RESEARCH

Proscovia Bazanya Namujju

Occurrence of infections with multiple human papillomavirus (HPV) types among Finnish and Ugandan women

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Occurrence of infections with multiple human papillomavirus (HPV) types among Finnish and Ugandan women

ACADEMIC DISSERTATION

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Dedicated
To my beloved grandfather Lauli Busulwa and my daughters
Cephas Petronela and Catherine Olive

Many are the plans in a man's heart, but it is the LORD's purpose that prevails.
Proverbs 19:21

Abstract

Cervical cancer (CC) is a major public health problem in women, especially in Sub-Saharan Africa. The prevalence of oncogenic, high-risk (hr) human papillomaviruses (hrHPVs), the sexually transmitted, necessary causative agents of CC, is on the increase due to increasing risk-taking sexual behaviour among the young. Despite the transient nature of HPV infection, infections of hrHPV types are associated with viral persistence and increased risk for cervical intraepithelial neoplasia (CIN) development. The reported overall efficacy of licenced, prophylactic HPV vaccines against CIN grade 3 is between 93 and 100% in developed countries. However, it is necessary to understand the distribution of genital (both low risk, lr and hr) HPV types, and factors that may interfere with the immunogenicity and efficacy of the prophylactic HPV vaccines in Sub-Saharan countries before mass HPV vaccination is implemented.

In this dissertation we use serum antibodies, a stable marker of cumulative (past and present) HPV exposure to study the occurrence of anogenital HPV infections and associated risk factors among fertile-aged Finnish and Ugandan women.

A cross-sectional study was conducted among Finnish pregnant women who donated blood samples to the Finnish Maternity Cohort (FMC) from 1995 to 2003 (Papers I and IV). Ugandan pregnant women were recruited in two phases in 2004 and again in 2008 (Papers I, II and IV). In a prospective study (Paper III) we used serum samples collected at baseline and at the end of the follow-up of the women from the Finnish Kuopio cohort. The samples were analysed for antibodies against 7 HPV types (6/11/16/18/31/33/45) using standard VLP-based ELISAs (Papers I, III) and a modified VLP-based ELISA (Paper IV), and using Multiplex serology for antibodies against 8 oncogenic HPV types (16/18/31/33/35/45/52/58) (Paper II). Other standard laboratory measurements were serum cotinine (smoking), HIV and *Chlamydia trachomatis* antibodies tests. In addition, questionnaire data for risk factors for acquiring genital HPV infections were available for the Ugandan women.

We found that seropositivity to multiple HPV types was highly prevalent in both Finnish (any type=44%, multiple types =22%) and Ugandan women (any type 57%, multiple types 33%). In both groups the prevalence of HPV was highest among the young women under 25 years of age. It was higher among Ugandan than Finnish

women. The most common virus types (in descending order) were: HPV16, 18, 31 and 33 (Finland) and HPV33, 16, 31, 45 (Uganda).

The risk of being seropositive for another given HPV type was significantly increased among women seropositive for a specific HPV type compared to seronegative women for that particular type (Paper I). Among HPV45- seropositive Finnish women the risk of double seropositivity for HPV18, 31, and 33 was significantly higher than among Ugandan women (Paper I). We also observed that age, education, parity and HIV were risk factors for hrHPV seropositivity, but HIV was the only independent, stand-alone risk factor of seropositivity for multiple hrHPV types among Ugandan women (OR 1.7, 95%CI 1.0-2.8) (Paper II).

In the prospective study, baseline HPV16 seropositive women had a significantly increased risk of seroconversion to phylogenetically related HPV types 31 and 33 compared to seronegative women. On the other hand, seropositivity for lrHPV types increased the risk of seroconversion to hrHPV types (OR 2.3, 95%CI 1.1-4.7) (Paper III). Moreover, we observed that low avidity HPV16 antibodies (suggesting acute infections or persistent low avidity) were common (18%) and associated with the risk of being seropositive for lrHPV types among Ugandan women (OR 2.2, 95%CI 1.01-8.4) (Paper IV).

