajous et al., 2005; Nyitray et al., 2008; Shin et al., 2004; Smith et al., 2007; Svare et al., 2002). The best anatomical sites for sampling in men appear to be the glans, corona, prepuce and shaft of the penis, considering adequacy in sampling and convenience (Auvert *et al*., 2010; Auvert *et al*., 2009; Bosch *et al*., 1996; Castellsague *et al*., 2002; Dunne *et al*., 2006; Franceschi *et al*., 2002; Munoz *et al*., 1996; Svare *et al*., 2002). Sampling from the corona sulcus and glans is essential for evaluating HPV in men due to the direct contact with the cervix (Dunne *et al*., 2006). Urethral sampling has the benefit in male circumcision studies as this anatomical site is probably not affected by circumcision status (Auvert *et al*., 2009). There is no consensus about the detection of HPV DNA in urine (Costa *et al*., 2009; D'Hauwers and Tjalma, 2009; Fife *et al*., 2003; Lazcano-Ponce *et al*., 2001; Sehgal *et al*., 2009). There is a high variability in the study designs and methodologies including sampling methods, PCR detection assays and collection sites in HPV and male circumcision studies (Table 1.3). A number of studies detected HPV DNA in semen (Giovannelli *et al*., 2007; Nielson *et al*., 2007a). Some authors recommend that a scrotal, peri-anal and anal sample should be included for optimal HPV detection (Giuliano *et al*., 2007). Some studies have been criticized for sampling bias by sampling only specific penile sites that are predisposed to HPV infection (Van Howe, 2007). This bias has been avoided in more recent studies by sampling the

whole penis and the scrotum or pooling data rather than a site analysis (Giuliano *et al*., 2009; Lajous *et al*., 2005; Muller, Chirwa, and Lewis, 2009).

Because HPV is a virus that infects the epithelium, specimen yield can be enhanced by abrasion with emery paper or other methods to dislodge more epithelial cells, followed by swabbing with a moistened Dacron swab (Fife *et al*., 2003; Hernandez *et al*., 2006; Weaver *et al*., 2004).

Self-sampling and non-invasive HPV sampling present an important alternative to boost uptake of testing for both men and women (Chin-Hong *et al*., 2008; Lack *et al*., 2005; Ogilvie *et al*., 2009).

Table 1.3 Summary of studies reporting on the association between male circumcision and genital HPV in men by sampling method and specimen collection sites

Study	Sampling Methods	HPV DNA Detection Assay	Urethra Meatus	Glans	Coronal Sulcus	Foreskin	Penile Shaft	Scrotum	Perianal Region	Samples Excluded
(Svare *et al*., 2002)	Swabs	PCR	No	Yes	Yes	No	Yes	Yes	Yes	No
(Castellsague *et al*., 2002)	Swabs	PCR	Yes	Yes	Yes	No	No	No	No	No
(Shin *et al*., 2004)	Cytobrush	PCR	Yes	Yes	Yes	Yes	Yes	Yes	No	No
(Baldwin *et al*., 2004)	Swabs	PCR	No	Yes	Yes	No	No	No	No	No
(Weaver *et al*., 2004)	Emery paper and swabs	PCR	Yes	Yes	No	Yes	Yes	Yes	No	Urine
(Lajous *et al*., 2005)	Swabs and cytobrush	PCR	Yes	No	Yes	No	Yes	Yes	No	No
(Vaccarella *et al*., 2006)	Cytobrush	PCR	Yes	Yes	Yes	Yes	Yes	Yes	No	No
(Nielson *et al*., 2007a)	Swabs	PCR	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Perianal
(Partridge *et al*., 2007)	Emery paper and swabs	PCR	Yes	Yes	No	Yes	Yes	Yes	No	Urine and finger nails
(Ng'ayo *et al*., 2008)	Swabs	PCR	No	Yes	Yes	No	Yes	Yes	yes	No
(Hernandez et al., 2008b)	Textured paper and swabs	PCR	No	Yes	Yes	Yes	Yes	Yes	No	Urine
(Hernandez *et al*., 2010)	Textured paper and swabs	PCR	No	Yes	Yes	No	Yes	Yes	No	No
(Lu *et al*., 2009)	Swabs	PCR	No	Yes	Yes	No	Yes	Yes	No	No
(Giuliano *et al*., 2009)	Swabs	PCR	No	Yes	Yes	No	Yes	Yes	No	No
(Ogilvie *et al*., 2009)	Emery paper and swabs	PCR	No	Yes	No	Yes	Yes	Yes	No	No
(Auvert *et al*., 2009)	Swabs	PCR	Yes	No	No	No	No	No	No	Urine
(Tobian *et al*., 2009)	Swabs	PCR	No	No	Yes	Yes	No	No	No	No
(Tobian et al., 2011)	Swabs	PCR	No	No	Yes	No	Yes	No	No	No
(Gray *et al*., 2010)	Swabs	PCR	No	Yes	Yes	No	Yes	No	No	No
(Muller, Chirwa, and Lewis, 2009)	Swabs	PCR	No	Yes	Yes	No	Yes	No	No	No

1.9 HPV and Human Immunodeficiency virus (HIV)

There is a strong relationship existing between HIV and HPV, both important sexually transmitted viruses. Cervical cancer has been labelled as an acquired immunodeficiency

syndrome (WHO-UNAIDS) defining disease. Numerous studies have consistently shown a high prevalence and incidence of anal and cervical HPV infection and HPV cancers in HIV-positive men and women (de Sanjose and Palefsky, 2002; Hessol *et al*., 2009; Jamieson *et al*., 2002; Klencke and Palefsky, 2003; Palefsky, 2009; Palefsky *et al*., 1999; Sun *et al*., 1995). This strong association between HPV and HIV could be due to the overlap of the risk and behavioural factors for HPV and HIV infections (Auvert *et al*., 2010). There is a high prevalence of hrHPV infection and low-grade cytological abnormalities in HIV-positive women. A high HIV viral load and low CD4 count are linked with an increased rate of HPV infection, type specific persistence and abnormal cytology, but not high grade cervical squamous intraepithelial lesions (Burg and Palefsky, 2009; Jong *et al*., 2008; Jong *et al*., 2009; Kitchener *et al*., 2007; Palefsky, 2006; Palefsky, 2007; Strickler *et al*., 2005).

HPV 16 is associated with immune status in HIV-seropositive women with prevalence increasing with severity of cervical lesions. HIV positive women with HSIL showed infection with other HPV types and multiple HPV types, while HPV16 was underrepresented in comparison with HIV negative population with HSIL (Burg and Palefsky, 2009; Clifford, Goncalves, and Franceschi, 2006; Clifford *et al*., 2005b; Fontaine *et al*., 2005; Fontaine *et al*., 2008; Strickler *et al*., 2003). There is a significant difference in the prevalence and concordance of HPV in HIV positive, HIV discordant and HIV negative partners (Mbulawa *et al*., 2009). A high rate of condylomas is observed in both HIV-infected heterosexual men, homosexual men and women (Abramowitz *et al*., 2007).

Men that are HIV-positive are more likely to be infected with HPV and develop anal intraepithelial lesions than HIV-negative men (Palefsky, 2006). The incidence of anal intraepithelial neoplasia (AIN), anal cancer and infection with multiple HPV types is high among HIV infected MSM (Damay et al., 2010b; Frisch et al., 2001; Palefsky, 2007). HIV negative and sexually active MSM have a high prevalence in all age groups of anal

squamous intraepithelial lesions (ASIL) possibly due to continuing HPV exposure (Chin-Hong *et al*., 2005; Chin-Hong *et al*., 2004). Associations between HIV co-infection and multiple HPV infection are also observed amongst heterosexual men (Auvert *et al*., 2010; Auvert *et al*., 2009; Muller, Chirwa, and Lewis, 2009; Sirera *et al*., 2006).

The HIV/AIDS epidemic in sub-Saharan Africa is expected to contribute to the high number of cervical cancer deaths: women infected with HIV are thought to be more likely to develop cervical lesions that can become cancerous. In some African countries, such as Kenya and Tanzania, HIV infected women had accelerated clinical progression of premalignant cervical lesions to invasive cervical cancer (Gichangi *et al*., 2003; Kahesa *et al*., 2008). This expected increase in cervical carcinoma has not been seen in many other African countries (Thomas, 2001).

The effects of HIV acquisition by HPV are not fully understood (Palefsky and Holly, 2003). HPV malignancies, other than cervical cancer, have increasingly been reported in recent years among HIV infected and uninfected men. Severe immuno-compromised persons and HIV-1 infected persons are at considerably higher risk of viral persistence, multiple HPV infections, low and high grade SIL (squamous intraepithelial lesions) and development to invasive cancers than immune-competent individuals (de Sanjose and Palefsky, 2002; Mbulawa *et al*., 2009; Orlando *et al*., 2008). These changes may be due to a deficient response of the immune system due to HIV or direct interaction between HIV and HPV (Pereira, Lacerda, and Barros, 2008). Several probable and non-exclusive explanations could account for an increased prevalence of HPV among HIV-infected individuals: HIV-induced immunodeficiency, immune response modulation to HPV, the reactivation of HPV from the basal layer facilitated by HIV or that HPV-induced immune activation facilitates HIV acquisition (Auvert *et al*., 2010; Palefsky, 2006). Anal HPV infection is independently associated with HIV acquisition (Chin-Hong *et al*., 2009).

Information as to the impact of highly active antiretroviral therapy (HAART) on HPV infections and HPV related disease is sparse and conflicting results have been reported (Heard, Palefsky, and Kazatchkine, 2004; Minkoff *et al*., 2010). Several papers reported that the incidence of HPV related cancers, in contrast with Karposi's sarcoma and non-Hodgekins lymphoma, did not decline with HAART and that anal cancer is increasing (Bower, Palmieri, and Dhillon, 2006; Bower *et al*., 2004; Chiao *et al*., 2005; Chin-Hong and Palefsky, 2002; Diamond *et al*., 2005; Hessol *et al*., 2007). This indicates that prolonged survival of persons with AIDS due to HAART might be associated with increased risk of some HPV induced cancers (Chaturvedi *et al*., 2009; Kreuter *et al*., 2009). Therapeutic HPV vaccines are promising as interventions for disease control for populations that are at risk such as HIV infected men and women (Burg and Palefsky, 2009; Chin-Hong and Palefsky, 2005; Palefsky, Gillison, and Strickler, 2006). Despite increasing knowledge of the natural history of HPV in HIV-positive men and women, the long term interaction between HIV co-infection and HPV tumourgenesis in an era of HAART is not fully understood.

1.10 HPV vaccines, treatment and preventative measures

In recent years there has been remarkable progress in the development of prophylactic vaccines against HPV. There is a general consensus that HPV vaccines are an unparalleled advancement towards the prevention of cervical cancer. HPV vaccination is a logical strategy worldwide, for the African continent and South Africa. HPV vaccination for low resource settings may be the only viable option for cervical cancer prevention and an effective vaccine could remove the need for screening programs (Cox and Cuzick, 2006; Stern, 2005). South Africa is one of the first African countries that registered highly effective HPV bivalent vaccine against the two main HPV types 16 and 18 in 2008 (Cervarix; Glaxo SmithKline). These vaccines are available in the private sector and there is no formalized immunization program for the country in place yet (Thomas, 2008). Cost

of the vaccine remains a problem for developing countries as currently the vaccine is priced at ZAR 700 (USD 100) per injection (Harries *et al*., 2009).

The FDA licensed an L1 virus-like particle (VLP) vaccine against HPV types 6, 11, 16 and 18 (Gardasil; Merck & Co.). This quadrivalent HPV vaccine is 99% effective against HPV 16 and 18 and equally effective against HPV 6 and HPV 11 related genital warts. It has been documented that the vaccine has been effective for up to 5 years (Braaten and Laufer, 2008). Waning immunity and cross protection of HPV vaccines need to be evaluated (Mariani and Venuti, 2010).

Virus-particle vaccines consist of recombinant HPV L1 capsid protein, which self-assembles into non-infectious virus-like particles. These particles elicit the production of antibodies that bind and neutralize infectious HPV virus.

The optimal target populations for HPV vaccinations have not been clearly defined. In the USA the FDA approved vaccination for all females between the ages of 9 to 26 years. Vaccination can be initialized at 9 years, but is recommended for girls aged 11 or 12 (Franco and Cuzick, 2008).

Encouraging immunogenicity and efficacy trials of HPV vaccines in males have been conducted. Immune responses to the quadrivalent HPV (qHPV) vaccine are comparable in men and women (Hillman *et al*., 2011). The HPV spread during oral sex is one of the main causes for the rise of oral and throat cancer. This provides an additional argument for boys to be vaccinated against HPV similarly to teenage girls to stop the spread of the disease.

HPV vaccination will prevent and reduce the burden of pre-cancer and cancer (Franco and Harper, 2005; Garnett *et al*., 2006). Concerns have been raised that women might decide not to be screened once they are vaccinated in both developed and developing

countries (Katz and Wright, 2006; Kulasingam, Pagliusi, and Myers, 2007). Most European countries, the United Kingdom, New Zealand, Australia and the United States have approved vaccination schedules that include HPV vaccine.

Treatment is focused on macroscopic (genital warts) or pathologic (precancerous) lesions caused by HPV infection. Specific antiviral therapy cannot eradicate HPV infection. The primary reason for treating genital warts is to improve symptoms, cosmetic concerns and ultimately, removal of the warts. If left untreated, visible genital warts can resolve without intervention, remain unchanged, or increase in size or number. Available therapies for genital warts include: cryotherapy, trichloroacetic acid (TCA), immunomodulators (administered topically, systemically or intra-lesionally) or surgical removal**.** These therapies will likely reduce, but will not eradicate, HPV infectivity (Stern et al., 2012).

Consistent condom use by male partners of sexually active women can reduce the risk for cervical and vulvo-vaginal HPV infection. Skin not covered by a condom remains vulnerable to HPV infection as the virus can still be transmitted through contact with areas of unprotected genital skin (Winer *et al*., 2006).

Several interventions are therefore needed for the prevention of HPV in men and there is currently no single fully protective measure available. Studies have shown the efficacy of male circumcision in HIV transmission (Auvert *et al*., 2005; Bailey *et al*., 2007; Gray *et al*., 2007) and could offer the same partial protection in reducing HPV infection in men, with resulting benefit to women.

1.11 HPV, other STIs and circumcision

Circumcision is the removal of a fold of skin that covers the glans (head) of the flaccid penis (Figure 1.6). Male circumcision is one of the oldest surgical procedures and is still the most common.

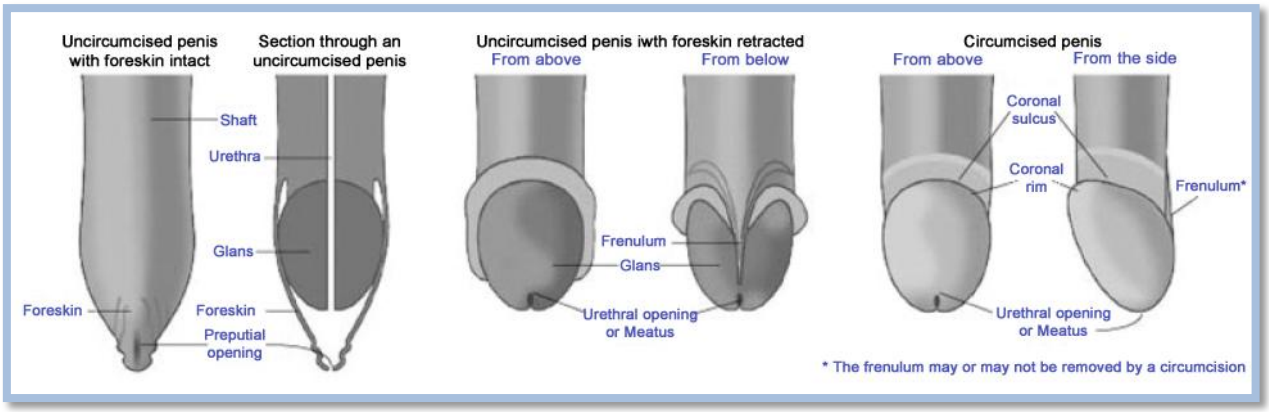

Figure 1.6 The uncircumcised and the circumcised penis, annotated to show the different anatomical parts (Morris, 2007)

Worldwide, about one in four men is likely to undergo circumcision for various reasons. Globally, 30%-35% of men are circumcised. The circumcision status is not always equated to medical circumcision, as there is a large variation in the cross-cultural and inter-individual techniques and the instruments employed, as well as the amount of foreskin removed. In Africa, approximately 68% of men are circumcised, and the majority, by traditional practitioners in the informal sector (Bailey and Egesah, 2006). It is a practice observed mostly for cultural and religious reasons and less often for health reasons (Figure 1.7) (WHO, 2007a).

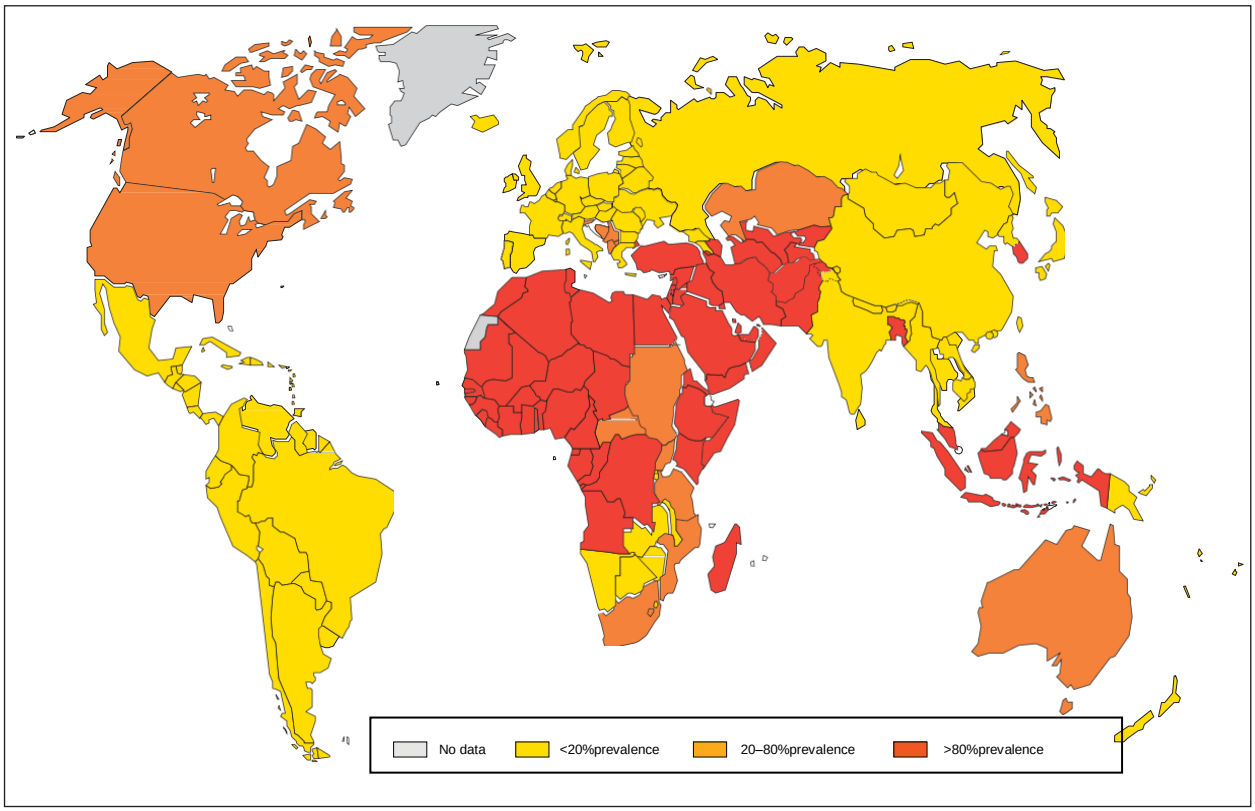

Figure 1.7 The prevalence of male circumcision (WHO, 2007a).

The benefit of male circumcision (MC) in halving HIV incidence in men is drawing attention to the role of circumcision in preventing other sexually transmitted infections, including HPV (Chin-Hong, 2008; Morris, 2007). Recent studies have shown that circumcision reduces HIV infection by 50% to 60% in heterosexual African men (Auvert *et al*., 2005; Bailey *et al*., 2007; Gray *et al*., 2007). Given the overwhelming evidence that male circumcision reduces susceptibility to HIV infection, WHO/UNAIDS have published recommendations for countries to consider scaling up access to MC. A better understanding of the additional benefits or risks of MC, including protective effects of MC on other sexually transmitted infections (STIs) is needed (Boily *et al*., 2008).

Evidence that male circumcision is associated with a strongly reduced risk of penile HPV infection is rapidly accumulating (Baldwin et al., 2004; Castellsague et al., 2002; Golden and Wasserheit, 2009; Gray et al., 2009; Hernandez et al., 2008b; Lu et al., 2009; Tobian et al., 2009; Vaccarella et al., 2006). Preventing HPV infection via circumcision is

persuasive, not only because it may prevent penile warts and penile cancer, but also because of the impact it will have on preventing HPV malignancies in female and male sex partners of HPV infected men (Chin-Hong, 2008). The mechanism by which circumcision lowers risk of HPV infection is not fully understood. There are a few possible explanations: The uncircumcised foreskin is comprised of unkeratinized mucosal epithelium that amplifies the surface area vulnerable to HPV infection. Micro abrasions to the epithelium due to trauma during intercourse allow easier access of the virus to the basal cells that are the target cells for infection by HPV. In contrast, there is relatively little mucosal epithelium in circumcised men. Exposed areas like the epithelium of the glans penis (cornified after circumcision) and stratified squamous epithelium of the penile shaft are more resistant to micro trauma and subsequent infections in circumcised men than uncircumcised men (Chin-Hong, 2008). It is also suggested that the anoxic microenvironment of the sub-preputial space may encourage the growth of pro-inflammatory anaerobic bacteria. These anaerobes activate Langerhans cells to present HIV to CD4 cells. The lessening of the microbiome post circumcision may play a part in protection from HIV and sexually transmitted diseases like HPV (Price *et al*., 2010).

In uncircumcised men, the shaft of the penis and outer surface of the foreskin are protected by the keratinized squamous epithelium acting as protective barrier against HPV infection. The non-keratinized mucosal lining of the inner foreskin might be more susceptible to the virus although one study concluded that there were no significant differences in the keratin thickness of the inner and outer foreskin of healthy Ugandan men (Dinh et al., 2012). During sexual intercourse the foreskin is retracted, which exposes the inner mucosal surface to vaginal and cervical secretions and to HPV. When the foreskin returns to cover the glans, contact time is prolonged between any pathogens present and non-keratinized skin (Albero *et al*., 2012; Golden and Wasserheit, 2009).

Randomized trials have shown that male circumcision significantly decreases risk of HSV-2 infections, but had no effect on the incidence of syphilis (Tobian *et al*., 2009). In contrast, a review of male circumcision and ulcerative STI strongly indicates that circumcised men are at lower risk of chancroid and syphilis (Templeton *et al*., 2009; Weiss *et al*., 2006). A borderline protection effect of male circumcision on *T vaginalis* infection, with no protective effect for *N gonorrhoeae* infection has been demonstrated (Sobngwi-Tambekou *et al*., 2009).

1.12 HPV diagnostic assays for male specimens

Traditionally, testing for HPV has been the focus of screening programs for early detection and treatment of cervical cancer in women by cytology and histology. Adjunctive molecular tests are increasingly being used to risk-stratify women with abnormal biopsies. South African National screening policy states that a woman is entitled to three (at the ages of 30, 40 and 50) free pap smears in the public sector. Screening takes place through pap smears.

