This thesis is the basis for a joint degree of Doctor of Philosophy (PhD) between Karolinska Institutet and Makerere University.

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Thesis for doctoral degree (Ph.D.) 2009

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2009

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Implications for a Vaccination Strategy

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HUMAN PAPILLOMAVIRUS INFECTIONS AMONG SEXUALLY ACTIVE YOUNG WOMEN IN UGANDA: *IMPLICATIONS FOR A VACCINATION STRATEGY*

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MAKERERE UNIVERSITY

Kampala and Stockholm 2009

Makerere University

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ACADEMIC THESIS
The public defence for the degree of Doctor of Philosophy at Karolinska Institutet and Makerere University will be held at Makerere University, Davis Lecture Theatre Mulago, Kampala, Uganda. Monday October 26, **2009, 09.00 AM**

AKADEMISK AVHANDLING
som för avläggande av gemensam medicine doktorsexamen (*Doctor of Philosophy, PhD*) vid Karolinska Institutet och Makerere University offentligen försvaras på det engelska språket i Makerere University, Davis Lecture Theatre, Mulago, Kampala, Uganda, måndagen den 26 oktober, **2009, kl 9.00**

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ABSTRACT

Introduction: Information about the genital (human papillomavirus) HPV infection is needed to support the introduction of HPV vaccination in Uganda.
Objectives: (i) To estimate the prevalence, incidence, clearance and to evaluate the associated risk factors for the genital HPV infection. (ii) To evaluate in a pilot study the possibility of using filter paper to collect, store and transport cervical material for HPV DNA testing and genotyping.

Subjects and Methods: We conducted two clinic-based prospective cohort studies between September 2002 and December 2006. We consecutively recruited 1,275 sexually active women among those seeking services at Naguru Teenage Information and Health Centre (NTIHC) and 1,097 consecutive young primigravidae from those seeking pre-natal care at Naguru Health Centre (NHC) in Kampala, Uganda. Women were followed up for an average of 18.5 months (range 9.7-26.6). Detailed information on socio-demographic characteristics, reproductive and menstrual factors, sexual behaviour, history of sexually transmitted diseases of the women and their sexual partner(s), use of contraceptive methods and other lifestyle characteristics was obtained at baseline and follow up using interviewer administered standardized questionnaires. Cervical exfoliated cells were collected in Phosphate Buffer Saline (PBS) or PreservCyt solution as well as on filter paper. A sensitive PCR assay (SPF10/LiPA) was used to detect 42 different genital HPV types.

Results: In **Paper I,** only 32.4% of HPV types determined with filter paper were verifiable with PBS (*kappa* statistic = 0.18)**.** Multiple HPV types were detected in 54.1% of PBS compared to 15.3% of filter paper samples. Infections with ≥ 4 HPV types were 18.0% in PBS compared to2.7% in filter paper samples. In **Paper II**, the prevalence of HPV and HIV infections was 74.6% and 8.6%, respectively. High-risk HPV (HR-HPV) types were found in 51.4% of the women. The most frequently detected HR-HPV types were 51, 52, 18 and 16. Multiple infections were frequent. HIV positive women had a higher HPV prevalence (87.8%) and multiple infections (64.6%) than HIV negative women, 73.2% and 37.3%, respectively. Employment in the tertiary sector, lifetime number of sexual partners, a positive pregnancy test and detection of genital warts were significantly associated with HPV positivity. In **Paper III,** the incidence rate of HPV infections was 30.5 per 100 person-years. The risk for incident infections was not statistically significant among HIV positive compared to HIV negative women (Risk Ratio, [RR] = 2.8, 95%; 95% Confidence Interval [CI]; 0.9-8.3). Clearance for the individual HPV type was frequent; 42.3-100.0 % for HR-HPV types and 50-100% for low-risk types. HIV negative women cleared their infection more frequently than HIV positive women (clearance $_{\text{Adjusted}}$ = 0.2, 95% CI = 0.1-0.7). In **Paper IV**, the prevalence of HPV infections was 60% among young primigravidae. HPV 16 and 18 were detected in 8.4% and 5.8%, respectively, which was less frequent than HPV 51 (8.7%) and HPV 52 (12.1%). Although HPV infections were detected in 42.9% of women between the $1^{st}/2^{nd}$ and 3^{rd} trimesters, and 38.1% between pregnancy and delivery, 50.0% and 71.8% of HPV infections, respectively cleared, leaving the HPV prevalence unchanged in different periods of pregnancy.

Conclusion: The prevalence and incidence of high-risk HPV infections were extremely high in our study populations of young women in Kampala, Uganda. Clearance of HPV infections was frequent but had no effect on prevalence.

Key words: HPV, women, pregnancy, incidence, prevalence, risk factors, HIV, Uganda

Published by Karolinska Institutet and Makerere University Printed by Universitetsservice-AB
Cover photo by Bo Lambert

ISBN 978-91-7409-586-9

I dedicate this work to the thousands of young women who accepted to share their privacy with us during the conduct of our studies at Naguru Teenage Information and Health Centre and Naguru Health Centre.

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Conclusion: The prevalence and incidence of high-risk HPV infections were extremely high in our study populations of young women in Kampala, Uganda. Clearance of HPV infections was frequent but had no effect on prevalence.

Key words: HPV, women, pregnancy, incidence, prevalence, risk factors, HIV, Uganda

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals. Accepted and published papers are reprinted with permission from the publishers.

I. **Banura C**, Franceschi S, van Doorn LJ, Arslan A, Wabwire-Mangen F, Mbidde EK, Quint W, Weiderpass E. Detection of cervical human papillomavirus infection in filter paper samples: a comparative study. *J Med Microbiol. 2008; 57:253-255.*

II. **Banura C,** Franceschi S, van Doorn LJ, Arslan A, Wabwire-Mangen F, Mbidde EK, Quint W, Weiderpass E. Infection with human papillomavirus and HIV among young women in Kampala, Uganda. *J Infect Dis. 2008; 197:555-562.*

III. **Banura C,** Sandin S, van Doorn LJ, Quint Wim, Kleter Bernhard, Wabwire-Mangen F, Mbidde EK, Weiderpass E. Type–specific incidence, clearance and predictors of cervical human papillomavirus infections (HPV) among young women: a prospective study in Uganda. *(Submitted)*

IV. **Banura C**, Franceschi S, van Doorn LJ, Arslan A, Kleter B, Wabwire-Mangen F, Mbidde EK, Quint W, Weiderpass E. Prevalence, incidence and clearance of human papillomavirus infection among young primiparous pregnant women in Kampala, Uganda. *Int J Cancer 2008; 123:2180-2187.*

CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ALTAS	ASCUS and LSIL triage studies
ART	Anti retroviral therapy
ASCUS	Atypical squamous cell of undetermined significance
Bp	Base pair
Cis	Confidence Intervals
CIN	Cervical Intraepithelial Neoplasia
CT	Chlamydia Trachomatis
DDL	Delft's diagnostic laboratory
DEIA	DNA Enzymme Immuno Assay
DNA	Deoxyribonucleic acid
E	Early gene
ELISA	Enzyme linked immuno absorbent assay
FDA	Food and Drug Administration
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HIV	Human immunodeficiency virus
HR	High risk
HC2	Hybrid Capture 2
HSV 2	Herpes Simplex Virus Type 2
HSIL	High-grade squamous intraepithelial lesion
IARC	International Agency for Research on Cancer
L	Late gene
LBA	Line Blot Assay
LCR	Long Control Region
LiPA	Line probe assay
LR	Low risk
LSIL	Low-grade squamous intraepithelial lesion
LC	Long Control
MOH	Ministry of Health
NHC	Naguru Health Centre
NTIHC	Naguru Teenage Information and Health Centre
Ors	Odds Ratios
ORFs	Open reading frames
Rb	Retinoblastoma gene
RNA	Ribonucleic acid
RLB	Reverse Line Blot assay
RPR	Rapid Plasma Reagin
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
SPF	Short PCR Fragment
STDs	Sexually Transmitted Diseases
STIs	Sexually Transmitted Infections
URR	Upstream Regulatory Region
USA	United States of America

VIA	Visual inspection with Acetic Acid
VILI	Visual inspection with Lugol’s Iodine
VLP	Virus-like particles
WHO	World Health Organization

1 INTRODUCTION

The motivation to study genital human papillomavirus (HPV) infections originates from its etiological role in cervical cancer. Cervical cancer is the second most common cancer in women globally, with an estimated 493,000 incident cases and 273,000 deaths each year [1]. Cervical cancer is the most common cancer of women in sub Saharan Africa with an estimated age-standardized incidence rate of 31 per 100,000 [2] and mortality rate per 100,000 ranging between 23.8 and 34.6 in Western and Eastern Africa [3], respectively, where rates are among the highest in the world. The striking differences of cervical cancer incidence rates observed in the world are a reflection of two factors; either the underlying risk of transmission of certain high-risk human papillomavirus (HR-HPV) types, or the failure to prevent their clinical manifestations by effective screening programs [4].

The establishment of HPV as the necessary cause of cervical cancer made the development of prophylactic HPV vaccines for primary prevention a reality. For the first time, since 2006, two effective prophylactic vaccines are available, both based on virus-like particles (VLP) developed through recombinant DNA technologies. Gardasil®, a quadrivalent vaccine containing HPV 6/11/16/18 L1 VLPs from Merck & Co. Inc., and Cervarix®, a bivalent vaccine containing HPV16/18 L1 VLP vaccine from GlaxoSmithKline Biologicals [5, 6]. Three doses are recommended over a 6-month period, and the possible need for booster doses is not yet established. Both vaccines have been tested in humans in large randomized double blind placebo controlled trials and were found to be equally safe. Both also showed nearly complete protection against cervical pre-cancerous lesions caused by vaccine-related types during 6.4 years of observation so far [7]. The quadrivalent vaccine was also 100% effective in preventing external genital warts caused by HPV 6 and 11 [6]. The consistency of these observations strongly suggest that widespread use of these vaccines has the potential to significantly reduce cervical cancer deaths, particularly in women residing in resource-poor countries, where 80% of cervical cancer cases occur [1] and conventional screening has had, at best, an inconsistent impact on mortality reduction due to this cancer. Of major concern, though, are the costs of the vaccines in the context of competing priorities for immunization resources and barriers of reaching pre-adolescent girls who are the initial primary target of these vaccines. Nevertheless, with the advent of these vaccines, the prospects of prevention of infection of genital

HPV types 16 and 18, responsible for 70% invasive cervical cancers globally [8], have never looked more encouraging, but the decision to introduce these new vaccines into a given population will depend upon a number of factors including the local epidemiology and the burden of HPV-associated cervical disease.

Although Uganda is one of the countries in the world with a high age-adjusted cervical cancer incidence rate estimated at 45.8 per 100,000 [9] and a mortality rate estimated at 29.3 per 100,000 [1], information on the burden of the HPV infection, type distribution, and risk factors for infection is sparse. The few studies that have been conducted involved select groups [10-12], small numbers and different HPV detection assays with a wide range of sensitivities [13], which raises doubts about the generalizability of the results. Yet, this information is important to support the introduction of the HPV vaccine in Uganda. For this purpose, we conducted two clinic-based prospective cohort studies among young Ugandan women aged between 12-24 years.

In study aim 1, whose results are reported in **Paper 1,** we explored the feasibility of using filter paper for collection, storage and transportation of cervical specimens for detection of HPV DNA among sexually active young women free from cervical dysplasias. Filter paper has been used successfully to collect, store and transport dry blood spots for detection of many infectious [14, 15] and non-infectious [16, 17] diseases. The existing standard media for collection, storage and transportation of cervical specimens are both expensive and flammable or require constant refrigeration, conditions not easily fulfilled by many resource poor countries. Thus, positive results of filter paper smears would greatly facilitate the collection, storage and transportation of exfoliated cervical cells for detection of HPV DNA in resource poor countries like Uganda.

In study aim 2, whose results are reported in **Paper II**, we estimated the prevalence of HPV, type distribution and risk factors for HPV infections among young women seeking services at a primary care clinic providing services to young people with a view of estimating the HPV infection burden and the distribution of HPV types before the introduction of the HPV vaccination.

In study aim 3, whose results are reported in the submitted **Paper III,** we present the results of type-specific HPV incidence, clearance and risk factors for infection in a

cohort of sexually active young women with a view of making recommendations about screening with HPV testing and the management of LSILs among young women in Uganda.

In study aim 4, whose results are presented in **Paper IV,** we evaluated prevalence, incidence and clearance of genital HPV infections and associated risk factors among young primigravidae to explore the possibility of using pre-natal clinics in the future to evaluate HPV vaccination programs.

2 BACKGROUND

2.1 HPV AND CERVICAL CANCER

Certain types of HPV are recognized as definite human carcinogens [18]. The link between HPV and cervical cancer has been well established for over three decades [19]. There is compelling accumulated evidence that an HPV infection is necessary for the development of invasive cervical cancer [18]. Case-control studies show odds ratios (ORs) of the order of ≥250 for infection with HR-HPV and cervical cancer [20]. Natural history studies show that CIN of any grade is caused by an infection of genital HR-HPVs [21]. Moreover, HR-HPV types particularly HPV 16 become increasingly dominant as the grade of the CIN increase [22, 23]. Laboratory studies demonstrate that genital HR-HPVs encode two potent oncogenes, E6 and E7 that, respectively, disable cell cycle control mediated by p53 and retinoblastoma (Rb) genes [24]. With optimal testing systems and cervical cancer specimens drawn from several countries around the world, investigators found HPV DNA in 99.7% cervical cancer cases [25]. Deviations from this estimate are largely explained by the quality of specimens and the sensitivity of HPV detection assays. Thus, HPV has the highest attributable fraction so far reported for a specific cause of any major human cancer [26]. It is estimated that without secondary prevention, cervical cancer develops in only about 1% of all women who acquire an HPV infection [27], clearly suggesting that cervical cancer is a rare complication of HR-HPV infections, and while it may be necessary for development of cervical cancer, it is definitely not sufficient [28, 29]. Other factors including the HPV type, host and environmental factors probably play a role in the progression from infection to the development of cervical cancer.

2.2 GENOME ORGANIZATION

Papillomaviruses are non-enveloped, double-stranded DNA containing viruses of the Papillomavirus genus belonging to the *papillomaviridae* family [30]. HPV is made up of approximately 8,000 base pairs (bps) with eight overlapping open reading frames (ORFs) (*Figure 1*) [31]. The HPV genome is comprised of the early (E), and late (L) regions as well as an upper regulatory region (URR) [32]. The early region consists of six genes (E1, E2, E4, E5, E6 & E7) required for viral replication and these genes may have transformational potential [33]. The L1 and L2 genes encode the major and minor capsid proteins, respectively [32]. The URR contains sequences that control

transcription.

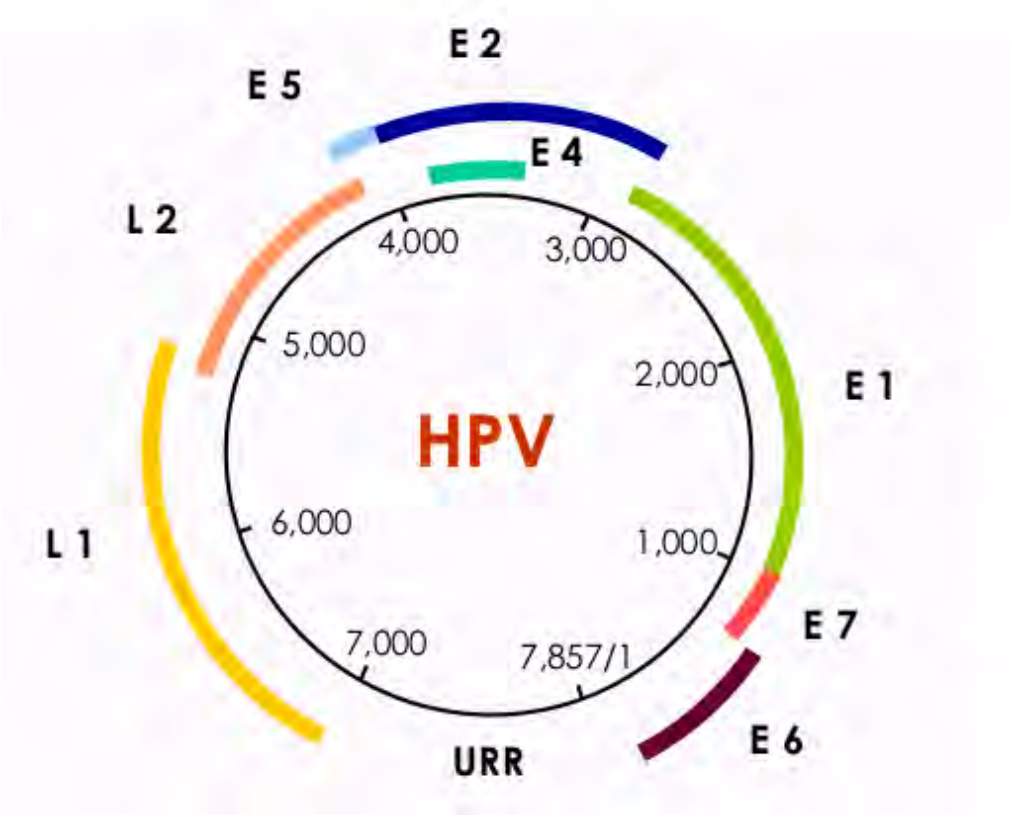

Figure 1: Schematic presentation of the HPV genome showing the arrangement of the early E or non-structural genes, the capsid genes (L1 and L2) and the upstream regulatory region [34]. Reproduced with permission from the publishers.

2.3 LIFE CYCLE AND CARCINOGENESIS

Unlike all other virus families, the lifecycle of genital HPV requires the availability of the basal epithelial cells of the mucosa of the anogenital tract that are still able to proliferate. It is hypothesized that HPV accesses the underlying basal epithelium through naturally thin basal epithelial layers such as those found in the transformation zone of the cervix in young women or through micro abrasion in the epithelium produced during sexual intercourse [35]. The nature of the cell surface receptor that allows initial attachment of the HPV virus to the cells is still debatable [36]. However, internalization of virions into the basal cells seems to occur through endocytosis [37-39]. Inside the cell, it appears that the papillomavirus uncoats by the disruption of intra capsomeric disulphide bonds as a result of reducing environment of the cell allowing viral DNA to be transported to the nucleus [40]. Initially, the viral genome replicates to a low copy number of about 100 virions and can persist in the basal epithelial cells in episomal form for varying periods of time [41]. Although the actual pattern of viral

gene expression in the basal cells is not well defined, it seems that E1 and E2 are expressed to maintain the viral DNA in episomal form while the viral genes E5, E6 and E7 enhance the proliferation of the infected cells and their lateral expansion [42, 43] (*Figure 2)*. As the basal cells continue to proliferate, the supra-basal cells infected with HPV continue to express E6 and E7, blocking the exit of daughter cells from the cell cycle [44, 45]. In the upper layers of the mucosa, where the basal cells reach the stage of terminal epithelial differentiation, E1 [46, 47], E2 [47], E6 [48] and probably E7 genes [48, 49] are expressed, finally resulting in replication of the genome, assembly, maturation and release of the viral particle.

On average, it takes approximately 20 years between the HPV infection and malignant transformation leading to the development of cervical cancer [50]. Viral integration into the host genome is considered to be a critical event in malignant transformation. Viral DNA integration assures the persistent expression of the HPV oncoproteins E6 and E7 in the basal and parabasal cells of the anogenital epithelium [51]. These oncoproteins interact with two cellular tumour suppressor gene products, p53 and Rb genes required for regulation of the cell-cycle progression in response to DNA damage [52, 53]. Together, they create a cellular environment in which normal checks on cell-cycle control are lost, allowing mutations to occur. It is the accumulation of mutations that promotes carcinogenesis [54].

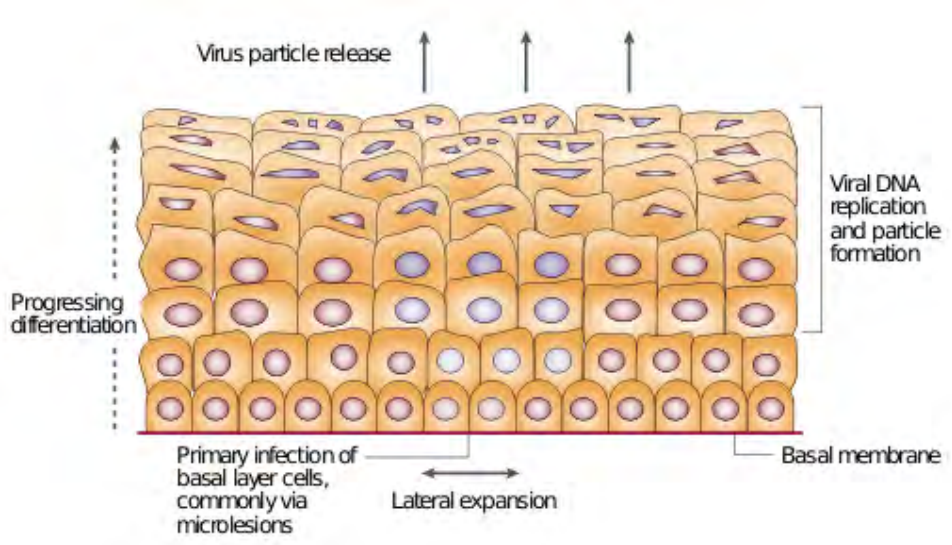

Figure 2: Schematic representation of different phases of an HR-HPV infection [43] Reprinted with permission from the publishers

2.4 CLASSIFICATION

2.4.1 Classification of HPV

More than 100 HPV types have been described from humans and classified based on sequence analysis of PCR products derived from the L1 ORF [55]. An HPV type is defined as a genome when the L1 gene sequence is at least 10% different from any other type, and each HPV is identified by a number based on the order of their discovery [56]. Isolates of a type whose L1 gene sequence differs by 2-10% are very rare and are referred to as subtypes. Presently, subtypes are known for a few HR-HPV types (68 and 82) and LR HPV types (5, 8, 20, 34, 44 and 54). The gene sequence of the L1 ORF of a detected type is compared to reference gene sequences, and relationships of different types are graphically presented as phylogenetic trees (*Figure 3*) [57]. The main branches also called *genera* are the major phylogenetic assemblages that share about 60% nucleotide sequence identity with the LI ORF and are identified by a Greek letter such as Alpha, Beta, Delta, Gamma etc [55]. The minor branches, also called *species*, are reserved for phylogenetic associations of multiple HPV types within a genus that share between 60% and 70% nucleotide sequence identity and considerable biological similarity e.g. HPV 6 and 11 [55]. Phylogenetic groupings predict the natural history and carcinogenicity of individual HPV types, as corroborated by data from case-control studies of cervical cancer [20, 58].

Researchers continue to describe new isolates of HPV genotypes in humans. A new isolate of HPV differs from the closest known type by more than 10% of the L1 gene [59]. Within each genotype, new subtypes differ by between 2% and 10%, and variants differ by only 2% maximally [55]. However, where there is greater intratypic divergence in the non-coding URR, variants may differ by as much as 5% [60].

About 40 HPV types regularly or sporadically infect the mucosal epithelial surfaces of the genital tract [34]. The HPV types that infect the cervix belong to the Alpha group 5, 6, 7, 9 and 11, which contains approximately 30 HPV types [43, 55]. HPV 16 and 18, the two most common types that cause 70% of cervical cancer worldwide, belong to Alpha 9 and Alpha 7, respectively [61].

The alpha group HPV types are further subdivided into HR and LR types according to their potential to cause cancer [20, 55]. HR–HPV types dominated by HPV 16 and 18, with their close relatives consisting of HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and

66 are the main cause of cervical cancer globally [18]. The LR types include HPV 6, 11, 40, 42, 43, 44 and 54. HPV 6 and 11 types cause 100% of condylomata acuminate, or genital warts and are frequently associated with LSIL [62]. While genital warts are benign lesions that pose no risk of malignant progression, they cause significant embarrassment, and anxiety [63, 64], and their management could become economically burdensome [65].

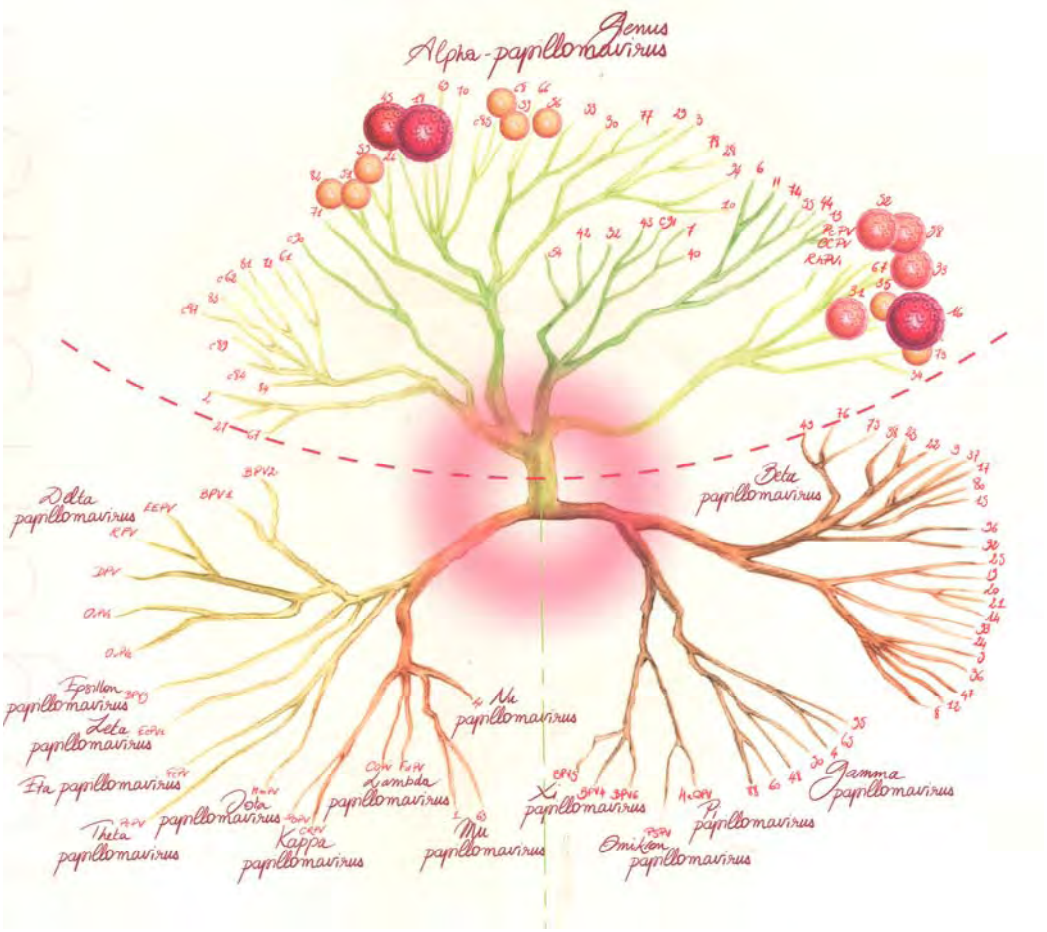

Figure 3: A phylogenetic tree showing the relationship between the different genera and species of HPV. The size of the ball is proportional to cervical cancer cases attributed to the oncogenic HPV type [66]. Reprinted with permission from the publishers.