Sizeable study cohorts allowed thorough comparison of variables. The pregnant women studied represent the majority of sexually active young/fertile-aged women. Type-specificity of the antibody tests and stability of the HPV antibodies allowed analysis of cumulative HPV infections during a period of more than five years. There were, however, also limitations in our studies; the cross-sectional nature of the two studies did not allow us to assess the order of acquisition of HPV infection, and in the prospective study the exact acquisition time could not be determined. We also lacked HPV DNA data and CD4 counts to distinguish current HPV infections from past infections, and to assess the immunological status of the HIV-positive study subjects, respectively.

Our results indicate that the risk of acquiring multiple HPVs is high among women exposed to any HPV type, but especially among women already infected with HPV16 and HPV18. Viral clearance in individuals with multiple HPV infections is exclusively determined by the HIV status. Therefore prophylactic mass HPV vaccination will be crucial in the control of the hrHPV types and associated disease burden in the young women of countries like Uganda, where HIV is endemic. Data on HPV vaccine efficacy and its determinants in Sub-Saharan Africa is urgently needed.

Tiivistelmä

Kohdunkaulansyöpä on suuri naisten kansanterveydellinen ongelma, erityisesti Saharan alapuolisessa Afrikassa. Syöpävaarallisten, korkean riskin (hr), ihmisen papilloomavirus (HPV) tyyppien, jotka ovat kohdunkaulansyövän syy, esiintyminen on noususssa nuorten kasvaneen seksuaalisen riskikäyttäytymisen vuoksi. Ohimenevästä luonteestaan huolimatta, hrHPV infektioihin voi liittyä pitkittymistä ja lisääntynyt riski kehittää kohdunkaulansyövän esiaste (CIN). Lisensoitujen, ennaltaehkäisevien rokotteiden raportoitu kokonaisteho CIN luokka 3 muutosta vastaan on kehittyneissä maissa 93 ja 100% välillä. On kuitenkin välttämätöntä tuntea genitaali-infektioita aiheuttavien HPV tyyppien (sekä matalan riskin, lr että hr tyypit), ja tekijöiden, jotka voivat häiritä ennaltaehkäisevien rokotteiden immunogeenisuutta ja tehoa, esiintyvyys ennen kuin HPV joukkorokotukset Saharan alapuolisessa Afrikassa pannaan toimeen.

Tässä väitöskirjatyössä käytimme seerumin vasta-aineita, stabiilia kumulatiivisten (aikaisempien ja nykyisten) HPV altistusten markkeria tutkittaessa anogenitaalisten HPV infektioiden esiintymistä ja niihin liittyviä riskitekijöitä fertiili-ikäisillä suomalaisilla ja ugandalaisilla naisilla.

Teimme poikkileikkaustutkimuksen suomalaisilla raskaana olevilla naisilla, jotka olivat antaneet verinäytteen Finnish Maternity Cohort (FMC) aitiysneuvolaseerumipankkiin 1995 - 2003 (Työt I ja IV). Ugandalaiset raskaana olleet naiset rekrytoitiin kahdessa vaiheessa 2004 ja 2008 (Työt I, II ja IV). Prospektiivisessa tutkimuksessa (Työ III) käytimme seeruminäytteitä, jotka oli kerätty seurannan alku- ja päätösvaiheessa naisilta, jotka kuuluivat suomalaiseen Kuopio kohorttiin. Näytteet analysoitiin 7 HPV tyypin (6/11/16/18/31/33/45) vasta-aineiden suhteen käyttäen standardi VLP-pohjoista ELISA menetelmää (Työt I, III) ja muunneltua VLP-pohjoista ELISAa (Paper IV), ja Multiplex serologiaa kahdeksan syöpävaarallisen HPV tyypin (16/18/31/33/35/45/52/58) vasta-aineiden mittaamiseen (Työ II). Muita standardi laboratoriomittauksia olivat seerumin cotiniini (tupakointi), HIV and *Chlamydia trachomatis* vasta-ainetestit. Lisäksi, ugandalaisilta naisilta oli käytössä kyselytietoa genitaali HPV-infektioiden saannin riskitekijöistä.