The majority of molecular testing for HPV has been optimized and validated for female specimens only. Men are typically screened clinically with a visual inspection for lesions. There are no specific tests for HPV in men that are FDA approved for clinical use. The following molecular tests can be used for the diagnosis of HPV in male specimens:

1.12.1 Polymerase chain reaction (PCR) – Target amplification

PCR is a powerful tool for the epidemiological investigation of HPV infection or cervical cancer. PCR has defined the natural history of HPV infection more clearly. All HPV types are closely related and thus assays can be easily designed to target the conserved regions of its genome or the regions that best distinguish between different HPVs (Denny and Wright, 2005), HPV can be detected by PCR with in-house developed assays that

use consensus primers to amplify a broad spectrum of HPV types in a single PCR. There are two principal approaches used in the detection of HPV by PCR, consensus PCR and type-specific PCR. The primers which have been developed, include degenerate primers MY09/11 or its derivative PGMY09/11; pU-1M/pU-2R; and consensus primers $GP5^{+}$/GP6+, SPF1/SPF2 (Coutlee *et al*., 2005). These primers target conserved regions of the HPV genome, such as the L1 capsid gene (Coutlee *et al*., 2005).

1.12.2 Genotyping

The reverse line blot assay from Roche Molecular Systems (Branchberg, NJ) is one of the first widely used prototype methods. This assay uses L1 consensus primer-based PCR with PGMY09/11 primers. The assay detects 37 different HPV types, 14 of which are high-risk genotypes and 11 low-risk types. The assay is commercialized as the Linear Array HPV Genotyping Test. Probes for multiple HPV types are preset on a membrane strip and the PCR amplicon is hybridized onto the strip and then followed by visual detection. The results are read with the unaided eye based on a visible band in specific areas of the hybridization strip.

Another commercially available test, INNO-LiPA HPV Genotyping Extra (Innogenetics, Ghent, Belgium), also utilizes the principles of reverse line blot hybridization. The INNO-LiPA test amplifies HPV DNA with SPF10 primers at the L1 region. The probes are fixed to a membrane strip in sequence-specific lines and visualized as purple/brown bands. The INNO-LiPA test distinguishes between 24 low and high risk HPV types.

Genotyping methods are less suited for high throughput processing, require more hands on time and are less amenable to automation (Cuschieri and Cubie, 2005; Wong *et al*., 2008). Most HPV genotyping systems for clinical use are PCR-based. The limitation of genotyping is that it is time-consuming, expensive, high levels of expertise are needed and cross contamination is widespread. An automated or semi-automated assay is

needed for the processing of the samples and for detection is advantageous (Fernandez-Olmos *et al*., 2008).

1.12.3 HPV viral load

HPV viral load has been suggested as a biomarker for HPV molecular testing. It has been proposed that high HPV viral loads are a result of active viral replication and proviral persistence (Keegan *et al*., 2009). Use of HPV viral load for identifying risk remains controversial, as the input number of cells per sample has not been standardized. HPV DNA and a human gene (β-globin) can be amplified for each specimen. The HPV DNA signal obtained can be adjusted according to the amount of cellular DNA present and expressed as HPV DNA copies/cell (Coutlee *et al*., 2005). Real time quantitative PCR (qPCR) assays are characterized by high reproducibility and precision, although, the accuracy of the data largely depends on several factors such as sample preparation, quality of standards, controls and inclusion of housekeeping genes (for example β-globin).

1.12.4 mRNA

More recently, HPV RNA testing has become an important alternative approach for molecular diagnosis of HPV. It is different from DNA assays, which only test for the presence of viral genomes, in that it detects HPV genome expression and viral oncogenic activity in infected cells (Villa and Denny, 2006). One shortcoming of testing for HPV DNA is that it may detect transient HPV infections which can result in over reporting. mRNA tests allow monitoring of persistent expression of oncogenes such as E6 and E7, known to be involved in the malignant process. mRNA testing may be a more specific approach and appropriate for persistence risk-evaluation. A commercially available detection kit for E6/E7, utilizing multiplex nucleic acid sequence based amplification (NASBA), is available for real-time detection of HPV 16, 18, 31, 33 and 45 E6/E7 full-length mRNA transcripts. It is limited in the number of HPV types detected, but serves as a valuable tool in monitoring

HPV infections that produce proteins with transforming potential (PreTect HPV-proofer, Norchip) (Cuzick *et al*., 2006; Molden *et al*., 2007)**.**

1.13 Rationale for the research

Worldwide, prevalence of HPV among women is high and it is the most common sexually transmitted infection in the world. The spread of HPV infection is wider in Africa (Clifford *et al*., 2005a; Clifford *et al*., 2005b). Genital cancer has been linked to hrHPV infection, with cervical cancer being the most common cancer affecting women worldwide. Extensive research in the HPV field has focused on infections in women because of the association between HPV infections and cervical cancer. There is little known about the natural history and clinical course of HPV infections in men. Observational studies (Gray *et al*., 2009) have suggested HPV prevalence is lower in men who are circumcised, but such association has not yet been demonstrated in a randomized control trial. It has been proposed that HPV entry and persistence is more efficient in the inner mucosal surface of the prepuce than in the keratinized penile surface of circumcised men (Hernandez *et al*., 2010). Studies of HPV in men are necessary to provide an understanding of HPV transmission, characterization and carcinogenesis in order to prevent disease in men and women. Very few epidemiological studies have investigated HPV infection among men in South Africa.

This study aims to: 1) Improve our knowledge of HPV infections and establishing the prevalence of HPV among young South African men; 2) to establish if there is significance in the relationship between HIV and HPV co-infections; and 3) to gain insight into the role of the foreskin, how it might affect HPV transmission and if the foreskin tissue (particularly inner foreskin tissue) is vulnerable to HPV acquisition.

1.14 Research objectives

1) The first objective of this study is to investigate the association and prevalence of hrHPV, lrHPV infection with male circumcision among young men in a randomized controlled trial.
2) The causal association between hrHPV or lrHPV and HIV acquisition and interaction will be explored.
3) The third objective is to evaluate the role of the male foreskin in HPV infection prevalence and transmission. It will compare subtype-specific HPV positivity among the following sampling sites: the inner surface of the foreskin, the outer surface of the foreskin, the corona sulcus and the inner and outer foreskin tissue. The effect of genital washing, sampling methods and its implications for HPV distribution will be investigated

CHAPTER 2: THE EFFECT OF MALE CIRCUMCISION ON THE PREVALENCE OF HIGH-AND LOW-RISK HUMAN PAPILLOMAVIRUS IN YOUNG MEN: RESULTS FROM A RANDOMIZED CONTROLLED TRIAL CONDUCTED IN ORANGE FARM, SOUTH AFRICA

2.1 Introduction

Human Papillomavirus is the most common sexually transmitted infection worldwide (Trottier and Franco, 2006), with a estimated global prevalence among women of 10.4% (de Sanjose *et al*., 2007). HPV prevalence among African women is disproportionately high, ranging from 22.1% to 46.0% (Clifford et al., 2005a; de Sanjose et al., 2007; Smith et al., 2008). In a study of genital HPV prevalence among heterosexual men on 5 continents, Vardas and colleagues found the prevalence of any HPV type at 21.0% (18.7% penis, 13.1% scrotum, 7.9% perineal / perianal region) (Vardas *et al*., 2011). In excess of 100 HPV genotypes have been identified, with about 40 infecting the mucosal epithelium. These HPV genotypes are divided into high-risk and low-risk genotypes, according to their association with cervical lesions. The high risk HPV (hrHPV) types are more frequently found in premalignant or malignant lesions and are associated with cancers of the cervix, vulva, vagina, anus, and penis (Baldwin et al., 2004; Castellsague, 2008; Castellsague et al., 2002; Cogliano et al., 2005; Gravitt et al., 2000; Gustavsson et al., 2009; WHO-IARC, 2007; WHO, 2007b).

An underlying association between cervical cancer and hrHPV has been well established (Bosch *et al*., 1995; Castellsague *et al*., 2002; Koutsky *et al*., 1992; Lehtinen *et al*., 2001; Morris, 2007; Rozendaal *et al*., 2000), as demonstrated by the estimated worldwide prevalence of hrHPV in cervical carcinomas of 99.7% (Walboomers *et al*., 1999). Cervical cancer is the most widespread cancer affecting women in developing countries and more than 70% of cases of cervical cancer occurring in this region have been attributed to hrHPV genotypes 16 and 18 (de Sanjose *et al*., 2007; Munoz *et al*., 2004; Schiffman *et al*., 2007). Therefore, any intervention reducing the acquisition or transmission of HPV will also significantly reduce the burden of disease, especially in developing countries (Castellsague *et al*., 2002).

Ninety percent of genital warts (GWs), also called condylomata acuminate is caused by low-risk HPV (lrHPV) genotypes 6 and 11 (Garland *et al*., 2009). LrHPV prevalence in women has been estimated at 13.6% in Sub-Saharan Africa (Clifford et al., 2005a), Comparatively little is known about the epidemiology and natural history of lrHPV infection and GWs in sub-Saharan Africa. LrHPV prevalence shows considerable regional variation, such as 11% in the Gambia , 28% in Tanzania and can range from 1.2% to 30.0% depending on geographical region, target population, and sexual behaviour (Smith *et al*., 2008). Although treatment helps to eliminate the virus, GWs generally not painful, but they do itch and with frequent new lesions or recurrences causing in patient distress (Garland *et al*., 2009; Lacey, 2005). GWs can cause a decline in health-related quality of life (Woodhall *et al*., 2008). The psychological burden of GWs have the largest impact on quality of life compared with other clinical forms of HPV, mainly due to impact on sexuality, self-image, and partner transmission (Pirotta *et al*., 2009).

Multiple hrHPV and lrHPV infections may have important clinical and epidemiological implications. Infection with multiple HPV genotypes has been associated with anogenital warts (Brown *et al*., 1999; Chan *et al*., 2009; Nielson *et al*., 2009), the acquisition of new HPV type and HPV infection persistence (Kjaer *et al*., 2005), dysplasia (Fife *et al*., 2001), cervical intraepithelial neoplasia severity (Bello *et al*., 2009; Spinillo *et al*., 2009), cytological abnormalities in anal epithelium (Damay *et al*., 2010b), and HIV co-infection (Banura *et al*., 2008; Muller, Chirwa, and Lewis, 2009; Vajdic *et al*., 2009). Therefore, the investigation of lrHPV infection and the number of lrHPV genotypes detected in urethral swabs is key since being infected with one or multiple genotypes may result in a different clinical outcome.

Incidence and HPV type distribution in men with GWs was 2.35 cases per 1000 person-years, the highest incidence among men aged 18-30 years (3.43 cases per 1000 person-years), with HPV 6 (43.8%), HPV 11 (10.7%), and HPV 16 (9.8%) the genotypes most

commonly detected in GWs (Anic *et al*., 2011). In a study of men who have sex with men (MSM) infected with HIV, 74.6% had anal HPV infection, 68.7% had hr HPV infection, and 56.7% had multiple infections. Most frequent HPV types identified were HPV 44/55 (19.4%), HPV 53 (19.4%), HPV 16 (16.4%), HPV 39 (16.4%), and HPV 42 (14.9%) (Damay *et al*., 2010a). In a South African cohort of heterosexual men, the prevalence of anogenital HPV among study participants was 78%. In 81%, HPV 6, 11, 16 and 18 were prevalent as either single or combined infections. HPV types 6 and/or 11 were significantly higher among GW patients ($p<0.001$). HIV seropositivity was significantly associated with multiple HPV infections (OR=3.98, 95% CI 1.58 to 10.03) (Muller, Chirwa, and Lewis, 2009).

Since men are carriers of HPV and the main route of transmission to women is through sexual contact with infected men, it would be of benefit to reduce the HPV prevalence, not only in women, but in men as well. Recent observational studies have suggested that HPV prevalence was reduced among circumcised men compared to uncircumcised men (Baldwin *et al*., 2004; Castellsague *et al*., 2007; Castellsague *et al*., 2002; Giuliano *et al*., 2009; Hernandez *et al*., 2008b; Vaccarella *et al*., 2006) whereas other studies failed to find an association (Nielson et al., 2007b; Shin et al., 2004). However, two recent male circumcision (MC) trials have demonstrated a lower hrHPV prevalence and incidence among circumcised men by 32% to 35%. The authors argue that while male circumcision is primarily a male concern, one trial also reported benefits derived by female partners of circumcised men (Wawer et al., 2011). This protective effect of MC against acquisition could be explained by the role of the foreskin on HPV transmission. Viral access to the basal keratinocytes in the cornified epithelium of the circumcised penis is limited. There is no a difference between the keratinisation of the glans penis of circumcised men and uncircumcised men, but the mucosal epithelium of the inner prepuce is not keratinised which makes it more susceptible to microtrauma injury during intercourse (vaginal, anal and oral sex). It is postulated that poor genital hygiene, inflammatory effect of

accumulated smegma, can also assist in the harbouring of HPV infection (Gray *et al*., 2009). Consequently, the same protective effect of MC can be expected on lrHPV prevalence, but to date, very little is known about this association.

The objective of this study was to analyze the effect of medical male circumcision (MMC) on the prevalence of hrHPV and lrHPV infection by considering both hrHPV and lrHPV prevalence and the number of hrHPV and lrHPV genotypes detected among young men. The secondary objective was to identify other risk factors for hr HPV or lrHPV infection.

Data used were collected during the MMC randomized controlled trial (RCT) conducted in Orange Farm (South Africa), which demonstrated a partially protective effect of male circumcision on the acquisition of HIV (Auvert *et al*., 2005).

2.2 Methods

2.2.1 Collection of data

A randomized, controlled, intervention trial was carried out in Orange Farm and surrounding areas, a semi-urban region close to the city of Johannesburg. The recruitment of participants took place in the general population from July 2002 to February 2004. Information about the trial was disseminated in the community through meetings during the recruitment period. Precise oral and written information was delivered at the investigation centre to potential participants during a pre-screen visit. Participants were then informed that the impact of MC on the acquisition of sexually transmitted infections (STIs), including HIV, is not known (See Appendix A for Participant Information Sheet). The inclusion and exclusion criteria are listed in Table 2.1. The participants received a total of 300 South African Rand as compensation. 3274 uncircumcised men aged 18–24 years were recruited, randomized into 2 groups, and followed up through visits. MC was offered immediately after randomization to the intervention group and after the end of the

follow-up period to control group participants. During each follow-up visit at 3, 12, and 21 months, circumcision status was assessed by a nurse through genital examination. Blood samples were obtained and tested for HIV as well as HSV-2 at each visit. Questionnaires (Appendix B) allowed for data collection on background characteristics (age, ethnic group, level of education, number of lifetime sexual partners, and marital status) as well as reported sexual behaviour in the past 12 months (number of sexual contacts and frequency of condom use).

Table 2.1 Inclusion and Exclusion Criteria

Type	Criterion
Inclusion criteria	Be a male between the age of 18 and 24
	Wish to be circumcised
	Reside in the Orange Farm area or surrounding areas
	Be able to understand the nature of the trial
	Agree to be randomized to either of the two groups (intervention group and control group)
	Agree to come to three follow-up visits
	Agree to answer general health question and questions related to sexual activity
	Agree to have genital examinations
	Agree to give blood samples tested for HIV and syphilis
Exclusion criteria	Be medically circumcised
	Have had any contraindication to MC

After 262 consecutive days, from 7 March to 24 November 2005, a penile and urethral swab sample was collected by a nurse from each participant who reported for the 21-month follow-up visit. Molecular testing for HPV requires a small amount of cells from which DNA can be extracted. Compared to sample collection from the cervix, cell collection from the keratinized epithelium of male genitalia is a challenge. Penile cells were collected by rubbing a wet Dacron swabs on the shaft of the penis and urethral swabs were taken by inserting a urethral Dacron swab into the urethra. Participants were

included in the trial regardless of their HIV and HSV-2 status. All participants signed a written consent form for these tests (Appendix B). The urethra was chosen as a sampling site because the detection of HPV in this anatomical site is probably not affected by circumcision status. These swab samples were analyzed to assess the association between the prevalence of hrHPV strains and MC as well as lrHPV and MC status. An additional five hundred and ninety six participant follow-up visits were added to the original database analyzed to report results on the association between MC status and hrHPV, yielding a total of 1768 participant follow-up visits to assess the association between MC and both prevalence and number of lrHPV genotypes. A urethral swab sample was also collected at a follow-up visit ~6 weeks after circumcision from all control group participants who took part in a nested study designed to compare 2 circumcision methods. To ascertain that the detection of HPV was not affected by circumcision status, we used these swab samples to compare the prevalence of hrHPV among the nested study participants before and after circumcision. Finally, study participants were asked to give a first-void urine sample to test for urogenital *Neisseria gonorrheae*, the presence of which was used as a biological marker of sexual behaviour.

Condoms were provided in the waiting room of the investigation centre and were also provided by the counsellor.

The circumcisions were performed by three local general practitioners in their surgical offices. The general practitioners were experienced in MC practices. The procedure was standardized and used the forceps-guided method, as is widely practiced in South Africa, and was reviewed by the Department of Urology, University of the Witwatersrand Medical School, South Africa.

To ensure confidentiality, participants' files were kept in a locked room at the centre and each participant received a number that was used to identify all documents related to that

person. To ensure blinding of study personnel, the randomization group information was not available to the personnel in charge of counselling or collecting information in the centre during the participants' visits.

Questionnaires were checked at the end of each interview. Participants failing to be present for any follow-up visit were visited at home by trial staff and encouraged to come for the follow-up visits, or ascertained the reasons for dropping out.

Laboratory results were stored in a database that was independent of the one used to store the information related to each participant. During the study, no HIV results were available to the investigation centre or to the investigators, apart from the statistician in charge of the interim analysis. Specimens were processed using an anonymous identifying number and a linked laboratory number. HPV laboratory results were entered and reviewed from the raw data generated into a Laboratory Information System by two separate laboratory staff members.

2.2.2 Laboratory methods

All swabs were frozen at -20°C immediately after collection and kept frozen until processing. Parallel testing of 60 penile and 60 urethral swabs was performed to assess which anatomical site would yield adequate cell collection and to reduce the incidence of incomplete specimens. Based on the results (Table 2.2) all subsequent analyses were limited to the urethral swabs.

DNA was extracted from the urethral swabs by means of the MagNA Pure LC instrument (Roche) with the Roche MagNA Pure LC DNA Isolation Kit I. Swabs were lysed in 500 µL of the kit lysis buffer for 30 min at room temperature. The MagNA Pure external lysis protocol was used to extract DNA from the lysis buffer into 100 µL of eluate; 50 µL of the eluate was used for screening (Roche AMPLICOR HPV Test). This standardized

polymerase chain reaction (PCR)-based method can detect 13 hrHPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). The other 50 μL eluate was used for genotyping (Roche Linear Array Genotyping test). The test utilizes amplification by Polymerase Chain Reaction (PCR) and nucleic acid hybridization and detects 37 hrHPV and lrHPV anogenital genotypes of HPV: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 39, 70, 71, 72, 73, 81, 82, 83, 84, IS39 and CP6108 (Gravitt *et al*., 2000; Gravitt *et al*., 1998).

Both tests are standardized, qualitative *in vitro* tests and use biotinylated primers to define a sequence of nucleotides within the polymorphic L1 region of the HPV genome that is approximately 165 (Roche AMPLICOR HPV Test) or 450 (Roche Linear Array Genotyping test) base pairs long. A pool of HPV primers present in the Master Mix is designed to amplify HPV from 13 or 37 genotypes respectively. Capture probes sequences are located in polymorphic regions of the L1 bound by these primers.

In the Roche AMPLICOR HPV Test, the amplification product is used for reverse hybridization with the oligoprobe specific for the hrHPV group and beta (ß)-globin gene coating microtitration plate wells. The PCR product bound to the specific probes is detected through biotin by a streptavidin-conjugated antibody with HRP (horse-radish peroxidase) and visualized by colour reaction of the substrate containing hydrogen peroxidase and 3,3',5,5'-tetramethylbenzedine.

Roche Linear Array Genotyping test requires aliquots of denatured amplicon to be transferred to an appropriate well of a typing tray containing a single Linear Array HPV Genotyping Strip coated with HPV and ß-globin probe lines. After stringent washing to remove unbound material, streptavidin-HRP and substrate steps are applied to each strip. A blue coloured complex at the different probe positions can be visually read by comparing it to the Linear Array HPV Genotyping Test Reference Guide.

The following 23 genotypes are characterized as lrHPV: 6, 11, 26, 40, 42, 53, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and CP6108. A more conservative approach was used, which considered a subset of 11 genotypes (i.e., genotypes: 6, 11, 26, 40, 42, 53, 54, 55, 83, 84, CP6108), excluding any genotype having been labelled as of intermediate risk in the literature or not listed in the lrHPV genotypes list. The following HPV genotypes were considered as hrHPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) (Cogliano *et al*., 2005; de Villiers *et al*., 2004; Munoz *et al*., 2003; Zuna *et al*., 2007).

Because of the combined probe of the assay for HPV-52 and to be conservative, samples were classified as being positive for HPV-52 only when they were negative for genotypes 33, 35, and 58. Negative results with a negative internal ß-globin PCR control were excluded. Fifteen results (15/1768; 0.84%) with a negative internal ß-globin PCR control were excluded, four in the control arm (4/867; 0.46%) and eleven in the intervention arm (11/901; 1.2%). Genotyping was performed for all positive samples. An HPV-positive sample was defined as a sample in which at least 1 HPV genotype was detected. In some analyses we also considered multiple-HPV samples, defined as samples in which at least 2 HPV genotypes were detected.

Urine specimens were tested for *N. gonorrheae* by PCR (Roche COBAS AMPLICOR PCR) by the Specialized Molecular Unit at the National Institute for Communicable Diseases (NICD). One EDTA blood tube of 10 ml of venous blood was taken and immediately centrifuged at 400 g for 10 min, and five aliquots of plasma were frozen at −20 °C. The samples were identified only by the participant number and transported each week to the laboratory, where they were stored at −70 °C and tested.

An ELISA screen (Genscreen HIV1/2 version 2, Bio-Rad, France) and two ELISA confirmatory tests (Wellcozyme HIV recombinant, Abbott Murex, Dartford, United Kingdom, and Vironostika HIV Uni-Formm II plus O, bioMérieux, Boxtel, Netherlands)

were used to test plasma for HIV-1 infection. Samples that were positive on all three ELISAs were regarded as “positive” and all others as “negative”.

An HSV type 2 specific IgG assay was used to detect HSV-2 antibodies with an index cut-off value of 1.1 (Kalon HSV-2 gG2 assay; Kalon Biologicals Ltd., Aldershot, UK), according to the manufacturer’s recommendations. All serology tests were performed by the Serology Department at NICD.

2.2.3 Data analysis.

The data analysis of this study was done in two parts, firstly the effect of MC on the prevalence of hrHPV was analysed, followed by an analysis of the association of lrHPV with MC. The intention-to-treat (ITT) and as-treated (AT) prevalence rate ratios (PRRs) for hrHPV positivity, lrHPV positivity and *N. gonorrheae* positivity were estimated using univariate log Poisson regression. These analyses were repeated multivariately for hrHPV controlling for ethnic group, age, education, lifetime number of sex partners, marital status, number of non-spousal partners in the past 12 months, condom use, number of sex acts in the past 12 months and HIV status. Results are formulated in terms of PRRs of lrHPV positivity and Poisson mean ratios (PMRs) of the mean number of HPV genotypes, respectively, comparing men of the intervention group with men of the control group. The intention-to-treat and as-treated adjusted PRRs (aPRRs) and adjusted PMRs (aPMRs) for lrHPV were estimated controlling for the following covariates: ethnic group, age, education, lifetime number of sex partners, marital status, number of non-spousal partners in the past 12 months, condom use in the past 12 months, number of sex acts in the past 12 months, and HIV status and HSV2 status (Cogliano *et al*., 2005) at the 21-month visit. Analyses were repeated using as an outcome, the subset of 11 lrHPV genotypes.