2.4.2 Classification of HPV-induced cervical lesions

The Bethesda system first developed in 1988 and revised in 2002 replaced the old Papanicolaou (Pap) smear cytological classification [67]. Under the Bethesda system, all cytological abnormalities are classified as either LSILs or HSILs. LSIL includes normal cells as well as mild dysplasia and lumps moderate-to-severe dysplasia together

with carcinoma in situ. When cervical smears contain atypical cells that cannot be easily classified, the cells are labelled as atypical squamous cells of undetermined significance (ASCUS). Newer techniques such as the identification of $p16^{ink4a}$ biomarker in cervical samples are being developed to significantly improve reporting of cytology results by substantially reducing the number of cytology results labelled as ASCUS [68].

2.5 DETECTION TECHNIQUES AND TECHNOLOGIES

2.5.1 Molecular techniques

Molecular diagnostics envisage two different applications [69]. The first is the clinical application for the diagnosis of HPV infections in women at risk of HPV-associated disease, though detection of HPV DNA alone is not sufficient to make a diagnosis of cervical disease. The second application is HPV testing for epidemiological studies and vaccine trials, where the aim is to obtain the maximum information about the HPV status in the population and to monitor the course of infections in detail. In this thesis, we are mainly concerned with the second application.

Since the late 1980s, HPV tests using nucleic acid probes have been commercially available, but these tests were cumbersome to perform and did not achieve widespread use because they could not detect all HR-HPV types. Modern techniques use essentially three types of nucleic acid hybridization to detect HPV DNA; the direct probe, signal and target amplification methods [70, 71]. Of the direct methods, the Southern blot technique was used in earlier studies, but abandoned because it was time consuming, had low sensitivity, and required large amounts of highly purified DNA.

The most frequently used signal amplification technique is the Hybrid Capture™ 2 (HC2 Digene Corp., Gaithersburg, MD, USA) assay. HC2 is a non-radioactive signal amplification method based on hybridization of the target HPV-DNA to labeled RNA probes in a solution that uses two different probe cocktails to detect 13 HR- and 5 LR-HPVs [72, 73]. Although HC2 is the only molecular technique cleared by the Food and Drug Administration (FDA) in the USA for in vitro use [13], it has some limitations. For instance, HC2 does not permit type-specific identification of HPVs, is less sensitive than polymerase chain reaction (PCR) assays with a detection limit of approximately 5,000 genome equivalents [74], and has the potential of cross-reactivity of the two probe cocktails, which would reduce the clinical importance of positive results [75, 76].

In most epidemiologic studies, PCR is the most widely used technology to test for HPV DNA, which uses target amplification methods that allow for the multiplication of unique regions of the DNA so they can be detected. General or consensus primer–mediated PCR assays have been developed to screen for a broad spectrum of HPV in clinical specimens using a single PCR reaction. The primers target a conserved L1 region in different HPV genotypes [13]. During a PCR reaction, the viral genome fragment is amplified through repeated cycles of denaturation, primer hybridization, and primer extension. PCR assays are highly sensitive and can detect 10 to 100 DNA molecules in a specimen as well as produce as many as one million copies from a single-stranded DNA molecule after 30 minutes of amplification cycles. However, PCR assays cannot detect very low HPV viral loads, and as a result such specimens would always be erroneously classified as HPV DNA negative.

The choice of primers is critical in the detection of type-specific HPV types. Sequences of a variety of HPV consensus primers pairs that are common to most, if not all, anogenital HPV types have been published. These primers include GP5/6 [77] and its extended version GP5+/6+ [78], the short PCR fragment (SPF) 10 [79], the MY09/11 degenerate primers [80] and its modified version PGMY09/11 [81]. To detect amplified type-specific HPV genotypes from the PCR products, three reverse hybridization assays could be used. The first system is a line blot assay (LBA) PGMY based on the MY09/11 primer set, which amplifies a 450 bps fragment and can identify 27 different HPV genotypes. The second system uses a non-radioactive reverse line blot assay (RLB) [82] and is based on the GP5+/6+ primer set that amplifies a fragment of 150 bps to identify 37 different HPV genotypes. The third system is a line probe assay (LiPA) and is based on the SPF10 primer set which amplifies a fragment of only 65 bps designed to distinguish different HPV types in ELISA format [83], or in a reverse line-blot hybridization (LiPA) [82, 83]. SPF 10 amplimers are first tested in a micro titer plate general hybridization assay to detect HPV DNA positivity. Then, the positive samples are analyzed by SPF10/LiPA, which permits the identification of 25 different HPV genotypes [83].

Different primer sets exhibit differences in sensitivities in detecting individual HPV genotypes simultaneously, particularly when there are co-infections with multiple HPV types [84]. A validation panel organized by the World Health Organization (WHO) showed no differences in DNA detection for HPV 16, 18, 33 and 45 across different

PCR assays with different primer sets. However, differences were observed for HPV 6, 31, 35, and 52 [85], and that variation should be taken into consideration when interpreting results from different studies.

2.5.2 Non-molecular techniques

There are also non-molecular techniques that do not detect the actual presence of HPV DNA but identify the clinical manifestations of HPV infection on the cervix using visual and/or microscopic methods. For many resource poor countries, establishing quality national cytology-based screening programs is beyond their capacity and resources. As an alternative to cervical cytology, visual techniques have been successfully evaluated in resource poor countries and show promising results to support their use in screening programs [86]. However, the specificity of non-molecular techniques is still low compared to that of a good conventional cytology, quality assurance under field conditions remains a major challenge, and there is no universally accepted uniform reporting of test results. Until these problems are fixed, good conventional cytology remains the "gold standard" non-molecular technique.

2.6 ACQUISITION AND TRANSMISSION

2.6.1 Sexual transmission

The most common mode of transmission of ano-genital HPV infections that are important to cervical cancer is vaginal intercourse with an infected partner [87-89], although a few studies were unable to confirm this [90, 91]. This mode of transmission accounts for the majority of cervical HPV infections. Additional overwhelming evidence confirming sexual transmission comes from studies that documented the transmission of genital warts between sexual partners [92]; rarity of genital HPV infection among sexually inexperienced women [93]; the strong and consistent association between lifetime numbers of sexual partners and a positive trend of HPV prevalence with age in women [93]; and men, though less consistently [94]; the increased risk of HPV acquisition from new and recent sexual partners [95]; and the concordance of type specific and HPV-16 variant DNA in sexual partners [96]. Unlike the transmission dynamics for some bacterial STDs [97], HPV infections are not restricted to core groups (i.e. groups of highly sexually active individuals with many partners or "super spreaders"), as infection is also relatively common among moderately sexually active individuals [98, 99]. Moreover, migrant populations may

provide a bridging opportunity for sexual transmission between members of high and low prevalence sub-populations [100, 101].

2.6.2 Non sexual transmission

Non-sexual transmission of genital HPV infections through modes like skin-to-skin contact, fingers and sex toys has been evaluated and accounts for a very small proportion of genital HPV infections [102-104]. Though feasible, transmission through this route is uncommon [105, 106]. Although rarely, vertical transmission of HR-HPV may occur, probably transplacentally during gestation or through direct exposure to cervical and genital lesions during birth [107], as both HPV DNA and serum antibodies have been detected in both infants and children [108-112]. However, HPV DNA sample analysis has also shown positivity in children born to HPV-negative women and transmission from father to baby after birth [112, 113], which suggests that non-sexual horizontal transmission may also occur. In pre-adolescent children, genital HPV infections are often considered a sign of sexual abuse, though horizontal transmission may also play a role [114].

2.7 TRANSMISSIBILITY OF HPV INFECTION

HPV infections are easily transmitted [95, 102, 115]. Yet, there is currently no empirical data on its transmissibility. Using stochastic computer simulations, the probability of HPV transmission per coital act is estimated at 5%-100% with a median of 40% [116]. Alternatively, mathematical models [117] estimate the probability of male-to-female transmission at 60%, which, is identical to the observed per partner transmission probability of genital warts [92]. These results tend to suggest that while the probability of transmissibility of HPV is comparable to that of some bacterial STIs, it may be more transmissible than other viral STIs [118, 119]

2.8 RISK FACTORS

Numerous risk factors for acquisition and transmission of HPV infection in different study populations and geographical locations have been reported by several epidemiological studies [99].

2.8.1 Number of sexual partners and acquisition of new partners

The strongest and most consistent risk factor is the number of lifetime sexual partners. The risk of HPV exposure appears to increase with the number of lifetime sexual

partners [102, 120-122], although having sex with only one partner is also associated with HPV infection [123]. Additionally, serologic studies found a very strong correlation between the presence of serum HPV antibodies and the lifetime number of sexual partners [124-126]. Moreover, exposure to new partners is strongly associated with incident infection [93, 95]. In a longitudinal study of adolescents and young women, the risk for incident infection increased nearly 10 times for each new partner reported per month [95]. In studies that compared HPV prevalence among female prostitutes, women's attending the STD clinic and women from the general population whose risk of exposure to the HPV infection varies. The prevalence was highest in all age-groups among prostitutes, followed by women attending the STD clinic and the lowest among women from the general population [89].

2.8.2 Age and age of sexual debut

In all the regions of the world, age has been found to be a strong and consistent risk factor for HPV infections, as infection is consistently found in sexually active women below 25 years [8, 62, 127]. Young age at initiation of sexual intercourse is a risk factor for the acquisition of new cervical HPV infections [128, 129]. The risk of HPV infections appears to increase with the interval between menarche and first sexual intercourse after controlling for other determinants of infection [130]. There are several mechanisms for the association of time from menarche and HPV infections. The replication and differentiation of host cells, which is characteristic of squamous metaplasia, which occur in the cervical transformation zone of young women following menarche, favours HPV replication [131]. Furthermore, basal epithelial cells, which are the target cells for HPV infections, are the most accessible in the transformation zone, which appears to increase in size with time from menarche, independent of sexual and other reproductive factors [132]. Nevertheless, the overall association between age and HPV infection may just be a marker of other risky behaviours [98].

2.8.3 Sexual behaviours of male partners

The role of men as vectors of HPV types related to cervical cancer has been extensively evaluated in epidemiological studies [90, 128, 129, 133, 134]. HPV DNA was detected from the shaft of the penis, the inner surface of the prepuce, the distal urethra, the prepuce external surface, the glans penis, and the scrotum confirming men as carriers of HPV infection [135]. Further evidence supporting the role of males in the transmission of HPV comes from case-control studies of cervical cancer conducted by IARC, which

showed that penile and cervical HPV correlated strongly with cervical cancer incidence rates [90]. Thus, sexual behaviour of male partners is as critical for the woman's HPV acquisition as from her own sexual behaviour. In fact, the HPV prevalence and incidence in women has been positively associated with the women's estimates of their male partners' lifetime number of sexual partners [102], though awareness of whether a sexual partner has other partners has been shown to be poor [136]. Thus, women who only have sex with their husbands or spouses are still at risk of HPV exposure, since he may have been infected prior to marriage, or he may be exposed to other partners who are HPV positive and thus bring infection to the spouse.

2.9 OTHER FACTORS THAT INFLUENCE SUSCEPTIBILITY AND/OR INFECTIVITY

Several cofactors which appear to be directly related to the physiologic and/or immunologic state of the cervix, i.e. the cervical microenvironment, may enhance susceptibility and/or infectivity of HPV above the probability attributable to HPV alone [137].

2.9.1 Co-infection with other HPV types

Regardless of the stage of cervical pathology, natural history studies show that about 20%-30% of HPV infected women harbor multiple types acquired either concurrently or sequentially [138]. Co-infections may increase susceptibility to acquisition of incident HPV infections [139]. The presence of HPV 16 in the enrolment cervical specimen in one prospective study of young women in the USA, was associated with an increased risk of acquisition of other HPV types [140]. In another prospective study in Brazil, infection with any type of HPV at study entry increased the likelihood of acquisition of any other type of HPV at a later visit [141]. Although sexual co-transmission is one likely explanation, it is still not clear whether HPV influence each other's transmission. Some reports seem to suggest that some HPV types may use the same endocytosis pathway to enter cells [142]. Conversely, in one study, the acquisition of new HPV infection seemed to occur in a non-independent manner in presence of co-infection with other types [143]. The odds of acquiring concurrent infection with HPV 31, 39 and 45 was increased by 11–18 times in women infected with HPV 18 than in women without that type. Furthermore, the odds of acquiring subsequent HPV 58 was increased by 5–7 times in women with incident HPV 16 infections than those without that type [144]. These findings seem to suggest sero-

reactivity across HPV types [145] and probably cross protection by cross-neutralization for HPV 16, 31 and 33 [146].

2.9.2 HIV Immune-suppression

Clinical studies involving HIV positive women have consistently demonstrated a lot more prevalent and incident infections of any HPV type compared to high-risk HIV-negative women [147]. Prospective cohort studies have also consistently reported an increased incidence of SIL in HIV positive compared to HIV-negative women [148]. Some studies have suggested that HIV infection is the strongest risk factor for cervical cancer independent of the usual demographic and behavioural risk factors [149, 150]. There are several mechanisms of how HIV could interact with HPV infections. First, a substantial proportion of cervical HPV DNA detected in HIV-positive women might reflect reactivation of previously acquired quiescent infections, rather than recent sexual transmission [151]. Secondly, HIV may have a direct viral-viral interaction with HPV, given that both viruses infect macrophages [152]. Thirdly, in vitro studies have indicated that expression of the HIV *tat* protein may increase the expression of HPV E1 and L1 viral genes [153] and HPV 16 E7 transcription [154].

2.9.3 Co-infection with other STIs

Cervical infections with other STIs such as *Chlamydia Trachomatis* (CT) and *Herpes Simplex* virus type 2 (HSV 2), may increase susceptibility to HPV infection by cervical inflammation or micro abrasions in the epithelium resulting from sexual intercourse, which allow HPV direct access to basal epithelial cells [95]. It is also possible that STIs could enhance the oncogenic effect of an already established HPV infection by influencing local immune response [155]. The similarity of risk profiles between HPV and other STIs, however, makes it difficult to distinguish whether other STIs are just markers of exposure to HPV or act as true cofactors by increasing susceptibility or infectivity [156].

2.9.4 Use of condoms, spermicides and vaginal lubricants

The consistent use of male condoms provides partial protection against cervical HPV infections [50]. In a recent prospective study, the consistent use of male condoms by sexual partners of a cohort of newly sexually active women appeared to significantly reduce male-to-female genital HPV transmission and the incidence of LSIL [157]. Furthermore, in a meta-analysis of 20 published studies, the risk of developing HPV-

related cervical lesions was reduced by the use of male condoms [158]. The molecular mechanisms by which condoms prevent HPV-associated disease are unknown. However, it has been hypothesized that using condoms may decrease the amount of virus transmitted or the exposure to other co-factors that may be involved in development of disease, which in turn, may reduce the probability of developing an HPV-related lesion [159]. In individuals who are already infected, the regular use of male condoms has been shown to limit the spread of HPV to additional sites [96, 160]. However, because of the high infectivity of HPV, the protective effect of condoms could be significantly be diminished by multiple sexual acts in an ongoing relationship [121]. Thus, even after the prophylactic HPV vaccines become widely available, the consistent use of condoms by their sexual partners may protect women against infection with other HR-HPV types that could put them at risk for cervical cancer.

Laboratory studies have shown that a widely used vaginal spermicide, Nanoxynol-9 (N-9), known to disrupt the normal architecture of human genital epithelium [161], greatly increased susceptibility to HPV infections [162]. In contrast, Carrageenan, a polysaccharide present in some vaginal lubricants, prevented HPV infections even in the presence of N-9, suggesting that Carrageenan might serve as an effective topical HPV microbicide in the future [162]. If these findings are confirmed in clinical trials in human beings, Carrageenan might become a useful adjunct to the current prophylactic HPV vaccines, which target a narrower spectrum of HPV genotypes [163].

2.9.5 Male circumcision

For a long time, male circumcision was associated with the prevention of common sexually transmitted diseases [164]. A pooled analysis of the International Agency for Research on Cancer (IARC) data confirmed that circumcised men not only had a substantially lower risk of penile HPV infections than uncircumcised men, but also that their partners had a lower risk of HPV infections and a lower risk of developing cervical cancer [145]. The protective effect was more pronounced among women whose male partners engaged in high-risk sexual behaviour. Further evidence of the protective effect of male circumcision comes from two recent randomized controlled trials. One trial showed that circumcision of adolescent boys and men in a rural Ugandan population reduced the prevalence of HPV infection by 35% [165]. Another trial conducted in South Africa demonstrated a significant reduction in the prevalence of urethral HR-HPV infection after male circumcision [166]. The reduction of HPV

infection by means of circumcision may involve anatomical factors, cellular factors, or both. First, the retraction of the foreskin over the penile shaft during intercourse exposes the inner preputial mucosa to vaginal and cervical fluids through micro tears resulting from sexual intercourse particularly those that occur in the frenulum. The micro tears probably facilitate the access of HPV to the basal epithelium [167]. Secondly, in uncircumcised men, the inner mucosa of the foreskin is lightly keratinized, which may facilitate the access of HPV to underlying basal epithelial cells. Thirdly, in circumcised men, keratinization of the surgical scar probably deters HPV infection from accessing the basal epithelia [167].

2.9.6 Cigarette smoking

It is not clear whether cigarette smoking independently influences susceptibility and/or infectivity of HPV, although tobacco-related carcinogens of cigarette smoking directly damage the genetic material of cervical cells resulting in the development of cervical cancer [168]. In studies that have found a positive association between smoking and the acquisition of HPV infections, a positive association was attenuated after controlling for sexual behaviour [99, 169]. Only one prospective study found a significant positive association between current cigarette smoking and incident HPV infections even after controlling for sexual behaviour [102]. While smoking could truly increase susceptibility to HPV infection, it may be just a proxy measure to unmeasured sexual behaviours [170].

2.9.7 Pregnancy

There is no consensus among researchers about the relationship between pregnancy and HPV infections. Depending upon the study type, HPV detection in pregnant women was shown to be higher [171, 172] or similar to [173-175] those women who were not pregnant. During pregnancy, the transformation zone on the ecto cervix is enlarged which may facilitate the exposure of the basal epithelial cells to HPV, although hormonal factors may also be involved. On the other hand, some researchers suggest that physiologic processes during pregnancy modify the host-immune response, which in turn reactivate the quiescent HPV virus acquired earlier, resulting in increased detectability [176].

2.9.8 Hormonal Contraception

Although the use of oral contraceptives for more than 5 years is associated with increased risk of cervical cancer, there is no strong positive or negative association between HPV positivity and ever use or long duration use (5 years or more) of oral contraceptives according to a review of 19 studies [177]. There are a number of hypotheses, but little direct evidence about the ways in which oral contraceptives might influence cervical HPV infections [178]. The use of oral contraceptives is associated with an increased incidence of cervical ectropion, which means that the squamo-columnar junction, the site where HPV infection preferentially induces neoplastic lesions, is more exposed to HPV oncogenes E6 and E7 [178]. Secondly, estrogens and progesterone may also affect cervical cells directly, increasing cell proliferation and thus stimulate the transcription of HPVs. Most studies, however, have not reported an association between the acquisition of HPV infections and the use of oral contraceptives independent of sexual behaviour [177].

2.9.9 Genetic predisposition

Several reports indicate that genetic background is important in defining susceptibility to HPV infections, particularly polymorphism of the major histocompatibility complex (MHC) and p53 genes [179]. It is hypothesized that HPV type (and variant) distribution could have co-evolved with populations, hence the difference in geographical and racial distribution of HPV types [180-182]. Thus, HPV infections may be intimately associated with certain host Human Leukocyte Antigen (HLA) gene complexes [156]. Evidence from research on the familial clustering of cervical cancer generally supports the existence of moderate genetic influence [183-185].

2.10 GLOBAL BURDEN OF HPV INFECTIONS

2.10.1 HPV prevalence

Four recent publications have increased our understanding of the global status of HPV infections [3, 8, 127]. Two publications reported the global HPV prevalence. The most recent meta-analysis of 78 studies with a total sample of 157,879 women with normal cytology estimated the global adjusted HPV prevalence to be 10.4% (95% CI, 10.2 – 10.7) [8]. Using this adjusted prevalence, it was estimated that at any one point in time, about 291 million women in the world are infected with HPV [8]. Women from resource poor countries had a higher HPV prevalence than those from developed regions, estimated at 15.5% and 10.0%, respectively. Women from Africa, and

especially the East African region, had the highest prevalence in the world estimated at 31.6% [8]. The lowest HPV prevalence estimated at 6.2% was for women from the South Eastern Asian regions (*Figure 4*) [8].

The second comprehensive review was a pooled analysis conducted by IARC based on 15,613 women with normal cytology drawn from populations with low, intermediate and high incidences of cervical cancer [62]. Based on this analysis, the estimated overall age-adjusted HPV-DNA prevalence was 10.5%. The analysis also showed significant geographic variations in prevalence. HPV prevalence among women from Sub-Saharan Africa was approximately 5 times higher than for women in Europe, and rates for women in South America and Asia were between those of Europe and Sub-Saharan Africa.

The heterogeneity of laboratory assays employed for HPV DNA detection and populations studied could explain the variations [13]. However, additional factors could also influence variations in the prevalence rates. The lack of screening programs, sexual behaviours of both men and women (such as early age at first marriage to older men or to men with several concurrent partners) and poor hygienic conditions might be some of those factors in Africa [186]. Concurrent HIV infection, a known risk factor for increased HPV prevalence, could explain the extremely high rates in the East African region [187]. Disparity in implementation and management of cervical lesions in screening programs in North, Central and South America [27] and change in sexual behaviours in Asia may be important factors to explain the variations in those regions [188].

While a large group of women with HR-HPV types and normal cytology may be identified by HPV testing in screening programs, these women have a low absolute risk for cervical cancer, even if their risk is higher than for HPV-negative women [189].

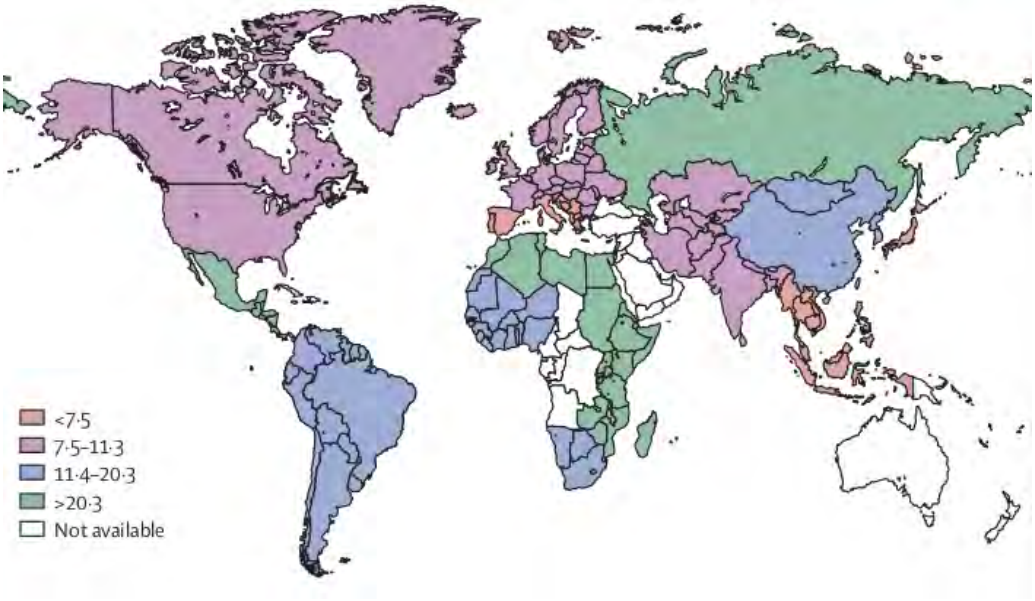

Figure 4: Estimated HPV Prevalence among adult women with normal cytology in the world regions [8]. Reprinted with permission from the publishers.

2.10.2 Age-specific prevalence rates

Globally, infections with HPV are strongly associated with age [8, 62, 127]. Except for the Asian regions, the predominant age-specific pattern of HPV prevalence in all major regions of the world is a bimodal curve, which has an early peak in young women, then declines in middle aged women and has a second peak in older women (*Figure 5)*. The second peak in older women does not reach that found in young women [190], as cross-sectional studies have demonstrated a 6–8fold difference in HPV prevalence in younger compared to older women [191-193].

The first peak is consistently found in women aged ≤25 years, which reflects the rapid acquisition of HPV infection soon after the initiation of sexual intercourse [93, 102, 194]. The sharp decline in middle aged women is consistent with viral transience as well as low incidence in older ages [195].