Havaitsimme, että seropositiivisuus useilla HPV tyypeille oli hyvin yleistä sekä suomalaisilla (mikä tahansa HPV tyyppi=44%, useampi tyyppi=22%) että ugandalaisilla naisilla (mikä tahansa HPV tyyppi=57%, useampi tyyppi=33%). Molemmis-

sa ryhmissä HPV esiintyvyys oli korkein nuorilla, alle 25 -vuotiailla. Esiintyvyys oli korkeampaa ugandalaisilla kuin suomalaisilla naisilla. Yleisimmät HPV tyypit olivat (alenevassa järjestyksessä): HPV16, 18, 31 ja 33 (Suomi) ja HPV33, 16, 31, 45 (Uganda).

Riski olla seropositiivinen tietylle HPV tyypille oli kohonnut niiden naisten joukossa, jotka olivat seropositiivisia jonkin toisen, spesifisen HPV tyypin suhteen verrattuna tämän HPV tyypin suhteen seronegatiivisiin naisiin (Työ I). HPV45 seropositiivisilla suomalaisnaisilla riski olla tupla- seropositiivisia sen ja HPV tyyppien 18, 31, ja 33 suhteen oli merkitsevästi korkeampi kuin ugandalaisnaisilla (Työ I). Havaitsimme myös, että ikä, koulutus, pariteetti ja HIV olivat hrHPV seropositiivisuuden riskitekijöitä, mutta että HIV oli ainoa itsenäinen useamman hrHPV tyypin seropositiivisyyden riskitekijä ugandalaisnaisilla (OR 1.7, 95%CI 1.0-2.8) (Työ II).

Prospektiivisessa työssä alkuhetkellä HPV16 seropositiivisilla naisilla oli merkitsevästi kohonnut riski serokonvertoida fylogeneettisesti sukulais-HPV tyyppien 31 ja 33 suhteen verrattuna seronegatiivisiin naisiin. Toisaalta seropositiivisuus lrHPV tyyppien suhteen lisäsi riskiä serokonvertoida hrHPV tyyppien suhteen (OR 2.3, 95%CI 1.1-4.7) (Työ III). Lisäksi havaitsimme, että ugandalaisnaisilla matalan aviditeetin HPV16 vasta-aineet (mahdollisesti merkkinä akuutista infektiosta tai pitkittyneestä matalasta aviditeetista) olivat yleisiä (18%) ja liittyivät riskiin omata vastaaineita lrHPV tyypeille (OR 2.2, 95%CI 1.01-8.4) (Työ IV).

Kookkaat tutkimuskohortit mahdollistavat muuttujien perinpohjaisen vertailun. Raskaana olevat naiset edustavat enemmistöä seksuaaliaktiivisesta nuorista/fertiiliikäisistä naisista. HPV vasta-aineiden tyyppispesifisyys ja stabiilius mahdollistivat analyysit HPV infektioiden kumuloitumisesta yli viiden vuoden aikana. Tutkimuksellamme oli myös rajoituksensa, kahden työn poikkileikkausluonne ei mahdollistanut saatujen HPV-infektioiden järjestyksestä, eikä prospektiivisessakaan tutkimuksessa tarkkaa infektion saantiajankohtaa voitu määrittää. Meiltä puuttui HPV DNA tiedot, ja CD4 määriä koskevat tiedot, jotta olisimme voineet erottaa tuoreet HPV infektiot aikaisemmista infektioista, ja arvioida immuniteetin tilaa HIV-positiivisilla tutkittavilla.

Tuloksemme osoittavat, että useiden HPV infektioiden riski on korkea naisilla, jotka ovat altistuneet yhdellekään HPV tyypille, mutta erityisesti tyypeille HPV16 ja HPV18. Virusten poistumiseen yksilöillä, joilla on useita HPV tyyppien aiheuttamia infektioita, vaikuttaa HIV positiivisuus. Tämän vuoksi ennaltaehkäisevät joukkorokotukset ovat keskeisen tärkeitä kontrolloitaessa hrHPV tyyppien aiheuttamaa tautitaakkaan nuorilla naisilla, Uganda tapaisissa maissa, joissa HIV on endeeminen.

Tietoa HPV rokotteen tehosta ja siihen vaikuttavista tekijöistä Saharan alapuolisessa Afrikassa kaivataan kipeästi.