To assess the potential impact of HIV acquisition, which is reduced by MC and is associated with HPV infection (Ng'ayo *et al*., 2008), these analyses were repeated after

excluding subjects who underwent HIV sero-conversion during the follow-up period (*n*=25). To evaluate a possible imbalance between the groups, analyses were repeated after controlling for propensity score coded in quintiles (Rosenbaum and Rubin, 1983). Percentages were compared with Fisher's exact test and means with the Mann-Whitney test. Statistical analyses were performed using the statistical package SPSS for Windows (version 8; SPSS) and the R programming language (version 2.6.1) and using STATA 9.0 (StataCorp. 2005. Stata Statistical Software: Release 9. College Station, TX: StataCorp LP).

2.2.4 Ethics

The research protocol was reviewed and approved by the University of Witwatersrand Human Research Ethics Committee (Medical) on 22 February 2002 (protocol study M020104). The trial was also approved by the Scientific Commission of the French National Agency for AIDS Research (ANRS; protocol study no. 1265; 2002, decision no. 50), and authorization was obtained from the City of Johannesburg, Region 11, on 25 February 2002. This trial has been registered at http://www.clinicaltrials.gov/ under the number NCT00122525.

2.3 Results

The results from the 60 urethral and penile swabs tested for specimen adequacy is summarized in table 2.2. HPV prevalence varied by anatomical site: 25% in urethral specimens and 11.37% from the penile shaft. ß-globin positivity ranged from 96.67% (urethral) to 55% (penile). For ß-globin positive samples, HPV DNA positivity was 25.86% and 21.21% in the urethral and penile specimens respectively. For ß-globin negative samples, the observed prevalence was 3.70% in the penile swabs, but there were none observed in the urethral swabs. In absolute terms, HPV DNA positivity was higher in ß-globin positive samples compared with ß-globin negative samples.

Table 2.2 Positivity to Human Papillomavirus according to beta-globin status and sample collection site

Site	N	HPV %	Beta-globin positive %	HPV% in beta-globin positive	HPV % in beta-globin negative
Urethral	60	25.00 (15/60)	96.67 (58/60)	25.86 (15/58)	-
Penile	60	11.67 (7/60)	55.00 (33/60)	21.21 (7 /33)	3.70 (1/27)

Table 2.3 shows the baseline characteristics of the initial 1264 participants from whom a urethral swab sample was collected at the 21-month visit and analysed for hrHPV, reported by randomization group. These characteristics were similar in the 2 groups; the only significant difference was in HIV status. The mean (median) durations of follow-up in the intervention and control groups were 644 (637) and 649 (637) days, respectively.

Table 2.3 Background characteristics, reported sexual behaviours, and HIV prevalence at the 21-month visit (Auvert et al., 2009).

Characteristic	Control group (n=627)	Intervention group (n=637)	*P*
Ethnic group			0.77[a]
Sotho	55.7%	54.9%	
Zulu	29.2%	28.4%	
Other	15.2%	16.6%	
Age < 21 years	34.0%	29.8%	0.12[a]
Primary level of education completed	98.9%	98.6%	0.80[a]
Married or living as married[b]	4.0%	5.2%	0.49[a]
Reported sexual behaviour			
Mean (median) number of lifetime partners	4.7 (4.0)	4.2 (4.0)	0.10[c]
Mean (median) number of non-spousal sex partners[b]	0.90 (1.0)	0.9 (1.0)	0.48[c]
Mean (median) number of sex-acts[b]	10.0 (5.0)	11.6 (5.0)	0.98[c]
Consistent condom use with non-spousal sex partners[b,d]	25.0%	26.0%	0.84[a]
HIV-positive	7.3%	3.9%	0.010[a]

NOTE.Data are percentage of subjects, unless otherwise indicated.

[a]Chi-square or Fisher's exact test, as appropriate.

[b]During the past 12 months.

[c]Kruskal-Wallistest.

[d]Among those having had sexual intercourse during the past 12months.

As indicated in Figure 2.1, the percentage of each of the 13 hrHPV genotypes was always lower in the intervention group than in the control group. In the intention-to-treat comparison, the differences were significant for genotypes 18, 31, 45, 52, 56, 58, and 68.

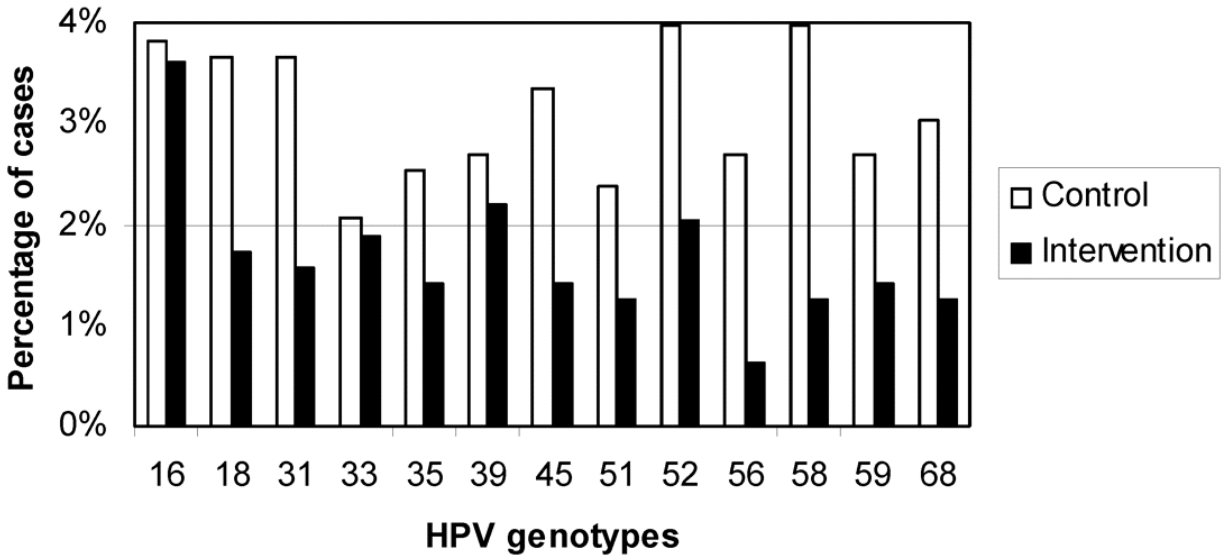

Figure 2.1 Distribution of high-risk human papillomavirus genotypes as a function of randomization groups (Auvert et al., 2009).

Table 2.4 presents the intention-to-treat univariate association between the prevalence of hrHPV and MC at the scheduled 21-month visit. HrHPV prevalence was significantly lower among men in the intervention group. It shows that the protective effect of MC on hrHPV was higher in the as-treated analysis than in the intention-to-treat analysis. The protective effect was lower in both analyses when potential confounders were controlled for, including HIV status and reported sexual behaviour cofactors. HrHPV was associated with HIV status in both analyses, with adjusted PRRs (aPRRs) of 2.2 (95% confidence interval [CI], 1.5–3.3) and 2.2 (95% CI, 1.5–3.2), respectively. When those who underwent HIV sero-conversion during the follow-up period were excluded from the analysis, the results remained practically unchanged from those shown in table 2.4, with *P* values <.009 and a relative variation in PRRs and aPRRs of <5.2%. The aPRRs were almost identical when the analyses were adjusted for propensity score in addition to the other covariates.

The prevalence of multiple hrHPV types was 7.0% (89/1267; 95% CI, 5.7%–8.6%). It was significantly lower in the intervention group than in the control group (4.2% vs. 9.9%; PRR, 0.43 [95% CI, 0.28–0.66]; *P*<.001). Among men with at least 1 hrHPV, the multiple hrHPV prevalence was also lower among those in intervention group (44.3% vs. 28.7%; PRR, 0.64 [95% CI, 0.45–0.94]; *P*=.020).

During the study period, 371 men in the control group were circumcised and underwent urethral swab sampling before and after MC. The average (median) duration between the 2 swab collections was 59 (43) days. As expected, the hrHPV prevalences were not different for the 2 samplings (23.7% vs. 23.9%; *P*>.99, sign test). The proportions of men with multiple hrHPV infections did not differ significantly (10.2% vs. 12.1%; *P*=.40, sign test). These results indicate that the as-treated effect of MC on hrHPV prevalence shown in Table 2.4 cannot be attributed to easier detection of hrHPV by urethral swab sampling in uncircumcised men.

Table 2.4 Association between the prevalence of high-risk human papillomavirus (hrHPV) and male circumcision (Auvert et al., 2009)

	hrHPV prevalence % (positive/total)	PRR (95% CI) [P]	
		Unadjusted	Adjusted[a]
Randomization group			
Control	22.3% (140/627)	1.00 (reference)	1.00 (reference)
Intervention	14.8% (94/637)	0.66 (0.51–0.86) [0.002]	0.68 (0.52–0.89) [0.004]
Circumcision status			
Uncircumcised	23.2% (144/621)	1.00 (reference)	1.00 (reference)
Circumcised	14.0% (90/643)	0.60 (0.46–0.79) [<0.001]	0.62 (0.47–0.80) [<0.001]

Note. CI, Confidence Interval; PRR, Prevalence rate ratio

[a] Adjusted for ethnic group, age, education, number of lifetime partners, marital status, number of non-spousal partners in the past 12 months, condom use in the past 12 months, number of sex acts in the past 12 months and HIV status.

As indicated in table 2.5 the prevalence of *N. gonorrhea* was similar in the 2 groups. Among men in the control and intervention groups, the median lifetime numbers of sex

partners were 4.1 and 4.2 (*P*=.49, Kruskal-Wallis test), and the proportions of subjects who consistently used condom were 17.4% and 19.7%, respectively (*P*=.45, Fisher's exact test). These findings suggest that the protective effect of MC on hrHPV cannot be attributed to a difference in sexual behaviour between the 2 groups.

Table 2.5 Association between the prevalence of *Neisseria gonorrhea* and male circumcision (Auvert et al., 2009).

	N. gonorrhea prevalence % (proportion positive)	**PRR (95% CI) [P]**	
		Unadjusted	**Adjusted[a]**
Randomization group			
Control	10.3% (62/601)	1.00 (reference)	1.00 (reference)
Intervention	9.1% (56/613)	0.89 (0.62–1.27) [0.51]	0.87 (0.60–1.26) [0.46]
Circumcision status			
Uncircumcised	10.0% (60/598)	1.00 (reference)	1.00 (reference)
Circumcised	9.4% (58/616)	0.94 (0.65–1.35) [0.73]	0.93 (0.64–1.34) [0.69]

Note. CI, Confidence Interval; PRR, Prevalence rate ratio

[a] Adjusted for ethnic group, age, education, number of lifetime partners, marital status, number of non-spousal partners in the past 12 months, condom use in the past 12 months, number of sex acts in the past 12 months and HIV status.

The 1753 participants from whom a urethral swab was collected at the 21-month visit and analyzed were included in the lrHPV analysis. The mean (median) durations in days of follow-up among the intervention and control group were 653 (637) and 648 (637), respectively.

Table 2.6 reports the study population's baseline characteristics, reported sexual behaviour and HIV, HSV-2, and lrHPV prevalences at the 21-month visit. Median age was 21.2 years (mean: 21.2, 95% CI: 21.2–21.3). The most frequent sexually transmitted infection (STI) was lrHPV. Compared to control group participants, men from the intervention group were slightly older (mean: 21.3 versus 21.1, $P < .04$) and reported more frequently having had a sexual partnership in the last year. Level of education, living with a partner, number of lifetime sexual partners, and consistent condom use were not

statistically different between the two groups. HIV and lrHPV prevalences at the 21-month visit were significantly lower in the intervention group in comparison with the control group. The mean number of lrHPV genotypes was 0.25 (95% CI: 0.21–0.30).

Table 2.6 Background characteristics, reported sexual behaviour, HIV, HSV-2 and lrHPV prevalence of the study population (Auvert *et al*., 2011)

	Control N=863	**Intervention N=890**	**P (1)**
Background Characteristics			
Ethnic group (%) Sotho Zulu Other	53.2 33.1 13.7	54.2 28.3 17.5	.02
Lives with a partner (%) (2)	4.7	5.5	.61
Aged 21 and older (%)	50.1	55.1	.04
Primary level of education completed (%)	98.8	(98.0)	.18
Reported sexual behaviour			
Mean (median) number of lifetime partners	4.3 (4.0)	4.4 (4.0)	.33
Had at least one sexual partnership in the past 12 months (%)	71.2	77.9	.004
Mean (median) number of partnerships in the past 12 months (2)	1.4 (1.0)	1.3 (1.0)	.70
Mean (median) number of sexual intercourses in the past 12 months (2)	13.3 (5.0)	13.9 (6.0)	.41
Consistent condom use in the past 12 months (%) (2)	23.6	24.9	.70
Sexually transmitted infection prevalence at the 21-month visit			
HIV positive (%)	7.2	4.6	.03
HSV-2 positive (%)	8.6	10.6	.17
Low-risk HPV positive (23 genotypes) (%)	15.8 (n=136)	8.5 (n=76)	<.001
Low-risk HPV positive (11 genotypes) (%)	12.4 (n=107)	6.6 (n=59)	<.001

(1) Fisher's exact for percentage comparison and Mann-Whitney test for mean comparison;

(2) Among those having at least one sexual intercourse in the past 12 months (437 control and 497 intervention participants).

The number of hrHPV and lrHPV genotypes were correlated (ρ = 0.67, P < .001). Among hrHPV-infected men, 56.2% (95% CI: 50.7–61.6) were infected with lrHPV. Among lrHPV-infected men, 85.9% (95% CI: 81.1–90.6) were infected with hrHPV. Figure 2.2 indicates that the percentage of each of the 23 lrHPV genotypes was always lower among men

from the intervention group compared to men from the control group. In the ITT comparison, the difference was significant for genotypes 40, 42, 53, 70, 84, and CP6108. Among the 212 lrHPV-infected men, 51.4% (95% CI: 44.6–58.2) were infected with more than one lrHPV genotype, for a maximum of 11 genotypes.

Figure 2.2 shows the distribution of the number of lrHPV genotypes among lrHPV-infected participants The prevalence of lrHPV infection was significantly lower among intervention group participants compared to control group participants (8.5% versus 15.8%; ITT aPRR: 0.54, 95% CI: 0.41–0.72, P<.001, and AT aPRR: 0.53, 95% CI: 0.40–0.70, P<.001). Figure 2.3 shows the distribution of the number of lrHPV genotypes among lrHPV-infected participants.

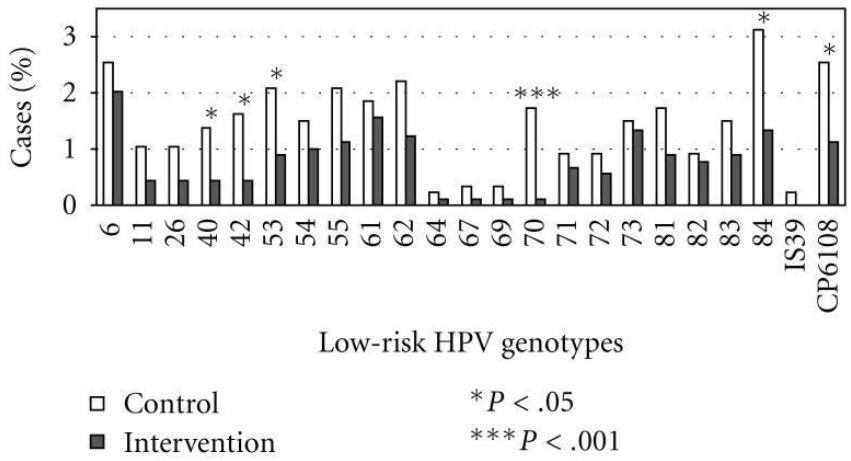

Figure 2.2 Distribution of the low-risk HPV genotypes as a function of randomization groups among the 1753 participants (intention to treat population) (Auvert *et al*., 2011).

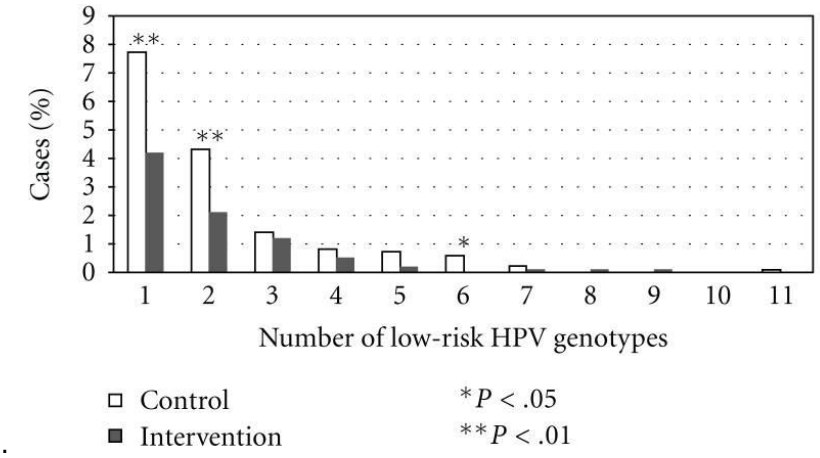

Figure 2.3 Distribution of the number of low-risk HPV genotypes collected in each urethral swab as a function of randomization group among the 1753 participants (intention to treat population) (Auvert *et al*., 2011).

Table 2.7 shows the univariate and multivariate PMRs of the number of lrHPV genotypes by MC status at the scheduled 21-month visit. In both univariate and multivariate ITT and AT analyses, the mean number of lrHPV genotypes in the intervention group was significantly lower, about half that of the control group. The association was slightly stronger in the AT analyses.

Table 2.7 Intention-to-treat (ITT) and As-treated (AT) Poisson Mean Ratios (PMRs) of the number of low-risk HPV (lrHPV) genotypes among the 1753 participants (Auvert *et al*., 2011).

	Number of participants (lrHPV infected)	**Mean of lrHPV genotypes**	**PMR (95% CI)**	**ITT aPMR (1) (95% CI)**	**AT aPMR (1) (95% CI)**
Circumcision status					
Randomization group			1	1	not applicable
Control Intervention	863 (136) 890 (76)	0.33 0.18	0.53 (0.44–0.64) P<.001	0.54 (0.44–0.66) P<.001	-
Circumcision status			1	not applicable	1
Uncircumcised Circumcised	855 (136) 898 (76)	0.35 0.16	0.45 (0.37–0.55) P<.001	-	0.46 (0.37–0.56) P<.001
Background characteristics					
Ethnic group			1	1	1
Sotho Zulu Other	941 (116) 538 (66) 274 (30)	0.24 0.27 0.26	1.12 (0.91–1.37) P=.29 1.05 (0.80–1.37) P=.75	1.05 (0.86–1.30) P=.63 1.00 (0.76–1.30) P=.98	1.04 (0.84–1.28) P=.77 1.06 (0.81–1.39) P=.56
Age			1	1	1
Under 21 21 and older	831 (82) 922 (130)	0.20 0.30	1.53 (1.26–1.85) P<.001	1.08 (0.88–1.32) P=.48	1.10 (0.90–1.35) P=.27
Primary level of education			1	1	1
Uncompleted Completed	28 (6) 1725 (206)	0.54 0.25	0.47 (0.28–0.78) P=.004	0.50 (0.30–0.84) P=.009	0.48 (0.29–0.81) P=.006

Reported sexual behaviour					
Number of lifetime sexual partners			1	1	1
two or less	586 (49)	0.14	2.24 (1.76–2.84)	1.68 (1.30–2.16)	1.69 (1.31–2.18)
more than two	1167 (163)	0.31	P<.001	P<.001	P<.001
Condom use in the past 12 months[(2)]			1	1	1
Inconsistent	695 (99)	0.32	0.48 (0.33–0.68)	0.66 (0.46–0.95)	0.68 (0.47–0.99)
Consistent	223 (19)	0.15	P<.001	P=.03	P=.04
Sexually transmitted infection prevalence at the 21-month visit					
HIV			1	1	1
negative	1650 (175)	0.21	5.10 (4.11−6.34)	3.29 (2.57–4.22)	3.20 (2.49–4.10)
positive	103 (37)	1.05	P<.001	P<.001	P<.001
HSV-2			1	1	1
negative	1585 (173)	0.22	2.68 (2.15−3.36)	1.49 (1.15–1.93)	1.52 (1.17–1.96)
positive	168 (39)	0.59	P<.001	P=.002	P=.002

(1) Adjusted for ethnic group, age, education, number of lifetime sexual partners, condom use in the past 12 months, and HIV and HSV2 statuses at the 21-month visit
(2) Among those having at least one sexual intercourse in the past 12 months.

Results remained unchanged when the more restrictive definition of 11 lrHPV genotypes was considered, with a twofold decrease of the mean number of lrHPV in the intervention group compared with the control group (0.10 versus 0.21; ITT aPMR: 0.51, 95% CI: 0.39−0.66, P<.001).

Mean number of lrHPV genotypes significantly increased with the number of lifetime sexual partners (ITT aPMR: 1.05, 95% CI: 1.02–1.09, P=.001). Prevalent HIV and HSV-2 infections at the 21-month visit were associated with an increased number of lrHPV genotypes (ITT aPMR: 3.40, 95% CI: 2.65–4.36, P<.001 and ITT aPMR: 1.49, 95% CI: 1.15–1.92, P=.002 respectively). Conversely, mean number of lrHPV genotypes was lower for participants having completed the primary level of education (ITT aPMR: 0.47, 95% CI: 0.28–0.79, P=.004) and those reporting consistent condom use (ITT aPMR: 0.65, 95% CI: 0.45–0.93, P<.02).

When participants who HIV sero-converted during follow-up were excluded from the analyses, the effect of MC on number of lrHPV remained unchanged (ITT aPMR: 0.53, 95% CI: 0.43–0.65, P<.001, and AT aPMR: 0.47, 95% CI: 0.38–0.58, P<.001). This implies that the effect of MC on lrHPV is independent of the effect of MC on HIV.

The aPMRs were almost identical when the analyses were adjusted for the propensity score in addition to the other covariates (ITT aPMR: 0.55, 95% CI: 0.45–0.66, P<.001, and AT aPMR: 0.46, 95% CI: 0.38–0.5, P<.001). This suggests that a possible imbalance between groups due to a differential follow-up had no effect on the association of lrHPV infection with MC.

2.4 Discussion

An independent and partial protective effect of MC was demonstrated on the prevalence of hrHPV and lrHPV. This effect on hrHPV was shown on the prevalence and not incidence because of the available biological samples in this MC trial. When the analysis was adjusted for possible confounding factors, such as sexual behaviour and condom use, the outcome remained unchanged. Given the results of the propensity analysis, the randomization and the absence of obvious differences between groups in *N. gonorrhea* prevalence or sexual behavioural characteristics, the difference in hrHPV prevalence between the 2 groups is likely attributable to MC. Therefore, the difference observed is probably the consequence of dissimilarity in hrHPV incidence between circumcised and uncircumcised men. In this study hrHPV prevalence is likely a proxy for hrHPV incidence, as HPV prevalence rises as a function of age among young men (Okesola and Fawole, 2000).

HPV-type co-infections can have an impact of HPV vaccination and disease in men due to potential HPV type competition followed by type replacement. It is important to conduct studies on multiple HPV infections, HPV distribution, associations between HPV types in multiple infections among men and type competition in men. Type competition may result from unknown biological kinetics, whereby infection with one HPV type inhibits the acquisition or facilitates persistence of other HPV types (Rositch *et al*., 2012).

No effect of MC on some hrHPV genotypes, such as 16 and 33 could be established. The apparent disparity in the effect of MC for different genotypes can be the result of true variation or random variation. This should be further investigated, for example, by comparing the results of the present study with those of other MMC trials conducted in other African countries (Bailey *et al*., 2007; Gray *et al*., 2007).