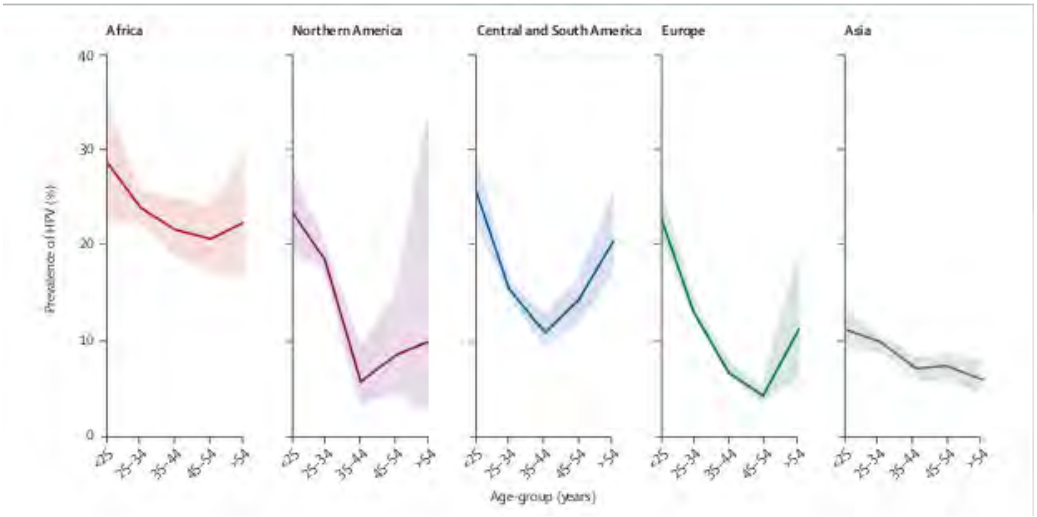

Figure 5: Age specific prevalence of HPV among women with normal cytology, by world region [8]. Reproduced with permission from the publishers.

Several explanations have been proposed for the second peak. First, follow-up studies have shown that new HPV DNA can be detected in all age-groups which suggest that new infections could either be acquired by middle aged women or that there is reactivation of infections acquired in early life [35]. In support for the acquisition of new HPV infections, behavioural surveys have demonstrated a high rate of new sexual partners in women and men aged 40 years and above [196-198]. Moreover, there is a difference of close to 10 years in age intervals in which the second peak is observed in the United States (35–44 years) compared to Europe (45–55 years), which seems to suggest behavioural factors rather than a biological effect linked to menopause or senescence [199]. Nevertheless, the reactivation of latent HPV infection due to gradual loss of type-specific immunity or a sudden loss due to hormonal influences during postmenopausal years in older women may also be a plausible explanation [200]. In the absence of any sexual activity, a second peak in the incidence rate has been observed in some older women populations [197] which support the re-activation of infection acquired earlier rather than the acquisition of new infection. Several researchers have also proposed a possibility of a cohort effect [201]. Early indication that there could be a cohort effect are shown by elevated antibodies against HPV 16 in young Finnish women [202], an elevated prevalence of HPV 16 in Swedish women under 35 years [203], and extremely high HPV prevalence rates among young women in North America [102] and the United Kingdom [189]. Confirmation of this proposition, however, would be difficult because it would require analysis of stored

representative samples from women of different eras using the same HPV DNA testing protocols. Recent studies also suggest that the second peak in the older category may be due to an increase in low-risk HPV types during peri-menopausal years resulting from under representation of HR-HPV types following effective treatment of high-grade cervical lesions in older women [204, 205]. It is possible that one or more of these factors could be responsible for the second peak in different geographical regions, but more research is needed to fully understand this phenomenon.

2.10.3 Incidence rates

Similar to prevalence, incidence rates of cervical HPV infections in young women are high and remain high with the acquisition of each new sexual partner [35, 102, 130, 206]. Although the cumulative risk of infection with any type of HPV infection appears to be high throughout the life of a woman, studies have shown that incidence rates decline with age [35].

Some key issues relating to incident HPV infections are still unresolved. Like other viral infections, HPV establishes persistent infections characterized by continuous low levels of viral replication with periodic reactivation of latent infection following apparently disease free intervals [207]. Consequently, in cohort studies, it is not possible to ascertain whether an active infection is truly new, because at present an incident infection cannot be unequivocally differentiated from reactivation of prevalent or latent infection [208]. Secondly, it is unclear whether the differences between latent and active cervical infection are qualitative or quantitative. Yet, a clear understanding of latency may help to explain the observed variation in world HPV trends, particularly the second peak in older women.

2.10.4 Clearance or persistence of HPV infections

Clearance of HPV infections is primarily determined by the host immune response, particularly a cell-mediated immune response [120, 209]. Whether HPV infections are cleared completely, are self-limited or are suppressed in long-term latency is still unclear. One of the major unresolved issue relating to the natural history of HPV infections is how often short-term viral clearance leads to long-term viral latency. Latency implies that no HPV-DNA is detected by the current conventional molecular tests, but that very small foci of cells maintain DNA at low DNA copy numbers. The existence of the latent state is supported by studies in immunosuppressed individuals

where immnosupression represents a risk factor for HPV viral persistence and cervical lesion progression [210]. However, the frequency of latency among immuno competent individuals, how long it lasts, and what causes re-emergence into detectable state is still unknown. Answers to these questions will greatly influence prevention strategies reliant on HPV DNA detection methods.

Natural history studies among young women where short interval measurements are made followed by HPV genotyping, show that up to 90% cervical infections (with or without cytological abnormalities) are no longer detected by sensitive HPV DNA testing assays within 1-2 years [120, 211-214]. Whether an HPV infection is cleared or not seems to be linked to the HPV type. Generally, infections with HR-HPV types clear less than with LR-HPV types [58]. Specifically, infections with HPV 16 and HPV 18 clear less than other HR or even low-risk types. Co-infections with multiple HPV types seem to reduce clearance rates compared to infections with a single HPV type [138]. HPV infections that last 2 years are less likely to clear and can last many more years (persist) as shown in a 24 month follow-up study of the ASCUS-LSIL Triage study (ALTS) [215].

2.10.5 HPV type distribution

Among HPV positive women with normal cytology, HPV 16 and 18 are the most common types all over the world, with estimated prevalence rates of 2.5% and 0.9%, respectively representing 32% of infections (23% due to HPV 16 and 8.5% to HPV 18) [8].

The distribution of HPV types varies in different geographic regions, which might be related to biologic interplay between different HPV types or variants and host immunogenetic factors (e.g. HLA polymorphisms) [216]. For instance, epidemiological studies have shown that compared to Europe, HPV positive women in Sub-Saharan Africa were significantly less likely to be infected with HPV 16 but more likely to be infected with other high- and low-risk HPV types [62]. It has been hypothesized that HPV 16 may be less influenced by immune status than are other HR-HPV types [151]. Impairment in cellular immunity in populations in Sub-Saharan Africa through cervical inflammation, parasitic infection, malnutrition and probably HIV could somehow contribute to the higher penetrance of HR-HPV types other than HPV 16. While the prevalence of other HR-HPV types increased with diminished

immunity as measured by CD4+ T-cell lymphocyte level, the prevalence of HPV 16 remained unchanged among HIV infected women [217].

Other notable geographical variations are observed in the ranking positions of the most frequent HPV types as well as the frequency of other HR-HPV types [8]. Fortunately, the particularities in the HPV-type distribution seen among women with normal cytology have only limited relevance to prophylactic vaccines against cervical cancer, knowing that HPV-16 is the type more likely to progress and becomes increasingly dominant with increasing severity of lesions than other HPV types [206, 218, 219].

2.11 PROPHYLACTIC HPV VACCINES

Since 2006, two effective prophylactic HPV L1 VLP vaccines whose goal is to reduce the incidence of HPV-related genital disease are available [5, 6]. Both vaccines are based on the recombinant expression and self-assembly of the major capsid protein, L1, into VLPs that resemble the outer capsid of the whole virus. The HPV VLPs contains no DNA and are not live/attenuated viruses. One of the vaccines is Garda sil®, a quadrivalent HPV 6/11/16/18 L1 VLP vaccine, which is delivered by intra-muscular injection as a 0.5-mL dose in a three-shot immunization protocol at 0, 2 and 6 months. The other vaccine is Cervarix®, a bivalent HPV16/18 L1 VLP vaccine, which is delivered by intra-muscular injection in a three-shot immunization protocol at 0, 1, and 6 months as a 0.5 mL dose. Efficacy and safety data of these vaccines[5, 6], as well as current follow-up data of the bivalent vaccine up to 6.4 years is available [7]. The vaccines are currently licensed for use in young people up to age 26 years but are likely to be more effective in pre-adolescents and adolescents before the age of sexual debut when HPV exposure occurs. In this regard, in June 2006, the Program for Appropriate Technology for Health (PATH) received a 5-year grant from the Bill & Melinda Gates Foundation to oversee pilot HPV vaccine introduction projects in 4 developing countries including Uganda [220]

2.12 CERVICAL HPV INFECTIONS AND ITS CLINICAL MANIFESTATIONS IN UGANDA

Cervical; cancer is the most frequent cancer in all women and the 2nd most common cancer among women aged between 15 and 44 years in Uganda.[3]. A complete data set for incidence of cervical cancer in Uganda during four time periods (1960–1966, 1967–1971, 1991–1994 and 1995–1997) was recently published [221]. Compared to

the USA, the age-adjusted cervical cancer incidence rate per 100,000 is 6 times higher in Uganda (45.8 vs. 7.7), and the age-adjusted death rate is 12.7 times higher (29.3 vs. 2.3) [1, 9]. The incidence rate is probably an underestimate, as many women at risk do not access health care. Screening is "opportunistic", offered only to those women who visit health units for other reasons and who probably represent a low risk group. As a result, over 80% of patients diagnosed with cervical cancer at the national referral hospital present with advanced disease [222]. Available data show that the HIV infection is associated with an earlier onset of cervical cancer [223], and about 77% of invasive cervical cancers are attributed to HPV 16 or 18 [12].

Data is not yet available on the burden of HPV infections and its clinical manifestations in the general population in Uganda. However, a few studies conducted in different populations across the country show very high prevalence rates of HR-HPV infections (*Table 1*). The prevalence of HR-HPV infection among young women aged 12–24 years in Kampala was 51.4% [224] and 43.0% among primigravidae aged 14-24years [175]. Among women in a rural district of Bushenyi, the prevalence of HR-HPV using HC2 was 17.2% [10]. The prevalence of HPV 16 or 18 was 80% in archival cervical cancer biopsies [12]. A population based cohort study of 606 rural women aged 15-49 years in the rural Rakai district using HC2 found HR-HPV prevalence of 19% [225]. An earlier cross-sectional study in the same district had found the overall prevalence of HPV using HC2 of 22%; 15.4% in 324 HIV-negative and 52% in 71 HIV-positive women [226]. Furthermore, a cross-sectional study conducted among women attending a sexually transmitted clinic in Kampala, found 18% of women infected with either HPV 16 or HPV 18 [11]. Antibodies against HPV-16 were significantly associated with cervical cancer in a case-control study [227]. In a cross-sectional study, preliminary findings from 16 scrapes of women with normal or dysplastic ecto-cervical epithelium revealed that while HPV 16 DNA was detected in only 12.5% of biopsies of women with normal cytology, it was detected in all 3 biopsy specimens of cervical cancer [228].

Data from prospective cohort studies in Uganda is exceedingly sparse. Apart from studies presented in this thesis, the only other prospective cohort study on HPV infection published recently evaluated the incidence and clearance of 13 HR-HPV infections and their determinants among 1,055 women [229]. In this study, using HC2 the estimated incidence rate of HR-HPV was 17.3 per 100 person-years among 147

HIV positive compared to 7.0 per 100 person-years among 908 HIV negative women. Incident HR-HPV infections were associated with HIV positivity, young age, many lifetime and recent sex partners, as well as women with high awareness of AIDS [229]. Clearly, all these studies show that both prevalent and incident HR-HPV infections are common in different female populations of Uganda in keeping with published studies from elsewhere in the world.

Table 1: Summary of prevalence rate of HPV infections among different populations in Uganda

Reference	Population and sample	HPV prevalence	
Banura et al.[224]	Clinic-based cohort of 1,275 young women aged 12-24 years seeking services at a teenage clinic in Kampala	Overall HPV prevalence HR-HPV types *HPV 16* *HPV 18*	74.6% 51.4% *10.6%* *10.7%*
Banura et al. [175]	Clinic-based cohort of 1,097 primigravidae (14-24 years) attending antenatal clinic at Naguru Health Center	Overall prevalence HR-HPV types *HPV 16* *HPV 18*	60.0% 43.0% *8.4%* *5.8%*
Asiimwe et al. [10]	Population-based survey of rural women 18-49 years	HR-HPV types	17.2%
Odida et al. [12]	186 archival cervical cancer biopsies	*HPV 16 or 18*	*80.0%*
Safaeian et al. [225]	606 women with paired and self-collected swabs	HR-HPV types	19.0%
Serwadda et al. [226]	Cross-sectional study of 960 rural women aged 15-59 years	Overall HPV prevalence	16.7%
Blossom et al. [11]	Cross-sectional study of omen in STD clinic in Kampala (18-55 years)	Overall HPV prevalence *HPV 16 and 18*	46.2% *18.0%*
Newton et al.[227]	Case-control study	Prevalence of antibodies against HPV 16 (OR = 2, 95% CI = 1.2 – 3.1)	27.0%
Bounaguru et al. [230]	Cross-sectional study of 16 scrapes of women with normal or dysplastic ecto-cervical epithelium	Prevalence of HPV 16 in women with normal cytology Prevalence of HPV 16 in specimens of cervical cancer biopsies	25.0% 100.0%

3 AIMS OF THE THESIS

I. To evaluate the feasibility of using filter paper for collection, storage and transportation of cervical exfoliated cells for HPV-DNA detection and genotyping (*Paper 1, Published*).

II. To estimate prevalence rates and risk factors for HPV and sub-type distribution and other selected sexually transmitted infections among sexually active young women seeking services at Naguru Teenage Information and Health Centre (*Paper 2, Published*).

III. To evaluate type-specific HPV incidence, clearance and associated risk factors among young women (*Paper 3, Submitted*).

IV. To estimate the prevalence, incidence and clearance of HPV infection between the $1^{st}/2^{nd}$ and 3^{rd} trimester of pregnancy and between pregnancy and delivery among young primigravidae seeking pre-natal care at Naguru Health Centre (*Paper 4, Published*).

4 STUDY SITES, SUBJECTS, AND METHODS

4.1 STUDY SITES

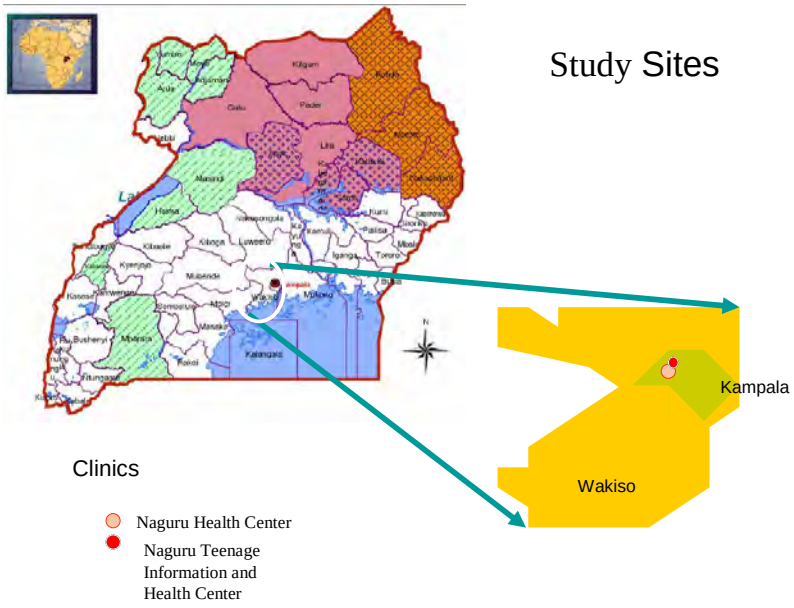

Figure 6: Map of Uganda showing study sites

Studies that resulted in **Paper I, II** and **III** were conducted at Naguru Teenage Information and Health Centre (NTIHC); an urban clinic that provides free "youth-friendly" services to both males and females aged 10-24 years. Until recently, NTIHC was located about 3 Kilometers east of the Kampala city centre but has since relocated. It is a primary care unit administered by the Kampala City Council. On a daily basis, for six days a week, the centre offers voluntary HIV counseling and testing, family planning counseling and services, treatment for sexually transmitted infections and referrals, condom promotion and distribution, antenatal and post natal care, post-abortal care, and treatment for other common minor ailments. In addition, the centre provides recreational activities for young people and on a typical day, 100 to 150 young people are provided with different services [231].

The study that resulted in **Paper IV** was conducted at Naguru Health Centre (NHC) located in the same compound as NTIHC. It is a 20-bed government funded primary care facility (level IV in the Uganda health care delivery system) that was recently upgraded to district hospital status. It provides free health care to adults and children

principally the low-income earners residing in the eastern suburbs of Kampala City and the surrounding districts. The facility provides on a daily basis, antenatal, maternity and theatre facilities, family planning counseling and services, treatment for sexually transmitted infections and referrals, condom promotion and distribution, prevention of HIV from mother-to-child services and general out-patient clinics for minor ailments. Twice a week, the centre provides HIV treatment and support services and post-natal services once a week. An estimated 200 patients obtain services from the outpatient clinics on a typical day.

4.2 STUDY DESIGNS

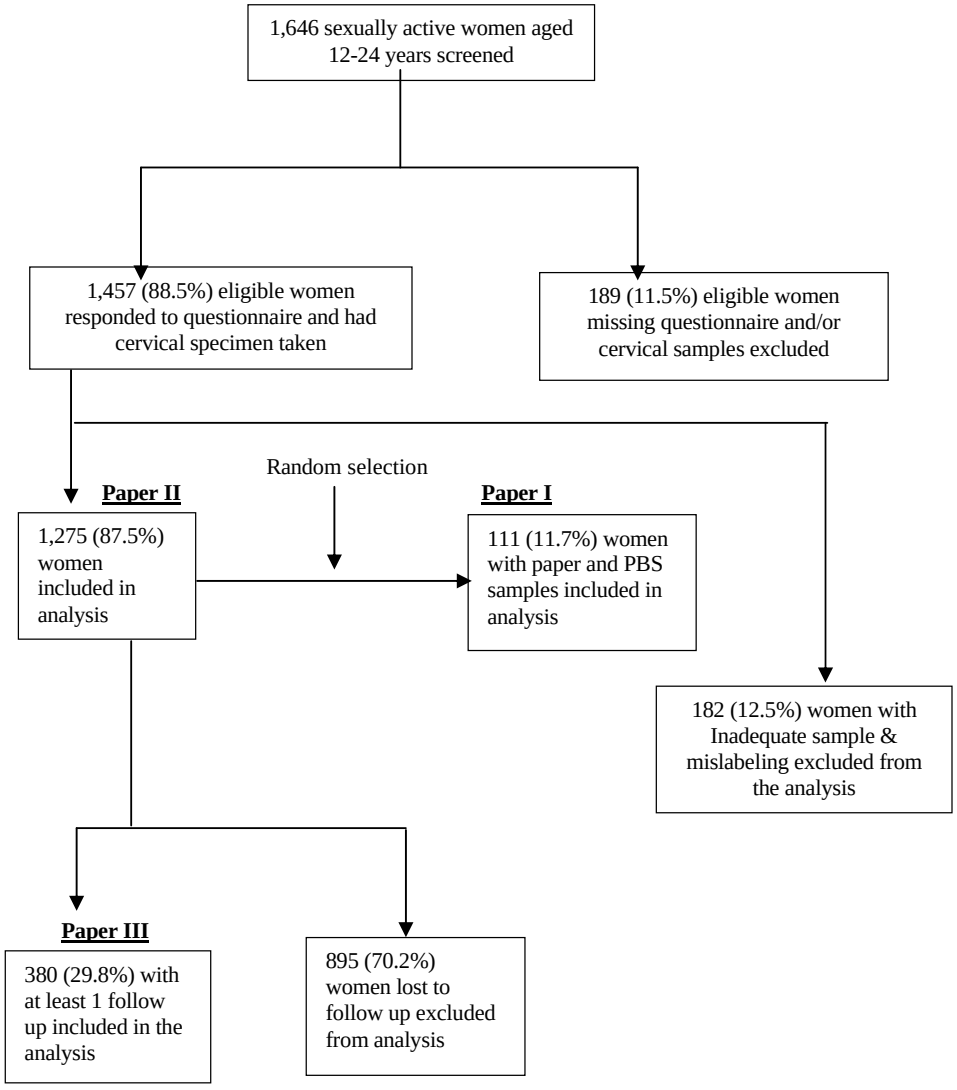

Figure 7: Selection of women who participated in Studies I, II and III reported in **Papers I, II, and III**.

We conducted two clinic-based prospective cohort studies. Figure 7 shows the selection of women for analysis of in **Papers I, II, and III**. The selection of women for analysis in **Paper IV** is shown in the published paper.

4.3 STUDY SUBJECTS AND SAMPLING METHODS

The young women reported in **Paper I, II** and **III** were selected from sexually active women seeking services at NTIHC and living within a 20 km radius of the centre. The studies were conducted in two phases. Between September and December 2002, we conducted a pilot study. The purpose of the pilot was to test our recruitment strategies, specimen collection, handling, transportation and storage before shipment to the Netherlands for HPV testing and genotyping. We used the lessons learned during the pilot to design and conduct the main study between February 2003 and December 2006. Some of the lessons learned were: (1) how to approach, screen and recruit the young women, (ii) who to employ as study staff, (iii) participant flow without long delays (iv) reasonable number of women to recruit per day, (v) how to fit the study into existing services offered by NTIHC without disrupting services.

The women reported in **Paper IV** were young primigravida aged 14-24 years selected from women seeking antenatal care at NHC and living within a 20 km radius of the health centre for the previous 6 months. The study was conducted between May 2004 and December 2006.

For each study, trained interviewers explained the study aims and procedures to potential participants as they waited to receive different services. All sexually active women aged between 10-24 years were eligible. Interested women were given detailed explanation about the study according to study protocols. After obtaining written informed consent, or assent, if they were minors, we consecutively recruited 1,646 women for studies whose results are reported in **Paper II** and 1,097 women for **Paper IV**. However, for **Paper I**, a random sample of 111 women was selected from the 951 HPV positive women whose results are presented in Paper II (Sample size calculation for all the studies is presented in Appendix 1). The women whose results are presented in **Paper III** comprised of women in Paper II followed up at least once.

4.4 DATA COLLECTION PROCEDURES

4.4.1 Questionnaires

We developed questionnaires to collect baseline and follow-up data based on published literature on HPV infections. The questionnaires were pre-coded and closed-ended and were pre-tested for content validity among young women attending a family planning clinic at Mulago Hospital. After pre-testing, the questionnaires were modified to incorporate the findings. The questionnaires were developed in English, which we subsequently translated into the common local dialect (Luganda). The translation process involved independent staff not working on our research team. We hired a staff conversant with the Luganda dialect and working with young people to translate the questionnaires from English to Luganda. Then we hired another independent medical worker conversant with the Luganda dialect to back-translate the Luganda version of the questionnaires into English. We then pre-tested the Luganda questionnaires among young women attending another "youth-friendly" primary care facility (Kawempe Adolescent Clinic) for content validity. After pre-testing, we made modifications accordingly.

Based on the lessons learned from the pilot study, we recruited, trained and used young women as interviewers. We conducted a 5-day training workshop for the interviewers and used the finalized English and Luganda questionnaires to standardize the understanding of the questions and the way questions would be asked. In study aims II, III and IV whose results are reported in **Papers II, III** and **IV**, we used the standardized interviewer administered closed-ended questionnaires at baseline and in subsequent follow-up visits to obtain detailed information on socio-demographic characteristics, cigarette smoking, use of illicit drugs, reproductive and menstrual factors, sexual behaviour, history of STDs of women and their partner(s) and use of contraceptive methods. Each interview lasted approximately 10 minutes.

4.4.2 Follow-up visits

We planned 3 follow up visits in study aim III and study aim IV, whose results are reported in **Paper III** and **Paper IV**, respectively. As reported in **Paper III**, the follow-up visits were scheduled between 6-12 months, 13-18 months, 19-24 months. However, 29 women who turned up after 24 months from baseline were not turned away, although this was beyond the visit window and therefore were unscheduled visit. According to the results reported in **Paper IV,** the follow-up visits depended on the

trimester of pregnancy at recruitment. The 425 women recruited in the 1st or 2nd trimester (< 26 weeks) were scheduled for one follow-up visit during the 3rd trimester of pregnancy (32-40 weeks of pregnancy) and another visit at least 6 weeks after delivery. The 562 women recruited in the 3rd trimester were scheduled for only one visit at least 6 weeks after delivery.

4.4.3 Collection of biological samples

In study aims I, II, III, and IV whose findings are reported in **Papers I, II, III** and **IV,** qualified midwives were re-trained in the performance of pelvic examinations and recording of abnormalities according to a standardized protocol [232]. From each woman in study aim I reported in **Paper I**, a sample was taken from the cervix by rotating (360º) a sterile cotton swab (Copan International, Brescia, Italy) thrice in the cervix, and smearing it within a 0.5-1.0 cm diameter on a labeled 3 MM Whatman™ filter paper (The Lab Depot, Inc., Dawsonville, GA) cut to the size of a small glass slide (5x2 cm). The cervical material remaining on the cotton swab was then placed in a labeled 15 ml tube holding 5 ml phosphate buffer saline (PBS, pH 7.2). Filter paper samples were air dried, placed into auto-seal (ziplock) plastic bags (International Plastics Inc.*)* and stored at room temperature (25-30º C) at Makerere Medical School, whereas PBS samples were stored at minus 20º C until shipment on dry ice to Delft's Diagnostic Laboratory (DDL), Vooburg, the Netherlands for DNA extraction and HPV analysis.

In **Paper II,** we reported that, after visual inspection of the vulva, a non-lubricated sterile speculum was inserted and cervical exfoliated cells were collected with a sterile cotton swab taken by rotating it thrice at 360 degrees in the cervix, which was then placed in a labeled 15 ml holding tubes with 5 mls of PBS (pH 7.2). Samples were temporarily kept at 4º C for an average of six hours and then transferred to a minus 20º-Centigrade freezer until shipment to DDL, Vooburg, the Netherlands for HPV DNA extraction and analysis. Before the speculum was removed, visual inspection with freshly prepared 5% acetic acid (VIA) and with Lugol's Iodine (VILI) was performed [233].