Avainsanat: ihmisen papilloomavirus, HPV, vasta-aineet, immuunikatovirus, HIV, seroprevalenssi, serokonversio, vasta-aineen sitoumuskyky, raskaana oleva nainen

Contents

List of original publications

This dissertation is based on the following original articles referred to in the text by their Roman numerals:

I   **Namujju PB**, Surcel HM, Kirnbauer R, Kaasila M, Banura C, Byaruhanga R, Muwanga M, Mbidde EK, Koskela P, Lehtinen M. Risk of being seropositive for multiple human papillomavirus types among Finnish and Ugandan women. Scand J Infect Dis. 2010; 42: 522-6.

II   **Namujju PB**, Waterboer T, Banura C, Muwonge R, Mbidde EK, Byaruhanga R, Muwanga M, Surcel HM, Pawlita M, Lehtinen M .Risk of seropositivity to multiple oncogenic human papillomavirus (HPV) types among HIV positive and HIV negative Ugandan women. J Gen Virol. 2011;92: 2776-83.

III   Palmroth J, **Namujju P**, Simen-Kapeu A, Kataja V, Surcel HM, Tuppurainen M, Yliskoski M, Syrjänen K, Lehtinen M. Natural seroconversion to high-risk human papillomaviruses (hrHPVs) is not protective against related HPV genotypes. Scand J Infect Dis. 2010; 42:379-84.

IV   **Namujju PB**, Hedman L, Hedman K, Banura C, Mbidde EK, Kizito D, Byaruhanga RN, Muwanga M, Kirnbauer R, Surcel HM, Lehtinen M. Low avidity of human papillomavirus (HPV) type 16 antibodies is associated with increased risk of low-risk but not high-risk HPV type prevalence. BMC Res Notes. 2011 6; 4:170.

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Abbreviations

Ab	antibody
Ag	antigen
AI	avidity index
AIDS	Acquired Immunodeficiency Syndrome
AP	associated protein
APC	antigen presenting cells
ASCUS	atypical squamous cells of undetermined significance
ASR	age standardized incidence rate
CA	cervical adenocarcinoma
CC	cervical cancer
CI	confidence interval
CIN	cervical intraepithelial neoplasia
CIS	carcinoma in situ
CMI	cell-mediated immunity
CTL	cytolitic T cell
DNA	Deoxyribonucleic acid
E1-E7	early proteins
ECM	extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Authority
FMC	Finnish Maternity Cohort
HC	hybrid capture
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPV	human papillomavirus
hr	high-risk
HSIL	high-grade squamous intraepithelial lesion
HSPG	heparin sulphate proteoglycans
HSV	herpes simplex virus
IARC	International Agency for Research on Cancer
ICC	invasive cervical cancer
ICTV	Internation Committee on Taxonomy of Viruses
IFN	interferon
IgG	immunoglobulin class G
IS	international standard
KC	Kuopio Cohort
L1/L2	late proteins
LBC	liquid based cytology
lr	low-risk

LSIL	low-grade squamous intraepithelial lesion
MFI	median fluorescence intensity
MHC	major histocompatibility complex
NK	natural killer cells
OD	optical density
OR	odds ratio
ORF	open reading frame
P53	protein 53
Pap	Papanicolau
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIN	personal identification number
pRb	retinoblastoma tumor suppressor protein
PsVs	pseudovirions
RNA	ribonucleic acid
RR	relative risk
SCC	squamous carcinomas
SIL	squamous intraepithelial lesion
STD	sexually transmitted disease
STI	sexually transmitted infection
TGF	tumour growth factor
TNF	tumour necrosis factor
VIA	visual inspection with acetic acid
VILI	visual inspection with Lugol's iodine
VLP	virus-like particle
WHO	World Health Organization

1 INTRODUCTION

Cervical cancer (CC) is the third most common cancer among females worldwide with 529,800 new cases and 275,100 deaths in 2008 (Ferlay et al. 2010, Jemal et al. 2011). The highest incidence (>80% of cases) and the highest mortality of CC occur in developing countries, most notably Sub-Saharan Africa (Sankaranarayanan 2006, Jemal et al. 2011, Arbyn et al. 2011) with no organized population based CC screening/treatment programmes. In the developing countries, most women only seek health care at the symptomatic/final stages of CC, which results in high mortality (Wabinga et al. 2003), while in the developed countries both incidence of and mortality from CC have markedly decreased due to population-based organized cytological screening programmes (Bulk et al. 2005, Arbyn et al. 2007, 2009). The incidence of cervical adenocarcinoma (CA) is, however, on the increase, especially among young women (Bray et al. 2005, Bulk et al. 2005, Pettersson et al. 2011). Sampling of the columnar epithelial cells giving rise to CA is difficult with the available screening techniques and new oncogenic, high-risk (hr) human papillomavirus (HPV) types infecting especially these cells have emerged (Bray et al. 2005, Castellsague et al. 2006).