The protective effect of MC demonstrated in this study, corresponds in magnitude to what could have been expected from observational studies. Castellsague and colleagues (Castellsague *et al*., 2007) found in their meta-analysis an odds ratio of 0.56 (95% CI, 0.39–0.82), whereas Baldwin (Baldwin *et al*., 2004) reported an adjusted relative risk of 0.44 (95% CI, 0.23–0.81). Hernandez (Hernandez *et al*., 2008b) determined that uncircumcised men had an increased risk of nearly a 2-fold of oncogenic HPV infections (relative risk, 1.96 [95% CI, 1.02–3.75]). This study presents clear evidence that MC lowers the risk of heterosexual hrHPV acquisition among men.

HrHPV remains a major public health concern because of its contributory association with malignancies, especially cervical cancer in women. These findings show why MC has long been thought to be protective against cervical cancer (Morris, 2007). MC reduces the risk of hrHPV infection among men and as a result reduces the exposure of women to hrHPV. Consequently, the risk of cervical cancer is lowered because of the causal link between hrHPV and cervical cancer among women (Bosch et al., 1995; Castellsague et al., 2002; Koutsky et al., 1992; Lehtinen et al., 2001; Morris, 2007; Rozendaal et al., 2000; Wawer et al., 2011).

Three randomized controlled trials have shown that MC has a partial protective effect on the acquisition of HIV by males in Africa (Auvert *et al*., 2005; Bailey *et al*., 2007; Gray *et al*., 2007). In each case, the trial was terminated as the results were conclusive and it was considered unethical not to offer MC to the control groups in advance of the planned trial end date. The effect of MC on HPV reinforces the recommendation of the World

Health Organization and the Joint United Nations Programme on HIV/AIDS for the implementation of MC programs in countries with a high prevalence of HIV infection, a low prevalence of MC, and a high acceptance of MC (UN, 2007). While the cost of HPV vaccine remains a problem in Africa, the protective effect of MC may be an alternative to HPV vaccines in terms of genotype coverage and target-group age range.

There was a strong independent association found between lrHPV urethral infection and MC. lrHPV infection was analyzed as a prevalence rate and in terms of number of lrHPV detected in urethral swabs, because it was assumed that being infected with one genotype was different from being infected with two or more genotypes (Chan *et al*., 2009; Muller, Chirwa, and Lewis, 2009; Nielson *et al*., 2009). Results showed that the risk of being infected with one lrHPV genotype (PMR) and with at least one genotype (PRR) consistently decreased among men from the intervention group. They did not differ significantly from control group participants in terms of sexual behaviour, apart from reporting more frequently at the 21-month visit having had at least one sexual partnership in the previous12 months.

Few observational studies have observed this association but results remained inconsistent (Baldwin et al., 2004; Giuliano et al., 2009; Muller, Chirwa, and Lewis, 2009; Nielson et al., 2007b; Svare et al., 2002). Only one of these studies found statistically significant association of circumcision status with the prevalence of lrHPV infection (Giuliano *et al*., 2009). However, Tobian and colleagues (Tobian *et al*., 2009) have published similar results, analyzing data collected in another African population and using a different HPV swabbing site. A 34% reduction in lrHPV prevalence among circumcised men was reported. For this reason, it can be concluded that the protective effect of MC on hrHPV infection can be generalized to lrHPV genotypes as well.

In accordance with observational studies, this work highlighted some other risk factors of lrHPV, such as the number of lifetime sexual partners (Giuliano et al., 2009; Hernandez et

al., 2008a; Nielson et al., 2007b; Shin et al., 2004; Vaccarella et al., 2006) and being older than 21 years old (Kjaer *et al*., 2005), although the role of age may vary (Giuliano et al., 2009; Nielson et al., 2007b; Vaccarella et al., 2006). The data also presented evidence that supports the protective role of condom use on hrHPV and lrHPV infections (Baldwin et al., 2004; Kjaer et al., 2005; Nielson et al., 2007b; Vaccarella et al., 2006).

Possible mechanisms by which circumcised men are less likely to be infected with HPV could be explained by a reduced acquisition of new infection or an increased clearance of pre-existing infection, because the absence of foreskin may reduce the risk of auto-reinfection at the urethral site (Gray *et al*., 2009).

This study has some limitations. First, biological samples were not collected throughout the follow-up period, so the hrHPV and lrHPV status at inclusion are not known. The reduced effect of MC on HPV infection could be not rigorously demonstrated due to the lack of data on HPV status at baseline. This information would have allowed a comparison of HPV incidence as a function of MC status and HPV prevalence between intervention groups at inclusion. As some participants were already infected by HPV at inclusion, the effect on prevalence measured undervalued the true effect of MC. However, MC was randomly assigned, and controlling for the propensity score did not affect the results. Second, participants were not blinded to the intervention and this may have led to sexual behaviour change and bias. Finally, hrHPV and lrHPV were detected in urethral swab samples, a method that is likely to miss infections (Giuliano *et al*., 2007). The prevalence of HPV infections in this cohort is likely underestimated, because the rate of detection in the urethra is significantly lower than that in the glans, corona sulcus, or penis shaft (Aguilar *et al*., 2006; Giuliano *et al*., 2007). This underestimation would be equally distributed among the two groups of randomization. However, there is no risk of non-differential misclassification, as there were no difference when the urethral HPV prevalences before and after circumcision in a subsample of participants were compared.

This underestimation would have no effect on PRRs and PMRs. Despite this loss of power, this study demonstrated a significant protective effect of MC against HPV infection. A critique of the laboratory methodology is that due to the initial screening for 13 hrHPV types only, followed by reflex genotype testing on the positives, some HPV (especially lrHPV) genotypes might have been missed.

2.5 Conclusion

RCT are the strongest level of evidence to determine the causal association between and outcome and an intervention. The data from this RCT has shown an inverse association between MC and genital lrHPV and hrHPV prevalence in men. Given the protective effect of MC on HIV, the results provide new arguments to endorse MC as a preventative procedure to reduce disease burden in both men and women. A shortcoming of this study is that it is limited to HPV prevalence. HPV disease prevalence is a result of both persistence of infection and incidence. It does not address whether circumcision influences acquisition and/or the ability of the host to clear HPV infection. More studies should assess the role of MC on acquisition and clearance of both lrHPV and hrHPV. A second limitation is that results are derived from baseline analyses and that no long term follow up of participants were done to identify possible new HPV infections.

CHAPTER 3: THE ASSOCIATION OF ONCOGENIC AND NONONCOGENIC HUMAN PAPILLOMAVIRUS WITH HIV INCIDENCE

3.1 Introduction

Human papillomavirus genotypes that infect the genital track in humans are divided on the basis of their oncogenic potential into “high-risk” (hrHPV) and “low-risk” (lrHPV) genotypes (Castellsague, 2008). lrHPVs are most commonly associated with lesions such as genital warts that are non-malignant (Monk and Tewari, 2007), whereas hrHPV are found in the majority of premalignant or malignant lesions of the genitals and are associated with cancers of the cervix, vulva, vagina, anus, penis, head and neck (Baldwin et al., 2004; Castellsague, 2008; Monk and Tewari, 2007; Morris, 2007; WHO, 2007b).

The prevalence of genital HPV among both sub-Saharan African males and females is high. Cervical cancer is 99% attributable to hrHPV (Walboomers *et al*., 1999). Cervical is the leading cause of cancer mortality among women in Southern African women (Castellsague, 2008). Cervical cancer is a recognized AIDS-defining illness and there is a strong correlation between HIV and invasive cervical cancer (Castro, 1992). In Africa, both infections contribute significantly to the burden of morbidity and mortality; therefore it is of great public health importance to conduct research to highlight the epidemiologic and aetiologic association of HIV and HPV. Recent published epidemiological data show that the probability of HIV transmission in African countries is amplified by HPV (Auvert *et al*., 2011; Mbulawa *et al*., 2009; Veldhuijzen *et al*., 2012). There are several mechanistic explanations why HPV and other STIs can aid HIV uptake: 1) it can increase the viral burden in the genital tract; 2) it can increase the concentration of HIV in genital secretions and increase viral diversity; 3) it may play a role in mucosal disruption and inflammation to facilitate HIV acquisition (Cohen, 2006)

The focus of most studies exploring the link between HIV and genital HPV was on the effects that HIV infection has on the prevalence, incidence, and the distribution of HPV genotypes. HIV-positive status is found to be strongly associated with higher HPV prevalence in both sexes (Castellsague, 2008; Giuliano et al., 2008c; Ng'andwe et al.,

2007; Ng'ayo et al., 2008; Partridge and Koutsky, 2006; Safaeian et al., 2008b), higher hrHPV prevalence (Aynaud et al., 1998; Giuliano et al., 2008c; Mayaud et al., 2001; Ng'andwe et al., 2007; Partridge and Koutsky, 2006; Safaeian et al., 2008b), higher HPV incidence (Safaeian *et al*., 2008a), higher prevalence of infections with multiple HPV genotypes (Aynaud et al., 1998; Giuliano et al., 2008c; Ng'andwe et al., 2007; Ng'ayo et al., 2008; Partridge and Koutsky, 2006; Safaeian et al., 2008a), and higher frequency of HPV lesions in multiple locations (Aynaud *et al*., 1998).

The inconsistency in both the clinical progression and transmission of HIV infection has driven a search for cofactors influencing replication of the virus. Host immune and genetic factors and replication kinetics influence the progression of HIV disease. Co-infections between diverse exogenously and sexually acquired infectious agents and HIV have emerged to influence the rate of HIV replication and transmission (Cohen, 2006). Genital herpes simplex virus type 2 (HSV-2) is highly prevalent worldwide and an increasingly important cause of genital ulcer disease (Klug et al., 2009). Increased HSV-2 prevalence in developing countries contributes to an increase in the proportion of GUD and attributes to HIV and HSV-2 asymptomatic shedding within the genital tract (Mahiane *et al*., 2009; Paz-Bailey *et al*., 2007). This observed synergistic association between HIV and HSV-2 can be used as a model for investigating the co-pathogenic existence between HIV and HPV. HPV and HSV-2 share similarities as for both, dormancy or persistence are key factors in management and diagnosis, both cause lesions that can aid uptake of HIV and prevalence for both viruses can be reduced by MC (Mahiane *et al*., 2009; Tobian *et al*., 2009)

The objectives of this study were: 1) to explore the association of hrHPV and lrHPV with HIV incidence in men, and 2) to evaluate HPV as a risk factor or confounder for HIV acquisition in a young male cohort from South Africa, a group which is presumably at high risk for STIs such as HPV and HIV.

Longitudinal data were used and collected in Orange Farm (South Africa), an area of high HIV prevalence (DOH, 2008) during a male circumcision randomized controlled trial, which demonstrated a reducing effect of male circumcision on the acquisition of HIV (Auvert *et al*., 2005).

3.2 Methods

3.2.1 Collection of data

Detailed methodologies of the trial (ANRS-1265) have been described in Chapter 2.

3.2.2 Laboratory methods

Detailed laboratory methods of the trial (ANRS-1265) have been described in Chapter 2.

3.2.3 Data analysis

The main analyses were performed on a subset of 1683 participants who were HIV negative at inclusion and were tested for both HPV and HIV at the 21-month visit. They represent 1683 of 2949 participants (57.1%) who were tested for HIV at the 21-month visit. The associations of hrHPV and lrHPV statuses with age were tested using logistic regression, controlling for ethnic group, education, circumcision status, and condom use. HIV incidence rate and HIV incidence rate ratios (IRRs) were estimated among participants by a piecewise exponential proportional hazards model implemented using univariate log-Poisson regression (Berry, 1983; Frome, 1983; Holford, 1980). The effect of lrHPV status on HIV incidence was tested after controlling for hrHPV status. The multivariate effect of hrHPV status on HIV incidence was estimated controlling for the following covariates: ethnic group, age, education, number of lifetime partners, condom use in the past 12 months, circumcision status, HSV-2 status at inclusion, HSV-2

acquisition during follow-up, and the status at the 21-month visit for the following sexually transmitted diseases: *N. gonorrhoeae*, *C. trachomatis*, and *T. vaginalis*.

To estimate the mean duration between HIV acquisition and swab collection for HPV testing, the date of HIV acquisition was estimated for each participant as the date at midpoint between the last follow-up visit with an HIV-negative test result and the first follow-up visit when HIV infection was detected.

The confidence intervals [CI] of the percentages were calculated using Bayesian estimation (Newcombe, 1998). Statistical analyses were performed using the statistical package SPSS for Windows version 8 (SPSS, Chicago, IL) and R version 2.6.2.

In two ancillary intention-to-treat analyses, we assessed whether the additional data on HIV and HPV had any impact, first on the estimated effect of male circumcision (MC) on HIV incidence and secondly on the estimated effect of MC on HPV prevalence. These effects were recalculated using the methods described in publications from the same trial (Auvert *et al*., 2009; Auvert *et al*., 2005).

3.2.4 Ethics

The research protocol was reviewed and approved by the University of Witwatersrand Human Research Ethics Committee (Medical) on February 22, 2002 (protocol study no. M020104). The trial was also approved by the Scientific Commission of the French National Agency for AIDS Research (ANRS; protocol study no. 1265; 2002, decision no. 50), and authorization was obtained from the City of Johannesburg, Region 11, on February 25, 2002. This trial has been registered at http://www.clinicaltrials.gov under the number NCT00122525.

3.3 Results

3.3.1 Population Characteristics

The background characteristics of the 1683 participants included in this study are presented in Table 3.1. The proportion of participants infected with HPV increased with age (*P* linear trend = 0.0024). The prevalence of hrHPV was significantly higher than the prevalence of lrHPV (*P* < 0.001, McNemar test). The number of hrHPV genotypes and lrHPV genotypes among those infected with at least 1 HPV genotype were correlated (*P* < 0.001, Spearman rank correlation test). The prevalence of lrHPV and hrHPV were also correlated (*P* < 0.001, Fisher exact test). Among the participants infected with a hrHPV, 70.5% (95% CI: 65.2 to 75.6) were also infected with a lrHPV, and among all those infected with a lrHPV, 87.3% (95% CI: 82.7 to 91.1) were also infected with a hrHPV. The distributions of hrHPV and lrHPV genotypes are listed in Table 3.2. As shown in this table, the 2 most frequent hrHPV were genotypes 16 and 18, and HPV 6 was by far the most frequent lrHPV. After controlling for age, ethnic group, education, circumcision status, sexually transmitted infections, and condom use, hrHPV and lrHPV statuses were significantly associated with number of lifetime partners with *P* values for linear trend of 0.025 and 0.022, respectively.

Table 3.1 Background Characteristics, Reported Sexual Behaviour, and Prevalence of HPV of the Study Population (Auvert *et al*., 2010).

Background Characteristics	**n = 1683**
Ethnic group % (95% CI)	
Sotho	53.9 (51.6 to 56.3)
Zulu	30.5 (28.4 to 32.8)
Other	15.6 (13.9 to 17.4)
Mean (median, IQR) of age (yrs)	20.7 (21.19–22)
Primary level of education completed	98.5 (97.8 to 99.0)
Married or living as married % (95% CI)[*]	4.3 (3.3 to 5.6)
Circumcised % (95% CI)	51.3 (48.9 to 53.7)
Reported sexual behaviour	
Mean (median, IQR) number of lifetime partners	5.2 (4.2–6)
Mean (median, IQR) age (yrs) of last sexual partner	20.0 (20.18–22)
Consistent condom use with non-spousal sex partners % (95% CI)[†‡]	25.2 (22.4 to 28.2)
Sexually transmitted infections	
HPV-positive % (95% CI)	19.1 (17.3 to 21.1)
lrHPV-positive % (95% CI)	14.0 (12.4 to 15.7)
hrHPV-positive % (95% CI)	17.3 (15.6 to 19.2)
At least two hrHPV % (95% CI)	7.0 (5.9 to 8.3)
HSV-2 at recruitment % (95% CI)	3.6 (2.7 to 4.6)
Became HSV-2 positive % (95% CI)	4.2 (3.3 to 5.3)
T. vaginalis[§] % (95% CI)	2.2 (1.6 to 3.0)
N. gonorrhoeae[§] % (95% CI)	9.8 (8.4 to 11.3)
C. trachomatis[§] % (95% CI)	2.8 (2.0 to 3.6)

[*]At some time during the past 12 months.
[†]During the past 12 months.
[‡]Among those having had sexual intercourse during the past 12 months.

Table 3.2 Distribution of hrHPV and lrHPV Genotypes among the study population (Auvert *et al*., 2010).

High-risk genotype	% (95% CI) (n) n = 1683
16	3.5 (2.7 to 4.6) (59)
18	3.1 (2.3 to 4.0) (52)
31	2.2 (1.6 to 3.0) (37)
33	1.4 (0.9 to 2.1) (24)
35	2.1 (1.5 to 2.8) (35)
39	2.8 (2.1 to 3.7) (47)
45	2.1 (1.5 to 2.8) (35)
51	1.8 (1.2 to 2.5) (30)
52	2.5 (1.8 to 3.3) (42)
56	1.7 (1.1 to 2.4) (28)
58	2.0 (14 to 2.8) (34)
59	1.9 (1.3 to 2.6) (32)
68	1.8 (1.2 to 2.5) (30)
Low-risk genotype	**% (95% CI) (n) n = 1683**
6	10.9 (9.4 to 12.4) (183)
11	0.7 (0.4 to 1.2) (12)
26	0.7 (0.4 to 1.2) (12)
40	0.8 (0.5 to 1.3) (13)
42	1.0 (0.6 to 1.5) (16)
53	1.4 (0.9 to 2.0) (23)
54	1.1 (0.6 to 1.6) (18)
55	1.4 (0.9 to 2.0) (23)
61	2.6 (1.9 to 3.5) (44)
62	1.5 (1.0 to 2.1) (25)
64	0.1 (0.02 to 0.4) (2)
66	2.4 (1.7 to 3.2) (40)
67	0.2 (0.07 to 0.6) (4)
69	0.2 (0.04 to 0.5) (Partridge et al.)(Partridge et al.)(Partridge et al.)(Partridge et al.)
70	0.7 (0.3 to 1.1) (11)
71	0.7(0.4 to 1.2) (12)
72	0.5 (0.3 to 1.0) (9)
73	1.3 (0.8 to 1.9) (22)
81	1.2 (0.7 to 1.8) (20)
82	0.8 (0.5 to 1.3) (14)
83	1.1 (0.7 to 1.7) (19)
84	1.8 (1.2 to 2.5) (30)
IS39	0.1 (0.02 to 0.4) (2)
CP6108	1.5 (1.2 to 2.5) (26)

3.3.2 HIV Prevalence and Incidence

During follow-up, 33 of 1683 participants (2.0%) became HIV positive. At the 21-month follow-up visit, HIV prevalence among those hrHPV negative was 1.1% (15 of 1391) and 6.2% (18 of 292) among those hrHPV positive [RR = 5.72 (2.88 to 11.3) $P < 0.001$].

HIV incidence was higher among those with at least 1 HPV genotype compared with those with no detected HPV in univariate analysis: 5.26 per 100 person-years versus 0.96 per 100 person-years; (IRR = 5.45, 95% CI: 2.72 to 10.9; $P < 0.001$) and when controlling for covariates (aIRR = 4.55, 95% CI: 2.23 to 9.28; $P < 0.001$).

When controlling for hrHPV status, lrHPV status was not associated with HIV incidence (aIRR = 1.13, 95% CI: 0.40 to 3.16; $P = 0.82$). Table 3.3 indicates the effect of hrHPV status and covariates on HIV incidence. In the univariate and multivariate analyses, HIV incidence was significantly higher among hrHPV-positive participants. HIV incidence increased with the number of hrHPV genotypes involved in multiple infections in univariate analysis (Figure 3.1). Figure 3.2 shows the distribution of the number of hrHPV genotypes among the 1683 participants for those with at least 1 hrHPV genotype. In multivariate analysis, among those positive for at least 1 hrHPV genotype, HIV incidence increased on average by a factor of 1.55 (95% CI: 1.12 to 2.14; $P = 0.0074$) when the number of hrHPV genotypes increased by 1 unit.

These results also suggest an overall substantial effect of the HSV-2 status on the HIV epidemic. It confirms that there is an association between HSV-2 infections and HIV acquisition.

Table 3.3 Risk Factors of HIV Sero-incidence and Associated IRRs (Auvert *et al*., 2010).

	IR Per 100 Person-Years n = 1683	Univariate IRR (95% CI)*	Multivariate IRR (95% CI)*
hrHPV			
Negative	1.01 (0.23 to 4.48)	1	1
Positive	5.45 (1.29 to 22.99)	5.41 (2.72 to 10.78) *P* < 0.001	3.76 (1.83 to 7.73) *P* < 0.001
Circumcision status			
Uncircumcised	3.62 (0.86 to 15.19)	1	1
Circumcised	1.18 (0.24 to 5.72)	0.32 (0.15 to 0.72) *P* = 0.0058	0.29 (0.13 to 0.67) *P* = 0.0039
Age group			
21-year old and younger	1.26 (0.28 to 5.67)	1	1
More than 21 years old	3.41 (0.81 to 14.31)	2.71 (1.26 to 5.83) *P* = 0.011	2.32 (1.02 to 5.25) *P* = 0.044
Ethnic group			
Sotho	2.14 (0.50 to 9.14)	1	1
Zulu	1.94 (0.43 to 8.83)	0.91 (0.41 to 2.04) *P* = 0.82	0.86 (0.38 to 1.96) *P* = 0.71
Other	3.05 (0.64 to 14.61)	1.43 (0.59 to 3.45) *P* = 0.431	1.65 (0.66 to 4.11) *P* = 0.28
Primary level of education completed			
No	3.81 (0.36 to 39.83)	1	1
Yes	2.14 (0.52 to 8.85)	0.56 (0.08 to 4.14) *P* = 0.57	0.43 (0.06 to 3.29) *P* = 0.421
Number of lifetime partners			
0	1.31 (0.19 to 8.86)	1	1
1–4	2.37 (0.55 to 10.34)	1.82 (0.42 to 7.87) *P* = 0.42	1.19 (0.26 to 5.53) *P* = 0.82
>4	2.39 (0.55 to 10.30)	1.83 (0.41 to 8.10) *P* = 0.43	0.95 (0.20 to 4.58) *P* = 0.94
Consistent condom use			
No	3.04 (0.72 to 12.82)	1	1
Yes	0.97 (0.14 to 6.85)	0.32 (0.07 to 1.37) *P* = 0.12	0.53 (0.12 to 2.33) *P* = 0.40
HSV-2			
Stayed HSV-2 negative	1.46 (0.35 to 6.12)	1	1
HSV-2 positive	5.93 (0.98 to 35.78)	4.05 (1.21 to 13.60) *P* = 0.024	3.02 (0.85 to 10.66) *P* = 0.087
Became HSV-2 positive	14.47 (3.24 to 64.67)	9.89(4.53 to 21.60) *P* < 0.001	6.23 (2.65 to 14.64) *P* < 0.001
T. vaginalis			
Negative	2.18 (0.53 to 9.02)	1	1
Positive	2.76 (0.26 to 29.08)	1.27 (0.17 to 9.31) *P* = 0.82	0.93 (0.11 to 7.49) *P* = 0.94
N. gonorrhoeae			
Negative	2.06 (0.50 to 8.58)	1	1
Positive	3.56 (0.70 to 18.26)	1.73 (0.67 to 4.49) *P* = 0.26	0.95 (0.35 to 2.56) *P* = 0.92
C. trachomatis			
Negative	2.13 (0.51 to 8.81)	1	1
Positive	5.30 (0.75 to 37.65)	2.49 (0.59 to 10.43) *P* = 0.21	1.30 (0.27 to 6.12) *P* = 0.74

*Obtained with a piecewise exponential model, which was implemented using a Poisson log-linear regression.
IR, incidence rate.