In **Paper III** and **IV**, we reported that, after visual inspection of the vulva during all visits, a non-lubricated sterile speculum was inserted, and cervical exfoliated cells were collected in a labeled vial containing ThinPrep PreservCyt (Cytyc Corp, Boxborough,

MA) preservation solution for liquid-based cytology and testing for HPV, *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG). To collect exfoliated cells from the endo- and ecto-cervix, the cytobrush (Medscand Medical AB, Malmo, Sweden) was inserted deep into the endocervical canal and rotated gently in a clockwise direction five times. The cytobrush containing cellular material was then placed in a vial containing PreservCyt solution (Cytyc Corp) and rinsed by pushing it into the bottom of the vial 10 times. The cytobrush was then discarded. The vial was closed tightly and kept temporarily at room temperature until shipment on dry ice to DDL, Vooburg, the Netherlands for DNA extraction and HPV analysis. Cervical abnormalities in liquid-based cytology were read at the Cytology Department of the Slotervaart Hospital, Amsterdam, the Netherlands, and classified according to the 2001 Bethesda Classification [67].

The women reported in **Papers I, II, III** and **IV** (after delivery) who had external genital warts were treated with 2% podophyllin paint. The treatment for genital warts was deferred for all pregnant women until after delivery. Women found with cervicitis and vaginal discharges received a one-week syndromic treatment including antibiotics, metronidazole, and a topical anti-fugal medication, in accordance with the National STD management protocols. The women were also asked to talk to their sexual partners and to come back for review after treatment. In **Paper II**, three women were suspected to have cervical cancer on VIA/VILI, and after biopsy, two were found to be histologically normal, whereas a histologically-confirmed stage IIIB cervical cancer was detected in the 3rd; a 23-year-old woman, who subsequently underwent palliative radiotherapy. Two women suspected to have cervical cancer and reported in **Paper IV** proved cancer free at liquid-based cytology (1 normal and 1 LSIL) and were neither biopsied nor treated.

A urine sample for pregnancy testing was collected from all the women and 4 ml of blood was collected in heparinized tubes from women who consented to HIV and syphilis testing after pre-test counseling as reported in **Papers II, III** and **IV**.

4.5 LABORATORY TESTS

4.5.1 DNA extraction and HPV analyses

In study aim I, whose findings are reported in **Paper I,** part of the filter paper was punched (~4 mm circle) and transferred to a 0.5 ml micro centrifuge tube containing

50µl distilled water. DNA was released by boiling it for 5 minutes. DNA was isolated from 200 µl of the suspension containing the cervical cells by the MagNa Pure Long Control (LC) instrument (Roche Applied Science, Indianapolis, IN) using the Total NA isolation kit from the PBS samples reported in **Papers I** and **II**, and exfoliated cell samples in PreservCyt solution (Cytyc) reported in **Papers III** and **IV**. Thereafter, DNA was eluted in 100 µl water and 10 µl was used for each PCR reaction reported in **Papers I, II, III,** and **IV**. Every PCR reaction included positive and negative controls to monitor the DNA isolation, PCR, HPV detection and genotyping procedures. Strict laboratory precautions and quality assurance/quality control measures were followed to avoid cross-contamination and carry-over PCR assay.

The short PCR fragment (SPF)10 primer set was used to amplify a broad spectrum of HPV genotypes, as described earlier [77, 81]. This primer set amplifies a fragment of 65 bps from the L1 region of HPV. Reverse primers contain a biotin labeled at the 5'end, enabling the capture of the reverse strand onto a streptavidin coated microtiter plate. Captured amplimers are denatured by alkaline treatment and the captured strand is detected by a defined cocktail of digoxigenin-labelled probes, detecting a broad spectrum of HPV genotypes. This method is designated HPV DNA enzyme immunoassay (DEIA) providing an optical density value.

The same SPF10 amplimers were used to identify the HPV type by reverse hybridization on a reverse hybridization line probe assay (LiPA), containing probes of 25 different HPV types including HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, and 74. Samples which were positive at (SPF)10 PCR primer reported in **Papers II**, **III** and **IV** but not revealing any of the afore-mentioned types, were provisionally classified as HPV X and subjected to a second reverse hybridization assay for 17 additional types (26, 30, 55, 61, 62, 64, 67, 69, 71, 82, 83, 84, 85, 87, 89, 90 and 91). In all studies, HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68/73 and 82 were considered high - risk types [66], and the rest were considered low-risk types.

4.5.2 Testing for HIV and Syphilis

HIV testing was performed at NTIHC using rapid tests following the National HIV Rapid Testing Algorithm consisting of Determine (Abbot Diagnostics Inc., Abbot Park, IL, USA) as the screening test, Statpak (ChemoBio Diagnostics Systems, Medford,

NY) as the confirmatory test and Unigold (Orgenics, Waltham, USA) as the tie-breaker [234]. For quality assurance/quality control measures, all HIV positive tests on rapid tests were re-confirmed by ELISA (Cambridge BioScience, Cambridge, UK) or PCR assays (Roche Molecular Systems, Pleasanton, CA) at Makerere Medical School. Women who were HIV positive were referred to health institutions offering treatment, care and support services.

Syphilis testing was routinely performed on all women who consented to HIV testing and at the request of the attending clinician using a commercially available standard Rapid Plasma Reagin (RPR) 18 mm card (Quorum Diagnostics, Sacramento, CA) test, following the manufacturer's instructions. Women with reactive RPR were referred to established laboratories for confirmation before treatment was offered to them and they were asked to talk to their partners.

4.5.3 Testing for Pregnancy

Pregnancy testing was offered to all the women who participated in the studies reported in **Papers I, II, III** and **Paper IV** (after delivery). The woman's urine was tested using a commercially available human chorion gonadotrophin dipstick pregnancy test (Cypress Diagnostics, Langdorpsesteenweg, Belgium) following the manufacturer's instructions, and the results were communicated to each woman immediately.

4.5.4 Testing for *Chlamydia Trachomatis* (CT) and *Neisseria Gonorrhea* (NG)

Isolated DNA for HPV analysis was also used for CT and NG testing in women reported in **Paper IV,** as described earlier [235, 236]. The CT detection and genotyping (Ct-DT) assay (Roche Diagnostics, Indianapolis, IN) was performed in the Netherlands according to the manufacturer's instructions. Briefly, the CT detection and genotyping assay is a multiplex broad-spectrum PCR for the criptic plasmid and the VD2-region of the *omp1* gene. Like HPV testing, CT-amplimers generated by PCR from both the criptic plasmid and the *smp*l amplimers are simultaneously detected in a DEIA test. The target of amplification of NG is the *cppB* gene 16, and the NG amplimers are then detected in a DEIA test.

5 STATISTICAL ANALYSES

5.1 Paper I

To compare the agreement between filter paper and PBS samples for detection of the overall HPV positivity, and the presence of each individual HPV type and multiple-type infections from the same woman, we calculated the kappa statistic (κ). The kappa statistic is a measure of the percent agreement between two rates that occur beyond chance, which ranges between 1 when there is complete agreement and 0 if the agreement is less than or equal to chance agreement. Thus the range for kappa > 0.75 is interpreted as excellent agreement, 0.40 to 0.75 as fair to good and <0.4 as poor agreement.

5.2 Paper II

We computed odds ratios (ORs) and corresponding 95% confidence intervals (CIs) by different women's characteristics using unconditional, multiple logistic regression models, considering HPV infection as the dependent variable in STATA version 9.0 (StataCorp, Texas). We used unconditional, multiple logistic regression models to adjust for age, HIV status and lifetime number of sexual partners. To test for trends in the prevalence of HPV with age, age at first intercourse, and lifetime number of sexual partners, we assigned an ordinal score to grouped values and then treated this score as continuous in the logistic regression model.

We took advantage of the large sample size of women to estimate the proportion of young women who had probably been infected by HPV 16 or 18 in single years, of age between 16 ≤ and ≥ 21 years, we: 1) computed the age-specific prevalence of HPV 16 or 18; and 2) estimated the cumulative incidence of the same types assuming a 1-year persistence of 30% [237]. For instance, the cumulative incidence of HPV 16 at any year of age was computed by adding the prevalence of that HPV type in the year of age in question, and in the previous year, minus the fraction of infections that we assumed to be carried over from one year to the next (i.e., 30%).

5.3 Paper III

We analyzed incidence and clearance of HPV infection for all women who had at least one follow-up visit. An HPV type was defined as a combined outcome if any HPV - type was present. We defined HPV 16 - related types as HPV 16, 31, 33, 35,

52, and 58 and HPV 18 - related types as HPV 18, 39, 45, 59 and 68. Single and multiple infections were defined without considering uncharacterized HPV types (HPV X). We computed relative risk (RR) for HPV incidence and clearance using a Poisson regression dividing the follow up time in 6 - month windows for risk time and events.

For incidence analysis, the person-time at risk was measured for each specific HPV type or combined set of HPV types from the first time a woman tested negative until a positive test or the last visit.

A woman was considered completely cleared of infection if all the HPV types she had at baseline cleared during the follow-up period. For type-specific clearance analysis, the person-time at risk was measured for each specific type or combined set of types among HPV positive women until the first negative test or the last visit. For each HPV type or group of HPV types, each woman was counted only once in order to avoid problems of correlated data having several measurements for each woman, which resulted in a loss of only 3 types for 3 different women, and thus was unproblematic.

Women positive for HPV X at baseline who tested positive for a known HPV type at follow-up visit were considered to have developed a new infection in the analysis of incidence but were excluded from the analysis of clearance as the persistence of HPV X could not be known. For each HPV-type, we fitted Poisson models including a time covariate only and models adjusting for HIV status, age at baseline (12 - 17, 18 - 20, 21 - 24 year), positivity for genital warts or syphilis (yes/no), and lifetime number of sexual partners (1, 2, 3, $\geq$4).

We evaluated prevalent cytological abnormalities only on follow-up visits, as the medium used to collect baseline specimens was not suitable for cytological analysis. Subjects were classified as normal or low - grade squamous intraepithelial lesions (LSILs) as no high-grade squamous intraepithelial lesions or as invasive cervical cancer cases were diagnosed during follow-up. We also evaluated incident cytological abnormalities according to type-specific HPV infections at follow-up (HPV 16 infected vs. non-infected, HPV 18 infected vs. non-infected, High-risk infected vs. non-infected, Low-risk infected vs. non-infected, HPV 16-related types

infected vs. non-infected, HPV 18-related vs. non-infected and infection with any HPV type vs. non-infected) among women with a baseline HPV negative sample with a valid follow-up cytological sample.

All tests of statistical hypotheses were based on a two-sided 5% level of significance with corresponding 95% confidence intervals. All analyses were made using the SAS software version 9.2 (SAS Institute, Inc.). Procedure genmod was used to fit the Poisson regression.

5.4 Paper IV

We computed odds ratios (ORs) and corresponding 95% confidence intervals (CIs) by different women's characteristics using unconditional, multiple logistic regression models, considering HPV infection as the dependent variable in STATA software (version 9.0; STATA Corp.). We used unconditional, multiple logistic regression models to adjust for age, HIV status and lifetime number of sexual partners. To test for trends in the prevalence of HPV with trimester of pregnancy at recruitment, age, education, and age at first intercourse, we assigned an ordinal score to grouped values and then treated this score as continuous in the logistic regression model.

We then evaluated the incidence and clearance of HPV between the $1^{st}/2^{nd}$ and 3^{rd} trimesters of pregnancy, and between the earliest visit during pregnancy and the visit after delivery. In a few instances where more than one follow-up visit after delivery was available from the same woman, we chose the earliest one. The incidence was calculated for any HPV type that had not been detected at the baseline visit using women as a unit of analysis. Conversely, we used infections as the denominator of the clearance because of our interest in clearance of individual HPV types. Women positive for HPV X at recruitment who tested positive for a known HPV type at follow-up visit were considered to have a new infection in the analyses of incidence, but were excluded from the analyses of clearance as the persistence of HPV X types could not be known. The association between selected baseline characteristics and the incidence and clearance of HPV was evaluated in all women who had at least one follow-up visit, preferentially choosing the visit after delivery. The incidence and clearance of cytological abnormalities was evaluated before and after delivery.

6 ETHICAL CONSIDERATION

All our studies included young women who were minors (under 18 years according to the Ugandan laws), and therefore we had several ethical issues to address. Following the National Guidelines for Conducting Research among Human Subjects [238], the women in our study were treated as emancipated minors. Thus, we did not seek consent from their parents or guardians for them to participate in our studies. If our studies had excluded minors, it would have reduced the value of information obtained, as sexual activity starts early in Uganda [198, 239].

All study participants signed an informed consent form, or assent form for minors, according to the recommendations of the ethical review committee of the Higher Degrees Research and Ethics Committee of the Faculty of Medicine of Makerere University, the Uganda National Council of Science and Technology Committee on Study on Human Subjects and the Institution Review Board of the International Agency for Research on Cancer.

We obtained the necessary administrative approvals from the Kampala City Council and the management of the clinics where the studies were conducted.

In accordance with the principle of justice, we used a hierarchy of risks and benefits to define the specific protection required for the young women who participated in our studies. We knew that the principle threats to the health and well-being of young people in general, and young women in particular, are social and behavioral at the personal level and from peers. Thus, the information provided to the young women throughout the conduct of our studies emphasized and promoted protective behaviors and attempted to reduce harmful behaviors. We think that the questionnaire information was likely to increase self-understanding of the risk from the woman's own behavior, and by raising such understanding; our studies may have facilitated the process of seeking care. Involvement in the informed consent or assent process may have increased the sense of self-control, as well as the decision-making capacity of the women. We believed that potential embarrassment and disclosure of sensitive information such as sexual and other behaviors by study staff were the likely risks in our studies. However, disclosure resulting from research settings is a rare phenomenon. Whenever we encountered women who had been sexually abused or

who were using illicit drugs, we disclosed this information with permission from the woman to the management of NTIHC for follow-up.

Pelvic examination was a procedure that could have caused anxiety, some discomfort, and in some cases, distress to the young women. To reduce their anxiety, trained midwives explained procedure in detail to the women to reassure them that the examination was not harmful.

Inevitably, the process of HIV testing would have been very stressful to the young women, irrespective of the subsequent test result. However, we followed local protocols approved by the Ministry of Health, which included trained counselors offering pre- and post-test counseling, as well as condom education and distribution. Regrettably, at the beginning of our studies, free anti-retroviral therapy (ART) services were offered in only a few institutions. Before starting therapy, the woman was required to have laboratory results of her immunological status (CD4+ T-lymphocytes) and HIV viral load, the costs of which were out of reach of most of the women who participated in our studies. Access to ART services only improved towards the end of our studies. Consequently, we referred HIV positive women to institutions offering care and support services for continued management, but later, to institutions where they could access ART services. On the other hand, all HIV positive pregnant women were referred to programs that offered services for HIV prevention of mother-to-child transmission, and we encouraged all women to come with their partners for voluntary counseling and testing for HIV as couples. We ensured that women with STDs received appropriate treatment based on the national STD treatment protocols and also encouraged them to talk to their partners about STD treatment, whenever it was necessary.

Participation in our studies was voluntary. We only reimbursed the women their transportation costs based on locally acceptable rates.

7 SUMMARY AND DISCUSSION OF RESULTS

7.1 PAPER I

We found an overall prevalence of 82.9% for any type of HPV in PBS compared to 32.4% in paper samples and only 32.4% were positive in both. Comparing the agreement of 22 different resulted in a *kappa* statistic=0.18.). Twenty-two different HPV types were detected in 225 infections in PBS samples, but only 18 types were found in 68 infections in paper samples. The most frequently detected high-risk types were HPV 52 (22.5% in PBS, 9.0% in paper and 7.2% in both samples), HPV 18 (18.9% in PBS, 2.7% in paper and 0.9% in both samples) and HPV 16 (16.2% in PBS, 4.5% in paper and 2.7% in both samples). The most frequently detected low-risk type was HPV 6 (20.7% in PBS, 9.0% in paper and 7.2% in both samples). There was perfect agreement between filter paper and PBS samples for HR-HPV 39 and LR-HPV types 43, 44 and 40, which were absent in both samples. Most *kappa* statistic for the detection of individual HPV types fell between 0.2 and 0.4, and hence suggested relatively poor agreement. Multiple-type infections were detected in 54.1% of PBS samples compared to 15.3% of paper samples and only 12.6% in both. Infections with $\geq$ 4 HPV types was also relatively common (18.0%) in PBS samples, but not in paper samples (2.7%).

Our results differ from those of the only published study conducted by researchers in India [240], whose results seemed much more favorable to the filter paper samples. The disagreement between our study and theirs could have resulted from differences in viral load of cells and HPV DNA obtained in the two studies. In their study, all the women had cervical dysplasia or carcinoma, whereas we included in our study young women who were free of cervical dysplasias at visual inspection with acetic acid and on account of their age, were unlikely to harbor cervical carcinoma. Furthermore, while Indian researchers tested for HPV 16 only, we tested for 25 HPV-types, using a highly sensitive and well-validated PCR-based assay [83]. On the contrary, DNA extraction was performed in a similar way in both studies. The likely step where variation between their study and ours could have arisen was smearing the swab on filter paper because it is difficult to control the spot size. As a result, it would not be known how many cells were placed on the filter paper, and how representative the smeared sample was of the entire cervical was sample.

7.2 PAPER II

One thousand four hundred and fifty seven of 1,646 (88.5%) eligible women were interviewed and subsequently underwent pelvic examination and collection of exfoliated cervical cells. One hundred and eighty two samples were inadequate as a result of contamination during transportation or because of mislabeling, leaving 1,275 (87.5%) women available for analysis. The median age was 20 years (range 12–24 years). Seventy seven percent (77.0%) of the women had never been married but 47.0% had been pregnant. Seventy seven percent (77.0%) of the women reported at least secondary education. Eighty one percent (81.0%) of women were Christians and 19.0% Muslims. Only 2.5% of the women reported having smoked cigarettes and 6.2% had accepted money or gift in exchange for sexual intercourse.

Nine hundred and fifty of 1,275 (75.0%) women agreed to be tested for HIV; of these women, 82 (8.6%) were HIV positive. Only 467 women were screened for syphilis, because the test kit was unavailable during certain periods of the study. Eighteen women (3.9%) were positive for syphilis.

We found an overall HPV prevalence of 74.6% for one HPV type or more. HR types were found slightly more often (51.4%) than LR types (39.8%). Among HR types, HPV 52 (13.2%), 51 (12.3%), 18 (10.7%), and 16 (10.6%) were the most frequently detected, whereas, among LR types, HPV 6 (15.5%) and 11 (13.3%) predominated. Ninety-five women who were HPV X-positive at the first reverse hybridization assay were retested for 14 additional types, and 57 could be assigned to a specific type or more, most frequently HPV 30 (9 women), HPV 61 (9 women), and HPV 67 (10 women). At the end of the two testing rounds, only 3.0% of all the women, and 0% HIV-positive women, could be assigned any HPV type, and 521 (54.8%) of the HPV positive women had multiple type infections.

A comparison of the prevalence of different HPV types by HIV status was possible for 950 women whose HIV status was known. Study participants who were HIV-positive showed a higher HPV prevalence (87.8%) than HIV-negative ones (73.2%). The largest difference was found in respect to multiple-type infections, which were detected in 37.3% of the HIV-negative and 64.6% of the HIV-positive women. The mean number of HPV types detected in the HPV-positive women without HIV infection was 2.1 (range 1-10); in the women with HIV infection, the mean number of types detected

was 2.8 (range, 1-9) [*t* test, 3.88; P < .001]. Among the HIV-negative women, multiple-type infections most frequently detected included the combination of HPV-16 or-18 and other HR types (13.8% of the women), whereas combinations of HR types that did not include HPV 16 or 18 predominated (22.0%) among the HIV-positive women. Small percentages of women were affected only by HPV 16 and/or 18 or by LR types (2.5% among the HIV-negative and 1.2% among the HIV-positive, respectively). Only 3.8% of the HIV-negative and 6.1% of the HIV-positive women had only LR-HPV types.

HPV 16 represented 7.2% of all infections in the HIV-negative women and 7.5% of all infections in the HIV-positive women. The distribution of other HPV types was similar in the 2 groups of women with the exception of HPV 18, which was underrepresented among the HIV-positive women ($\chi 2 = 4.15$; P = 0.42).

After adjustment for HIV positivity, age group and lifetime number of sexual partners, a significant trend of a decreasing HPV infection prevalence with increasing age emerged (P < .004). HPV positivity was significantly associated with HIV positivity (OR = 2.4, 95% CI = 1.2-4.8), employment in a tertiary sector (OR = 1.6, 95% CI = 1.1-2.2), lifetime number of sexual partners (OR for ≥4 vs. 1 partner = 2.2, 95% CI = 1.5-3.2), a positive pregnancy test (OR =1.7, 95% CI = 1.1-2.7) and detection of genital warts at study visit (OR =2.0, 95% CI = 1.1-3.8) but not to age at first sexual intercourse (OR for < 15 years vs. 18-24 years = 1.0, 95% CI = 0.7-1.5 and OR for 16–17 years vs. 18-24 years = 1.0, 95% CI = 0.7-1.4). The association was much stronger for multiple-type (OR=3.9, 95% CI = 1.9-7.9) than for single–type infection (OR=1.8, 95% CI = 0.8-3.9) as shown in Table 2.

Table 2: Risk of HPV-infection comparing HIV negative and HIV positive women, odds-ratios (OR) and associated two-sided 95% confidence intervals and p-value and chi-square statistics for the test of OR=1.

HPV types	HIV-	HIV+	Chi^2	P-value	OR (95% CI)
HPV 16	93 (10.7)	15 (18.3)	4.3	0.04	1.8 (1.0 -3.3)
HPV 18	105 (12.1)	8 (9.8)	0.4	0.53	0.7 (0.3 – 1.5)
HPV 33	83 (9.6)	16 (19.5)	7.9	0.005	2.1 (1.2 – 3.9)
HPV 51	92 (10.6)	15 (18.3)	4.4	0.035	1.8 (1.0 – 3.3)
HPV 52	105 (12.1)	17 (20.7)	5.0	0.025	1.8 (1.0 – 3.3)
Other HR	255 (29.4)	33 (40.2)	4.2	0.04	1.5 (0.9 – 2.4)
HPV 6	133 (15.3)	13 (15.9)	0.02	0.90	1.1 (0.6 – 2.1)
HPV 11	110 (12.7)	14 (17.1)	1.3	0.26	1.5 (0.8 – 2.8)
Other LR	213 (24.5)	35 (42.7)	12.8	<0.001	2.4 (1.5 – 3.9)
Single Infections	272 (31.3)	19 (23.2)	2.4	0.13	1.8 (0.8 – 3.9)
Multiple Infections	336 (38.7)	53 (64.6)	20.8	<0.001	3.9 (1.9 – 7.9)

Religion, education level, smoking status, marital status, parity, number of sexual partners in the last year, acceptance of money or gifts in exchange for sexual intercourse, use of hormonal contraceptives or male condoms, and history of sexually transmitted diseases were not statistically significantly associated with HPV positivity.

An estimate of cumulative incidence for infection with HPV 16 and HPV 18 showed that for each type, in no single age did the prevalence of infection exceed 15%. The cumulative incidence by age 18 was 31.9% for HPV 16, 27.0% for HPV 18, and rose approximately 50% for each type by age 21.

To our knowledge, our study was the largest study ever conducted among young women in Africa. Our results show that 3 out of 4 women were HPV-positive, and 51.4% harbored HR-HPV types. Multiple-type infections were more frequent than single type infections. Other high-risk types did not often accompany HPV 16 or 18. An HPV infection prevalence of higher than 70% had only been reported in studies of HIV-positive women [241], yet only 8.6% the women in our study were HIV positive. Several studies from Sub-Saharan Africa in general [242, 243] and the East African region in particular [147, 187, 244], had previously reported HPV prevalence rates ranging between 25% and 40%, which were much lower than our findings. The HPV prevalence rate in our study also differed from the only previously published survey conducted in one of the rural district of Uganda whereHC2 was used to detect HR-HPV

infections [226]. In this study, the prevalence of infections with HR-HPV among women aged 15-29 years was 15.4% in 324 HIV-negative and 52% among 71 HIV-positive women, which was much lower than in our study, despite the higher prevalence of HIV infection (18%) than in our clinic-based sample in Kampala. It is worth noting, however, that we used a highly sensitive PCR assay (SPF10/LiPA), which has a much higher sensitivity than the HC2 used in their study [13]. Furthermore, the differences in study designs particularly the age range of the women in our study compared to theirs, might be responsible for the observed variations. Given that HPV infections tend to be acquired very early after sexual intercourse, perhaps because of its high transmissibility [116], previous studies have shown a very high prevalence of HPV infections in the years following the initiation of sexual intercourse [192, 237, 245]. Like in many different populations around the world, we found an inverse association between the prevalence of HPV infection and age even in our very young population of women, which is in keeping with previous studies [8] and also in keeping with the concept of rapid acquisition of HPV infections.

The strongest risk factors for HPV positivity were the self-reported lifetime number of sexual partners, HIV positivity, the presence of genital warts, and pregnancy. However, reported condom use by male partners was not associated with HPV positivity as consistent use was rarely reported by the women in our studies, though it has been suggested that condoms confer partial protection against HPV transmission [158].

In keeping with published studies, HPV 52 was the most common HR- type [8], and HPV 6 and 11 were the most common LR-HPV types. Other frequent HR-HPV types detected included HPV 16 and 18 and 51. Among HR-HPV types, HPV 18, 51, and 52 were detected slightly more frequently than HPV 16, as were LR-HPV types 6 and 11. The proportion of HPV infections due to HPV 16 seemed to be lower among young women in Uganda compared with populations outside Sub-Saharan Africa, in keeping with previous work on HPV distribution in different populations [8, 62]. All HPV-types, except for HPV 18, were detected much more in HIV-positive than HIV-negative women. The most striking difference we found was a much higher frequency of multiple type HPV infections among the HIV-positive compared to the HIV-negative women, which was similar to the results of a large meta analysis [241].