Genital infection with hrHPVs is very common (Koutsky 1997, de Sanjose et al. 2007) with peak prevalence in women under 30 years of age (Kjaer et al. 2007, Smith et al. 2008). The most notable hrHPV types are 16 and 18 (Durst et al. 1983, Boshart et al. 1984). In 90% of immuno-competent individuals clear these viruses spontaneously within 18 months of infection (Bulkmans et al. 2007, Stanley 2010a). Persistent hrHPV infections in 10% of women may progress via various grades of cervical intraepithelial neoplasia (CIN) to cervical cancer (CC). HPV16 /18 are responsible for >70% of CC (Lehtinen et al. 2001, IARC Monograph 2007, Li et al. 2011).

Multiple genital HPV infections are common (Thomas et al. 2000, Rousseau et al. 2003, Soto-De et al. 2011). Twenty to 50% of infected women harbour multiple hrHPV types (Liaw et al. 2001, Cuschieri et al. 2004, Clifford et al. 2005, Vaccarella et al. 2010, Chaturvedi et al. 2011, Weaver et al. 2011). Occurrence of multiple hrHPV types is associated with persistent infection and increased risk of cervical cancer (Ho et al. 1998, Kjaer et al. 2005, Trottier et al. 2006).

Quadrivalent (HPV6/11/16/18) or bivalent (HPV16/18) virus-like particle (VLP) vaccines comprising the major L1 structural proteins of the non-oncogenic and/or oncogenic HPV types have been licenced (Markowitz et al. 2007, FDA

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www.fda.gov/news release . 2010, EMA www.ema.europa. eu/ PDFs_Gardasil 2006 accessed 2010, EMA, www.europa.eu/humandocs/PDFs/ Cervarix 2007 accessed 2010). They have both shown 100% immunogenicity and more than 90% efficacy against persistent HPV16/18 infections and HPV16/18 associated CIN2/3 (Harper et al. 2006, Villa et al. 2006a,b, Paavonen et al. 2009, Lehtinen et al. 2012) in the developed countries. The vaccines are being administered in developing countries (Anon 2011) but so far no HPV vaccine trials have been completed in Sub-Saharan Africa. To establish most efficient mass HPV vaccination programmes in the latter, it is necessary to understand the distribution of HPV types and factors which may interfere with the immunogenicity and the efficacy of the vaccines in Sub-Saharan Africa.

The stability of serum HPV antibodies makes serology a useful tool for epidemiological studies on HPV occurrence. Concomitant seropositivity to multiple HPV types has been reported (Carter et al. 2000, Kaasila et al. 2009, Porras et al. 2010, Merikukka et al. 2011), but the protective role of antibodies acquired from natural HPV infections is unclear. Some studies have reported increased risk of acquiring new HPV infections in individuals exposed to one HPV type (Lehtinen et al. 2006, Kaasila et al. 2009, Merikukka et al. 2011) whereas others have reported protection against new infections (Ho et al. 2002, Malik et al. 2009). Factors affecting the co-existence/acquisition of different HPV types need to be understood for optimal use of HPV vaccines (Tornesello et al. 2007).

2 LITERATURE REVIEW

2.1 Biology of HPV

Human papillomavirus is a member of the Papillomaviridae family as designated by the International Committee on Taxonomy of Viruses (ICTV) that infects only humans (ICTV Virus Taxonomy 2009). Like all papillomaviruses, HPV establishes productive infections only in the epithelium of the skin or mucosa. Molecular biology has made it possible to study the interactions of viral gene expression and replication with the host cell phenotype (IARC Monographs 2007). Major breakthroughs in the understanding of the viral oncoprotein, most notably the E5/6/7 proteins, activities of hrHPV types to the integrity of cellular DNA and cell-cycle control were made already in the 1990's.