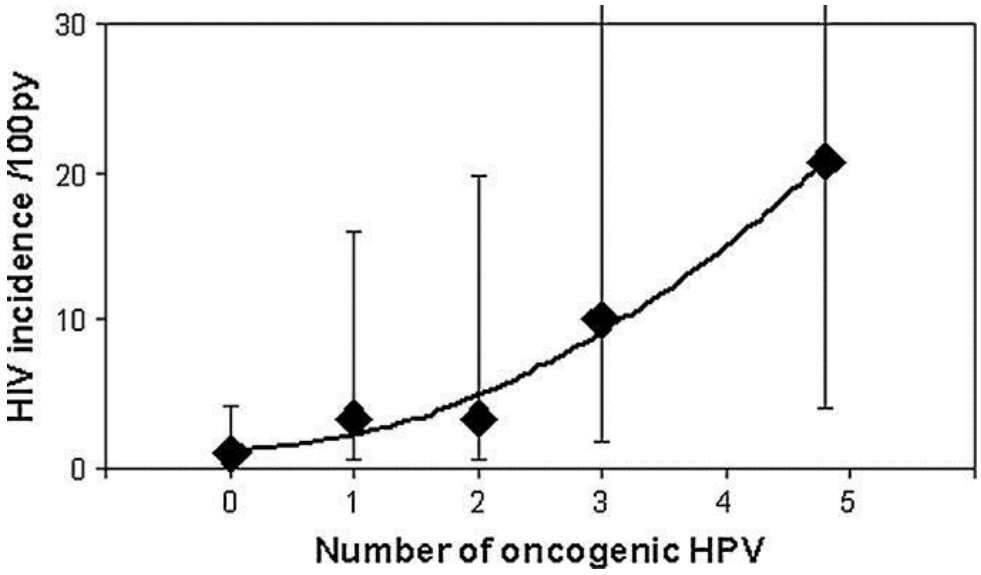

Figure 3.1 Distribution of HIV incidence as a function of the number of hrHPV genotypes. The data for the number of hrHPV from 4 to 8 have been combined. The error bars represent the 95% CI of the HIV incidence. A second degree polynomial curve has been fitted to the graphics. (py indicates person-years) (Auvert *et al*., 2010).

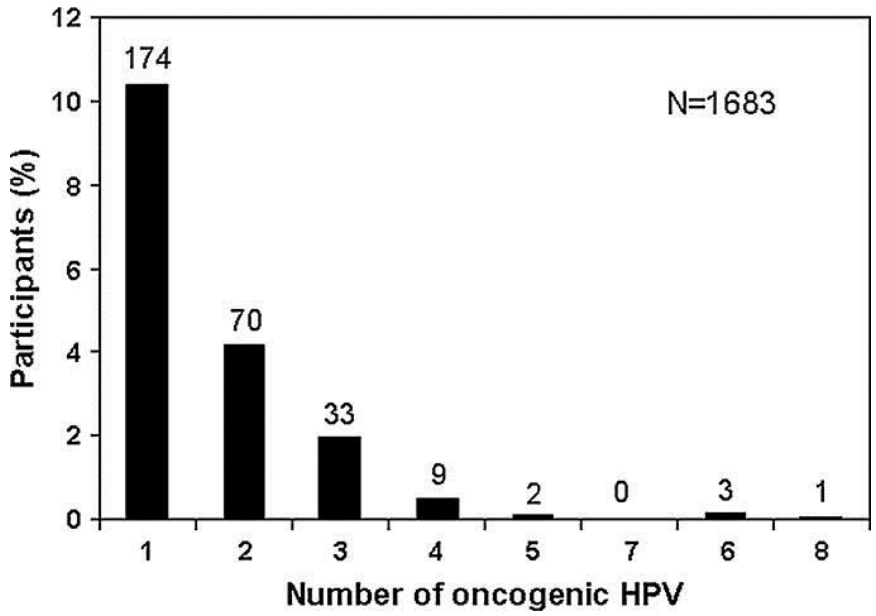

Figure 3.2 Distribution of the number of hrHPV genotypes among the 1683 participants for those with at least 1 hrHPV genotype. The number of participants with any HPV genotype (82.7%, 1391 of 1683) is not represented on the figure (Auvert *et al*., 2010).

The mean duration between the estimated date of HIV acquisition and the date of the swabbing for the detection of HPV was 10.2 months (interquartile range; 4.6 to 13.6 months).

With the additional follow-up visits and HIV results, there were 21 HIV infections (incidence rate 0.82 per 100 person-years) in the intervention group and 52 (2.1 per 100 person-years) in the control group, corresponding to an HIV IRR of 0.40 (95% CI: 0.24% to 0.66%; $P < 0.001$). The prevalences of hrHPV among the MC intervention and MC control groups were 14.5% (129 of 890) and 22.1% (191 of 863), respectively, with a prevalence ratio of 0.65 (0.52 to 0.82) ($P < 0.001$). Almost identical estimates were reported in the initial publications: 0.40 for HIV risk ratio and 0.66 for the HPV prevalence rate ratio (Auvert *et al*., 2009; Auvert *et al*., 2005).

3.4 Discussion

Data from this trial demonstrated an important association of oncogenic HPV with HIV incidence among young African men. However, the data did not show any association of nononcogenic HPV with HIV incidence.

This study has main 5 limitations. First, as with any observational study, no causal relationship between HPV and HIV could be concluded from the findings. Second, although urethral sampling has been shown to be unaffected by circumcision status (Auvert *et al*., 2009; Weaver *et al*., 2004), it has the shortcoming of underestimating the presence of genital HPV (Aguilar *et al*., 2006; Giuliano *et al*., 2007). This underestimation is not expected to change the positive association with HIV incidence, but it could be argued that the strength of this association may vary with swabbing sites. HrHPV was the most prevalent sexually transmitted infections in this cohort despite this underestimation. Third, the collection of genital swabs for HPV testing was done at the last follow-up visit, which is on average several months after HIV infection. It is therefore possible that, among the subsets of participants who contracted hrHPV during follow-up, some got infected with HPV after HIV acquisition. This will tend to dilute the strength of the association between HPV and HIV. Fourth, the Roche Linear Array genotyping assay used for screening in this study, detects mainly. This needs to be considered in the

comparison hrHPV and lrHPV prevalence. Lastly, HPV lesion detection was not performed in this study.

Several nonexclusive and plausible explanations could account for these findings. First, both sexually transmitted viruses, HIV and HPV share the same transmission route and behavioural risk factors. However, after controlling for these sexual behavioural variables, this association remains strong. Second, the results could be partly due to HIV infection facilitating HPV acquisition or HPV reactivation from the basal cell layer in the epithelium due to a compromised immune system. This explanation is unlikely as the participants in this study were recently infected with HIV and in all probability still had healthy immune systems. Thirdly, it could be argued that female partners co-infected with HIV and HPV- may have shed hrHPV more easily than HIV-negative women. HPV lesions are more likely to be dysplasic when the immune system is depressed (Steben and Duarte-Franco, 2007) and latent HPV infection can reactivated in immune compromised individuals (Aubin *et al*., 2007; Aynaud *et al*., 1998; Strickler *et al*., 2005). The average age of the female partners of the study participants was 3 years older than the median age of sexual debut reported in the same community, which is about 17 (Pettifor *et al*., 2005). This makes this last explanation improbable: female partners who had their sexual debut on average 3 years earlier, making it unlikely that they already had a compromised immune system due to HIV infection. Fourth, the findings could indicate that hrHPV, but not lrHPV, facilitates HIV acquisition.

There are several points in favour of the latter explanation: 1) there is strong evidence of a association between HIV and HPV acquisition, as shown in various cross-sectional studies (Ng'andwe *et al*., 2007; Ng'ayo *et al*., 2008; Safaeian *et al*., 2008b); 2) two other longitudinal studies have shown that HPV facilitates HIV acquisition among MSM in the United States (Chin-Hong *et al*., 2009) and among Zimbabwean women (Smith-McCune, 2009), with the latter demonstrating a differential effect between hrHPV and lrHPV on HIV

acquisition; 3) hrHPVs facilitating HIV acquisition is biologically plausible. The likelihood of hrHPV increasing the susceptibility to HIV infection was first suggested in 2002 (Clarke and Chetty, 2002). The arguments are that (*a*) hrHPV infection of basal cell epithelia could activate cell-mediated immune responses through the recruitment of macrophages and T lymphocytes (Coleman *et al*., 1994; Nicol *et al*., 2005; Stanley, 2009), these are HIV target cells and may facilitate HIV uptake; (*b*) cytokines could be stimulated by genital HPV (Gage *et al*., 2000; Nicol *et al*., 2005), which can facilitate HIV transcription and replication; and (*c*) HPV infection can lead to persisting inflammation and immune system activation (Behbahani *et al*., 2007). hrHPV is more likely to result in a persistent infection (versus lrHPV) (Brown *et al*., 2005; Franco *et al*., 1999; Rowhani-Rahbar *et al*., 2007), escalating the probability of HIV acquisition if there is really HPV-induced immune activation.

This study provides additional evidence of the strong interaction between HPV and HIV; however it does not give a definite explanation of the association of HPV and HIV incidence. There is a need for further studies to investigate this association, using, for example, data from other longitudinal male circumcision trials (Bailey *et al*., 2007; Gray *et al*., 2007) or the data collected during the COL-1492 trial (Marais *et al*., 2008), conducted on South African female sex workers. Testing the hypothesis that hrHPV facilitates HIV acquisition is complex as it entails the demonstration of a causal association. The validation of this hypothesis could require testing the potential protective effect of available HPV vaccines on HIV acquisition.

CHAPTER 4: HPV PREVALENCE ON THE FORESKIN AND IN FORESKIN TISSUE AND THE EFFECT OF WASHING WITH SOAP ON HPV DETECTION ON THE FORESKIN SURFACE

4.1 Introduction

The foreskin is a double-layered, retractable fold of skin and mucous membrane that covers the glans penis completely and protects the glans and urinary meatus from abrasion, irritation and foreign material. It is also described as the prepuce. The complete foreskin is at least a third of the total penile skin. The foreskin is tethered to the underside of the glans by the frenulum. The outside of the foreskin is a continuation of the skin on the penile shaft, but the inner foreskin is a mucous membrane (Figure 4.1). The sub-preputial epithelia of the inner foreskin are wet mucosal epithelia. The mucocutaneous zone occurs between the outer and inner foreskin (Anderson, Politch, and Pudney, 2011; Cold and Taylor, 1999). This moist cavity in uncircumcised men is a primary infection site for HPV (Flores et al., 2008b; Smith et al., 2007).

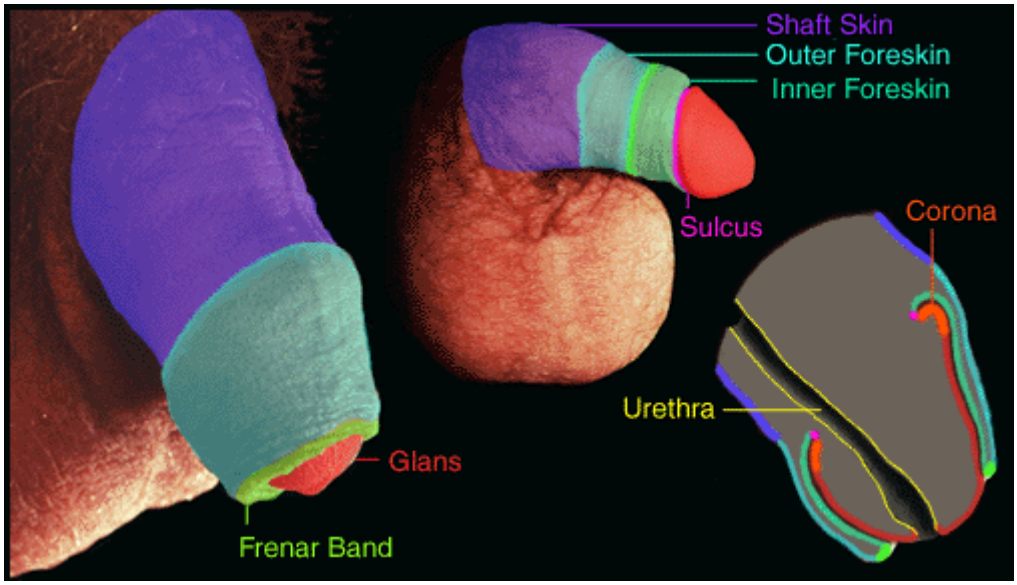

Figure 4.1 Foreskin with colour-coding of anatomical features. Foreskin covering the glans, the foreskin when retracted and close-up of the foreskin covering the glans (Illustrations Copyright ©1995,1996,1997 Derrick Townsend, published online http://www.noharmm.org, Author: T. Hammond. Accessed 21 December 2011)

A robust inverse association between medical male circumcision (MMC) and the HPV prevalence in men has been established in both HIV-negative and HIV-positive men (Albero *et al*., 2012). The prevalence and incidence of hrHPV were reduced by MMC in HIV-negative men and their female partners, however there was not the same effect seen

between HIV-positive men and their female partners. The mechanism by which circumcision lowers risk of HPV infection and leading to protection remains undefined and not fully understood and further studies investigating the foreskin mucosa are needed (Tobian and Gray, 2011; Wawer *et al*., 2011). The decrease in HPV acquisition after circumcision suggests a role for the foreskin in HPV transmission. There are a few possible explanations: 1) the uncircumcised foreskin is comprised of unkeratinized mucosal epithelium that amplifies the surface area vulnerable to HPV infection. Micro abrasions to the epithelium due to trauma during intercourse allow easier access of the virus to the basal cells that are the target cells for infection by HPV. In contrast, there is relatively little mucosal epithelium present on the penis of circumcised men. 2) Exposed areas like the epithelium of the glans penis (cornified after circumcision) and stratified squamous epithelium of the penile shaft are more resistant to infection in circumcised than uncircumcised men (Chin-Hong, 2008). The male genital mucosal immunology, microenvironment and HIV/HPV interaction are poorly understood and highly speculative to date (Prodger et al., 2012). A larger foreskin surface area was found to be associated with an increased risk of HIV acquisition. An explanation for this finding is that a larger foreskin area would be linked with an increased number of HIV immune cells (Kigozi *et al*., 2009). Studies comparing foreskin keratin thicknesses yielded conflicting results (Dinh *et al*., 2010; McCoombe and Short, 2006; Patterson *et al*., 2002; Qin *et al*., 2009). Two studies found the outer foreskin more keratinized that the inner foreskin, one study found that inner foreskin keratin was thicker than that of the outer foreskin. Dinh *et al*. found no difference between the keratinization of the inner and outer parts of the foreskins. The results from these studies confirm that the protective mechanisms of male circumcision are multi-factorial and involve more that keratin alone (Anderson, Politch, and Pudney, 2011; Dinh, Fahrbach, and Hope, 2010).

Foreskin tissue sampling in conjunction with genital swab sampling could provide an opportunity to generate data to characterize HPV infection in men. Sampling the whole

foreskin could also provide novel insights into the underlying mechanism of how male circumcision can decrease HPV transmission, infection, clearance and persistence.

The effect of genital hygiene on the detection of human papillomaviruses (HPVs) on the penis is largely unknown (Hernandez *et al*., 2010; Widdice *et al*., 2010). For example, the extent to which HPV genotypes detected on the penile surface reflect tissue infection or surface contamination has not been investigated. Moreover, because epidemiological evidence suggests that the foreskin may be particularly vulnerable to sexually transmitted infections, including HPV (Golden and Wasserheit, 2009; Hernandez et al., 2008b; Tobian and Gray, 2011; Veldhuijzen et al., 2010), exploring this question is further warranted. The objectives of this study were to a) assess the effect of washing with water and soap on the detection of HPV genotypes on the foreskin surface layers, b) compare the HPV genotypes detected on the foreskin surface after washing with those detected in the foreskin tissue and c) describe the spatial distribution of HPV infection in the foreskin tissue. These findings will provide clearer information as to how male circumcision and the foreskin play a role in HPV transmission.

4.2 Methods

4.2.1 Enrolment

The study was approved by the Committee on Human Studies of the University of the Witwatersrand (M070367). Written, informed consent was obtained from all volunteers (Appendixes C and D). Study participants were recruited among men coming for male circumcision surgery, in the context of the ANRS-12126 study, which is ongoing and aims to roll-out male circumcision in the township of Orange Farm (South Africa) as described elsewhere (Auvert *et al*., 2010).

Eligible men had to fulfill the following inclusion criteria: a) be enrolled in the ANRS-12126 study, b) understand the nested study's aims and procedures, c) volunteer for participating d) be at least 20 years old and e) display no penile abnormalities. A total of 26 participants were included.

4.2.2 Specimen collection

Before and after washing the foreskin with water and soap, biological material was collected on the outer and inner surfaces of the foreskin by a trained nurse by swabbing with pre-wetted Dacron tip swabs (Sterile Dryswab™, tubed, standard Polyester, Medical Wire MW102D) with sterilized P-600 emery paper attached to the shaft (4 swabs for each participant). Prior to swabbing, the nurse exfoliated a surface of approximately 0.5cm by 2cm, parallel to the axis of the penis. To avoid swabbing the same surface area twice, swabbing before and after washing was performed on different areas of the foreskin surface. The washing of the glans penis, coronal sulcus, inner and outer foreskin surface, and shaft was carried out by participants themselves under the supervision of a nurse. The washing procedure was explained using a video. Upon completion of the circumcision surgery, performed according to the sleeve resection method, the inner and outer foreskins were separated by the surgeon using different sterilized instruments to prevent cross-contamination. The isolated layers were then placed in separate plastic tubes (Sterile, 50 mL conical BD Falcon™ tube, Becton Dickenson) and immediately snap frozen on dry ice. All tissue and swab samples were transported frozen to the laboratory on the day of collection and were stored at -80°C until processing. Complete inner and outer foreskins were weighed before processed.

4.2.3 Swab testing

To extract the biological material collected, the emery paper and swab tips were incubated in 500 µL DNA MagNA Pure lysis buffer for 30 minutes, at room temperature. Using the external lysis protocol, DNA extraction was performed on the lysate with the MagNA Pure

instrument (Roche) with the Roche MagNA Pure LC DNA Isolation Kit I. Input volume on the instrument was 350 µL lysate with a 100 µL elution containing HPV DNA. HPV genotyping was then conducted on 50 µL eluted DNA, using the Linear Array HPV genotyping test (Roche), according to manufacturer's instructions.

4.2.4 Tissue testing

On each of the 52 foreskin layers collected (inner and outer foreskin for 26 participants), 12 round tissue punches of 4 mm diameter were harvested using a sterile carbon steel hollow punch (Groz Engineering Tools, Germany). This was done in a standardized way of three horizontal rows with four punches each (Figure 4.2 and Figure 4.3).

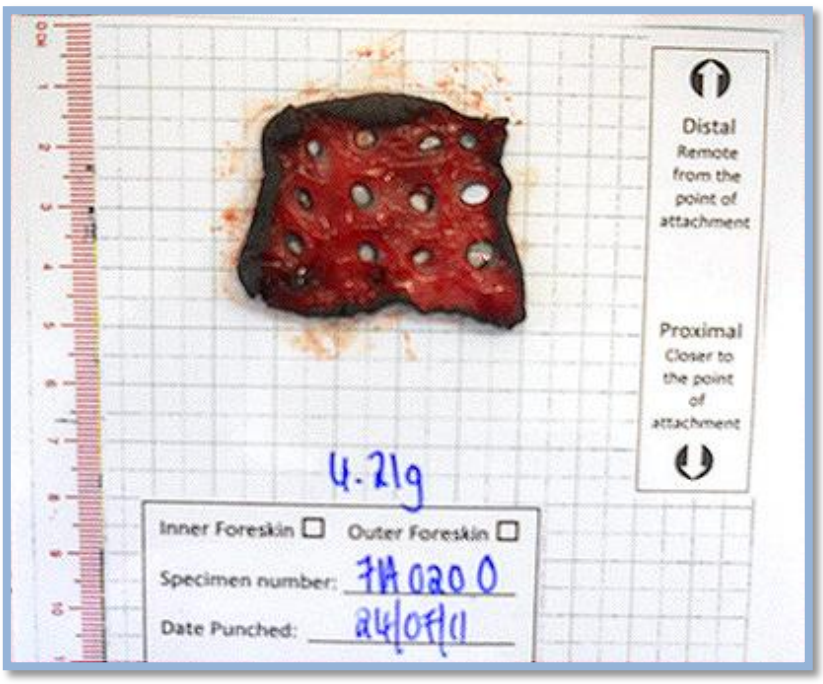

Figure 4.2 Punched Outer Foreskin

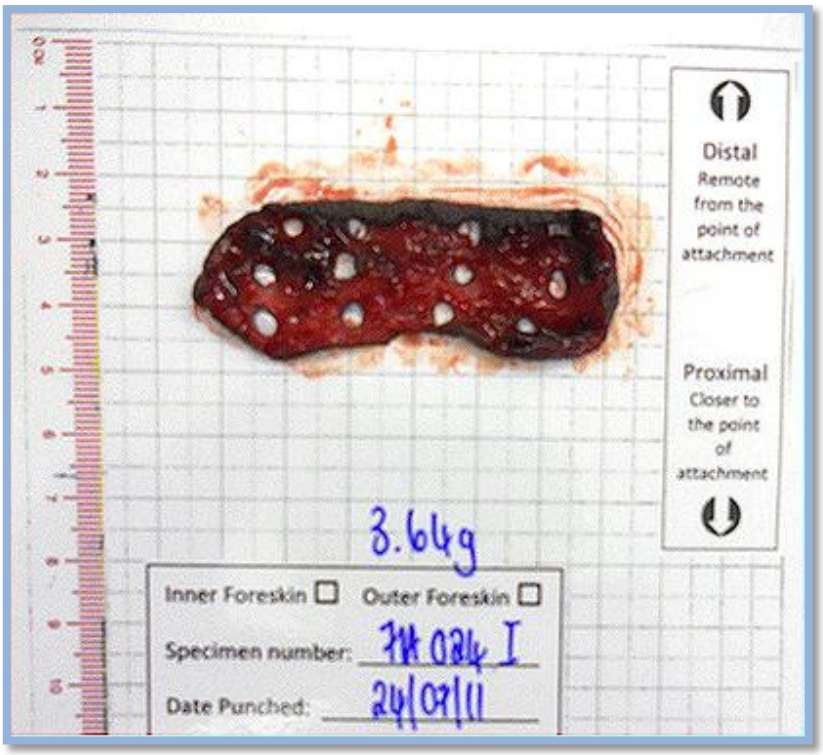

Figure 4.3 Punched Inner Foreskin

The mechanical disruption of skin samples is difficult as the intact skin is naturally resistant to shearing forces and often results in incomplete sample processing and sample loss (Berglund *et al*., 2007). Historically, this technique has been difficult and often required large amounts of starting tissue (Dobbeling *et al*., 1997). Concerning the extraction of DNA from foreskin tissue, the full thickness skin punch method optimized in this study, offered a foreskin tissue sample with an intact, three-dimensional architecture that contained a multitude of cell types (keratinocytes, fibroblasts, melanocytes, Langerhans cells, nerve fibers and macrophages, along with smooth muscle tissue and endothelial cells) (Cole *et al*., 2001). It was therefore decided not to separate the dermal and epidermal layers of the biopsy. This method was developed and optimized for small volume human foreskin biopsies and can be easily adopted for downstream testing for the presence of HPV DNA in tissue.