Furthermore, in our study, a larger proportion of infection with multiple HR-HPV types in the HIV-positive women included neither HPV 16 nor 18.

Our estimates of cumulative incidence from prevalence data suggest that by approximately age 18, about 70% are not yet infected with HPV 16 or 18, the most oncogenic HPV types. Furthermore, our estimates of cumulative incidence were relatively robust to the assumption of 12-month persistence in the 10%-40% range shown by different cohort studies [211, 246].

7.3 PAPER III

Of the 1,275 women enrolled in the study at baseline, only 380 (29.8%) women had at least one follow-up visit. Compared to women with adequate follow-up, the women with inadequate follow-up were slightly younger at study entry (>18 years 85.5% vs. 90.3 %), less likely to have genital warts (7.6% vs. 8.2%), less likely to be pregnant at time of examination (11.8% vs. 15.0%) but more likely to be HIV positive (6.2 vs. 5.8%) and more likely to have initiated sex before 15 years (15.0% vs.10%). There was no difference at baseline between women who were adequately followed up and those who were not with respect to HPV positivity (74.6% vs. 74.7%) and the number of lifetime sexual partners (≥ 2 or more; 74.8% versus 74.4%). The median time to the 1st follow up visit was 18.5 months, with an inter-quartile range of 9.7 to 26.6 months.

The overall prevalence with any HPV type at baseline of 380 women followed up at least once was 75.3% with cumulative positivity (i.e. for women who were followed more than once) of 86.6%. Except for HPV 34, all the common high- and low-risk types were detected. The 5 most common high-risk types cumulatively detected in descending order were HPV 51 (n = 76, 20.0%), HPV 52 (n = 71, 18.7%), HPV 16 (n = 58, 15.3%), HPV 18 (n = 56, 14.7%) and HPV 33 (n = 52, 13.7%). Of the most common low-risk types, HPV 6 (n=94, 24.7%), HPV 11 (n = 62, 16.3%) and HPV 66 (n = 35, 9.2%) were frequently detected. Cumulatively, high-risk HPV types (68.2%) were more frequently detected than low-risk types (55.0%). Single infections (55.3%) were slightly more common than multiple infections (47.6%) as were HPV 16 related-types (48.6%) compared to HPV 18-related types (28.2%). Cumulatively, 75 (19.7%) infections were uncharacterized (HPV X) after the first round of testing and went through a second round of testing for an additional 17 types. Thirty-three (33) women

could be assigned one or more specific types after the second round of testing, leaving 42 (11.1%) women as HPV X.

Sixty-nine women had an incident HPV infection during 226 person-years of observation, reflecting an incidence density of 30.5 per 100 person-years. Type - specific incidence rates ranged between 0.8 and 4.2 per 100 person-years of observation for HR-HPV types and between 1.1 and 3.4 per 100 person-years for common low-risk types. Incident HR-HPV types (20.9 per 100 person-years) were more frequent than low - risk HPV types (10.6 per 100 person-years). Incident infections with HPV 16 - related were twice as likely as HPV 18 - related types (10.8 and 5.6 per 100 person-years, respectively). Incident infections not statistically significantly associated with HIV, age at study entry or lifetime numbers of sexual partners.

Overall, 143 women with prevalent infections cleared their infections during 526 person-years of observation, reflecting complete clearance for any HPV type of 27.2 per 100 person-years. Clearance for specific types ranged between 42.3 and 100.0 per 100 person-years for HR and 50 and 100.0 per 100 person-years for LR-HPV types. Women with HR-HPV types (42.2 per 100 person-years) cleared their HPV infections as much as those with LR-HPV types (47.3 per 100 person-years). However, women with HPV-16-related types (51.0 per 100 person-years) cleared their infections less than those with HPV 18-related types (65.5 per 100 person-years). HIV-negative women cleared their HPV infections more than HIV-positive women (RR = 0.3, 95% CI = 0.1–0.8) and the difference was statistically significant. However, HPV clearance was not associated with age at study entry, lifetime number of sexual partners, and multiplicity of infections.

Incident HPV infections were common in both HIV-positive (rates between 2.2 and 15.4 per 100 person-years) and HIV-negative women (rates between 0.8 and 4.2 per 100 person-years). Compared to HIV-negative women, the risk for incident infections was elevated for LR-HPV types (RR = 3.3, 95% CI = 1.4–7.8) and HPV 16-related types (RR = 3.0, 95% CI = 1.3–6.9) in HIV-positive women and the difference was statistically significant. There was no difference in risk for HPV 18-related types (RR = 1.8, 95% CI = 0.5–6.2); single infections (RR = 1.1, 95% CI = 0.5–2.3), multiple infections (RR = 2.2, 95% CI = 0.6–7.6) and HPV–other X (RR = 0.9, 95% CI = 0.5–1.8) among HIV-positive compared to HIV-negative women. Clearance was

statistically significantly less for any HPV and multiple infections among HIV positive women compared to HIV negative women (adjusted clearance = 0.2, 95% CI = 0.1–0.7). There was no statistically significant difference in clearance of any high- or low-risk HPV types, HPV 16 - related, HPV 18 - related and single infections between HIV positive and negative women.

Three hundred seventy-eight 378/380 (99.5%) women with at least one follow up visit provided a sample for liquid-based cytology. Twelve samples were inadequate, leaving 365 samples for cytological analysis; of these 365 samples, 53 (14.5%) had LSILs. None of the women had HSIL or cancer. One hundred and seventy-three (173) women provided at least 2 samples for cytologic analysis of which, 5 and 3 samples were inadequate at the 1st and 2nd follow-up visits, leaving 168 and 170 samples, respectively, available for cytological analysis. The prevalence of LSILs at the 1st cytologic sample was 15/168 (8.9%) and declined to 9/170 (5.3%) at the 2nd visit. Evaluation of incident LSIL was possible for 150/155 (96.8%) women who were HPV DNA negative at baseline. Twenty-two (22) of 150 (14.7%) developed LSILs between baseline and the 1st follow up. Incident LSIL was not statistically significant different for HPV 18 infected women compared with women not infected with HPV 18.

The observed high incidence rate of HPV infections among the young women in our study is similar to findings described in previous studies [102, 247, 248]. A woman's risk for incident infection was defined by her HIV serostatus. Indeed, HIV-positive women were twice as likely as HIV-negative women to have incident infection with high- or low-risk HPV types in agreement with published studies. The difference in risk for incident HPV 16 and HPV18 infections was not statistically significant comparing HIV-positive and HIV-negative women. Our results differ from those of a population-based study in a rural district of Uganda, which found a 3fold-elevated risk for HPV 16 and HPV 18 among HIV positive compared to HIV negative women [229]. The study population in their study consisted of both young and old women aged 15 - 55 years compared to our young women aged 12-24 at baseline could explain the difference between our findings and theirs. However, the limited sample size in our study could have limited our ability to detect a difference. Despite the limited age range of the women in our study, we found a higher incidence rate for HR- than for LR-HPV types, similar to studies with a wide age ranges [241].

We observed higher rates of clearance of both high- and low-risk HPV types, which is consistent with transience. Our findings differ from studies conducted among adult populations, which found more 12-month clearance for low-risk types than for high-risk types but no clear difference between infections with high-risk types other than HPV 16 [249]. The different sensitivity of HPV DNA detection assays used in other studies compared to ours and the age of the population of women studied might explain the observed differences. The women's characteristics associated with clearance of HPV infections is not consistent across studies [250]. However, women infected with HIV consistently clear their infection less than HIV negative women [175, 229], similar to what we found in our study. Age at study entry, lifetime number of sexual partners, and multiplicity of infections at the beginning of follow-up were not associated with clearance. Type-specific clearance was high though only 27.2% of the women had complete clearance of all their HPV infections by the end of approximately 27 months of follow up. However, considering the median time to the first follow-up in our study was 18.5 months, and this period was probably long enough for a woman to clear an infection and get re-infected with the a different or even the same HPV type. Thus, it is possible that our observed clearance was likely to be of subsequent incident infections rather than the initial prevalent infections.

Despite the high incidence of HPV infections, the incidence of LSIL was relatively low (14.5%), which suggests that many young women who were infected with HPV had no cellular changes. In a study of 496 adolescents and young women, Moscicki *et al*. (2001) [95] found that only 25% of those who acquired HPV developed LSILs. It is believed that LSIL is merely a manifestation of HPV infection. In our study, LSIL seemed to be more frequent among women infected with HPV-18 related types than those not infected with those types. The decline in prevalent LSIL from 8.9% to 5.3% between 2 visits underscores the transient nature of HPV infection and associated cervical lesions among young women.

7.4 PAPER IV

One thousand ninety-seven primigravidae accepted to participate, but essential information (e.g. age) was missing for 15, and exfoliated cervical cell samples were missing or inadequate in 34 and 61 women, respectively. Thus, 987 women were included in the baseline analysis. Of these, 985 women accepted to be tested for HIV, and 72 (7.3%) were HIV-positive. The median age was 19 (range, 14–24 years).

Overall, 60% of our study participants were positive for one HPV type or more. HR types were found more often (43.0%) than LR types (23.0%). Among HR types, HPV 52 (12.1%), 51 (8.7%), and 16 (8.4%) were the most frequently detected, whereas, among LR types HPV 66 (6.5%) and 6 (5.5%) predominated. One hundred and one women (10.2%) had samples that were HPV X-positive at the first reverse hybridization assay, which went through a second round of testing for 17 additional types. After this second round, 55 women could be assigned to a specific type or more, most frequently HPV 61 (12 women) and HPV 55 (9 women), whereas 4.7% of all women (1.4% of HIV-positive women) could not be assigned any HPV type.

Study participants who were HIV-positive showed a higher HPV prevalence (72.2%) than HIV-negative ones (58.9%) (P = 0.027) and the largest difference was found with respect to multiple infections, which were detected in 24.5% of HIV-negative and 45.8% of HIV-positive women (P < 0.001). The mean number of HPV types detected among HPV-positive women was higher among HIV-positive women (2.4, range 1-10) than among HIV-negative women (1.8, range 1-12) (Student's *t*-test = 2.79, $p = 0.005$).

After adjustment for age group, there was a tendency of HPV positivity to decline with age, but the trend was not statistically significant. HPV positivity was statistically significantly associated with HIV positivity (OR = 1.8, 95% CI = 1.1-3.1), cytological abnormalities ([OR = 3.8, 95% CI = 2.4-5.9] and [OR = 3.5, 95% CI = 1.6-7.7] for the presence of atypical squamous cells of undetermined significance [ASCUS] and LSIL, respectively), and young age at first sexual intercourse (OR for ≤ 15 versus 18-24 = 1.6, 95% CI = 1.1-2.4). In addition, a higher HPV prevalence was detected among women who presented with genital warts (OR = 3.1, 95% CI = 1.6-6.1) and *Chlamydia trachomatis* [CT] (OR = 3.1, 95% CI = 1.5–6.2). However, there was no statistically significant difference in HPV prevalence among women who tested positive for syphilis (OR = 2.4, 95% CI = 1.0–5.5) and *Neisseria Gonorrhea* [NG] (OR = 2.7, 95% CI = 1.0–7.2). HPV positivity was not associated with the trimester of pregnancy when the baseline visit took place, educational level, marital status, and multiple sexual partners in the last year. When the risk factors for positivity for HR- and LR-HPV types were assessed separately, no significant heterogeneity was found.

Of 425 women recruited in the 1st/2nd trimester of pregnancy, 105 women had a follow-up visit and adequate cervical cell samples during the 3rd trimester and hence, contributed to the comparison of the HPV prevalence between the 1st/2nd and 3rd trimester (mean interval between visits: 16 weeks). Of 562 women recruited during the 3rd trimester of pregnancy, 149 women had a follow-up visit and adequate cervical samples after delivery. After the addition of 140 women recruited during the 1st/2nd trimester of pregnancy, 289 women contributed to the comparison of the HPV status between pregnancy and after delivery (mean interval between visits: 48 weeks). Overall, 334 women had at least one follow-up visit and an adequate sample either during pregnancy (45) or after delivery (289).

Women who were not adequately followed up were younger (OR for 14-18 versus 21-24 years = 1.9, 95% CI = 1.3–2.7) and had a lower educational level (OR for illiterate or primary versus secondary or more = 1.4, 95% CI = 1.1-1.9) than women adequately followed-up. Conversely, women with and without adequate follow-up did not differ significantly by any other characteristics, including positivity for HPV (OR = 1.2, 95% CI = 0.9-1.5), HIV (OR = 1.0, 95% CI = 0.6-1.6) and other sexually transmitted infections (Syphilis, OR = 1.5, 95% CI = 0.6-3.3; CT, OR = 1.2, 95% CI = 0.6-2.1; NG, OR = 1.0, 95% CI = 0.4-2.3).

Overall, 42.9% and 38.1% of the women respectively, acquired new HR and LR HPV infection(s) during pregnancy and after delivery. Similar to the prevalence, the incidence of HR types was greater than the incidence for LR types. Consequently, the net effect of new infection(s) and clearance showed that the prevalence of HPV16/18, high- and low-risk types, and all HPV types between the 1st/2nd and 3rd trimesters, and between pregnancy and after delivery, did not change significantly. Fifty percent of individual HPV infections cleared between the 1st/2nd and 3rd trimesters, and 71.8% cleared between pregnancy and after delivery.

The comparison of liquid-based cytological findings during pregnancy and after delivery was possible among 217 women. Of the 171 women cytologically normal at baseline, 9.4% (95%CI = 5.4%-14.7%) developed ASCUS or LSIL, whereas 46 women who had ASCUS or LSIL, 76.1% (95% CI = 61.2%-87.4%) became cytologically normal. Thus, the prevalence of ASCUS/LSIL decreased from 21.2% before to 12.4% after delivery ($\chi 2 = 5.95$; $p = 0.015$).

The incidence of HPV infection did not vary with the time interval between visits and the number of sexual partners in the interval between visits, but was related inversely and significantly with age (OR for 21-24 versus 14-18 years = 0.3, 95% CI = 0.1-0.5). Cytological abnormalities (OR = 1.9, 95% CI = 1.1-3.4) and the presence of genital warts, CT or NG (OR = 2.1; 95% CI = 1.1-4.1), but not of HIV infection (OR = 1.5, 95% CI = 0.6-3.4) were significantly associated with increased HPV incidence.

A long interval between two visits (OR = ≥12 versus ≤5 months = 7.8, 95% CI = 3.8-16.2), the presence of HIV infection (OR=0.5, 95% CI = 0.3-0.9), and two or more sexual partners in the interval between visits (OR = 0.1, 95% CI = 0.0–0.7) were significantly associated with HPV clearance. Clearance was not statistically significantly differently less frequent for HPV 16/18 (OR = 0.6, 95% CI = 0.3-1.3) and other HR-HPV types (OR = 0.6, 95% CI = 0.4–1.1) than for LR types. One woman seroconverted to HIV between the $1^{st}/2^{nd}$ and 3^{rd} trimester and 5 seroconverted between pregnancy and delivery.

Our large study shows a high prevalence of HPV infections (60%) among the young primigravidae; 7.3% of whom were HIV positive. In over two-thirds of HPV-positive women, high-risk types were involved, most notably HPV 16, 51 and 52, and multiple type infections were common (43.6% of HPV infections). However, itisimportant to note that the HPV prevalence at baseline was similar in women who were seen in different periods of pregnancy. In a similar study conducted about the same time among equally young nonpregnant women attending a teenage clinic in the same health centre in Kampala [224], the prevalence of HPV (74.6%) and HIV (8.6%) were slightly higher than among the women included in this study.

The aim of the follow-up in our study was to assess changes in HPV prevalence between early and late pregnancy and after delivery. High parity, young age at first birth [251] and the long-term use of oral contraceptives [252] are associated with an increased risk of cervical cancer that is not totally eliminated by careful adjustment for sexual behaviour. Although multiparous women and hormonal contraceptive users did not show a higher HPV prevalence than women with fewer or no children and non-users in a large cross-sectional study of women aged 15-59 [253], endocrinological or lifestyle changes during pregnancy and after delivery might affect, at least temporarily, the acquisition or clearance of HPV infections. During pregnancy, high progesterone

levels, in conjunction with high estrogen levels, down regulate cell-mediated immunity, which would be harmful to the fetus [254], but are essential for the clearance of HPV infections [255]. A reduced humoral response to HPV 16 has also been reported in pregnant women [256].

There is no consensus about the HPV prevalence in pregnancy compared to a nonpregnancy state [171, 172, 257]. Inconsistencies may derive from differences in the selection on nonpregnant women and the use of a variety of HPV detection methods. However, in two prospective cohort studies, researchers used sensitive PCR-based assays to detect a broad range of HPV types. Nobbenhuis *et al* [174] compared pregnant and nonpregnant women with cytological abnormalities in the Netherlands. Pregnant women showed nonsignificantly reduced clearance rates of HR-HPV types compared to nonpregnant women during the 1st trimester of pregnancy, but compensatory higher clearance rates after delivery. Minkoff *et al*. [173] evaluated the impact of pregnancy in 628 HIV positive women in the United States among whom the baseline HPV prevalence (32.8% for HR- and 33.3% for LR-HPV types) was high, as in our study women. In addition, as in our study, women acted as their own controls, i.e. they were assessed before, during and shortly after pregnancy. The prevalence and copy number of HR- and LR-HPV types did not significantly differ between pregnancy and either the pre- or post-pregnancy periods. The HPV incidence, however, was significantly lower during pregnancy than after delivery.

In our study, we found a high HPV incidence during pregnancy and after delivery, but clearance was also frequent, especially when the interval between 2 visits was relatively long. Among study women seen at a 12-month interval or more, 90% of HPV infections had cleared, i.e. a finding consistent with what has been reported among nonpregnant women [258].

Young age, cytological abnormalities and presence of sexually transmitted infections other than cervical HPV were associated with acquisition of a new HPV infection. Clearance was lower among HIV-positive women. HPV 16/18 and other HR-HPV types also showed lower clearance than LR-HPV types, but the difference was not statistically significant. Very few women reported more than 1 sexual partner in the interval between 2 examinations, but a strong association of multiple partners with reduced clearance suggested that re-infections with the same types might have masked

the infection clearance in some women. Despite a substantial level of HPV incidence and clearance, HPV prevalence during early and late pregnancy and during pregnancy and after delivery, was similar. These findings suggest a balance between the acquisition and clearance of HPV infection during and after pregnancy among our study women.

Our study included the largest number of liquid-based cytology tests ever reported among pregnant women. Despite the high burden of HR-HPV infections, and the reading of the cytology by an experienced cytologist in the Netherlands, no high-grade lesions were detected. Cytology results became available months after the baseline visit and, therefore, women with ASCUS or LSIL were neither biopsied nor treated. Nevertheless, clearance of HPV was not significantly different in women with or without cytological abnormalities. Three-quarters of ASCUS and LSIL detected during pregnancy, and nearly the same percent of HPV infections, disappeared after delivery.

8. GENERAL DISCUSSION

8.1 METHODOLOGICAL CONSIDERATIONS

8.1.1 Study design and participants

The overall aim of the studies included in this thesis was to obtain data on the natural history of infections with HPV among the young women of Uganda in preparation for the introduction of prophylactic HPV vaccines. We specifically estimated the prevalence, incidence, clearance and evaluated risk factors for HPV infection as well as assessed the HPV type distribution among the young women who participated in our studies. We also explored the feasibility of using filter paper in collecting, storing and transportation of cervical exfoliated cells for HPV-DNA extraction and genotyping. To our knowledge, the sample sizes of women described in **Paper II** and **Paper IV** and cytological examination reported in **Paper IV** were the largest studies so far conducted among young women in Africa.

Using stringent design criteria, we selected our study samples from young women seeking services at NTIHC **(Papers 1, II** and **III)** and prenatal services at NHC **(Paper IV)**. We chose to conduct our studies in these two clinics for practical and logistical reasons. NTIHC is located in the compound of NHC, and both clinics are located within 15 minutes of the national referral hospital at Mulago hospital. Thus, whenever necessary, women could be referred for expert management or other services that were not provided at NTIHC or NHC. Our research protocols required us to obtain clinician-collected cervical specimens under a high degree of privacy and confidentiality that was provided by both facilities. The closeness of the two clinics enabled us to recruit women concurrently for studies presented in **Papers I, II** and **Paper IV** and conduct concurrent follow-up for **Paper III** and **Paper IV**. For continuity of services, we chose to conduct our studies in facilities, which were already providing HIV counseling and testing and later, treatment with anti retroviral therapy services. Finally, specimens that required immediate and constant refrigeration were stored at Makerere Medical School, which we could easily reach within about 15 minutes.

8.1.2 Internal Validity

In epidemiological studies, internal validity refers to the degree to which conclusions drawn from the study population relate to the source population. Thus, assessing the

internal validity requires an evaluation of bias, confounding and chance, which could have distorted the interpretation of our findings.

8.1.3 Bias

Bias refers to systematic errors that distort estimates of the study from the truth. In our studies, we were concerned with two important types of biases: selection bias and information bias.

Selection bias

To exclude selection bias, we evaluated the procedures used to select the women and factors that could have influenced their participation in our studies.

Although the women who provided cervical samples compared in **Paper 1** were consecutively enrolled, random sampling was used to select the 111 filter paper and corresponding PBS samples from 951 HPV positive samples (Paper II), eliminating the probability of selection bias. In **Papers II** and **IV**, we recruited consecutively the women who participated in our studies. This was a non-probability method of selecting the women. By so doing, we may have introduced selection bias, as the women who participated in our studies may have been different from those who refused to participate. However, the refusal rate in our studies was very small (less than 2%), which would not alter the findings in significant ways. Additionally, the age distribution and sexual behaviors of women who refused to participate in our studies was not significantly different from those who did, excluding potential selection bias in **Papers II** and **IV**. Moreover, the target age range for the women in our studies was quite narrow (between 12-24 years), and we captured women in all the ages. Nonetheless, women below 15 years were under-represented in **Paper II** perhaps because of shyness to divulge information about their sexuality and sexual behaviors.

Selection bias due to loss to follow-up

Regrettably, significant proportions of women in **Paper III** (70.2%) and **Paper IV** (61%) were lost to follow-up, which may have potentially introduced selection bias. We had anticipated significant loss to follow-up during the design of our studies and increased the sample size to accommodate the loss. However, the loss was more than we anticipated. In **Paper III,** the women were lost to follow-up because they could not be located, declined to have further specimens collected or were back in school.

However, the women who had inadequate follow-up were not significantly different from those who had adequate follow-up with respect to HIV positivity and the number of lifetime sexual partners two consistent and strong risk factors for HPV infections. Conversely, the women with inadequate follow-up were slightly younger, less likely to have genital warts, less likely to be pregnant at the time of examination, but more likely to be HIV positive. It is highly unlikely that the loss to follow-up was related to their HPV status because the women were blinded to their HPV status throughout the data collection period. Therefore, the potential bias due to significant loss to follow up would have affected HPV positive and HPV negative women uniformly given that we were able to show statistically significant associations similar to other published studies [122].

In **Paper IV,** women were lost to follow-up mainly because they chose to seek obstetric care elsewhere, terminated pregnancy voluntarily or otherwise, declined to have further specimens collected or could not be located. The women who did not have adequate follow-up were younger, had a lower education level than women who were adequately followed up. However, the baseline positivity to HPV, HIV and other STIs were not significantly different between women followed up adequately and those who were not. Similar to **Paper III**, the women were blinded to their HPV status throughout the data collection period. Therefore, it is unlikely that loss to follow-up was due to HPV status. Accordingly, we think the potential resultant bias, if any, would have uniformly affected HPV positive and HPV negative women. Consequently, we were able to show significant associations similar to published studies.

Information bias

To minimize interviewer bias in **Papers II, III** and **IV,** we trained the interviewers before data collection and used standardized closed-ended structured questionnaires to collect all information at baseline and follow up. Additionally, the interviewers were blinded to the HPV status of the women throughout data collection and so obtained data from HPV positive and HPV negative women uniformly. We acknowledge that recall bias on self-reported past behaviors of the women such as number of sexual partners and other risky behaviors was highly likely in our studies. However, both HPV positive and HPV negative women would have had the same problems of recalling their past behaviours as the women were blinded to their HPV status

throughout data collection. Thus, any recall bias, if any, would have uniformly distorted the risk estimates towards unity among both HPV positive and HPV negative women and thereby leading to underestimation of the true risk estimates.

It is likely that some women may have known the relationship between the visible external genital warts and HPV infection in **Papers II, III** and **IV**. However, the proportion of women with visible external genital warts (as opposed to visible external plus vaginal and cervical warts) in our studies was too small to impact the results in significant ways.

Misclassification of exposure

Stability of samples during transportation and storage is extremely important to avoid degradation by endogenous enzymes (endonucleases) resulting in false negative results. In studies I and II whose results are presented in **Papers I** and **II**, we ensured that PBS samples were kept cold on frozen ice packs for an average of 6 hours after collection, transported samples on frozen ice packs and kept frozen at minus 20 degrees Centigrade until shipment on dry ice to the DDL Laboratory in the Netherlands for HPV analysis. In studies III and IV whose results are presented in **Papers III** and **IV**, we used a preservation solution (PreservCyt) which adequately preserves nucleic acids for molecular diagnosis even after prolonged storage at ambient temperatures [259].

In **Papers I, II, III** and **IV**, we used a highly sensitive PCR assay for detection of HPV DNA, and the analysis was done in an established laboratory (DDL, Vooburg, The Netherlands) by experienced technicians making under-detection of HPV infections unlikely in our studies. Every PCR reaction included positive and negative controls to monitor the DNA isolation, HPV detection and genotyping procedures. However, we acknowledge that the SPF10 primer set used in the PCR assay does not efficiently differentiate HPV 68 from HPV 73 and probably from HPV 39. Hence, those types could have been under-estimated in our studies. For the important common HPV types 16, 18 and other HR-HPV types, it was unlikely that they were under-estimated by the PCR primer set used. Thus, in **Papers III** and **IV,** new infections were unlikely to be infections previously missed, and cleared infections were unlikely to be due to errors in follow-up samples or failure to detect them.