2.1.1 Classification of HPV

HPV belongs to the Papillomaviridae family (ICTV Virus Taxonomy 2009). Currently, 120 different HPV genotypes that infect humans have been classified (Bernard et al. 2010) and are allocated a type number according to the order of discovery. The taxonomy is based on comparison of the nucleotide sequences and homology of the L1 ORF. A new type is named if the difference in the DNA sequence of the L1 gene is > 10% from that of the closest HPV type known (de Villiers et al. 2004).

Approximately 40 HPV types infect the epithelial lining of the anogenital mucosae and cause warts and cancerous lesions of the cervix, vulva, vagina and anus in women and the penis and anus in males (IARC monograph 2007, Stanley 2010b). The HPV types that belong to the genus Alphapapillomavirus are predominately detected in the mucosal epithelium (de Villiers et al. 2004). The oncogenic HPV types associated with genital intraepithelial neoplasia and cancer are referred to as thirteen high-risk types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68), these are further grouped according to their phylogenetic relatedness into clade A7 (HPV18, 39, 45, 59, 68), clade A9 (HPV16, 31, 33, 35, 52, 58) and clade A5 (HPV51, 69) species (de Villiers et al. 2004, Schiffman et al. 2009). Those HPVs found in benign genital warts (condylomata accuminata) are referred to as "low-risk" (lr) types and belong mostly to clade A10 (HPV6, 11, 13, 44, 55) (Munoz et al. 2003).

2.1.2 Structure and genome of the papillomavirus

Papillomavirus is a small (52-55nm diameter), non-enveloped, epitheliotropic, double stranded DNA virus. The virion comprises double-stranded DNA, and a capsid with the major (L1) and minor (L2) proteins of 55kDa and 70 kDa, which comprise 80% and 20% of the total virion protein content, respectively (Doorbar 2005). The L1 and L2 proteins assemble into capsomers, during the generation of progeny virions (Fehrmann and Laimins. 2003).

Figure 1. The genome structure and functional parts of the open reading frame (ORF) of HPV

- E1-Rreplication
- E2- replication/transcription
- E4- viral release
- E5- immune evasion
- E6-binds P53
- E7- binds pRb
- LCR- regulatory elements
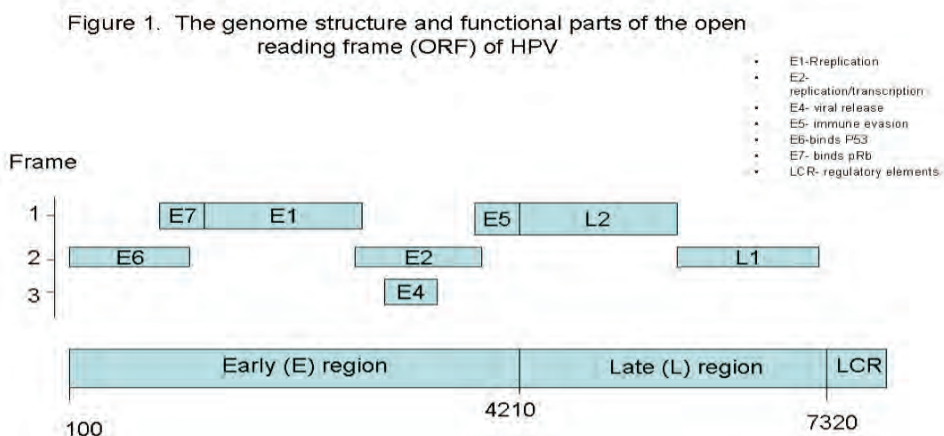
Figure 1. Genome structure of HPV

The genome of all HPV types comprises eight open reading frames (ORFs) (E1, E2, E4, E5, E6, E7, L1, and L2) that are transcribed from a single DNA strand of about 8,000 base pairs. There are three functional parts in the genome; the early (E) region that encodes the proteins (E1-E7) responsible for viral replication (E1, E2), transcriptional regulation (E2) and cellular transformation (E5, E6, E7); the late (L) region that encodes the structural proteins (L1 and L2) that are necessary for virion assembly; and the long control region (LCR), which contains cis-regulatory elements that are necessary for the replication and transcription of viral DNA (Figure 1). The viral proteins E1 and E2 act as factors that recognize the origin of replication and E2 is the major regulator of viral transcription. E4 encodes a protein required for final stages of replication, while the E5 protein functions in both early and late phases (Doorbar et al. 2005). The most important oncogenic HPV proteins are E6

and E7, which target a number of regulators of the cell cycle most importantly p53 and p105Rb respectively (Münger and Howley 2002).