Each tissue punch was transferred into a 2ml Sarstedt tube and incubated for 2 hours at room temperature in 300 µL MagNA Pure Tissue lysis buffer. Homogenization per lysed

punch was performed using one 5 mm Stainless Steel Bead (Qiagen) per tube and the MagNA Lyser instrument (Roche). Disruptions and homogenization are achieved through the beating and grinding effect of beads on the sample material as they are shaken together in the tube. It is critically important to release the nucleic acids from the sample. This process shears the high molecular weight cellular proteins and carbohydrates that might reduce the binding of DNA to the MagNA Pure magnetic particles. On the other hand, too extensive disruption and homogenization will lead to the shearing of high-molecular weight genomic DNA. Efficient disruption of the tissue in the lysis buffer was achieved with three 5000 rpm pulse cycles of 20 seconds each. Long disruption cycles may cause degradation of DNA by heat stress. Although DNA tends to be more resistant against heat stress than against mechanical stress, disruption cycles of more that 25s were avoided. Sarstedt tubes were placed briefly on ice between cycles. Homogenization was followed by a brief centrifugation at 14 000 rpm (resolutions per minute). Following centrifugation, 80 µL of the tissue lysate supernatant was immediately transferred as input volume to the MagNA Pure instrument (Roche). The DNA II Tissue protocol was used for DNA extraction in combination with the MagNA Pure LC DNA Isolation kit II (Tissue). Tissue DNA was eluted in 200 µL and tested for HPV as described above.

4.2.5 Statistical analysis

The Roche Linear Array assay identifies 37 HPV genotypes (Refer to Chapter 2, section 2.2.2). The assay can only indirectly detect HPV 52 using a mixed probe (52/33/35/58), genotype 52 was included in this analysis only for one subject who tested positive for the mixed probe and negative for HPV 33, 35 and 58. The three subjects with indeterminate HPV 52 statuses were considered negative for this genotype.

HPV genotypes detected from the pre-washing swab collection on the inner and outer foreskin surfaces were pooled for each patient to obtain the number of genotypes

detected before washing. Similarly, the post-washing swab collection results for each patient were pooled to obtain the number of genotypes detected after washing. In the main analysis, the genotypes detected in the inner and outer foreskin tissues were also pooled for each patient. The Wilcoxon signed-rank test was used to compare: a) the number of genotypes detected on the surface before washing with the number obtained after washing and b) the number of genotypes obtained on the surface after washing with the number obtained in the foreskin tissues. 95% confidence intervals were estimated by bootstrap with 5000 replications. For this study, high-risk HPV genotypes were comprised of genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66. In total, five swabs (4.8%; 5/104) were beta-globin negative (internal control). These five swabs were all collected on the inner foreskin surface, four before washing, and one after washing. These swabs were nonetheless included in the analyses because at least one HPV was detected on each of them, indicating a valid test result.

To describe the spatial distribution of HPV in each layer of the foreskin tissue, we defined a localized, extended or generalized infection as having 1 to 3, 4 to 9 or 10 to 12 punches infected with the same genotype, respectively. The punches obtained from the inner and outer foreskin layers were analyzed separately using these three categories and the results were then pooled, for each patient.

4.3 Results

The median (range) participants' age was 25 (20-47). Only 2 participants presented with visible HPV lesions. The average weight of the outer foreskins and inner foreskin was 5.40g (3.23 -14.3g) and 2.97g (1.06-7.5g), respectively. The emery board-swab method offered an efficient sampling method where all swabs collected had adequate ß-globin internal controls or HPV genotypes amplifying.

4.3.1 Swabs

A total of 20 different HPV genotypes were found from all 26 participants combined before washing with water and soap (6, 16, 26, 35, 39, 51, 52, 53, 54, 58, 59, 61, 62, 66, 68, 69, 72, 81, 84, CP6108) (Figure 4.4). For all study participants, the percentage of participants infected with any individual HPV genotype obtained from the swabs analysis before washing was 50.0% (13/26; 95% CI=30.8% to 69.2%) and with multiple HPV genotypes was 46.2% (12/26; 95% CI=26.9% to 65.4%). Percentage of participants infected with high-risk HPV was 26.9% (7/26; 95% CI=11.5% to 46.2%).

For all study participants combined, a total of 18 genotypes were found after washing with water and soap (6 hrHR and 12 lrHPV): 6, 16, 26, 35, 51, 53, 54, 58, 59, 61, 62, 66, 67, 68, 71, 72, 81, CP6108). The percentage of participants infected with any individual HPV genotype obtained from the swab analysis after washing was 7.69% and with multiple HPV genotypes was 26.92%. The distribution of these genotypes found among all participants combined is indicated in Figure 4.4. The HPV genotype found in the largest number of participants was HPV genotype 61 (7 participants), followed by HPV 6 (6 participants) and genotypes 54, 62, 66 and 84 (4 participants). This figure shows that the more participants had specific HPV genotypes being detected on the foreskin surface before washing as opposed to after washing for 14 (63.6%) different genotypes. Equal number of participants was found pre- and post-washing for 6 genotypes (27.3%) and for 2 genotypes, more participants were found after washing as opposed to before washing (9.1%). The percentage lrHPV and hrHPV genotypes removed by washing are 68.5 and 53.5%, respectively. Four HPV genotypes (39, 52, 69 and 84) were completely removed by washing in all participants' penile swabs.

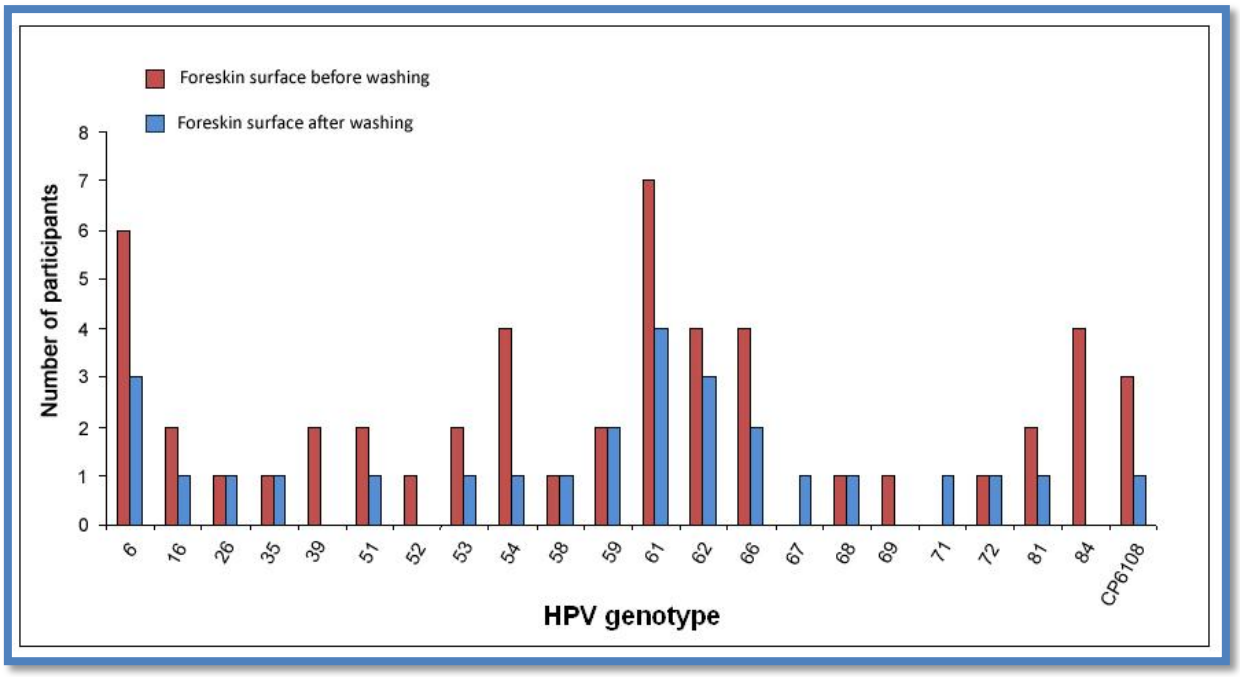

Figure 4.4 HPV genotype distribution among the 26 subjects on the foreskin surface before and after washing with water and soap.

The total cumulative number of HPV genotypes detected on all subjects on the foreskin surface before and after washing, and in the foreskin tissue are indicated in Table 4.1. Washing of the penis significantly decreased the average number of detected HPV genotypes in total by 49.0% (1.9 genotypes vs. 0.96 genotypes; 95% CI=19.1% to 73.0%; p=0.018). The average number of genotypes detected on the surface after washing, however was not significantly different from the average number detected in the foreskin tissues (1.2 vs. 0.96; p=0.23).

Table 4.1 Variation of the accumulative number of HPV genotypes detected among the 26 subjects by sampling method

	Foreskin surface swabbing before washing	Foreskin surface swabbing after washing	Foreskin tissue explants
Number of HPV genotypes			
Range per subject	0 – 12	0 – 6	0 – 9
Cumulative number among subjects	49	25	52
Average per subject (95% CI)	1.9 (0.96 to 3.0)	0.96 (0.38 to 1.7)	1.2 (0.54 to 2.1)
P-value[1]	0.018	0.23	NA

[1]Comparison with the number of HPV genotypes per subject detected in the foreskin tissue using the Wilcoxon signed-rank test NA = not applicable

Among the HPV genotypes detected on the foreskin surface of each participant before washing, 63.3% (31/49; 95% CI=44.2% to 85.1%) were not detected after washing, and 53.1% (26/49; 95% CI=28.0% to 73.5%) were detected in the foreskin tissue. Two participants presented with hrHPV 16, but no hrHPV 18 was detected in this cohort.

Among the 25 HPV genotypes detected on the foreskin surface after washing, 72.0% (18/25; 95% CI=50.0% to 90.5%) were detected in the foreskin tissue, and 28.0% (7/25; 95% CI=8.7% to 57.9%) were not detected before washing. This last proportion is smaller than the proportion of HPV genotypes detected before washing which were not detected after washing (28.0% vs 63.3%; p=0.003).

4.3.2 Foreskin tissue

A total of 15 different HPV genotypes were found in the outer foreskin tissue (16, 26, 35, 39, 51, 52, 54, 58, 59, 61, 62, 66, 68, 84 and CP6108) and a total of 17 different HPV genotypes were found in the inner foreskin tissue (11, 16, 26, 35, 51, 52, 53, 54, 58, 59, 61, 62, 66, 68, 71, 84 and CP6108) (Table 4.2). A total number of 312 tissue punches were tested per outer and inner foreskin - 164 (52,6%) and 242 (76.6%) punches tested positive for HPV respectively.

Table 4.2 Distribution of HPV genotypes found in outer and inner foreskin tissue explants, indicating the number of positives per genotype.

	HPV11	HPV16	HPV26	HPV35	HPV39	HPV51	HPV52/33/35/58	HPV53	HPV54	HPV58	HPV59	HPV61	HPV62	HPV66	HPV68	HPV71	HPV84	HPV CP6108	Total
Outer Foreskin tissue																			
Number of positive participants		1	1	1	1	1	2		1	1	1	3	2	2	1		1	2	21
Number of punches positive		12	12	1	2	12	18		2	10	12	13	21	15	8		2	24	164
Inner Foreskin tissue																			
Number of positive participants	1	1	1	1		1	3	1	3	1	2	4	3	3	1	1	2	2	31
Number of punches positive	7	12	12	8		12	17	8	6	10	18	31	30	25	12	6	7	21	242

The percentage of participants infected with any individual HPV genotype in the outer and inner foreskin tissue was 11.5% and 19.2%, respectively. Multiple HPV genotypes were detected in 15.4% of outer foreskins and on 23.0% of inner foreskins.

When comparing the number of participants being infected by HPV genotypes either on the foreskin surface after washing or in the foreskin tissue, it was found that an equal number of participants were infected for 9 (40.9%) different genotypes, a higher number of participants were infected by HPV in the foreskin tissue than on the foreskin for 4 (18.2%) different genotypes and a lower number of participants were infected by HPV in the foreskin tissue than on the foreskin for 7 (31.2%) different genotypes. Sampling was limited to small selected areas for both the tissue and swab specimen, this might be the reason why some HPV genotypes were not detected in the foreskin tissue and only on the foreskin surface and *vice versa*.

Among the 52 total number of tissue samples showing positivity for any HPV (see Table 4.2), 14 (26.9%; 95% CI=15.7 to 39.2%) were localized (1-3 tissue punches containing the same genotype), 15 (28.8%; 95% CI=17.9% to 43.1%) were extended (4-9 tissue punches

containing the same genotype) and 22 (42.3%; 95% CI=29.4 to 56.9%) were generalized (10-12 punches containing the same genotype). Hence, the overall results indicate that more than 2/3 of the HPV genotypes detected in the foreskin tissues were characterized as either extended or generalized infections. These extended or generalized infections were equal or more prominent, except for 3 genotypes (HPV 39, 52 and CP6108) on the inner foreskin than the outer foreskin tissue. The total number of positives per genotype is further subdivided into number of participant positive for this genotype and the number of punches positive for HPV per participant. (Figure 4.5). This highlights the specific susceptibility of the inner foreskin for HPV infections. There was no link found between the weight of the foreskin and the prevalence of HPV per participant.

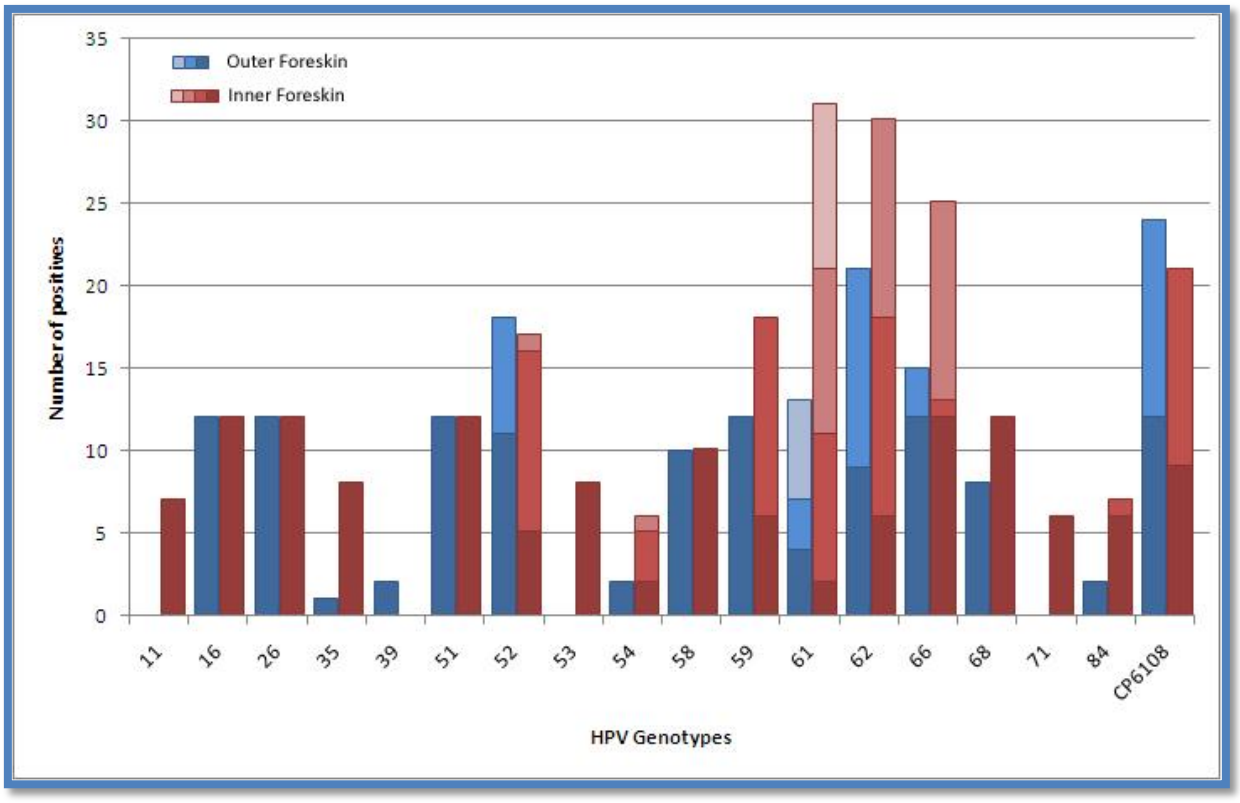

Figure 4.5 Distribution of HPV genotypes found in outer and inner foreskin tissue explants, indicating the number of positives per genotype out of 12 punches. Different shades per column indicate different participants.

4.4 Discussion

Data collection for the previous two chapters did not control for contamination from recent sexual partners. Despite the small sample size in this study, the primary finding is that washing with water and soap significantly reduces, by almost half, the total accumulative number of HPV genotypes detected on the foreskin surfaces of the 26 study participants. To the best of our knowledge, such a result has not been previously reported, although one study has found that genital hygiene practices reduces HPV detection among men (Widdice *et al*., 2010). The second finding is that the total accumulative number of HPV genotypes detected on the foreskin surface after washing is similar to the total number detected in the foreskin tissue. Hence, it can be recommended that HPV infection assessed by swabbing the foreskin surface should be performed after washing with water and soap for a more accurate assessment of HPV infection of the foreskin.

Our main result is plausible. Indeed the HPV genotypes present on the foreskin surface can have three origins: a) infection of the foreskin tissue, b) contamination from a recent infected sexual partner or c) contamination from the host himself. In this context, washing with water and soap is likely to remove most HPV viral particles present on the surface because of a contamination, leading to the observed reduction of the number of HPV genotypes detected on the surface. Two HPV genotypes were detected on the surface of the foreskin after washing, but not before. This could be because two different areas were swabbed before and after washing and that these two genotypes were limited to the area swabbed after washing.

The foreskin is critical to acquisition of HPV. The HPV distribution and higher HPV DNA positivity of single and multiple HPV genotypes on the inner foreskin found as opposed to the outer foreskin, highlighted the specific vulnerability of the inner foreskin to HPV infections. Foreskin tissue as a result of MMC provides an opportunity to evaluate an immunological model to clarify the mechanisms of HPV infections. This study is unique,

albeit some limitations. Firstly, it was conducted on a limited sample size. Although the numbers was small, a large amount of HPV testing was done on a diverse number of samples from each patient. This sample size was chosen based on cost, logistics and due to the invasive nature of the sample collection (MC). However, the results obtained were statistically significant, indicating that the results are unlikely due to chance. Secondly, swabbing before and after washing with water and soap was intentionally performed on different areas of the foreskin surface to avoid swabbing the same area twice. This could at least partly explain the variability for each subject between the HPV genotypes detected on the foreskin surface before and after washing. However, it cannot explain the difference observed before and after washing for the average number of genotypes detected per participant.

The fact that washing with water and soap can modify the number of HPV genotypes detected with swabbing has some implications for future studies. Primarily, it indicates that foreskin HPV testing should be performed after washing the surface with water and soap. Otherwise, HPV genotypes detected on the foreskin surface may vary depending on the host's hygiene habits, which could bias epidemiological and clinical studies. For example, a lower HPV prevalence detected on the penile surface among those frequently washing their penis with water and soap could be attributed to an effect of genital hygiene on HPV infection, while it could be due, at least partly, to an effect of hygiene on the presence and detection of HPV genotypes on the surface of the penis. Another important issue is the extent to which HPV genotypes that can be removed with washing are still infectious. In addition, because HPV viral load can predict persistence of HPV infection, (Xi *et al*., 2011), it will be interesting to study whether HPV genotypes removed by washing have a different viral load than those not removed by washing.

This study calls for further research on the effect of washing with water and soap on HPV detection using swabbing. In particular, it may be necessary to define a standard washing

procedure before penile swabbing. Because testing multiple sites increases the likelihood of HPV DNA detection (Weaver *et al*., 2004), it may be also necessary to compare HPV detection on the overall penis surface before and after washing. Further studies should be conducted to attempt to identify the origin of HPV genotypes removed by washing, and determine whether these genotypes are autologous (re-infection) or heterologous (from an infected partner). Finally, longitudinal studies are necessary to understand what proportion of transient infections are in fact only surface contaminations and by studying HPV transmission dynamics, HPV genotypes identified can be better understood. Such research is necessary to re-evaluate commonly used methods of HPV detection among men and improve our general understanding of the mechanisms of penile HPV infection.

CHAPTER 5: DISCUSSION

There is much to learn about HPV and its interaction with the human host. In South Africa, knowledge about HPV and HPV disease as well as the virus' interaction with HIV is of great importance and a major public health concern. Countries with high incidences of HIV also have the highest incidence of HPV causing cancers (Giuliano, van der Loeff, and Nyitray, 2011). This association is predominantly true in southern Africa, where HIV incidence and cervical cancer incidence are among the highest in the world (Nel et al., 2006). Interventions that can lower the rates of HIV infection, HPV infection, or both, are urgently needed. The burden of disease from genital warts is substantial, both in terms of the incidence of infection, cost of treatment, and the chronic nature of the condition. HPV vaccines are not readily accessible in developing countries and the affordability of these vaccines remains a problem (Saxenian, 2007). Men are not routinely screened for HPV infections and these infections are mainly asymptomatic, therefore heterosexual men may act as silent reservoirs for continued HPV transmission.

Results from this research conducted in this and other circumcision trials demonstrated that MC is an immediately available and cost-effective method to reduce HPV infection. This study will add value to existing knowledge on HPV in South African men. It has provided added insight into three areas: 1) circulating HPV genotypes amongst young South African men, 2) the role of circumcision in preventing hrHPV in men, 3) HIV and HPV association. However, these results indicate that protection is only partial, therefore the promotion of safe sex practices and other prophylactic interventions for example condom use and vaccination are also important considerations.

This study is the first randomized controlled trial to show a reduction in the prevalence of urethral hrHPV after MC in South Africa. In an intention-to-treat analysis, the prevalence of hrHPV among the intervention groups and control groups were 14.8% (94/637) and 22.3% (140/627), respectively. The results identified a strongly protective effect of MC on HPV with a Prevalence Rate Ratio (PRR) of 0.66 (0.51-0.86) (*P*=.002). Controlling for

propensity score and confounders (ethnic group, age, education, sexual behaviour, marital status and HIV status) had no effect on the results. Unfortunately, the effect of MC on specific genotypes, such as 16 and 33 could not be established and should be further investigated; in light of the fact that genotype 16 is one of the most prevalent genotypes causing cervical cancer. Nonetheless, MC was shown in this study to reduce HPV infection and strengthens the WHO-UNAIDS recommendation to implement MC programs in targeted countries.

This study also investigated the association of lrHPV infection with MC. Compared to the control group, lrHPV prevalence and mean number of genotypes were significantly lower among the intervention group, 8.5% versus 15,8%; aPRR: 0.54, *P*<.001 and 0.33 versus 0.18; aPMR: 0.54, *P*<.001, respectively. (aPRR is adjusted Prevalence Rate Ratio and aPMR is adjusted Poisson Mean Ratios). The mean number of lrHPV genotypes increased with the number of lifetime sexual partners and decreased with education level and consistent condom use. The results show a reduction in lrHPV infection among circumcised men. This implies that by reducing the frequency of HPV infection among men, the risk of exposure in their female sexual partners will be reduced. There is therefore an indirect, but substantial beneficial effect of male circumcision for women.

The second objective was to explore the association of oncogenic (high risk) and non-oncogenic (low risk) HPV with HIV incidence. The prevalence of hrHPV and lrHPV was 14.0% (95% CI: 12.4 -15.7) and 17.3% (95% CI: 15.6 -19.2), respectively. When controlling for hrHPV status, lrHPV status was not associated with HIV incidence (aIRR (adjusted Incidence Rate Ratio) = 1.13, 95% CI: 0.40 to 3.16; *P* = 0.82). When controlling for all covariates, HIV incidence increased significantly with hrHPV positivity (aIRR = 3.76, 95% CI: 1.83 to 7.73, *P* < 0.001) and with the number of hrHPV genotypes (adjusted-*P* linear trend = 0.0074). Whilst no causal relationship between HPV and HIV can be

deduced from these findings, the study did reveal evidence of their interaction and warrants further investigation.