In **Papers II, III** and **IV** there were women with uncharacterized HPV X, because the SPF10 primer set could not detect them and we excluded them from the analysis of clearance in **Papers III** and **IV,** as persistence of HPV X could not be known. As a result, we may have underestimated clearance. However, the proportion of women with HPV X after the second round of testing HPV including HPV 26, 30, 55, 61, 62, 64, 67, 69, 71, 82, 83, 84, 85, 87, 89, 90, and 91 was small and could not have changed our results in significant ways.

In **Paper I**, to further minimize misclassification of exposure, the same specimen smeared on filter paper was placed in PBS and subsequently used for HPV-DNA detection. Similarly, in **Paper IV**, the same woman was used as her own control, meaning that comparison of HPV-DNA was made on specimens from the same woman collected at different times (during and after delivery).

The outcome of an HPV-DNA assay can vary depending on the menstrual cycle [260], which could have consequences on determining HPV-DNA and also influence the accuracy of HPV detection, particularly when multiple HPV genotypes are present at different concentrations [261]. As we reported in **Paper II** and **Paper IV**, multiple infections were frequent in our study populations and inevitably, cervical specimens were taken at different times of the woman's menstrual cycle. Consequently, some of HPV types in all our studies may have been under-estimated despite the highly sensitive PCR assay used.

Because of limited samples of incident infections, we used prevalent infections in **Papers III** and **IV** to evaluate clearance. We defined clearance as the first negative test following a positive result. Yet, Mosciski and colleagues (1998) [262] demonstrated that using a more conservative estimate of clearance (≥3 negative HPV tests) appreciably altered the estimates. Therefore, based on our definition, we may have over-estimated the clearance of HPV infections. Similarly, it is also possible that our definition of incident infections in **Papers III** and **IV,** based on only a single positive test, may have overestimated the incidence rates as some new infections could have been reactivation of latent infections acquired earlier.

During the administration of the questionnaire, the potential source of misclassification of exposure could have arisen from the women's' inability to understand the questions

or the women's willingness to disclose information on risky behaviors that they thought were socially undesirable, for example, questions on cigarette smoking, sex for money or gifts and lifetime number of sexual partners. To minimize the misclassification, we trained and closely supervised the interviewers, and both women and interviewers were blinded to the HPV status throughout the data collection period. Therefore, any misclassification would have uniformly affected HPV positive and HPV negative women.

8.1.4 Confounding

Confounding is the apparent distortion of the exposure, because an extraneous factor is mistaken for or mixed up with the actual exposure effect, which may be null. Usually, it is only possible to control for confounders during analysis only if data on them is collected. We are aware that controlling for known confounders during analysis depends on the accuracy of the data, as the non-differential misclassification of exposure to a confounder would lead to under estimation of the effect of a confounder and it would consequently attenuate the degree to which the confounder could be controlled for. To minimize confounding factors in **Papers II, III** and **IV**, we collected data from all potential confounders based on previous epidemiological studies on HPV infections. In designing our studies, we used inclusion/exclusion criteria to select the study women. Furthermore, during analysis, we used adjustment to permit multiple known confounding factors to be controlled for simultaneously in logistic regression models. However, we cannot completely rule out residual confounding resulting from inaccurate measurement of sexual behavior.

8.1.5 Chance

Inevitably, because studies cannot include entire target populations, estimates in any study may result by chance and not represent the ultimate true values, even if bias and confounding are excluded. Tests of statistical significance and confidence intervals are commonly used to evaluate chance. In our studies, we chose to use confidence intervals, because it is a more informative measure of chance. It is also important to note that statistically significant results do not mean that chance could not have accounted for the findings, only that such explanation was unlikely.

The large sample size in **Paper I** ensured our ability to make a valid comparison between filter paper and PBS samples and to rule out chance as the likely explanation

of our findings. Calculation of confidence intervals around the kappa statistic would not have provided a different interpretation to our findings given the large sample size.

In **Papers II** and **IV,** the large number of women in the calculation of prevalence rates increased the precision of our estimates yielding fairly narrow confidence intervals excluding unity, thus making chance the unlikely explanations of our findings. In **Papers III** and **IV** we had fewer women for analysis of incidence and clearance. As a result, we may have missed statistically significant differences where they existed. In variables where statistical significant results were obtained, the confidence intervals were fairly wide because of the small sample sizes. Nevertheless, we were able to confirm important associations of some of the exposure variables with narrow confidence intervals excluding unity, suggesting significant results.

8.1.6 External Validity

External validity means extrapolation of findings to other populations of similar individuals. The findings presented in this thesis were obtained from clinic-based samples of women, implying that the women who participated in our studies may have been different from the women in the general population. We acknowledge that a probability sample from the general population of young women at risk for HPV infection would have been ideal for our studies, but for logistical and practical reasons it was not feasible to design such a study. If we consider the number of lifetime sexual partners as the strongest risk factor for acquiring HPV infection and use it as a proxy measure to a representative sample of a general population, we think the rates in our clinic-based sample of women may be representative of women of similar age in the general population. In a recent Uganda HIV/AIDS Sero-Behavioral survey conducted among a representative sample of women aged 15-49 years, 21.5% of the women reported 4 or more lifetime sexual partners [198], which was similar to our finding in **Paper II** for the same variable (21.8%). Moreover, studies of STIs demonstrate that risk factors identified in clinic-based populations are largely confirmed in the general population samples [263]. Our results, therefore, could be generalized to the general population of women aged below 25 years to the extent that HPV testing techniques used are of comparable sensitivity as the one used in our studies.

8.2 REFLECTIONS ON ETHICAL ISSUES

We knew from the inception of our studies that we had ethical issues to address in the course of conducting our research studies. However, the solutions were not always easy. The examples will demonstrate our ethical dilemma.

Dealing with minors, we knew we would find sexually abused women who were likely to need further help beyond what the study staff could provide. At the beginning of our studies we arranged with NTIHC to handle such women, because the center had an existing collaboration with the police department. However, we needed a woman's permission before referral. We were rather surprised that once the abused women knew that the police would be involved, they would decline the referral. We discovered that the males within the homestead abused many of the sexually abused women causing the women to fear reprisal of reporting the crimes. Our studies, therefore, did not help sexually abused women, as we would have wished.

The HIV positive women identified in the course of our studies did not have access to anti retroviral therapy (ART) as we would have wished. To access ART, an HIV infected woman had to undergo expensive laboratory tests that most of the women could not afford. Secondly, the prevention of mother-to-child transmission program focused on the unborn child and not the mother. All the ART treatment the pregnant woman got was to stop the baby getting infected and not to treat her own infection. This issue was beyond our means to solve until almost at the end of our studies when ART services became available at NHC.

Ethically, we were compelled not to disclose the women's HIV status to anyone except with her permission. At post-test counseling, the HIV positive women in particular, the women told the study staff that they would disclose their HIV serostatus to no one not even their sexual partner (s) since they did not have any sign of infection yet. There was no law to compel us to disclose the woman's HIV status to her sexual partner(s). We only hoped that through pre- and post-test counseling, the women would demand for protected sex irrespective of HIV serostatus.

8.3 INTERPRETATION AND IMPLICATIONS

8.3.1 Implications for clinical work

Results from **Paper I** suggest that over two-thirds of infections with high-risk HPV types would have been missed in filter paper compared to PBS samples, and multiple type infections would also have been under estimated by threefold if only paper samples had been used. This finding implies that though filter paper samples could potentially facilitate the use of HPV testing in resource poor countries, for the moment, it should not be used until the reliability of the sampling method is developed to ensure equivalence with the standard liquid-based samples.

In our studies, the prevalence **(Paper II** and **Paper IV)** and incidence **(Paper III**) of HPV infections was extremely high among HIV positive compared to HIV negative women. We know from published literature, that the frequency and severity of histologically documented dysplasia increase with declining CD4+ counts [264] and involves other sites including the vagina, vulva and perineal region [265]. Therefore, clinicians caring for HIV positive women should routinely perform a pelvic examination to exclude severe cervical dysplastic lesions.

8.3.2 Implications for policy

Although the common HPV types among the women who participated in our studies **(Paper 11** and **Paper IV)** in descending order were HPV 52, 51, 16 and 18, the predominant HPV types in cervical cancer in Uganda are HPV 16 and 18 [12]. Therefore, the current prophylactic HPV vaccines are as relevant to the women in Uganda for prevention of cervical cancer as elsewhere in the world.

Given the high prevalence of genital warts found in our study population (**Paper II** and **Paper IV**), the potential added benefit of preventing most cases of genital warts by HPV vaccination including HPV types 6 and 11 (quadrivalent vaccine) should be considered in decisions about which vaccine to implement in Uganda.

Our results show that pregnancy does not seem to be a period of special vulnerability to HPV infection (**Paper IV**), but the burden of infection among young primigravidae in Uganda is very high. Thus, it would substantially reduce the cost-effectiveness of the administration of the prophylactic HPV vaccine if it were administered after delivery, as the majority of women are likely to be already exposed to HPV types contained in

the vaccine by the time they get pregnant because of the multiplicity of sexual partners frequently observed in the young women as reported in our Paper II.

Our studies show that HPV infections are simply too common to contemplate HPV screening in women below age 25 when there is currently no treatment (**Papers II, III, IV**) and cervical cancer screening programs are not routinely organized. Moreover, the purpose of screening is to detect lesions that have a high probability of becoming cancer. There are several arguments in favor of and against screening young women for cervical cancer despite the low incidence of cervical cancer. There are indications that increasing sexual activity in young women has resulted in high rates of CIN [266]. There is also evidence that the incidence of cervical cancer has increased in younger women (< 35 years) in some countries [1]. Conversely, there is a greater potential for detecting transient LSIL and ASCUS, likely a result of recently acquired genital HPV infections as clearly shown by the high rates of decline in the prevalence of LSIL in the women who participated in studies reported in **Papers III** and **Paper IV**, which would lead to additional unnecessary management costs. Our studies support the adaptation of the WHO recommendations to screen women at age 25 years and older [267]. Because the majority of cervical dysplastic lesions (ASCUS, LSIL) in young women are likely to be self-limited, consideration should be given to conservative follow-up by repeat Pap smears rather than by triage HPV testing or aggressive management with cryotherapy and the loop electro surgical excision procedure.

Our studies (**Paper II** and **Paper IV**) suggest that key messages about risk reduction behaviors, reduction in the number of sexual partners and the delayed onset of intercourse, which, should be promoted together with the introduction of the prophylactic HPV vaccines. These messages already conveyed in the context of HIV prevention are critical for the success of any intervention program for STIs. However, because the duration of HPV infectiousness is unknown and genital HPV infections are so common among women who have been sexually active, the value of disclosing a past diagnosis of HPV infection to future sex partners is unclear, although sexual partners should be encouraged to have a frank discussion about past STDs.

8.3.3 Implications for training

Although HPV is a sexually transmitted infection, it is not typically included in the existing education/counseling protocols for STI prevention. Therefore, there is a need

to develop educational/counseling materials and train or re-train health workers in how to offer such counseling to pre-adolescent girls, who are the initial target for the prophylactic HPV vaccines, and their parents or guardians. Studies have shown that the real challenge of HPV vaccines gaining acceptance among the general public and health care providers is a lack of understanding of HPV infection by health care workers [268]. Yet, it is through their understanding that they will promote the prevention of HPV infections. Therefore, there is a need to develop training materials to educate the educators.

There is a need to develop the laboratory capacity in HPV testing techniques and technologies through technology transfer to Uganda. Currently, there is limited in-country capacity to perform HPV DNA analysis and genotyping, therefore, the laboratory analysis from our studies were performed abroad. Yet following introduction of the HPV vaccination, there will be need for continued surveillance to evaluate the impact of vaccination on HPV-associated diseases, thus, implementation of laboratory facilities for HPV testing are justifiable.

8.4 IMPLICATIONS FOR AN HPV VACCINE STRATEGY

Our study population was comprised of young women likely to be targeted for HPV vaccination, at least initially. WHO recommends that girls aged between 9–13 years should be vaccinated [269]. The findings of our studies seem to indicate that the prevalence of HPV 16 or 18 among young women aged 12-16 years was approximately 10% of the women. Therefore, our studies support the WHO recommendation on when to initiate vaccination. However, the duration of protection of the HPV vaccine [269] and whether the girls will require a booster dose is not yet known.

In our studies, the young women had an extremely high prevalence and incidence of HPV infections. An important public health question that has no answer yet, at least for resource-poor countries, is whether vaccination programs should consider provisions for “catch-up” vaccinations. If so, what age-range should be covered by catch-up vaccinations? Our findings seem to indicate that women below 16 years could eventually be included in catch-up programs, but beyond that age, vaccination may be of little value, as many women would already be exposed.

We found a high prevalence of genital warts in or study populations. Currently, the HPV quadrivalent vaccine would possibly decrease the incidence of external genital lesions, assuming that the most common HPV types in the genital warts in the women in our studies were indeed HPV 6 and 11. However, the current dose scheduling of the quadrivalent vaccine at 0, 2 and 6 months may be even more logistically complicated to deliver to the target population than for the bivalent HPV vaccine given at 0, 1, and 6 months.

Should young men be vaccinated? A recent study seems to suggest that HPV vaccination also protects against vaccine-related types in males [270]. However, there are no studies that currently demonstrate that the HPV vaccination of males will result in less sexual transmission of HPV types contained in the vaccine. Mathematical models predict that if the HPV vaccine coverage is high, a female-only vaccination program is likely to protect males (who have sex with the vaccinated female partners) against HPV types contained in the vaccine through herd immunity [271]. Conversely, if vaccine coverage is low, as may occur in some resource poor countries, vaccination of both males and females may be more effective in preventing HPV-related cervical disease.

Our studies showed that multiple infection with HR-HPV types were frequent. Fortunately, the current prophylactic HPV vaccines have shown some cross-protection against other HPV 16- and 18-related types associated with cervical neoplasia [272]. Cross protection, even if limited, may help to prevent more cervical cancers particularly where screening programs are limited or non-existent. However, in developed countries where screening programs have been successfully implemented, modest cross-protection could not be sufficient to consider the discontinuation of screening programs in vaccinated women. In addition, in the absence of a broad cross-protection, there is a theoretical concern that HR-HPV types that rarely cause cervical cancer could occupy the niche vacated by types targeted by the prophylactic vaccines known as sero-type replacement [161]. So far, studies have shown that HPV 16 did not affect persistence of concurrent HPV infections, regardless of the type suggesting that the prevention or removal of HPV 16 is not likely to promote the risk of infection with other types [154]. Additionally, there is currently no direct evidence from vaccine trials to support the existence of this phenomenon. Only long-term follow-up of vaccinated women would rule out this phenomenon.

There is limited data to support or discourage the vaccination of HIV-positive women [269] Yet our results clearly show that both the HPV prevalence and incidence is higher in HIV-positive than HIV-negative women and infections do not clear as much as in HIV-negative women.

8.5 FUTURE RESEARCH

The studies presented in this thesis clearly demonstrate that there is a need for national baseline data to estimate the rates and risk factors for genital HPV incidence and prevalence in order to estimate the burden of infection and evaluate the likely public health impact of HPV vaccination in the future. Additionally, studies of risk factors of persistent HPV infection are critical because of the potential role of persistent infection in transmission dynamics of women and men as well as predicting the subsequent development of cervical neoplasia.

Future research should prioritize HPV vaccine-induced protection against HPV types contained therein. There is an obvious need to evaluate and describe the long-term impact of HPV vaccines under field conditions in poor settings, such as Uganda, as well as among subgroups of individuals that suffer from endemic nutrition deficiencies such as protein energy malnutrition, infections e.g. HIV, malaria and infestations e.g. intestinal worm infestations may affect the degree of protection.

As Uganda plans to introduce an HPV vaccination program in the near future, there is a need to design a surveillance program that will be linked to the HPV vaccination uptake over a long period of time to monitor the impact of vaccination. Clinics for young people and pre- natal clinics like the ones where our studies were conducted may be ideal sentinel sites to set up surveillance activities to monitor the early effects of the national vaccination program.

Further research on alternative sampling methods using filter paper or other simple, less laboratory-intensive, quick to perform in the field and cheap methods that do not require refrigeration is needed.

9 CONCLUSIONS

Study aim I was to evaluate the feasibility of using filter paper for the collection, storage and transportation of cervical exfoliated cells for HPV-DNA detection and genotyping

- Our results do not support, for the moment, the use of filter paper for the collection of cervical specimens until a standardized procedure is developed to ensure equivalence with liquid-based samples.

Study aim 2 was to estimate the prevalence rates and risk factors for HPV and sub-type distribution and other selected sexually transmitted infections among sexually active young women seeking services at Naguru Teenage Information and Health Center

- Infections with HPV (74.6%) were extremely frequent in our study population.
- The prevalence of HIV infection (8.6%) was also high (National average is 6.4% for individuals aged 15-49 years)
- HPV 52, 51, 18, and 16 were the most common HR-HPV types, and HPV 6 and HPV 11 were the most common low-risk HPV types in our study population
- The lifetime number of sexual partners, concurrent pregnancy and presence of genital warts and employment in a tertiary sector were associated with HPV positivity.

Study aim 3 was to evaluate type-specific HPV incidence, clearance and associated risk factors among young women

- The incidence of HPV infections was high but type-specific clearance was also frequent. Thus, HPV infections in young women appears to be transient
- Incident HPV infections were common in both HIV-positive (rates between 2.2 and 15.4 per 100 person-years) and HIV-negative women (rates between 0.8 and 4.2 per 100 person-years) and were not statistically significantly associated with age at study entry or lifetime numbers of sexual partners.

Study aim 4 was to estimate the prevalence, incidence and clearance of HPV infection between the 1st/2nd and 3rd trimester of pregnancy and between pregnancy and delivery among young primigravidae seeking pre-natal care at Naguru Health Center).

- The prevalence of HPV infections among the young primigravidae was high (60%)
- HPV prevalence remained unchanged in different periods of pregnancy, thus pregnancy does not seem to be a special period of vulnerability to HPV infections
- HPV infections were transiently increased during pregnancy, but declined during the post-partum period.

10 ACKNOWLEDGEMENTS

This thesis is a culmination of several years of hard work, and along the way many people directly or indirectly have contributed in many ways to the process of conducting the studies and writing this thesis. Below is a list of people whom I wish to express my sincere gratitude

Dr Elisabete Weiderpass Vainio, my main supervisor, I am indebted to you for the unending support and optimism in all situations. Despite the distance and the many simultaneous tasks you had to accomplish, you still managed to provide effective supervision. I feel lucky to have had you as a supervisor.

Dr Edward Katongole–Mbidde, my co-supervisor and Coordinator of the degenerative diseases sub-program at Makerere Medical School, thank you for your fantastic intellectual and personal support and for encouraging me to join the research world!

Dr Fred Wabwi-Mangen, my co-supervisor at Makerere Medical School, thanks for all your help, support and encouragement.

Professor Bo Lambert, my mentor, thanks for your guidance, support and for being a good friend. It is always a pleasure to speak to you.

Dr Maxwell Donald Parkin, special thanks from the bottom of my heart for introducing me to the people at IARC.

Dr Silvia Franceschi, thank you for taking an interest in my work. Your knowledgeable input in all the publications was an invaluable contribution. I feel honored to have worked with you.

Annie Arslan, for providing a pleasant and friendly working atmosphere during my visits at IARC and for the tremendous time you spent helping me clean up the databases and for providing impressive statistical expertise.

Perdrix-Thoma Trudy and *Margarethe Meo*, thank you for excellent secretarial assistance during the preparation of the manuscripts.

Sven Sandin, for your patience in helping me organize the database and excellent statistical skills in the statistical analysis of the fourth article. It was a pleasure working with you.

Professor Chris C Whalen, I sincerely feel fortunate for having you as a friend. You never let me down whenever I needed books on Epidemiology and Biostatistics.

Grace Svilar and *Alice Cantini*, thank you for your warm hearts and being good friends.

Prof. Nelson Sewankambo, thank you for your encouragement that saw me through the roller-coaster ride for the last several years I have spent as a PhD student.

Mr Steven Kijjambu, thank you for your encouragement and support.

Assoc. Prof. Elly Katabir, thank you for your guidance and support.

Associate Prof. Florence Mirembe, thank you for your continued encouragement and support and for being there for me when times were rough.

Associate Prof. Henry Wabinga, thank you for availing me with office space at the Kampala Cancer Registry.

To other PhD students, in particular, *Micheal Odida, Jackson Orem, Twaha-Mutyaba, and Ateenyi-Agaba* for our time together in the degenerative diseases sub-program.

Prof. Deirdre Carabine, thank you for your understanding and support.

To the committed study staff and in particular *Susan Nalubya, Annie Achunge, Rose Aliro, Rose Kusemererwa, Betty Mbabazi, Lilian Mwanje, Susan Mbatabe.* Thank you for your dedication to work. It was a job well done!

NHTIC and NHC staff and administration: thank you for your support and making me feel welcome throughout the conduct of the studies.

Leen Jan van Doorn, Wim Quint, and Bernhard Kleter of DDL Laboratory, Vooburg, The Netherlands: thank you for performing the HPV analyses.

To my fantastic children *Bernadette and Denis*: thanks for giving me the reason to wake up everyday and the energy to go on during all these years. Thanks for your love today and always.

Thanks to my extended family of children *Nicholas, Mathew, Paul, Isaiah, Linda Lucille, Leon, Angela, Agatha, Victoria, Jonathan and Vanessa.*

To my siblings *Celine, Margaret, Charles, Tadeo, Philip and Agnes:* When the going got tough, I knew I could count on you. Thank you for being there for me.

Thanks to my dear brother and friend *Rev. Dr Gerald Banura (RIP).* You never lived long enough to see me write this thesis. I miss your wise counsel.

My parents *Paul K. Banura (RIP) and Victoria K. Banura (RIP),* I will forever be indebted to your love, guidance and support. Thank you for teaching me to aim high no matter what.

Special thanks to the *Director and staff at the School of Graduate Studies*, Makerere University.

Special thanks to *Karolinska Institutet, in particular Dr Stefan Peterson and Prof Hans Rosling (IHCAR)* for providing the framework of the research collaboration between Makerere University and Karolinska Institutet and to the Department of Medical Epidemiology and Biostatistics for welcoming me as a research student.

Last but not least, special thanks to the Swedish International Development Cooperation Agency (SIDA) for providing financial support.

11 REFERENCES

1. Ferlay J, Bray F, Pisani M, et al. Editors. GLOBOCAN 2002 Cancer Incidence. Mortality and Prevalence Worldwide. IARC Press: Lyon, 2004.
2. Parkin D, Ferlay J, Hamdi-Cherif M, et al. Cancer in Africa: Epidemiology and Prevention. *IARC Scientific Publications No. 153*. IARC Press: Lyon, 2003.
3. Castellsague X, de Sanjose S, Aguado T, et al. HPV and Cervical Cancer in the World. 2007 Report. *WHO/ICO Information Center on HPV and Cervical Cancer (HPV information Center)*. Geneva: WHO, 2007. Available at: www.who.int/hpvcentre
4. Parkin D, and Bray F. Chapter 2:The burden of HPV-related cancers. *Vaccine* 2006, **24 (Suppl 3)**:S3-S11.
5. Harper D, F Franco EL, Wheeler CM, et al. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomized controlled trial. *Lancet* 2004; **364**:1757-65.
6. Villa L, Costa RL, Petta CA, et al. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16 and 18) L1 virus-like particle vaccine in young women: a randomized, double-blind placebo-controlled multi center phase II efficacy trial. *Lancet Oncol* 2005; **6**:271-78.
7. Harper D, Gall S, Naud P, et al. Sustained immunogenicity and high efficacy against HPV 16/18 related cervical neoplasia: Long-term follow up through 6.4 years in women vaccinated with Cervarix. *Presented at the Meeting of the Society of Gynecologic Oncology*. Tampa, Florida, USA, 2008. Abstract published in Gynecologic Oncology.
8. de Sanjose S, Diaz M, Castellsague X, et al. Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis. *Lancet Infect Dis* 2007; **7**:453-59.
9. Curado M, Edwards B, Shin HR, et al. editors. Cancer Incidence in five continents. *Vol. IX. IARC Scientific Publications No. 160.* IARC Press: Lyon 2008.
10. Asiimwe S, Whalen CC, Tisch DJ, et al. Prevalence and predictors of high-risk human papillomavirus infection in a population-based sample of women in rural Uganda. *Int J of STD & AIDS* 2008; **19**:605-10.
11. Blossom D, Beigi RH, Farrell JJ, et al. Human papillomavirus genotypes associated with cervical cytologic abnormalities and HIV infection in Ugandan women. *J Med Virol* 2007; **79**:758-65.
12. Odida M, de Sanjose S, Quint W, et al. Human papillomavirus type distribution in invasive cervical cancer in Uganda. *BMC Infectious Agents and Cancer* 2008; **8**:85.
13. Iftner T, & Villa LL. Chapter 12: Human papillomavirus technologies. *J Natl Cancer Instit* 2003; **31**:80-8.
14. Beebe J, and Briggs LC. Evaluation of enzyme-linked immunoassay systems for detection of human immunodeficiency virus type 1 antibody from filter paper disks impregnanted with whole blood. *J Clin Microbiol* 1990; **28**:808-10.
15. Gupta B, Jayasuryan N, and Jameel S. Direct detection of hepatitis b virus from dried blood spots by polymerase chain reaction amplification. *J Clin Microbiol* 1992; **30**:1913-6.
16. Bickel H, Bachmann C, Beckers R, et al. Neonatal mass screening for metabolic disorders. *Eur J Pediatr* 1981; **137**:133-9.