2.1.3 Lifecycle of HPV

Mechanisms of viral entry

HPV replicates only in epithelial cells. The virus enters basal epithelial cells through micro-wounds or micro-abrasions. This is why early age at first sexual debut, coarsed sex and other sexually transmitted infections (STIs) enhance virus entry (Stanley 2010a). Receptors such as glycosaminoglycans (GAGs), most notably heparan sulphate proteoglycans (HSPG) which are frequently found in the extracellular matrix (ECM) and on the surface of most cells, are suggested to be the sites of initial attachment for HPV virions (Sapp and Bienkowska-Haba 2009). Other studies suggest that the virus first binds heparan at the basement membrane, and later to a cellular receptor on the keratinocyte (Kines et al. 2009).

HPV entry into the target keratinocyte is a very slow process, which can take up to 12 hours (Sapp and Day 2009). The L1 antibodies can block the virus attachment, but may also interfere with post attachment interactions (Stanley 2010a). Neutralizing HPV L1 antibodies needed to block the basement membrane binding are effective at very low concentrations (Stanley 2010a). However, higher HPV antibody levels of high affinity are required for cross-protection.

Viral replication

Once inside the cell, the HPV life-cycle strictly follows the differentiation of the host keratinocytes. First, the viral DNA is uncoated and its genome is transported to the nucleus taking advantage of the cellular machinery to replicate its genome with a high degree of proof reading. In the nucleus, the viral DNA-binding proteins E2 and E1, responsible respectively for viral DNA transcription and replication are first expressed. At this stage, the infected cells usually contain small numbers of viral copies (10-200 copies per cell) (Doorbar 2007). The proliferating cells move towards the outer layer of the epithelium, and the viral replication is strictly controlled to prevent the viral genome being amplified and expressed too early in order to avoid immune recognition. When the host keratinocyte reaches the S-phase in the differentiation compartment of the epithelium, viral E4 protein activates the productive phase of the viral replication and the genome copy number increases up to 1,000 copies per cell. At this stage, the L1 and L2 genes are transcribed and the corresponding proteins are translated and serve as structural proteins that assemble into capsids, which encapsidate the viral genomes forming the progeny viral particles.

The virions are sloughed off with the dead squamous cells of the host epithelium (Stanley 2007) for further transmission of the new virions (Figure 2).
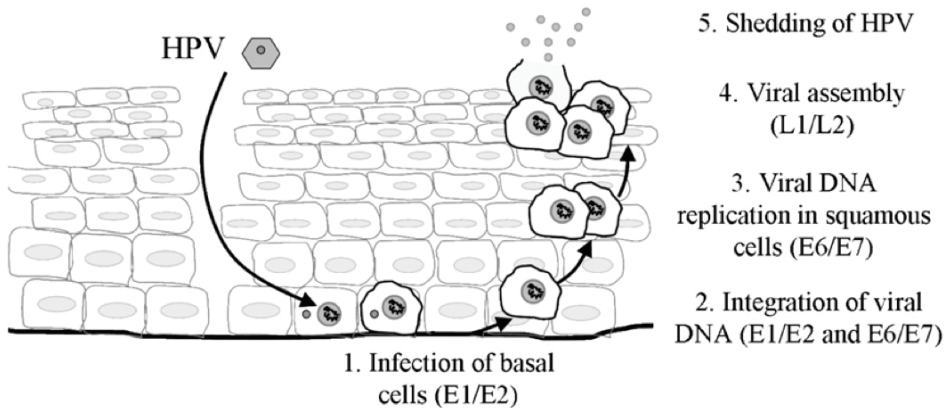
Figure 2. Life cycle of genital human papillomavirus (Gynecol Oncol 2008; 109:s15-s21). Reproduced with permission from the publishers

Viral transformation and oncogenic pathways

In the replicative cycle the viral E5, E6 and E7 proteins responsible for the transformation of infected cells are expressed in keratinocytes doomed to die. In contrast in the basal cells, the oncogenic E5, E6 and E7 genes are expressed to establish and maintain viral persistence in keratinocytes, which gradually turn from differentiating into transformed cells (Stanley 2010b).