The possibility that hrHPV could increase the susceptibility to HIV infection could be due to a number of explanations such as the fact that the foreskin is a vulnerable site for HPV infection. HPV replicates in keratinocytes which are “programmed to die” (squames). This exclusive intraepithelial viral infectious cycle exposes minimal amounts of replicating virus particles to the host surveillance cells. Thus viral release is non-inflammatory. It is also known that HPV infection interferes with local immune surveillance mechanisms such as inhibiting of interferon synthesis and signalling, delayed activation of adaptive immune responses and causes genetic instability. All of these factors can potentiate HIV-1 acquisition in men.

Proposed biological mechanisms, by which MMC offers protection against HPV acquisition, include reduction of virus entry through keratinized inner mucosal surface after circumcision, removal of moist environment under the foreskin and by removing the foreskin the range of microbes are reduced after MMC. Reduced genital mucosal inflammation caused by microbes leads can decrease susceptibility to HPV (Price et al., 2010). Another plausible argument is that circumcision removes the foreskin of the penis, which is rich in immune system cells, targeted by HIV and perhaps other viruses, to prevent acquisition.

This study also aimed to evaluate the role of washing before and after swab sample-taking and to compare specific HPV positivity among sampling sites. Washing with water and soap significantly decreased the average number of detected HPV genotypes per subject by 49.0% (1.9 vs. 0.96; 95% CI=19.1% to 73.0%; p=0.018) and the average number of genotypes detected on the surface after washing was not significantly different from the average number detected in the foreskin tissues (1.2 vs. 0.96; p=0.23). This correlation indicates that surface swabs taken in conjunction with an exfoliation step are

an adequate specimen as it generates results that emulate integrated HPV detection in the foreskin tissue. The origin of genotypes present on the foreskin surface could be from a number of different sources. Washing with water and soap is likely to remove most HPV viral particles present due to a contamination. This part of the study calls for further research in order to define a standard washing procedure before penile swabbing. This will improve our overall understanding to what extent genital washing influences penile HPV infection.

Together with the removal of possible contaminants with washing it is also important to pursue specific sites for detection and incidence or prevalence of HPV in men. With the possible bias in favour of MMC by evaluating only the corona sulcus and the urethra, sampling should include the entire penis and scrotum and pooling the results to avoid this bias.

More HPV genotypes were detected on the inner foreskin tissue than on the outer foreskin tissue. Extended or generalized infections were more prominent on the inner foreskin than the outer foreskin tissue. This highlights that the foreskin is critical to HPV infection and the specific susceptibility of the inner foreskin for HPV infections.

Investigating the connection between MMC and HPV is a methodological challenge. Various studies conducted show great variability in methodological concerns such as determining circumcision status, sampling from different genital sites, HPV outcome definition (clinical lesion versus infection with HPV infection), measures used for analysis, the sample collection method and assay used to detect HPV DNA (Albero et al., 2012).

Data from clinical trials indicate that MMC decreases genital ulcer disease, herpes simplex virus-2 (which is associated with an increased risk of HIV), *Trichomonas vaginalis* and bacterial vaginosis in their female partners. No evidence exists of a protective effect against *Chlamydia trachomatis* or *Neisseria gonorrhea* (Gray et al., 2008). There is weak

evidence that circumcision has a direct protective effect on HIV-1 infection in women, although an indirect benefit is likely (Giuliano, van der Loeff, and Nyitray, 2011). Efforts to increase the practice of male circumcision in areas with high rates of sexually transmitted infections, including Africa, could have a substantial benefit.

It has been claimed that the benefit of circumcision is overstated and premature and that condoms are much more effective, much cheaper and much less invasive (Van Howe, 2007). They warn about the misconception that circumcision is a reliable way to prevent STIs including HIV. Promotion of consistent safe sexual practices remains important. Some opponents of MMC state that it is not cost-effective, an unnecessary surgical procedure, causes physical, sexual and psychological harm and decreases sexual pleasure by removing most sensitive nerves of the penis, causes loss of erogenous tissue and is traumatic (Taylor, Lockwood, and Taylor, 1996).

It can thus be acknowledged that the prevention of any sexually transmitted infection is multifaceted and is also a behavioural problem. Decreased incidence and prevalence of high-risk HPV infection is likely to reduce the long-term risk for cervical cancer for women with circumcised male partners. Circumcision provides a useful intervention to prevent HPV infection and HPV disease. Increased medical circumcision services in South Africa offer an opportunity to reduce HPV infection in men with resulting benefit for women. Future studies should investigate if MC is associated with clearance of HPV as well as to explore the implications of MC in terms of long term protection against HPV.

APPENDIX A. MC STUDY PATIENT INFORMATION SHEET

Version 2

Evaluation of the impact of voluntary, safe male circumcision on STD infections in sub-Saharan Africa
A randomised controlled trial

MC study 01-07-2002

Participant Information sheet

Dear Sir,

You have been asked to take part in a clinical research study to assess the effect of a male circumcision. Male circumcision could reduce infection by STD's, but this has not been proven. It is therefore important to know that circumcision does NOT protect you from STD's. For this study we are looking for 3500 young men aged from 18 to 24 years.

Before you decide on your participation, it is important that you understand why this study is performed and what your participation in it involves. Please read the following instructions carefully, and if you have any question, don't hesitate to ask.

Background and purpose of the study

One of the possible reasons why the STD epidemic and HIV infections are high in some countries in Africa is the lack of male circumcision. Circumcision could partly prevent the infection by STD's. But it is not known if it does.

The main objective of this study is to evaluate if voluntary and safe male circumcision has a protective effect against infection with STD's and HIV.

Study surgery

The male circumcision will be performed in this study by trained general practitioners. They will follow the recommendations of the Department of Urology of the University of Witwatersrand. It will be done under local anaesthesia. It will be paid by the project.

Attribution of medication

In order to evaluate the effect of safe male circumcision, we will have to ask some of you to be circumcised at the beginning of the study, and others at the end of the study. The allocation of participants in the 2 groups will be done by chance.

Expected adverse events related to the study medication.

The circumcision surgery is safe. But it will not be possible to be circumcised if you are sick or if you have any disease that is incompatible with surgery.

Disadvantages of the participation in the study

An intravenous blood sample (1 tube of 20 cc) will be taken 4 times. This may cause a little bleeding and some pain for a few seconds.

When you are circumcised you will be asked to have no sexual contact in the 6 weeks after surgery. To have sexual contact before your skin of your penis is completely healed, could lead to infection if your partner is infected with a sexually transmitted disease. It could also be painful and lead to bleeding. If you desire to have

sexual contact in the 6 weeks after surgery, despite our recommendation, it is absolutely essential that your use a condom.

You could have some pain after the surgery. In very rare cases, the surgery could lead to infection or allergic reaction to the anaesthesia. Care will be taken to prevent and treat such side effects if they occur.

Advantages of the study medication and participation in the study

If this study shows that voluntary and safe male circumcision reduce transmission STD's or HIV, you will have contributed to important progress in the fight against STD's and HIV.

During the study you will have some medical examinations and biological tests that could detect diseases that will be treated free of charge.

Trained counsellors will be available for the duration of the study to discuss any problems or provide emotional support to you should it be required.

Course of the study

Before any study procedures can be performed, you will be asked to sign a consent form.

The duration of this study is 21 months and will include 4 visits.

At the first (initial) visit
Your general health is checked.
A blood sample (20cc) will be taken
You will have to answer a questionnaire.
You will know if you will be circumcised at the beginning or at the end of the study.

Safe male circumcision will then be performed for half of the participants (selected by chance).

At the second visit (3 months after the beginning)
You will have a medical examination
A blood sample (20cc) is taken

At the third visit (12 months after the beginning)
You will have a medical examination
A blood sample (20cc) is taken
You will have to answer a questionnaire

At the fourth visit (21 months after the beginning)
You will have a medical examination
A blood sample (20cc) is taken
You will have to answer a questionnaire
Safe male circumcision is then proposed for the participants who have not been circumcised at the beginning of the study.

At each visit you will receive information on sexually transmitted diseases and how to avoid these diseases. A swab will be used in case of a genital ulceration.

If you have not been circumcised at the beginning of the study, you will normally be circumcised at the end of the study. In case the study do not show any protective effect of male circumcision on sexually transmitted, you will be informed. It will not be useful for

you to be circumcised in this case. However should you still prefer to be circumcised you will then be circumcised.

Biological tests

A detection of infection by syphilis will be performed each time a blood sample is taken (at visits 1, 2, 3 and 4). If you are infected the result will be communicated to you and you will be treated. If a genital lesion due to syphilis or other treatable disease is detected at visits 1, 3 or 4, you will be informed and it will be treated.

HIV and herpes detection will be done each time a blood sample is taken. The results will be kept absolutely confidential and will not be linked to your name. It will be communicated only to the unit in charge of the analysis of the study located outside of South Africa.

In case of genital lesion, the swab will be analysed for HSV-2, syphilis and chancroid.

Responsibility of the participants

After you have decided to participate in this study, you are requested to be present at each visit. If you are moving out in the same town or in another town you are requested to indicate to the nearest STD clinics your name, your old and new addresses. To facilitate the course of this study you are requested to have with you at each visit the card that will be given to you at the first visit. It is important for the study that you continue to participate even if you move out.

The participation in this study is not expected to cause any restrictions to your daily living.

Participation in the study, withdrawal from the study and benefits

Your participation is completely voluntary, and if you decide to participate, you may withdraw from the study at any time without giving a reason and without incurring displeasure or penalty

Confidentiality

In the data stored in an electronic database and transferred to the persons in charge of the analysis of this study, you will be identified with a number. Your personal data (name and address) are retained at the study centre. Your personal data can be reviewed by local and foreign health authorities, as well as by an independent ethics committee. This is to compare original data with those analysed to ensure proper documentation of the study.

Payment

For your kind participation you will receive some money. You will receive 30, 40, 80 and 150 Rand at the visit 1, 2, 3 and 4, respectively. You will therefore receive a total of R 300 for your participation.

In case of injury due to your participation compensation will be offered. This compensation will be proposed by the persons in charge of this study and could be discussed with the ethical committee.

Long term evaluation

After the end of this study you could be asked to participate in a future evaluation. This following study will evaluate the long-term impact of the safe male circumcision that you have received during this study.

Schedule and supervision

Before the study is started, this Participant Information Form and Consent Form, including the study plan, have been approved by the Ethics Committee of the South African Medical Research Council and by South African medicines Control Council. These Ethic Committee and Authorities will also be informed of all possible changes in the study plan.

According to the study plan, the study will be started in 2002, and will be conducted in 3 towns of South Africa.

For further information and/or if you have any problems, please leave a message for us at the STDs clinics closest to your home.

You could also contact the following physician (directly or by leaving a message at the study center):

Name: Doctor Adrian Puren
Tel:(a) (011) 321 42 28, (b) 82 893 25 47
Reverse charges call can be made to anyone of these numbers

In case of emergency, please call the physician who is looking after you during this study. His name and telephone number will be given to you at the beginning of the study.

If you decide to participate in this study, please keep this Participant information form.

Thank you.

APPENDIX B. MC STUDY PATIENT QUESTIONNAIRES AND CONSENT

Version 2

Secretary

Initial Inclusion Visit

For Office Use Only:	
Date of Entry	
Data Capturer	

I1	Secretary's Initials	

I2	Centre	Orange Farm Station	1
		Venue 2	2
		Venue 3	3

I3	Date of previous Visit (From IDN Card)	Previous Visit						
		2nd last Visit						
		3rd last Visit						
		4th last Visit						
			D	***D***	***M***	***M***	***Y***	***Y***

I4	Has the Volunteer already been randomised?	Yes	1
		No	2

If yes, stop the inclusion visit

I5	Date of Initial Inclusion Visit (Today's date)							
			D	***D***	***M***	***M***	***Y***	***Y***

I6	IDN card cabinet has been checked for IDN card.	

I7	IDN card has been created.	

I8	Has this date been written on IDN Card?	Yes	1
		No	2

I9	Surname																				
I10	First Name																				
I11	Other Names																				
I12	IDN																				
I13	**Address:** Stand Number:																				
I14	**Address:** Street Name:																				
I15	**Address:** Area																				
I16	Own Telephone Number:																				
I17	Own Cell Number:																				
I18	Tel/Cell where message can be left:																				

Give this form with the IDN card to the nurse

1/20

Version 2

Initial Inclusion Visit
Nurse

I20	Initials of Nurse	

I21	Does the ID Photo correspond with the person?	Yes	1
		No	2

Clinical examination by the nurse

I22	Is the person circumcised?	Yes	1
		No	2

I23	Presence of a STD:	Yes	1
		No	2

If Yes:
ASK PERSON TO GET TREATMENT IN A STD CLINIC AND TO COME BACK FOR A NEW INITIAL VISIT. END INTERVIEW

I24	Fever:	Yes	1
		No	2

I25	Recent weight loss:	Yes	1
		No	2

I26	Skin in good condition:	Yes	1
		No	2

I27	Allergy to anaesthesia:	Yes	1
		No	2

I28	Diabetes:	Yes	1
		No	2

I29	Persistent skin rashes or flaky skin:	Yes	1
		No	2

I30	Coughing:	Yes	1
		No	2

I31	Shortness of breath:	Yes	1
		No	2

I32	Severe and persistent diarrhoea:	Yes	1
		No	2

I33	Extreme fatigue:	Yes	1
		No	2

I34	Severe headache:	Yes	1
		No	2

I35	Difficult or painful swallowing:	Yes	1
		No	2

I36	Large lymph nodes:	Yes	1
		No	2

I37	Swollen glands:	Yes	1
		No	2

I38	Anemia:	Yes	1
		No	2

I39	Oral trash:	Yes	1
		No	2

I40	haemophylus:	Yes	1
		No	2

I41	Other medical problem:	Yes	1
		No	2

If Yes: specify:	

I42	Can the volunteer be circumcised:	Yes	1
		No	2

If No: Explain why to the volunteer, and, if the problem can be solve, ask him to come back.

I43	Comments:

I44	Is the Initial Inclusion Criteria Satisfactorily?	Yes	1
		No	2

If no:

I45	Is the volunteer allowed to come back for a new inclusion visit?	Yes	1
		No (Already circumcised or 2nd try	2

Give this form with the IDN card to the interviewer

Version 2

Initial Inclusion Visit
Interviewer

If the nurse inclusion criteria are not satisfied, give the form and the IDN card to the facilitator

I50	Initials of Facilitator / Interviewer	

I51	Does the ID Photo correspond with the person?	Yes	1
		No	2

I52	How did you hear about this study?	From a Selector	1
		Poster	2
		Meeting	3
		Stable girlfriend	4
		Relative	5
	Specify:	Other	6

I53	Why do you want to volunteer for this study?	To participate in medical Research	1
		To improve my health	2
		Free Male Circumcision	3
		Free Medical Follow-up	4
		Money	5
	Specify:	Other	6

I54	Please provide the names & Addresses of 2 people who lives in Orange Farm, but not the same address as yours

Person 1

I55	Surname																			
I56	First Name																			
I57	**Address:** Stand Number:																			
I58	**Address:** Street Name:																			
I59	**Address:** Area																			
I60	Telephone Number:																			
I61	Cell Number:																			

I62	What is your relationship to person 1?	Immediate Family Member	1
		Other family member	2
		Friend	3
		Neighbour	4
		Stable girlfriend	5
	Specify:	Other	6

Version 2

Person 2

I70	Surname																			
I71	First Name																			
I72	**Address:** Stand Number:																			
I73	**Address:** Street Name:																			
I74	**Address:** Area																			
I75	Telephone Number:																			
I76	Cell Number:																			

I80	What is your relationship to person 2?	Immediate Family Member	1
		Other family member	2
		Friend	3
		Neighbour	4
		Stable girlfriend	5
	Specify:	Other	6

I81	Do you intend to move in the future?	Yes	1
		No	2

If Yes:

I82	When?	
I83	Where to?	

I84	Has counselling and information sheet been given?	Yes	1
		No	2

I85	Update the P-file.	

Give this form with the IDN card to the interviewer

5/20

Version 2

Initial Inclusion Visit
Facilitator:

I91	Initials of Facilitator	

I92	Does the ID Photo correspond with the person?	Yes	1
		No	2

I93	Are the interviewer criteria (not moving out of the area in the next 2 years) AND the nurse criteria satisfied?	Yes	1
		No	2

If yes

I94	Information sheet have been given and explained	Yes	1
		No	2

I95	The participant has understood the study:	Yes	1
		No	2

I96	The final inclusion visit has been described to the participant (Blood sample, inclusion questionnaire, and randomisation)	Yes	1
		No	2

I97	He accepts to come back with his ID, in more than 2 days but less than 2 weeks for the final inclusion visit	Yes	1
		No	2

I98	If result of randomisation will be 'Now', he is ready to be circumcised in the days following the final inclusion visit:	Yes	1
		No	2

I99	If result of randomisation will be 'Later', he is prepared to wait 21 months to be circumcised?	Yes	1
		No	2

I100	The volunteer accepts to come to the centre to indicate his new address in case of moving	Yes	1
		No	2

I101	The volunteer is clearly informed about the money (see money sheet):	Yes	1
		No	2

I102	Money given: …… (see money sheet)	Yes	1
		No	2

I103	Update the *facilitator money register*:	Yes	1
		No	2

Version 2

For all

I104	Initial inclusion visit passed:	Yes	1
		No	2

I105	Ask the volunteer to ask his friends to come	

I106	Update the IDN card (initial visit 'Passed', 'Failed', 'Failed and not allow to come back')	

I107	Comments:

If initial visit 'Passed':
Give to the secretary: Inclusion form and IDN Card

If initial visit 'Failed' or 'Failed and not allowed to come back':
Give to the secretary: IDN Card
Put in the data entry box: Inclusion form

Version 2

Final Inclusion Visit

F0	**Put sticker here**

Secretary

For Office Use Only:

Date of Entry	
Data Capturer	

F1	Secretary's Initials		
F2	Centre	Orange Farm Station	1
		Venue 2	2
		Venue 3	3
F3	Check that ID photo corresponds		
F4	Check that date of initial visit is at least 2 days ago?		
F5	Set of new stickers stapled to the folder		

F6	Today's Date							
		D	***D***	***M***	***M***	***Y***	***Y***	

F7	Has this date been written on the IDN card?	
F8	Has a P-file been created (copy information from initial visit form)?	
F9	Has a P-card been created with dates corresponding to M3, M12 and M21?	
F10	Has an Alphabetical card been created?	
F11	Has (6) stickers been put to the folder, P-file, P-card, alphabetical card, inclusion form and IDN card	

Put in the folder: P-card, P-file, alphabetical card, inclusion form and IDN card

Give to the interviewer: the folder with 4 documents:
Inclusion form,
P-card,
IDN card
Alphabetical card

8/20

Version 2

Final Inclusion Visit
Interviewer

F21	Initials of Facilitator / Interviewer	

F22	Does the ID Photo correspond with the person?	Yes	1
		No	2

F23	Has the volunteer signed 2 copies of the Consent Form (see below)?	

F24	Has the PN been written on the second copy of the consent form?	

F25	Inclusion questionnaire (see below)	

F26	Counselling (see counselling sheet)	

Give to the nurse: the folder with 4 documents:
Inclusion form,
P-card,
IDN card
Alphabetical card

Version 2

Final Inclusion Visit
Nurse

F31	Initials of Nurse		

F32	Does the ID Photo correspond with the person?	Yes	1
		No	2

F33	Has a blood sample been taken?	Yes	1
		No	2

F34	If blood been taken, Put sticker used here

F35	**Give the following Information to the respondent**	
	• The result of syphilis test will be delivered in an envelope at home	
	• If necessary: will be given a *STD ticket* (in the envelope) to be treated in a STD clinic	
	• Has shown photos of genital ulceration	
	• Has asked to come to the centre in case of genital ulceration	

F36	Update Blood and swabs register?	

F37	Update the check list?	

F38	Comments:

Give to the facilitator: the folder with 4 documents:
Inclusion form,
P-card,
IDN card
Alphabetical card

10/20

Version 2

Final Inclusion Visit
Facilitator

F41	Initials of Facilitator		

F42	Does the ID Photo correspond with the person?	Yes	1
		No	2

F43	Checks that the information sheet and consent form have been given	

F44	Does the volunteer understand the programmed visits?	Yes	1
		No	2

F45	Does the volunteer accept to be circumcised?	Yes	1
		No	2

F46	Does the volunteer accept the randomisation?	Yes	1
		No	2

If result of randomisation will be 'Now'

F47	He is ready to be circumcised in the days following the final inclusion visit	Yes	1
		No	2

If result of randomisation will be 'Later:

F48	He is ready to wait 21 months to be circumcised ?	Yes	1
		No	2

F49	The volunteer is clearly informed about the money (see money sheet):	

F50	The volunteer accepts to come to the centre to indicate his new address in case of moving	Yes	1
		No	2

F51	**Final inclusion satisfied** (= the participant can be randomised):	Yes	1
		No	2

F52	**IF "NO"**		
	• Write 'No' on the Folder, P-card, alphabetical card, P-file and destroy these 4 documents		
	• Is the volunteer allowed to come back for a new inclusion visit?	Yes	1
		No	2
	• Update the IDN card		
	• Give the IDN card back to the *secretary*		
	• Put the Inclusion form in the data entry box		

Version 2

F53	**IF "YES"**							
	• Proceed to the randomisation							
	• Date of randomisation: Today's Date							
		D	*D*	*M*	*M*	*Y*	*Y*	
	• Result of Randomisation					Now	1	
						Later	2	

F54	**IF "NOW" only**	
	• MC ticket given:	
	• Ask to the participant not to have sex in the 6 weeks following MC	
	• If problem after MC, go back to see the doctor or free phone number: on P-Card	
	• Update the *Randomisation register*	
	• Ask the participant to follow the advice that will be given by the GP	

F55	• Remind the participant to come back in 3, 12 and 21 months:	
	• Print out 3 Photographs (1 to be stapled to the folder and 2 to this form)	
	• Remind to come if moving:	
	• Remind to ask friends of the participant to come:	
	• Check P-card (date of next programmed visits: 3, 12, 21):	
	• P-card given to the participant:	
	• One copy of the consent from given to the participant (with the PN written):	
	• Remind to always come to the centre with ID and P-card	
	• Update IDN card	
	• Give money	
	• Update money register	
	• Update the *Programmed visit register*:	
	• Update P-file (final inclusion visit)	

F60	• Money given: …… (see money sheet)	

F61	Comments:

If randomisation:
Give back to the secretary, the folder with:
P-file,
Alphabetical card
IDN card
Put the inclusion form in the Data entry box

Version 2

Final inclusion questionnaire **Initial of interviewer : .....**

Section 1: Background characteristics

No.	Questions and filters	Coding categories	Skip to
Q 100	Where are you living ?	Council area 1 Private area 2 Squatter settlement 3 Site and service scheme 4 RDP 5	
Q 101	Have you ever been to school?	Yes 1 No 2	 → Q 104
Q 102	What is the last grade you have completed?	Grade: |__|__| *(Primary school: grade 1 to 7* *Secondary school : grade 8 to 12)*	
Q 103	Are you currently enrolled in a school?	Yes 1 No 2	
Q 104	What is your usual occupation? [*Probe: what kind of work do you do most of your time? record verbatim and then code]* _____ _____ _____	Work for a mining company 1 Domestic work outside your home 2 Domestic work at home 3 Selling things 4 Manual labour 5 Professional 6 Sex work 7 Scholar or Student 8 Unemployed 9 Retired 10 Specify: ..... Other 11	
Q 105	How many children (born alive) have you had?	Number |__|__|__|	
Q 106	What is your religion?	Protestant 1 Catholic 2 Muslim 3 African Traditional 4 Other 5	
Q 107	What ethnic group do you belong to?	Sotho 1 Tswana 2 Xhosa 3 Zulu 4 Other 5	