17. Garrick, M., Dembure P, and Guthrie R. Sickle-cell and other hemoglobinopathies: procedures and strategy for screening employing spots of blood on filter paper specimens. *N Eng J Med* 1973; **288**:1265-8.
18. IARC Monographs on the evaluation of carcinogenic risks to humans. *Human Papillomaviruses*, Vol. 90. International Agency for Research on Cancer: Lyon, 2007.
19. zur Hausen H. Human papillomaviruses and their possible role in squamous cell carcinomas. *Curr Top Microbiol* 1977; **78**:1-30.
20. Munoz N, Bosch FX, de Sanjose S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Eng J Med* 2003; **348**:518-27.
21. Kiviat N, Koutsky LA, Critchlow CW, et al. Prevalence and cytologic manifestations of human papillomavirus (HPV) types 6, 11, 16, 18, 31, 33, 35, 42, 43, 44, 45, 51, 52, and 56 among 500 consecutive women. *Int J Gynecol* Pathol 1992; **11**:197-203.
22. Clifford G, Smith JS, Aguado T, et al. Comparison of HPV type distribution in high-grade cervical lesions and cervical cancer: a meta-analysis. *Br J Cancer* 2003; **89**:101-5.
23. Clifford, G., Franceschi S, Diaz M, et al. Chapter 3: HPV type-distribution in women with and without cervical neoplastic diseases. *Vaccine* 2006; **24 (Suppl 3)**:S26-S34.
24. Munger K, Baldwin A, Edwards KM, et al. Mechanisms of human papillomavirus induced oncogenesis. *J Virol* 2004; **78**:11451-60.
25. Walboomers J, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol* 1999. **189**:12-9.
26. Parkin D. The global health burden of infection-associated cancers in the year 2000. *Int J Cancer* 2006; **118**:3030-44.
27. Kitchener H, Castle PE, Cox JT. Achievements and limitations of cervical cytology screening. *Vaccine* 2006; **24 (Suppl 3)**:S3/63-S3/70.
28. Munoz N, Bosch FX. Current reviews on the epidemiology of HPV and cervical cancer. *In*: Papillomavirus Reviews: Current Research on Papillomaviruses, 1996: 227-37.
29. Rothman K, Greenland S. Editors. Modern Epidemiology. Second Edition. Lippincott-Raven. 1998: 53-5.
30. International Commitee on Taxonomy of Viruses.
31. Klug A and Finch JT. Structure of viruses of the papilloma-polyoma type 1. I. Human wart virus. *J Mol Biol* 1965; **11**:403-23.
32. IARC Monographs on the evaluation of carcinogenic risks to humans. *Human Papillomaviruses, Vol. 64*. International Agency for Research on Cancer: Lyon, 1995.
33. Streenbergen R, de Wilde J, Wilting SM, et al. HPV-mediated transformation of the ano-genital tract. *J Clin Virol* 2005, **32 (Suppl 1)**:S25-33
34. Munoz N, Castellsague X, de Gonzalez AB, et al. Chapter 1: HPV in the etiology of human cancer. *Vaccine* 2006, **24 (Suppl 3)**:S3/1-S3/10.
35. Schiffman M, Kjaer SK. J Chapter 2: Natural history of anogenital human papillomavirus infection and neoplasia. *Natl Cancer Instit Monogr* 2003; **31**:14-9.
36. Giroglou T, Sapp M, Lane C, et al. Immunological analyses of human papillomavirus capsids. *J Virol* 2001; **9**:1783-93.
37. Culp T and Christensen ND. Kinetics of in vitro adsorption and entry of papillomavirus virions. *Virology* 2004; **319**:152-61.

38. Day P, Lowy DR and Schiller JT. Papillomavirus es infect cells via a clathrin-dependent pathway. *Virology* 2003; **307**:1-11.
39. Selinka H, Giroglou T, Snapp M. Analysis of the infectious entry pathway of human papillomavirus type 33 pseudovirus. *Virology* 2002; **299**:279-87.
40. Li M, Beard P, Estes PA, Lyon M. et al. Intracapsomeric disulphide bonds in papillomavirus assembly and disassembly. *J Virol* 1998; **72**:2160-7.
41. Oguchi T, Sato S, Xiao YH, et al. Usefulness of PCR in situ hybridization as a technique for morphological detection of human papillomavirus in uterine cervical neoplasia. *Eur J Gynecol*. Oncol 2000; **21**:585-7.
42. Wilson V, West M, Woytek K, et al. Papillomavirus E1 proteins: Form, function and features. *Virus Genes* 2002; **24**:275-90.
43. zur Hausen, H., *Papillomaviruses and cancer: From basic studies to clinical application.* Nature Reviews Cancer, 2002. **2**: p. 342 - 350.
44. Jeon S, Allen-Hoffmann BL and Lambert PF. Integration of human papillomavirus type16 into the human genome correlates with a selective growth advantage of cells. *J Virol* 1995; **69**:2989–97.
45. Flores E, Allen-Hoffmann BL, Lee D, et al. Establishment of the human papillomavirus type 16 (HPV-16) life cycle in an immortalized human foreskin keratinocyte cell line. *Virology* 1999; **262**:344–54.
46. Hubert W and Laimins LA. Human papillomavirus type 31 replication modes during the early phases of the viral life cycle depend on transcriptional and post transcriptional regulation of E1 and E2 expression. *J Virol* 2002; **76**:2263-73.
47. Stubenrauch F, Colbert AM & Laimins LA. Trans-activation by the E2 protein of oncogenic human papillomavirus type 31 is not essential for early and late viral functions. *J Virol* 1998; **72**:8115–23.
48. Thomas J, Hubert WG, Ruesch MN, et al. Human papillomavirus type 31oncoproteins E6 and E7 are required for the maintenance of episomes during the viral life cycle in normal human keratinocytes. *Proc. Natl Acad. Sci. USA* 1999; **96**:8449–54
49. Flores E, Allen-Hoffmann BL, Lee D, et al. The human papillomavirus type 16 E7 oncogene is required for the productive stage of the viral life cycle. *J Virol* 2000; **74**:6622–31.
50. Frazer I, Cox JT, Mayeaux EJ, et al. Advances in prevention of cervical cancer and other human papillomavirus-related diseases. *Pediatr Infect Dis* 2006; **25**:S65-S81.
51. Duensing S, Munger K. Mechanism of genomic instability in human cancer: insights from studies with human papillomavirus oncoproteins. *Int J Cancer* 2004; **109**:157-62.
52. Palefsky J and Jolly EA. Molecular virology and epidemiology of human papillomavirus and cervical cancer. *Cancer Epidemiol Biomarkers & Prev* 1995; **4**:415-28.
53. Yokota J, Sigmura T. Multiple steps in carcinogenesis involving alterations of multiple tumor suppressor genes. *FASEB J* 1993; **7**:920-5.
54. zur Hausen H. Papillomaviruses causing cancer: evasion from host cell control in early events in carcinogenesis. *J Natl Cancer Instit* 2000; **92**:690-8.
55. de Villiers E, Fauquet C, Broker TR, et al. Classification of papillomaviruses. *Virology* 2004; **324**:17-27.
56. Bernard HU, Calleja-Macias IE, and Dunn ST. Genome variation in human papillomavirus types: Phylogenetic and medical implications. *Int J Cancer* 2006; **118**:1071-6.

57. Chan S, Delius H, Halpern AL, et al. Analysis of genomicsequences of 95papillomavirus types: uniting typing, phylogeny, and taxonomy. *J Virol* 1995; **69**:3074-83.
58. Schiffman M, Herrero R, deSalle R, et al. The carcinogenicity of human papillomavirus types reflects viral evolution. *Virology* 2005; **337**:76-84.
59. de Villier E. Human pathogenic papillomavirus types: an update. *Curr Top Microbiol Immunol* 1994; **186**:1-12.
60. Ho L, Chan, SY, Chow V, et al. Sequent variants of human papillomavirus type 16 in clinical samples permit verification and extension of epidemiological studies and construction of a phylogenetic tree. *J Clin Microbiol* 1991; **29**:1765-72.
61. Lörincz A, Reid R, Jenson AB, et al. Human papillomavirus infection of the cervix: Relative risk associations of 15 common anogenital types. *Obstet. & Gynecol* 1992; **79**:328-37.
62. Clifford G, Gallus S, Herrero R, et al. Worldwide distribution of human papillomavirus types in cytologically normal women in the International Agency for Research on cancer HPV prevalence surveys: a pooled analysis. *Lancet* 2005; **366**:991-8.
63. McCaffrey K, Waller J, Forrest S, et al. Testing positive for human papillomavirus in routine cervical screening: examination of psychosocial impact. *BJOG* 2004; **111**:1437-43.
64. Harper M. Why Am I Scared of HPV? *CA Cancer J Clin* 2004; 2004; **54**:245-7.
65. Hu D, Goldie S. The economic burden of non-cervical human papillomavirus diseases in the United States. *Am J Obstet Gynecol* 2008; **500**:e1-e7.
66. Munoz N, Bosch FX, Castellsague X, et al. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *Int J Cancer* 2004; **111**:278-85.
67. Solomon D, Davey DD, Kurman R, et al. The 2001 Bethesda System: terminology for reporting results of cervical cytology. *JAMA* 2002; **287**:2114-9.
68. Wentzensen N, Hampl M, Herkert M, et al. Identification of high-grade cervical dysplasia by the detection of $p16^{ink4a}$ in cell lysates obtained from cervical samples. *Cancer* 2006; **107**:2307-13.
69. Snijders P, van den Brule AJ, Meijer, CJ. The clinical relevance of human papillomavirus testing: relationship between analytical and clinical sensitivity. *J Pathol* 2003; **201**:1-6.
70. Hubbard R. Human papillomavirus testing methods. *Arch Pathol Lab Med* 2003; **127**:940-5.
71. Molijn A, Kleter B, Quint W, et al. Molecular diagnosis of human papillomavirus (HPV) infections. *J Clin Virol* 2005; **32 (Suppl 1)**:S43-S51.
72. Bozzeti M, Nonnenmacher B, Mielzinska I, et al. Comparison between Hybrid Capture II and polymerase chain reaction results among women at low-risk for cervical cancer. *Ann Epidemiol* 2000; **10**:466.
73. Lörincz A. Hybrid Capture method for detection of human papillomavirus DNA in clinical specimens: a tool for clinical management of equivocal Pap smears and for population screening. *J Obstet Gynecol Res* 1996; **22**:629-36.
74. Cope J, Hildesheim A, Schiffman MH, et al. Comparison of Hybrid Capture tube test and PCR for detection of human papillomavirus DNA in cervical specimens. *J Clin Microbiol* 1997; **35**:262 -5.
75. Castle P, Schiffman M, Burk RD, et al. Restricted cross-reactivity of Hybrid Capture 2 with non-oncogenic human papillomavirus types. *Cancer Epidemiol Biomarkers & Prev* 2002; **11**:1394-9.

76. Poljak M, Marin IJ, Seme K, et al. Hybrid Capture II HPV test detects at least 15 human papillomavirus genotypes not included in its current high-risk probe cocktail. *J Clin Virol* 2002; **25 (Suppl 3)**:89-97.
77. Van den Brule, A Meijer CJLM, Bakels V, et al. Rapid detection of human papillomavirus in cervical scrapes by combined general primer-mediated and type specific polymerase chain reaction. *J Clin Microbiol* 1990; **28**:2739 -43.
78. Laconi S, Greco M, Pellegrini-Bettoli P, et al. One-step detection and genotyping of human papillomavirus in cervical samples by reverse hybridization. *Diagn Mol Pathol* 2001; **10**:200-6.
79. Kleter B, Doorn LJ, ter Schegget J, et al. Novel short fragment PCR assay for highly sensitive broad -spectrum detection of anogenital human papillomaviruses. *Am J Pathol* 1998; **153**:1731-9.
80. Manos MM, Waldman J, Zhang TY, et al. Epidemiology and partial nucleotide sequence of four novel genital human papillomaviruses. *J Infect Dis* 1994; **170**:1096-9
81. Gravitt P, Peyton CL, Alessi TQ, et al. Improved amplification of genital human papillomaviruses. *J Clin Microbiol* 2000; **38**:357-61.
82. Van den Brule A, Pol R, Fransen-Daalmeijer N, et al. GP5+/6+ PCR followed by reverse blot analysis enables rapid and high-thoroughput identification of human papillomavirus genotypes. *J Clin Microbiol* 2002; **40**:779-87.
83. Kleter B, van Doorn LJ, Schrauwen L, et al. Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. *Am J Microbiol* 1999; **37:**2508-17.
84. Chan P, Cheung TH, Tam AO, et al. Biases in human papillomavirus genotype prevalence assessment associated with commonly used consensus primers. *Int J Cancer* 2006; **118**:243-5.
85. Quint W, Pagliusi SR, Lelie N, et al. Results of the first World Health Organization Internationals Collaborative Study of detection of human papillomavirus DNA. *J Clin Microbiol* 2006; **44**:571-9.
86. Sankaranarayanan R, Graffikin L, Jacob M, et al. A critical assessment of screening methods for cervical neoplasia. *J Gynecol Obstet* 2005; **89 (Suppl 2)**:S4-S12.
87. Bleeker M, Hogewoning CJA, van den Brule AJC, et al. Penile lesions and human papillomavirus in male sexual partners of women with cervical intraepithelial neoplasia. *J Am Acad Dermatol* 2002; **47**:351-7.
88. Castellsagué, X., Bosch FX, & Muñoz N. The male role in cervical cancer. *Salud Publica Mex* 2003; **45 (Suppl. 3)**:S345–S353.
89. Kjaer S, Svare EI, Worm AM, et al. Human papillomavirus infection in Danish female sex workers decreasing prevalence with age despite continuously high sexual activity. *Sex Transm Dis* 2000; **27**:438-45.
90. Castellsagué X., Ghaffari A, Daniel RW. Prevalence of penile human papillomavirus DNA in husbands of women with and without cervical neoplasia: A study in Spain and Colombia. *J Infect Dis* 1997; **176**:353-61.
91. Franceschi S, Castellsagué X, Dal Maso L, et al. Prevalence and determinants of human papillomavirus genital infection in men. *Br J Cancer* 2002; **86**:705-711.
92. Oriel J. Natural history of genital warts. *Br J Vener Dis* 1971; **47**:1-13.
93. Kjaer S, Chackerian B, van den Brule A, et al. High-risk human papillomavirus is sexually transmitted: Evidence from a follow-up study of virgins starting sexual activity (Intercourse). *Cancer Epidemiol Biomarkers & Prev* 2001; **10**:101-106.

94. Patridge J, Koutsky LA. Genital human papillomavirus infection in men. *Lancet Infect Dis* 2006; **6**:21-31.
95. Moscicki A, Hills N, Powell K, et al. Risk for incident human papillomavirus infection and low-grade squamous intra-epithelial lesions development in young females. *JAMA* 2001; **285**:2995-3002.
96. Bleeker M, Hogewoning CJ, Berkhof, J, et al. Concordance of specific human papillomavirus types in sex partners is more prevalent than would be expected by chance and is associated with increased viral loads. *Clin Infect Dis* 2005; **41**:612-20.
97. Doherty I, Padian, NS, Marlow C, et al. Determinants and consequences of sexual networks as they affect sexually transmitted infections. *J Infect Dis* 2005; **191 (Suppl 1)**:S42-S54.
98. Aral S and Holmes, KK. Social and behavioral determinants of epidemiology of STDs: industrialized and developing countries. *In*: Holmes, KK, Mardh P-A, Sparling PF, et al. editors, Sexually transmitted diseases. New York: McGraw-Hill. 1999, 39-76.
99. Koutsky L and Kiviat NB. Genital human papillomaviruses. *In*: Holmes, KK, Mardh P-A, Sparling PF, et al. editors. Sexually transmitted diseases. New York: McGraw-Hill. 1999, 347-59.
100. del Amo J, Gonzalez C, Lozana J, et al. Influence of age and geographical origin in the prevalence of high risk papillomavirus in migrant female sex workers in Spain. *Sex Transm Infect* 2005; **81**:79-84.
101. Gonzalez C, Ortiz M, Canals J, et al. Higher prevalence of human papillomavirus infection from migrant workers from Latin America in Spain. *Sex Transim Infect* 2006; **82**:260-2.
102. Winer R, Lee SK, Hughes JP, et al. Genital human papillomavirus infection: Incidence and risk factors in a cohort of female university students. *Am J Epidemiol* 2003; **157**:218-26.
103. Coutlee F, Trottier AM, Ghattas G, et al. Risk factors for oral human papillomavirus in adults infected and not infected with human immunodeficiency virus. *Sex Transm Dis* 1997; **24**:23-31.
104. Fairley C, Gay NJ, Forbes A, et al. Hand-genital transmission of genital-warts? An analysis of prevalence data. *Epidemiol & Infect* 1995; **115**:169-76.
105. Marrazo J, Koutsky LA, Kiviat NB, et al. Papanicolau test screening and prevalence of human papillomavirus among women who have sex with women. *Am J Public Health* 2001; **91**:947-52.
106. Sonnex C, Strauss S and Gray JJ. Detection of human papillomavirus DNA on the fingers of patients with genital warts. *Sex Transm Dis* 1999; **75**:317-19.
107. Armbruster-Moraes E, Ioshimoto LM, Leao E. et al. Presence of human papillomavirus DNA in amniotic fluids of pregnant women with cervical lesions. *Gynecol Oncol* 1994; **54**:152-8.
108. Smith-Moraes E, Ioshimoto LM, Leao E. et al. Presence of human papillomavirus DNA in amniotic fluids of pregnant women with cervical lesions. *Gynecol Oncol* 1994; **54**:152-8.
109. Dunne E, Karem KL, Sternberg MR, et al. Seroprevalence of human papillomavirus type 16 in children. *J Infect Dis* 2005; **191**:1817-9.
110. Sinal S. Human papillomavirus infections of the genital and respiratory tracts in young children. *Semin Pediatr Infect Dis* 2005; **16**:306-16.
111. Syrjanen K and Puranen M. Human papillomavirus infections in children: the potential role of maternal transmission. *Crit Rev Oral Biol Med*, 2000; **11**:259 - 74.

112. Castellsague X, Drudis T, Canadas MP, et al. Human papillomavirus (HPV) infection in pregnant women and mother-to-child transmission of genital HPV genotypes: a prospective study in Spain. *BMC Infectious Diseases* 2009; **9**:74.
113. Rintala M, Grenman SE, Puraren MH. Transmission of high-risk human papillomavirus (HPV) between parents and infant: a prospective study of HPV in families in Finland. *J Clin Microbiol* 2005; **43**:376-81.
114. Stevens-Simon C, Nelligan D, Breese P, et al. Transmission of genital human papillomavirus infections in abused and non-abused pre-adolescent girls. *Pediatrics* 2000; **106**:645-9.
115. Brown D, Shew ML, Qadadri B, et al. A longitudinal study of genital papillomavirus in a cohort of closely followed adolescent women. *J Infect Dis* 2005; **191**:182-92.
116. Burchell A, Richardson H, Mahmud SM, et al. Modeling the sexual transmissibility of human papillomavirus infection using stochastic computer simulation and empirical data from a cohort of young women in Montreal, Canada. *Am J Epidemiol* 2006; **163**:534-43.
117. Barnabas R, Laukkanen P, Koskela P, et al. Epidemiology of HPV 16 and cervical cancer in Finland and the potential impact of vaccination: mathematical modeling analyses. *PLoS* 2006; **3**:e138.
118. Anderson R. Transmission dynamics of sexually transmitted infections. *In*: Holmes, KK, et al. Sexually transmitted diseases. New York: McGraw-Hill. 1999, 25-37.
119. Wald A, Langenberg, AG, Link K, et al. Effect of condoms on reducing the transmission of herpes simplex virus type 2 from men to women. *JAMA* 2001; **285**:3100-6.
120. Ho GYF, Bierman R, Beardsley L et al**.** Natural history of cervicovaginal papillomavirus infection in young women**.** *N Eng J Med* 1998; **338**:423-*8***.**
121. Ho G, Studentsov YY, Bierman R, et a. Natural history of human papillomavirus type 16 virus-like particle antibodies in young women. Cancer Epidemiol Biomarkers & Prev, 2004; **13**:110-16.
122. Peyton C, Gravitt PE, Hunt WC, et al. Determinants of Genital Human Papillomavirus Detection in a US Population. *J Infect Dis* 2001; **183**:1554-64.
123. Ley C, Bauer HM, Reingold A, et al. Determinants of genital human papillomavirus infections in young women. *J Natl Cancer Instit*1991; **83**:997-1003.
124. Wang S, Schiffman M, Herrero R, et al. Determinants of human papillomavirus 16 serological conversion and persistence in a population-based cohort of 10,000 women in Costa Rica. *Br J Cancer* 2004; **91**:1269-74.
125. Nonnenmacher B, Pintos J, Bozzetti MC, et al. Epidemiologic correlates of antibody response to human papillomavirus among women at low risk of cervical cancer. *Int J STD AIDS* 2003; **14**:258-65.
126. Stone K, Karem KL, Sternberg MR, et al. Seroprevalence of human papillomvirus type 16 infection in the United States. *J Infect Dis* 2002; **186**:1396-402.
127. Smith J, Melendy A, Rana RK, et al. Age-specific prevalence of infection with human papillomavirus in females: a global review. *J Adol Health* 2008; **43**:S5-S25.
128. Bosch FX, Castellsague X, Munoz N, et al. Male sexual behavior and human papillomavirus DNA: Key risk factors for cervical cancer in Spain. *J Natl Cancer Instit* 1996; **88**:1060–7.

129. Muñoz N., Castellsague X, Bosch FX, et al. Difficulty in elucidating the male role in cervical cancer in Colombia, a high-risk area for the disease. *J Natl Cancer Instit* 1996a; **88**:1068–75.
130. Collins S, Mazloomzadeh S, Winter H, et al. Proximity of first intercourse to menarche and risk for papillomavirus infection: A longitudinal study. *Int J Cancer* 2005; **114**:498-500.
131. Singer A. The uterine cervix from adolescence to menopause. *Br J Obstet Gynecol* 1993; **82**:81-9.
132. Jacobson D, Peralta L, Farmer M, et al. Cervical ectopy and transformation zone measured by computerized planimetry in adolescents. *Int J Gynecol & Obstet* 1999; **66**:7-17
133. Castellsague X, Bosch FX, Munoz N, et al. Male circumcision, penile human papillomavirus infection and cervical cancer in female partners. *N Eng J Med* 2002; **346**:1105-12.
134. Skegg D, Corwin PA, Paul C, et al. Importance of male factor in cancer of the cervix. *Lancet* 1982; **2**:581-3.
135. Dunne E, Nielson CM, Stone KM, et al. Prevalence of HPV infection among men: A systematic review of the literature. *J Infect Dis* 2006; **194**:1044-57
136. Drumright L, Gorbach PM, Holmes, KK. Do people really know their sexual partners? Concurrency: knowledge of partner behavior and sexually transmitted infections within partnerships. *Sex Transm Dis* 2004; **31**:437-42.
137. Burchell A, Winer RL, de Sanjose S, et al. Chapter 6: Epidemiology and transmission dynamics of genital HPV infections. Vaccine 2006; **24 (Suppl 3)**: S3/52-S3/61.
138. Moscicki A, Ellenberg JH, Farhat S, et al. Persistence of human papillomavirus infection in HIV-infected and uninfected adolescent girls: risk factors and differences by phylogenetic types. *J Infect Dis* 2004; **190**:37–45.
139. Wideroff L, Schiffman MH, Hoover R, et al. Epidemiologic determinants of seroreactivity to human papillomavirus (HPV) type 16 virus-like particles in cervical HPV-16 DNA-positive and -negative women. *J Infect Dis* 1996a; **174**:937-43
140. Liaw KL, Hildesheim A, Burk RD, et al. A prospective study of human papillomavirus (HPV) type 16 DNA detection by Polymerase Chain Reaction and its acquisition and Persistence of other HPV types. *J Infect Dis* 2001; **183**:8-15.
141. Rousseau, M., Pereira JS, Prado JCM, et al. Cervical co-infection with human papillomavirus (HPV) types as a predictor of acquisition and persistence of HPV infections. *J Infect Dis* 2001; **184**:1508-17.
142. Bousarghin L, Touze A, Sizaret P, et al. Human papillomavirus types 16, 31, and 58 use different endocytosis pathways to enter cells. *J Virol* 2003; **77**:3846-50.
143. Thomas K, Hughes JP, Kuypers J, et al. Concurrent and sequential acquisition of different genital human papillomavirus types. *J Infect Dis* 2000; **182**:1097-102
144. Mendez F, Munoz N, Posso H, et al. Cervical co-infection with human papillomavirus (HPV) types and possible implications for the prevention of cervical cancer by HPV vaccines. *J Infect Dis* 2005; **192**:1158-65.
145. Wideroff Schiffman M, Haderer P, et al. Seroreactivity to human papillomavirus type 16, 18, 31 and 45 virus-like particles in a case-control study of cervical squamous intraepithelial lesions. *J Infect Dis* 1999; **180**:1424-28.

146. Combita A, Touze A, Bousarghin L, Christensen N, et al. Identification of two cross- neutralizing linear epitopes within the L1 major capsid protein of human papillomaviruses. *J Virol* 2002; **76**:6480-6.
147. de Vuyst H, Steyaert S, Renterghem LV, et al. Distribution of human papillomavirus in a family planning population in Nairobi, Kenya. *Sex Trans Dis* 2003; **30**:137-42.
148. Hawes S, Critchlow CW, Faye Niang MA, et al. Increased risk of high-grade cervical squamous intraepithelial lesions and invasive cervical cancer among African women with human immunodeficiency virus infections: relation with cervical dysplasia-neoplasia in African women. *J Infect Dis* 2003; **188**:555-63.
149. Franco E, Duarte-Franco E and Ferenczy A. Cervical cancer: epidemiology, prevention and the role of human papillomavirus infection. *CMAJ* 2001; **164**:1017-25.
150. Wright T, Jr, Ellerbrock TV, Chiasson MA et al. Cervical intraepithelial neoplasia in women infected with human immunodeficiency virus: prevalence, risk factors, and validity of Papanicolaou smears. New York Cervical Disease Study *Obstet Gynecol* 1994; **84**:591-7.
151. Strickler H, Burk RD, Fazzari M, et al. Natural history and possible reactivation of human papillomavirus in human immunodeficiency virus-positive women. *J Natl Cancer Instit* 2005; **97**(8):577-86.
152. Clarke B and Chetty R. Post modern cancer: the role of human immunodeficiency virus in uterine cervical cancer. *J Clin Pathol* 2002; **55**:19-24.
153. Dolei A, Curreli S, Marongiu P, et al. Human immunodeficiency virus infection in vitro activates naturally integrated HPV 18 and induces synthesis of L1 capsid protein. *J Gen Virol* 1999; **80**:2937-44.
154. Vernon S, Hart CE, Reeves WC et al. The HIV-1 tat protein enhances E2-dependent HPV 16 transcription. *Virus Res* 1993; **27**:133-45.
155. Schmauz R, Okong P, de Villiers EM, et al. Multiple infections in cases of cervical cancer from high incidence area in tropical Africa. *Int J Cancer* 1989; **43**:805-9.
156. Trottier H, Franco EL. The epidemiology of genital human papillomavirus infection. *Vaccine* 2006; **24 (Suppl 1)**:S4-S15.
157. Winer R, Hughes JP, Feng Q, et al. Condom use and the risk of genital human papillomavirus infection in young women. *N Eng J Med* 2006; **354**:2645-54.
158. Manhart L and Koutsky L. Do condoms prevent HPV infection, external genital warts, or cervical intraepithelial neoplasia? *Sex Transm* Dis 2002; **29**:725-35.
159. Hogewoning C, Bleeker MCG, van den Brule AJ, et al. Condom use promotes regression of cervical intraepithelial neoplasia and clearance of human papillomavirus: a randomized clinical trial. I*nt J Cancer* 2003; **107**:811-6.
160. Moscicki A. Impact of HPV infections in adolescent populations. *J Adol Health* 2005; **37**:S3-S9.
161. Culp T, Budgeon LR, Marinkovich MP, et al. Keratinocyte-secreted laminin 5 can function as a transient receptor for human papillomavirus by binding virions and transferring them to adjacent cells. *J Virol* 2006; **80**:8940-50.
162. Roberts J, Buck CB, Thompson CD, et al. Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan. *Nature Medicine* 2007; **13**:857-861.
163. Lowly D & Schiller JT. Prophylactic human papillomavirus vaccine. *J Clin Invest* 2006; **116**:1167-73.
164. Cook L, Koutsky LA, and Holmes KK. Circumcision and sexually transmitted diseases. *Am J Public Health* 1994; **84**:197-201.

165. Tobian A, Serwadda D, Quinn TC, et al. Male circumcision for the prevention of HSV-2 and HPV infections and Syphilis. *N Eng J Med* 2009; **360**:1298-309.
166. Auvert B, Sobngwi-Tambekou J, Cutler E, et al. Effect of male circumcision on the prevalence of high-risk human papillomavirus in young men: Results of a randomized controlled trial conducted in Orange Farm, South Africa. *J Infect Dis* 2009; **199**:14-9.
167. Szabo R and Short R. How does male circumcision protect against HIV infections? *Br Med J* 2000; **320**:1592-94.
168. Haverkos H, Soon G, Steckley SL, et al. Cigarette smoking and cervical cancer: Part I: a meta-analysis. *Biomed Pharmacother* 2003; **57**:67-77.
169. Winer R, Koutsky LA. *The epidemiology of human papillomaviruses infections.* Cervical cancer: from etiology toprevention., ed. S.K. In: Rohan T. Dordretch, The Netherlands: Kluwer Academic Publishers, 2004: 143 - 87..
170. Phillips A and Smith GD. Cigarette smoking as a potential cause of cervical cancer: has confounding been controlled? *Int J Epidemiol* 1994; **23**:42-9.
171. Fife K, Katz BP, Rosh J, et al. Cancer-associated human papillomavirus types selectively increased in the cervix of women in the first trimester of pregnancy. *Am J Obstet Gynecol* 1996; **174**:1487-93.
172. Hernandez-Giron C, Smith JS, Lorincz A, et al. High-risk human papillomavirus detection and related risk factors among pregnant and non pregnant women in Mexico. *Sex Transm Dis* 2005; **32**:613-8.
173. Minkoff H, Shen X, Watts, DH, et al. Relationship of pregnancy to human papillomavirus among human immune-deficiency virus-infected women. *Obstet Gynecol* 2006; **108**:953-60.
174. Nobbenhuis M, Helmerhorst TJM, van den Brule AJC, et al. High-risk human papillomavirus clearance in pregnant women: trends for lower clearance during pregnancy with a catch-up postpartum. *Br J Cancer* 2002; **87**:75-80.
175. Banura C, Franceschi S, Doorn L-J. et al. Prevalence, incidence and clearance of human papillomavirus infection among young primiparous pregnant women in Kampala, Uganda. *Int J Cancer* 2008; **123**:2180-7.
176. Fife K, Katz BP, Brizendine EJ et al. Cervical human papillomavirus deoxyribonucleic acid persists throughout pregnancy and decreases in the post-partum period. *Am J Obstet Gynecol* 1999; **180**:1110-4.
177. Green J, de Gonzalez AB, Smith JS, et al. Human papillomavirus infection and use of oral contraceptives. *Br J Cancer* 2003; **88**:1713-20.
178. de Villiers EM. Relationship between steroid hormone, contraceptives, HPV cervical intraepithelial neoplasia and cervical cancer. *Int J Cancer* 2003; **103**:705-8.
179. Maciag P and Villa LL. Genetic susceptibility to HPV infections and cervical cancer. *Braz J Med Biol Res* 1999; **32**:915-22.
180. Chan S, Ho L, Ong CK et al. Molecular variants of human papillomavirus type 16 from four continents suggests ancient pandemic spread of the virus and its co-evolution with humankind. *J Virol* 1992; **66**:2057-66.
181. Xi L, Kiviat NB, Hildesheim A, et al. Human papillomavirus type 16 and 18 variants: race-related distribution and persistence. *J Natl Cancer Instit* 2006; **98**:1045-52.
182. Yamada T, Manos MM, Peto J, et al. Human papillomavirus type 16 sequence variation in cervical cancers: a worldwide perspective. *J Virol* 1997; **71**:2463-72.
183. Magnusson P, Sparen P and Gyllensten UB. Genetic link to cervical tumors. *Nature* 1999; **400**:29-30.

184. Hemminki, K and Chen B. Familial risk for cervical tumors in full and half siblings: etiologic apportioning. *Cancer Epidemiol Biomarkers & Prev* 2006; **15**:1413-4.
185. Zelmanowicz A, Schiffman M, Hererro R, et al. Family history as a co-factor for adenocarcinoma of the uterine cervix: results from two studies conducted in Costa Rica and the United States. *Int J Cancer* 2005; **116**:599-605.
186. Bayo P, Bosch FX, de Sanjose S, et al. Risk factors of invasive cervical cancer in Mali. *Int J Epidemiol* 2002; **31**:202-9.
187. Mayaud P, Gill DK, Weiss HA, et al. The interrelation of HIV, cervical human papillomavirus and neoplasia among antenatal clinic attenders in Tanzania. *Sex Transm Infect* 2001; **77**:248-54.
188. Li L, Dai M, Clifford GM, et al. Human papillomavirus infection in Shenyang City, People's Republic of China: a population- based study. *Br J Cancer* 2006; **95**:1593-37.
189. Peto J, Gilham C, Deacon J, et al. Cervical HPV infection and neoplasia in a large population-based prospective study: the Manchester cohort. *Br J Cancer* 2004; **91**:942-53.
190. Herrero R, Hildesheim A, Bratti C, et al. Population-based study of human papillomavirus infection and cervical neoplasia in rural Costa Rica. *J Natl Cancer Instit* 2000; **92**:464-74.
191. Moscicki A, Palefsky J, Gozales J, et al. Human papillomvirus infections in sexually active adolescent females: prevalence and risk factors. *Pediatr Res* 1990; **28**:507.
192. Moscicki AB, Ellenberg JH, Vermund SH, et al. Prevalence of and risks for cervical human papillomavirus infection and squamous intraepithelial lesions in adolescent girls: impact of infection with human immunodeficiency virus. *Arch Pediatr Adol Med* 2000; **154**:127-34.
193. Ferreccio C, Prado, RB, Luzoro AV, et al. Population-based prevalence and age distribution of human papillomavirus among women in Santiago, Chile. Cancer *Epidemiol Biomarkers & Prev* 2004; **13**:2271-76.
194. Jacobs M, Walboomers JMM, Snidjers PJF, et al. Distribution of 37 mucosotropic HPV types in women with cytologically normal cervical smears: the age related patterns for high-risk and low-risk types. *Int J Cancer* 2000; **87**: 221-7.
195. Sherman M, Lorincz AT, Scott DR, et al. Baseline cytology, human papillomavirus testing, and risk for cervical neoplasia: A 10-year cohort analysis. *J Natl Cancer Instit* 2003b; **95**: 46-52.
196. Wellings K, Collumbien M, Slaymaker E, et al. Sexual behavior in context: a global perspective. *Lancet* 2006; **368**:1706-28.
197. Munoz N, Mendez F, Posso H, et al. Incidence, duration, and determinants of cervical human papillomavirus infection in a cohort of Colombian women with normal cytological results. *J Infect Dis* 2004; **190**:2077-87.
198. MOH and ORC Macro. Uganda HIV/AIDS Sero - Behavioral Survey 2004-2005. Claverton, Maryland, 2006.
199. Bosch F, Burchell AN, Schiffman M, et al. Epidemiology and natural history of human papillomavirus infections and type specific implications in cervical neoplasia. *Vaccine* 2008; **265**:K1-K16.
200. Lazcano E, Herrero R, Munoz N, et al. Epidemiology of HPV infection among Mexican women with normal cervical cytology. *Int J Cancer* 2001; **91**:412-20.
201. Herrero R, Hildesheim A, Brati C, et al. A population-based study of all grades of cervical neoplasia in rural Costa Rica. *J Natl Cancer Instit* 2000; **92**:464-73.

202. Laukkanen P, Koskela P, Pukkala E, et al. Time trends in incidence and prevalence of human papillomavirus type 6, 11, and 16 in Finland. *J General Virol* 2003; **84**:2105-9.
203. af Geijersstam V, Wang Z, Lewensohn-Fuchs I, et al. Trends in seroprevalence of human papillomavirus type 16 among pregnant women in Stockholm, Sweden during 1969-1989. *Int J Cancer* 1998; **76**:34-4.
204. Castle P, Jeronimo J, Schiffman M, et al. Age-related changes of the cervix influence human papillomavirus type distribution. *Cancer Res* 2006; **66**:1218-24.
205. Franceschi S, Herrero R, Clifford GM, et al. Variation in age-specific curves of human papillomavirus prevalence in women worldwide. *Int J Cancer* 2006; **119**:2677-84.
206. Castle P, Schiffman M, Herrero R, et al., A prospective study of age trends in cervical human papillomavirus acquisition and persistence in Guanacaste, Costa Rica. *J Infect Dis* 2005; **191**:1808-16**.**
207. Tyler K and Nathanson N. Fields Virology. 4th edition *In*: Fields, Knipe, DM and Howley PMJ. editors. Philadelphia: Lippincott Williams & Wilkins, 2001: 220-2.
208. Garcia-Pineres A, Hildesheim A, Hererro R. et al. Persistent human papillomavirus infections associated with a generalized decrease in immune responsiveness in older women. *Cancer Res* 2006; **66**:11070-6.
209. Scott M, Nakagawa M, and Moscicki AB. Cell-mediated immune response to human papillomavirus infections. *Clin Diagn Lab Immunol* 2001; **8**:209-20.
210. Petry K, Scheffel D, Bode U, et al. Cellular immunodeficiency enhances the progression of human papillomavirus–associated cervical lesions. *Int J Cancer* 1994; **57**:836-40.
211. Molano M, van den Brule A, Plummer M, et al. Determinants of clearance of human papillomavirus infections in Columbian women with normal cytology: A population- based, 5-year follow-up study. *Am J Epidemiol* 2003; **158**:486-94.
212. Richardson H, Kelsall G,Tellier P, et al. The natural history of type-specific human papillomavirus infections in female university students. *Cancer Epidemiol Biomarkers & Prev* 2003; **12**:485-90.
213. Woodman C, Collins S, Winter H, et al. Natural history of cervical papillomavirus infection in young women: a longitudinal cohort study. *Lancet* 2001; **357**:1831-36.
214. Stanely M. Immune responses to human papillomaviruses. *Vaccine* 2006; **24 (Suppl 1)**:S16-S22.
215. Plummer M, Schiffman M, Castle PE, Maucort-Boulch D, et al. A 2-year prospective study of human papillomavirus persistence among women with a cytological diagnosis of atypical squamouus cells of undetermined significance or low-grade intra epithelial lesion. *J Infect Dis* 2007; **195**:1582-9.
216. Hildesheim A, Wang SS. Host and viral genetics and risk of cervical cancer: a review. *Virus Res* 2002; **89**:229-40.
217. Strickler H, Palefsky JM, Shah KV, et al. Human papillomavirus type 16 and immune status in human immunodeficiency virus-seropositive women. *J Natl Cancer Instit* 2003; **95**:1062-71.
218. Kahn J. Vaccination as a prevention strategy for human-papillomavirus-related diseases. *J Adol Health* 2005; **37**:S10-S16.
219. Franceschi S and Clifford GM. Re: A study of the impact of adding HPV types to cervical cancer screening and triage tests. *J Natl Cancer Instit* 2005; **97**:938-9.

220. PATH. Cervical cancer vaccine. Available at:http://www.path.org/projects/cervical_cancer_vaccine.php. Accessed 26, August 2009.
221. Wabinga H, Parkin DM, Wabwire-Mangen F, et al. Trends in cancer incidence in Kyadondo County, Uganda, 1960 - 1997. *Br J Cancer* 2000; **82**:1585-92.
222. Mirembe F. The changing pattern of carcinoma of the cervix in Uganda. *In:* Proceedings of the Association of Obstetricians of Uganda. Kampala, 1993.
223. Sekirime SekirimeW and Gray R. HIV infection among Ugandan women with cervical cancer: a retrospective study. *Gynecol & Obstet Invest* 2007; **63**: 222-8.
224. Banura C, Franceschi S, Doorn LJ, et al. Infection with human papillomavirus and HIV among young women in Kampala, Uganda. *J Infect Dis* 2008, **197**:555-62.
225. Safaeian M, Kiddugavu M, Gravitt P, et al. Determinants of incidence and clearance of high-risk human papillomavirus infections in rural, Rakai, Uganda. *Cancer Epidemiol Biomarkers & Prev* 2008; **17**:1300-7.
226. Serwadda D, Wawer, MJ, Shah, KV, et al., Use of Hybrid capture assay of self-collected vaginal swabs in rural Uganda for detection of human papillomavirus. *J Infect Dis* 1999; **180**:1316-9.
227. Newton R, Bousarghin L, Ziegler, J, et al. Human papillomaviruses and cancers in Uganda. *Eur J Cancer Prev* 2004; **13**:113- 8.
228. Bounaguru F, Tornesello ML, Salatiello I, et al. *The Uganda study on HPV variants and genital ulcers.* J Clin Virol 2000; **19**:31-41.
229. Safaeian M, Kiddugavu M, Gravitt P, et al. Determinants of incidence and clearance of high-risk human papillomavirus infections in rural, Rakai, Uganda. *Cancer Epidemiol Biomarkers & Prev* 2008; **17**(6):1300-1307.
230. Bounaguru F, Tornesello ML, Salatiello I, et al. *The Uganda study on HPV variants and genital ulcers.* J Clin Virol 2000; **19**:31-41.
231. Naguru Teenage Information and Health Center, *Annual Report*. 2006: Kampala, Uganda.
232. Sankaranarayanan R, Basu P, Wesley RS, et al. Accuracy of visual screening for cervical neoplasia: results from an IARC multicentre study in India and Africa. *Int J Cancer* 2004; **110**:907-13.
233. Sellors J and Sankaranarayanan R. Colposcopy and treatment of cervical intra-epithelial neoplasia: A beginner's manual. Lyon: IARC, 2003.
234. Downing R, Otten RA, Marum, E, et al. Optimizing the delivery of HIV counseling and testing services: the Uganda experience using rapid HIV antibody test alogarithms. *J Acquir Immune Defic Syndr Hum Retrovirol* 1998; **18**:384-8.
235. Quint K, van Doorn LJ, Kleter B, et al. A highly sensitive, multiplex broad-spectrum PCR-DNA-enzymme immunoassay and reverse hybridization assay for rapid detection and identification of *ChlamydiaTrachomatis* serovars. *J Mol Diagn* 2007; **9**:631-8.
236. Ho B, Feng WG, Wong BK, et al. Polymerase chain reaction for the detection of *Neisseria Gonorrhea* in clinical samples. *J Clin Pathol* 1992; **45**:439-42.
237. Molano M, Posso H, Weidepass E, et al. Prevalence and determinants of HPV infection among Colombian women with normal cytology. *Br J Cancer* 2002; **87**:324-33.
238. UNCST. National Guidelines for Research Involving Humans as Research Participants. Uganda, 2007.
239. UBOS and Macro. Uganda Demographic and Health Survey 2006. Cleverton, USA, 2007.

240. Kailash, U, Hedau S, Gopalkrishna V, et al. A simple "paper smear" method for dry collection, transport and storage of cervical cytological specimens for rapid screening of HPV infections by PCR. *J Med Microbiol* 2002; **51**:606-10.
241. Clifford G, Goncalves AG, Franceschi, S et al. Human papillomavirus types among women infected with HIV: a meta-analysis. *AIDS* 2006; **20**:2337-2344.
242. Thomas J, Herrero R, Omigbodun AA, et al. Prevalence of papillomavirus infection in women in Ibadan, Nigeria: a population-based study. *Br J Cancer*, 2004; **90**:638-45.
243. Clifford G and Franceschi S. HPV in sub-Saharan Africa. *Papillomavirus Report* 2005; **16**:322-6.
244. Baay M, Kjetland EF, Ndhlovu PD, et al. Human papillomavirus in a rural community in Zimbabwe: the impact of HIV co-infection on HPV genotype distribution. *J Med Virol* 2004; **73**:481-5.
245. Kitchener H, Almonte M, Wheeler P, et al. HPV testing in routine cervical screening: cross-sectional data from the ARTISTIC trial. *Br J Cancer* 2006; **95**:56-61.
246. Giuliano A, Harris RB, Sedjo RL, et al. Incidence, prevalence and clearance of type-specific human papillomavirus infections: The Young Women's Health Study. *J Infect Dis* 2002; **186**:462-9.
247. Sellors, Karwalajtsy TL, Kaczorowski J, et al. Incidence, clearance and predictors of human papillomavirus infection in women. *CMAJ* 2003; **168**:421-5.
248. Oh Oh JK, Ju YH, Franceschi S, et al. Acquisition of new infection and clearance of type-specific human papillomavirus infections in female students in Busan, South korea: a follow-up study. *BMC Infectious Diseases* 2008; **18**:13.
249. Strickler H, Burk RD, FazzariM, et al. Natural history of human papillomavirus in human immuno-deficiency virus-positive women. *J Natl Cancer Instit* 2005; **97**:577-86.
250. Shew M, Fortenberry JD, Tu W, et al. Association of condom use, sexual behaviors, and sexually transmitted infections with the duration of genital human papillomavirus infection among adolescent women. *Arch Pediatr Adolesc Med* 2006; **160**:151-6.
251. International Collaboration of Epidemiological Studies of Cervical Cancer. Cervical carcinoma and reproductive factors: collaborative reanalysis of individual data on 16,563 women with cervical carcinoma and 33,542 women without cervical carcinoma from 25 epidemiological studies. *Int J Cancer* 2006; **119**:1108-24.
252. International Collaboration of Epidemiological Studies of Cervical cancer. Cervical cancer and hormonal contraceptives: collaborative reanalysis of individual data on 16,573 women with cervical cancer and 35,509 women without cervical cancer from 24 epidemiological studies. *Lancet* 2007; **370**:1609-21.
253. Vacarella S, Herrero R, Dai M, et al. Reproductive factors, oral contraceptive use and HPV infection: pooled analysis of IARC HPV Prevalence surveys. *Cancer Epidemiol Biomarkers & Prev* 2006; **15**:2148-53.
254. Druckmann J, Druckmann MA. Progesterone and the immunology of pregnancy. *J SteroidBiochem Mol Biol* 2005; **97**:389-96.
255. Dillner, J., M. Arbyn, and L. Dillner, *Translational mini-review series on vaccines: Monitoring human papillomavirus vaccination,*. Clinical and Experimental Immunology, 2007. **148**: p. 199 - 207.

256. Sethi S, Muller M, Schneider A, et al. Serologic response to E4, E6 and E7 proteins of human papillomavirus type 16 in pregnant women. *Am J Obstet & Gynecol* 1998; **178**:360-4.
257. Chang-Claude J, Schneider A, Smith E, et al. Longitudinal study of the effects of pregnancy and other factors on detection of HPV. *Gynecol Oncol* 1996; **60**:355-62.
258. Schiffman M, Castle PE, Jeronimo J, et al. Papillomavirus and cervical cancer. *Lancet* 2007; **370**:890-907.
259. Sherman M, M, Schiffman MH, Lorincz AT, et al. Cervical specimens collected in liquid buffer are suitable for both cytologic screening and ancillary human papillomavirus testing. *Cancer* 1997; **81**:89-97.
260. van Ham M, Melchers WJ, Hanselaar AG, et al. Fluctuations in prevalence of cervical papillomavirus in women frequently sampled during a single menstrual cycle. *Br J Cancer* 2002; **87**:373-6.
261. Quint W, Scholte G, van Doorn LJ, et al. Comparative analysis of human papillomavirus infections in cervical scrapes and biopsy specimens by general SPF10 PCR and HPV genotyping. *J Pathol* 2001; **194**:154-8.
262. Moscicki A, Shiboski S, Broering J, et al. The natural history of human papillomavirus infection as measured by repeated DNA testing in adolescents and young women. *J Pediatr* 1998; **132**:277-84.
263. Manhart L, Aral SO, Holmes KK, et al. Influence of study population on the identification of risk factors for sexually transmitted diseases using a case-control design: the example of gonorrhea. *Am J Epidemiol* 2004; **160**:393-402.
264. Palefsky J and Holly EA. Chapter 6: Immunosuppression and co-infection with HIV. *J Natl Cancer Instit Monogr* 2003; **31**:41-46.
265. Conley L, Ellerbrock TV, Bush TJ et al. HIV-1 and risk of vulvovaginal and perianal condylomata acuminate and intraepithelial neoplasia. *Lancet* 2002; **359**:108-13.
266. Ashley S, Rocconi RP, Straughn MJ, et al. Cervical intra-epithelial neoplasia in adolescent women: incidence and treatment outcomes. *Obstet & Gynecol* 2006; **108**:1369-74.
267. WHO, *Comprehensive Cervical cancer Control: A guide to essential practice.* 2006, Geneva, Switzerland. 11.
268. Mutyaba T, Mmiro FA, Weiderpass E. Knowledge, attitudes, and practices on cervical cancer screening among medical workers of Mulago hospital, Uganda. *BMC Medical Education* 2006; **6**:13.
269. WHO. Comprehensive Cervical cancer Control: A guide to essential practice. Geneva, Switzerland: WHO. 2006.
270. Block, Nolan T, Sattler C, et al. Comparison of immunogenicity and reactogenicity of a prophylactic quadrivalent human papillomavirus L1-virus-like particle in male and female adolescent and young adult women. *Pediatrics* 2006; **118**:2135-45.
271. Garnett G. Role of herd immunity in determining the effect of vaccines against sexually transmitted disease. *J Infect Dis* 2005; **191(Suppl 1)**:S97-S106.
272. Paavonen J, Jenkins D, Bosch FX, et al. Efficacy of a prophylactic adjuvanted L1 virus-like-particle vaccine against infection with human papillomavirus types 16 and 18 in young women: an interim analysis of a phase III double-blind randomized controlled trial. *Lancet* 2007; **369**:2161-70.
273. Hulley S and Cummings S. Designing Clinical Research: An epidemiologic approach. Baltimore, MD,USA: Williams & Wilkins. 1988.

12 APPENDICES

12.1 SAMPLE SIZE CALCULATION FOR STUDY AIMS I, II AND III

The study sample size was decided to ensure a precision of the estimated HPV prevalence of ±2.5% or better, ensuring that the 95% confidence interval is no wider than 5% (i.e. 0.05)

- We assumed the prevalence of HPV among urban- based women aged below 25 years to be 50%. The assumption was based on results of a study conducted in rural Uganda [226] which found the prevalence of HPV infection in HIV infected adolescents and young women to be approximately 75% among 15-19 year olds and 55% among 20 –29 year old, respectively. However, the estimate was based on a small sample size of adolescents and young women aged below 30 years. However, in HIV negative adolescents and young women, the HPV prevalence was significantly lower. The prevalence rate among the 15-19 year old was about 25% and about 20% in 20-29 year old women.

Note: Since the precision is lowest for 50% we did the sample size calculation assuming 50%. Higher or lower prevalence will only give us better precision.

According to Hulley & Cummings (1988) [273]

$$\mathbf{n \geq (z/m)^2 \times p(1 - p)}$$

where n = sample size

$z_\alpha = 1.96$ which is the standard normal deviate for α, when $\alpha = 0.05$

m = the intended precision in terms of ±m/100, e.g 0.025 for ±2.5%

p = prevalence

Thus, $n \geq [(1.96/0.025)^2 \times 0.5 \times 0.5] = 1{,}536$ women.

- During the pilot study, about 7.5% of eligible women refused to participate in our study. Therefore, we increased the calculated sample size by an additional 115 women giving us a total sample size of 1, 651 women.

12.2 SAMPLE SIZE CALCULATION FOR STUDY AIM IV

The study sample size was decided to ensure a precision of the estimated HPV prevalence of ±2.5% or better, ensuring that the 95% confidence interval is no wider than 5% (i.e. 0.05)

According to Hulley & Cummings (1988) [273]

$$\mathbf{n \geq (z/m)^2 \times p\,(1 - p)}$$

where n = sample size

z_α = 1.96 which is the standard normal deviate for α, when α = 0.05

W = width of the margin of error for 0.05 or 0.025 for two tailed test

Thus, $n \geq (1.96/0.025)^2 \times 0.2 \times 0.8) = 983$ women

We assumed that about 2% of women (19 women) would refuse to participate in the study and 10% (98) women would not return for the postnatal visit, which gave us a total sample size of 1,100 women.