Version 2

No.	Questions and filters	Coding categories	Skip to
Q 108	Where were you born?	Orange Farm area 1 Other urban area of South Africa 2 Rural area of South Africa 3 Other country 4	
Q 109	Where were you living 2 years ago?	Orange Farm area 1 Somewhere else in another country 2 Somewhere else in South Africa 3	→ Q111 → Q111
Q 110	If somewhere else in South Africa, specify the province.	Eastern Cape 1 Free State 2 Gauteng 3 KwaZulu-Natal 4 Mpumalanga 5 North-West 6 Northern Cape 7 Northern Province 8 Western Cape 9	
Q 111	How long have you stayed in the Orange Farm Area?	**duration** |__|__| YEARS and |__|__| MONTHS [Since birth, ENTER 98 YEARS] [If less than one month, ENTER 00 month]	
Q 112	How often did you have drinks containing alcohol, including beer, wine spirits, traditional beer, etc., in the last four weeks? Would you say? [*READ OUT*]	At least once a day 1 Less than once a day but at least once a week 2 Less than once a week but at least once a month 3 Not in the last month 4	
Q 113	Have you ever been married or living with someone as married ? (living as married = living in the same house)	Yes 1 No 2	→ Q 201
Q 114	Are you currently married or living with someone as married ? (living as married = living in the same house)	Yes 1 No 2	→ Q 116
Q 115	How many people are you currently married to or living as if married to?	NUMBER |__|__|	
Q 116	When were you married or living as married for the first time	**date** MONTH |__|__| YEAR |__|__|__|__|	

Version 2

No.	Questions and filters	Coding categories	Skip to
Q 117	How many persons have you been married to (or lived as married) in your whole lifetime?	NUMBER |__|__|	
Q 118	How old was your spouse when you first got married (or lived as married)?	AGE |__|__|	

Version 2

Section 2: Sexual behaviour

No.	Questions and filters	Coding categories	Skip to
Q 201	Have you ever had penetrative sexual intercourse? By penetrative sex I mean when the penis enters the vagina, anus or mouth. [*PROBE: include forced sex*]	Yes 1 No 2	→ Q 410
Q 202	How old were you when you had penetrative sexual intercourse for the first time?	AGE |__|__|	
Q 203	Did you use a condom the first time you had penetrative sexual intercourse?	Yes 1 No 2	
Q 204	How many different people did you have penetrative sex with up to now? [*PROBE: include all partners including clients and sex workers*]	NUMBER |__|__|__|	
Q 205	Have you ever had penetrative sexual intercourse with someone of the same sex as you?	Yes 1 No 2	
Q 206	Have you ever used a condom?	Yes 1 No 2	
Q 207	Have you ever been the victim of violent sexual aggression (rape or abuse)?	Yes 1 No 2	
Q 208	Have you ever given or received money in exchange for sex?	I gave money for sex 1 I received money for sex 2 No 3	→ Q 210
Q 209	In the last 12 months, how many times did you give or receive money for sex?	NUMBER |__|__|__|	
Q 210	How many PARTNERS did you have sex with IN THE LAST 12 MONTHS? (including partners you are married with and including partners you are living with as if married)	|__|__|__| PARTNERS If 0, skip to Q 410	

Version 2

No.	Initials →	.....	.....	.....	.....	.....	.....	.....	.....
Q 300	Sex of this person ?	Female : 1 Male : 2	Female : 1 Male : 2	Female : 1 Male : 2	Female : 1 Male : 2	Female : 1 Male : 2	Female : 1 Male : 2	Female : 1 Male : 2	Female : 1 Male : 2
Q 310	Are you living with this partner ?	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2
Q 311	Are you married with this partner ?	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2
Q 312	How old is this partner ?	..... years	..... years	..... years	..... years	..... years	..... years	..... years	..... years
Q 313	How many times did you have sex with this person in the last 12 months?	.....	.....	.....	.....	.....	.....	.....	.....
Q 314	How often did you use condom use with this partner in the last 12 months?	Never : 1 Sometimes : 2 Always : 3	Never : 1 Sometimes : 2 Always : 3	Never : 1 Sometimes : 2 Always : 3	Never : 1 Sometimes : 2 Always : 3	Never : 1 Sometimes : 2 Always : 3	Never : 1 Sometimes : 2 Always : 3	Never : 1 Sometimes : 2 Always : 3	Never : 1 Sometimes : 2 Always : 3
Q 315	When did you had sex for the first time? (dd mm yyyy)	- - / - - / - - - -	- - / - - / - - - -	- - / - - / - - - -	- - / - - / - - - -	- - / - - / - - - -	- - / - - / - - - -	- - / - - / - - - -	- - / - - / - - - -
Q 316	When did you had sex for the last time time? (dd mm yyyy)	- - / - - / - - - -	- - / - - / - - - -	- - / - - / - - - -	- - / - - / - - - -	- - / - - / - - - -	- - / - - / - - - -	- - / - - / - - - -	- - / - - / - - - -
Q 317	Have you ever give money for sex with this partner	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2	Yes : 1 No : 2

Version2

Section 4: Genital heath

No.	Questions and filters	Coding categories	Skip to
Q 410	How many times in the last week, have you washed your genitals with soap?	NUMBER |__|__|__|	
Q 411	Do you use something other than soap and water to wash your genitals?	Yes 1 No 2 **If Yes** please specify _____	
Q 412	Have you received injections during the last 12 months?	Yes 1 No 2	→ Q 414
Q 413	**If Yes** how many?	NUMBER |__|__|	
Q 414	Have you been hospitalised for an accident or a serious health problem during the last 5 years?	Yes 1 No 2	→ Q 416
Q 415	Have you received a blood transfusion during hospitalisation?	Yes 1 No 2	
Q 416	Some men experience ulceration in the genital area. During the last 12 months, have you noticed any sores?	Yes 1 No 2	→ Q 418
Q 417	How many episodes started in the last 12 months?	NUMBER |__|__|	
Q 418	During the last 12 months, have you attended the clinic for a health problem related to the genital area?	Yes 1 No 2	→ END
Q 419	How many time in the last 12 months?	NUMBER |__|__|	

Thank you very much

Version2

Evaluation of the impact of voluntary, safe male circumcision on sexually transmitted infections - A randomised controlled trial (MC study)

Consent form

The original consent form stapled to the inclusion form
One copy to be given to the participant by the facilitator
Write the Participant Number on the participant copy:

Participant number : |__|__|__|__|__|__|__|__|__|__|__|__|

I have been informed by the person whose signature is given below, about the nature of the study 'Evaluation of the impact of voluntary, safe male circumcision on sexually transmitted infections', as well as what effects and possible advantages and possible adverse events or risk to expect. I received a copy of the written participant information and I had sufficient opportunity to ask questions and do not have any further questions at the moment.

My participation in this study is voluntary. I may end my participation in the study at any time without suffering any disadvantages. I am not required to give reasons for my decision. During my participation I will accept and follow the instructions of the centre staff and GP. I confirm that the information I have given on my health is correct.

I have been informed of the surgery that could be performed. I have been informed of the questionnaires about my life and my sexual behaviour that I will have to respond to. I have been informed of the blood samples that will be taken, of the medical examination I will have and the laboratory test that will be performed confidentially and anonymously on biological samples. I have been informed that a trained counsellor will be available during the duration of the study should I need to speak to a counsellor.

I have been informed and agreed that the participant records may be handled, stored and transmitted electronically and that data transferred to the study centre are entered in an anonymous way.

I agree to participate in the above-mentioned study.

Participant's name (in block letters)	Age	Date	Participant's signature

Interviewer's name (in block letters)	Date	Interviewer's signature (The person who conducted the consent discussion)

Version2

Evaluation of the impact of voluntary, safe male circumcision on sexually transmitted infections - A randomised controlled trial (MC study)

Consent form

The original consent form stapled to the inclusion form
One copy to be given to the participant by the facilitator
Write the Participant Number on the participant copy:

Participant number : |__|__|__|__|__|__|__|__|__|__|__|__|__|

I have been informed by the person whose signature is given below, about the nature of the study 'Evaluation of the impact of voluntary, safe male circumcision on sexually transmitted infections', as well as what effects and possible advantages and possible adverse events or risk to expect. I received a copy of the written participant information and I had sufficient opportunity to ask questions and do not have any further questions at the moment.

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I agree to participate in the above-mentioned study.

Participant's name (in block letters)	Age	Date	Participant's signature

Interviewer's name (in block letters)	Date	Interviewer's signature (The person who conducted the consent discussion)

APPENDIX C. FORESKIN STUDY INFORMATION SHEET AND CONSENT

DISCOMFORT OR INCONVENIENCE ASSOCIATED WITH PARTICIPATION IN THE STUDY

There is no risk associated with participation in the study. There is the discomfort of having to donate a blood sample and of the collection of the swabs.

There are no benefits associated with participation in the study.

As it is the case for any participant of the main Bophelo Pele male circumcision study, if a curable sexually transmitted disease is diagnosed, free treatment will be provided to you immediately.

REIMBURSEMENT FOR STUDY PARTICIPATION

If you participate in this study, we will give you **R 40-00** for the time spent, which is about 2.5 hours, and for the discomfort caused by the collection of biological samples. However, the final decision of my inclusion in the study will be taken by the staff after the completion of the questionnaire. If you are not included after having answered the questionnaire, this questionnaire will be destroyed and you will receive only a reduced indemnity **R 10-00**. In this last case, no swabs will be taken.

ETHICS APPROVAL

This clinical study protocol has been submitted to the University of the Witwatersrand, Human Research Ethics Committee (HREC) and a written approval has been granted by that committee.

The study has been structured in accordance with the Declaration of Helsinki (last updated: October 2000), which deals with the recommendations guiding doctors in biomedical research involving human subjects. A copy may be obtained from me should you wish to review it.

The study, as part of the Orange Farm Part-2 Study, is sponsored by the French National Association of Aids Research (ANRS).

RESTRICTIONS CONCERNING MY PARTICIPATION IN THE STUDY

There are no restrictions in participating in this study. Nevertheless, please be completely truthful with me regarding the questionnaire since you might otherwise contribute to false research results.

You should fall within the age group of 20 to 39, as this is the age group targeted by the study.

SOURCE OF ADDITIONAL INFORMATION

For the duration of the study, you will be under the care of Daniel Shabangu (nurse).

If you want any information regarding your rights as a research participant, or if you have any complaints regarding this research study, you may contact (Prof. Cleaton-Jones), Chairperson of the University of the Witwatersrand, Human Research Ethics Committee HREC), which is

Investigator initials: Patient initials:

an independent committee established to help protect the rights of research participants at (011) 717 2301.

Your participation is completely voluntary and, if you decide to participate, you may withdraw from the study at any time without giving a reason and without incurring displeasure or any penalty

CONFIDENTIALITY

All information obtained during the course of this study will be kept strictly confidential and is fully anonymous. The questionnaire, the swabs and the blood sample are not linked to your name or any personal data such your date of birth or address. Data that may be reported in scientific journals will not include any information that identifies you as a participant in this study. This information will be reviewed by authorized representatives of the study.

The information might also be inspected by the University of the Witwatersrand, Human Research Ethics Committee (HREC) as well as auditors from the sponsoring agency. Therefore, you hereby authorize me to release your information to them.

In this case your information will be utilized only in connection with their obligations relating to this clinical study.

Any information obtained regarding your tests or state of health as a result of your participation in this study will be held in strict confidence. You will be informed of any finding of importance to your health or continued participation in this study, but this information will not be disclosed to any third party in addition to the ones mentioned above without your written permission.

Investigator initials: Patient initials:

a) to accept the collection of 2 swabs in your mouth and 10 swabs on your penis
b) to answer an anonymous questionnaire about yourself and your sexual behavior,
c) to accept that, after your circumcision, your foreskin will be sent to the to the National Institute of Communicable Diseases.
d) to accept that we record the presence, or not, of any genital infection on your penis.

After being analyzed in the laboratories listed above, your foreskin will be destroyed within 12 months.

The laboratories where your foreskin will be analyzed and then destroyed are located in Gauteng and are placed under the authority of the National Department of Health.

The following samples will be collected:
a) 10 swabs on your penis
c) 2 swabs in your mouth

After the collection of the first 7 swabs on your penis, your penis will be washed with water and soap before the collection of the 5 last penile swabs. Between the washing of your penis and the collection of the last swabs, you will have to wait 2 hours.

The swabs will be collected on the outer surface of the foreskin (2 swabs), on the inner surface of the foreskin (4 swabs), in the corona sulcus and on the inner surface of the cheeks (1 swab).

As recommended by a scientific report, the outer surface of the foreskin will be gently rubbed with emery paper (grade 600) before swabbing. This rubbing does not cause any pain, irritation or inflammation. Before doing this, we test the procedure on the back of your hand to prove to you that it does not cause any pain, irritation or inflammation. In addition, this rubbing will be done on a part of your foreskin that will be removed during circumcision.

Of the 10 swabs collected on your penis, 6 will be analyzed to detect the presence of any type of HPV. The 4 other penile swabs will be analyzed to detect the presence of any foreign biological material on your penis that could interfere with the HPV analysis. This could happen in case you had a recent unprotected sexual contact since a sexual partner's genetic material can still be detected on the penis several days after an unprotected sexual contact. This last analysis necessitates the collection of a mouth swab.

3 swabs (one swab collected in your mouth and 2 swabs collected on your penis) will be sent to a laboratory facility in the United states (University of California Los Angeles) for analyses.

The genital examination will be performed by a trained nurse and will allow us to assess whether you have any symptoms of a sexually transmitted infection.

STORAGE and RE-ANALYSYS OF SAMPLES

The swabs will be destroyed after being analyzed. As mentioned above, your foreskin will be destroyed within 12 months.

Investigator initials: Patient initials:

The Orange Farm part-2 study: a community study of male circumcision (ANRS-12126)

Biological properties of foreskins

Informed consent (English) for participants

I hereby confirm that I have been informed by the study nurse, of the nature, conduct, benefits and risks of the clinical study "Biological properties of foreskins".

I have also received, read and understood the above written information (Patient Information Leaflet and Informed Consent) regarding the clinical study.

I am aware that the results of the study, including personal details regarding age and diagnoses will be anonymously processed into a study report.

I am aware that my foreskin will be analyzed for scientific purpose at the National Institute of Communicable Diseases. I am aware that my foreskin will be destroyed within 12 months.

I am aware that 3 swabs will be send to a laboratory facility in the United States (University of California Los Angeles) for analyses.

I am aware that the final decision of my inclusion in the study will be taken by the staff after the questionnaire. If I am not included after having answered the questionnaire, this questionnaire will be destroyed and I will receive only a reduced indemnity.

In view of the research requirements, I agree that the data collected during this study be processed in a computerized system by the study staff.

I may, at any stage and without prejudice, withdraw my consent and participation in the study.

I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

Patient: Printed Name: Date of birth: _ _ / _ _ / 1 9 _ _

Signature Date and Time:

I, herewith confirm that the above patient has been fully informed about the nature, conduct and risks of the above study.

Study nurse: Printed Name:

Signature Date and Time

Investigator initials: Patient initials:

APPENDIX D. FORESKIN STUDY QUESTIONNAIRE

The Orange Farm part-2 study: a community study of male circumcision (ANRS-12126)

Biological properties of foreskins

Case Report Form v6

Before washing

		Sticker
1 CRF		FAxxx
2 Cotton swab	Mouth	FAxxx-M
2 Cotton swab	Inner Foreskin (top right)	FAxxx-IF1
1 Dacron swab	Corona sulcus (right part)	FAxxx-CS1
1 Dacron swab	Emery – Wetted swab Outer Foreskin (Lower right)	FAxxx-EO1
1 Dacron swab	Emery – Wetted swab Inner Foreskin (Lower right)	FAxxx-EI1

After washing

		Sticker
2 Cotton swab	Inner Foreskin (top left)	FAxxx-IF2
1 Dacron swab	Corona sulcus (left part)	FAxxx-CS2
1 Dacron swab	Emery – Wetted swab Outer Foreskin (Lower left)	FAxxx-EO2
1 Dacron swab	Emery – Wetted swab Inner Foreskin (Lower left)	FAxxx-EI2
1 Inner foreskin		FAxxx-IF
1 Outer foreskin		FAxxx-OF

Cotton swabs: to be dried → -20°C
Dacron swabs: → -20°C
Foreskin → -20°C

ANONYMOUS STICKER

F 6	Age of the volunteer (from the MC card) (must be in the range 20-39 yrs)	___

If younger than 20 years or older than 39 years, stop the procedure.

F 10	Is the volunteer fluent in Zulu?	Zulu 1 Sotho 2 Other language 3

If the volunteer is not fluent in Zulu or Sotho, stop the procedure.

Give to the volunteer the participant information leaflet.
Read the participant information leaflet to the volunteer.
Answer any question

F 12	Is the person willing to participate?	Yes 1 No 2

If the volunteer is not willing to participate, then stop the procedure and do not include him as a participant.

Verify that the volunteer has understood the study by asking him the following 6 questions. In case of a first incorrect answer, re-explain the study and ask the question again. If the answer is still incorrect, mark "incorrect answer" and do not include the volunteer.

F 13	*Is it possible that you may not be included after completion of this questionnaire?	Correct answer 1 Incorrect answer 2
F 14	* If included, are we going to take swabs on your penis?	Correct answer 1 Incorrect answer 2
F 16	* If included, are we going to take a swab in your mouth?	Correct answer 1 Incorrect answer 2
F 18	* If included, are we going wash your penis with water and soap?	Correct answer 1 Incorrect answer 2
F 22	* If included, where will your foreskin be transported and analyzed?	Correct answer 1 Incorrect answer 2
F 24	* If included, will your foreskin be destroyed after it is analyzed?	Correct answer 1 Incorrect answer 2

F 28	Did the person answer correctly the 6 last questions?	Yes 1 No 2

If the volunteer did not answer the last 6 questions correctly:
Stop the procedure
Indicate to the participant that he cannot be included

If the volunteer answered the last 6 questions correctly:

Indicate to the participant that he can be included
Give 2 information sheets to be marked with the initials of the participant
The 2 copies must also be marked with the initials of the investigator
Give 3 consent forms to be signed by the participant
The 3 copies must also be signed by the investigator
Give to the participant 1 copy of the information sheet and one copy of the consent form.

F 30	Date of the visit	DD I__I__I MM I__I__I YYYY I_2_I_0_I_0_I__I

F 31	The person has received oral information on the study.	Yes 1 No 2
F 32	The person had the opportunity to ask questions about the study and complete answers were given.	Yes 1 No 2

F 33	Has the participant signed 2 copies of the information leaflet and 3 copies of the consent form (One to be given to the participants, 2 copies to be kept with this CRF)	Yes 1 No 2

If the consent form is not signed, stop the procedure.

Questionnaire

F 40	*Have you ever had penetrative sexual intercourse? By penetrative sex I mean when the penis enters the vagina, anus or mouth. (*Include forced sex*)	Yes 1 No 2	→ Next → End

F 41	*Have you ever had penetrative sexual intercourse in the **last seven day**? By penetrative sex I mean when the penis enters the vagina, anus or mouth. (*Include forced sex*)	Yes 1 No 2	

If End

Indicate to the participant that his participation is completed
Give the participant R 10
Thank the participant for participating in this study

IF "looking for No" and Yes to F41:

Indicate to the participant that his participation is completed
Give the participant R 10
Thank the participant for participating in this study

IF "looking for No" and No to F41:

→ Indicate to the participant that he is recruited
→ Staple stickers to this CRF
→ Staple the MC card to this CRF.
→ Send the participant to the nurse

IF "looking for Yes" and No to F41:

Indicate to the participant that his participation is completed
Give the participant R 10
Thank the participant for participating in this study

IF "looking for Yes" and Yes to F41: Next

F 51	* Have you ever had penetrative sexual intercourse in the **last 48 hours**? By penetrative sex I mean when the penis enters the vagina, anus or mouth. (*Include forced sex*)	**Yes 1** No 2	→ Next → End

***I am going to ask you questions about your last sexual intercourse** *

F 53	* How many hours ago did you have sex for the last time?	Hours ago |__|__|	
F 55	* This last time, did you use a condom?	Yes 1 **No 2**	

I am going to ask you questions about what you have done between your last sexual intercourse and now

F 57	* Have you washed your penis **between your last sexual intercourse and now**?	Yes 1 No 2	→ Next → Write 0 to F 60
F 60	* How many times did you wash your penis **between your last sexual intercourse and now**?	|__|__|	**0** → End **1** → Next more than 1 → End

I am going to ask you questions about how you have washed your penis?

F 62	* What did you use? (READ OUT)	Water only 1 Water and soap 2 Other 3
F 64	* During this washing, did you pull your foreskin to uncover the glans ?	Yes 1 **No 2**

IF F51=Yes and F55=No and F60=0 → Recruitment
IF F51=Yes and F55=No and F60=1 and F64=2 → Recruitment

If not recruited

Indicate to the participant that his participation is completed
Give the participant R 10
Thank the participant for participating in this study

If recruited

→ Indicate to the participant that he is recruited
→ Staple stickers to this CRF
→ Staple the MC card to this CRF.
→ Send the participant to the nurse

Genital examination

F 70	Is the penis circumcised?	Yes 1 No 2

F 71	Inflammation of the penis	No inflammation 1 Balanitis 2 Other 3 DNK 8

F 72	Ulceration on the penis (Retract the foreskin)	No ulceration 1 Ulcerations 2 Vesicles 3 Ulcers and vesicles 4 Other ulcerative dermatitis 5 DNK 8
F 73	Warts on the penis	No warts 1 Warts present 2 DNK 8
F 74	Discharge from the urethra	No discharge 1 Purulent 2 Mucoid 3 Milking only 4 DNK 8
F 75	Examination of the scrotum	Normal scrotum 1 Abnormal scrotum 2 DNK 8
F 76	Ulceration on the scrotum	No ulceration 1 Ulceration 2 Vesicles 3 Ulcers and vesicles 4 Other ulcerative dermatitis 5 DNK 8

Biological samples

F 80	2 cotton swabs taken in the mouth? (-M)	Yes 1 No 2
F 81	2 cotton swabs taken on the inner surface of the foreskin (top right)? (-IF1)	Yes 1 No 2
F 82	1 Dacron swab taken on the right part of the corona sulcus (-CS1)	Yes 1 No 2
F 83	1 saline-wetted Dacron swab taken with Emery on the outer foreskin (Lower right) (-EO1)	Yes 1 No 2
F 83	1 saline-wetted Dacron swab taken with Emery on the inner foreskin (Lower right) (EI1)	Yes 1 No 2

Keep the glans uncovered
Ask the participant to wash his penis with water and soap
Dry gently.
Cover the glans with the foreskin

F 84	What is the current time?	Hours: _ _ Minutes: _ _

The participant has to wait two hour from the time noted above (F44)

F 85	2 cotton swabs taken on the inner surface of the foreskin (top left)? (–IF2)	Yes 1 No 2
F 86	1 Dacron swab taken on the left part of the corona sulcus (-CS2)	Yes 1 No 2
F 87	1 saline-wetted Dacron swab taken with Emery on the outer foreskin (Lower left) (-EO2)	Yes 1 No 2
F 88	1 saline-wetted Dacron swab taken with Emery on the inner foreskin (Lower left) (EI2)	Yes 1 No 2

Send the participant to the surgeon for male circumcision

F 90	The inner foreskin has been put in a tube? (-IF)	Yes 1 No 2
F 91	The outer foreskin has been put in a tube? (-OF)	Yes 1 No 2

Give the participant R 40
Thank the participant for participating in this study
Remove the MC card from this CRF

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