During the transformation process the E5 protein interacts with the 16kD subunit of the protein-pump ATPase, which inhibits the acidification of endosomes and the gap-junction intercellular communication in the keratinocytes. This facilitates activation of epidermal growth factor (EGF) receptor tyrosine kinase and its signaling pathway. Hence an increase in the mitogenic stimulus occurs following E5 expression from the growth factor receptor to the nucleus (DiMoio and Mattoon 2001, Auvinen et al. 2004). On the other hand, E5 also modulates expression of a number of host cell microRNAs, which suggests its role may be more complex (Greco et al. 2011)

E6 protein, in association with host E6-associated protein (AP), has ubiquitin ligase activity and ubiquitinates p53 to facilitate its proteosomal degradation. On the other hand, E7 protein of the oncogenic HPV acts as the primary transforming protein. E7 protein competes for pRb binding, and enables transcription factor E2F to transacti-

vate its targets, thus pushing the cell cycle forwards. The net effect shut off of the main cancer-controlling genes p53 and pRB leads to cellular transformation.

2.1.4   HPV Transmission

The main route for the transmission of genital HPV types is sexual contact. Approximately, 95% of individuals infected with genital HPV acquired the virus through sexual contact (Fairley 1993, Kjaer et al. 2001). Several studies have reported sexual debut and number of sexual partners as the most notable risk factors for the acquisition of genital HPVs (Dillner et al. 1996, Auvinen et al. 2005, Barthell et al. 2009, Malik et al. 2009, Luovanto et al. 20. 2011). Hernandez et al. (2008) the estimated overall HPV transmission rate from penis to cervix and from cervix to penis, to be 60/100 and 210/100 person years respectively. In a longitudinal couple study the overall transmission probability was calculated to be 42% (95% CI 36-47) per partnership and 0.80% (0.6-1.0) per act (Burchell et al. 2006a,b, 2010). The highest prevalences of single and multiple HPV infections based on both HPV DNA and serology are found in young adult women under <30 years of age (Mendez et al. 2005, Kaasila et al. 2009, Banura et al. 2010).

Vertical transmission is an infrequent mode of HPV transmission. A study in Denmark showed that women who were virgins at baseline and engaged in sexual activity during follow-up, became HPV16 antibody and/or HPV16 DNA positive (Kjaer et al. 2001). There is, however, evidence that 15-18% of genital and 10-21% of oral HPV infections can be transmitted from an infected mother to an infant between birth and six months after delivery (Medeiros et al. 2005, Rintala et al. 2005).

Sexual transmission in children associated with sexual abuse has been identified as a mode of HPV transmission. Increased numbers of HPV-infected children parallels the rise in childabuse cases in the US (Unger et al. 2011, Hobbs 2011). Non-sexual transmission also occurs, e.g. contact with infected urogenital secretions from sharing towels or bathing together (Unger et al. 2011). Transmission probability estimates for other than sexual transmission do not exist.

2.1.5   Risk factors for genital HPV infections

Potential risk factors for exposure to HPV are all related to sexual behaviour and viral entry facilitated by micro-wounds.

Since HPV is spread by skin/mucosa to skin/mucosa contact the potential factors of transmission are related to sexual behaviour, and anything which affects the integrity of the body surface causing micro-wounds may lead to increased acquisition of HPV (Almonte et al. 2008, Veldhuijzen et al. 2010, Louvanto et al. 2011a). Susceptibilty to the infection, however, depends on the host's resistance because not all exposed persons become infected. The proximate determinants of genital HPV transmission are presented here. Co-factors/confounding factors, and host immune response are considerd together with the natural history of an established infection.

Sexual debut There is evidence from follow up studies which shows that the first sexual intercourse is strongly associated with HPV infections. In follow-up studies young women who became sexually active tested HPV positive and those who remained virgins remained HPV negative (Kjaer et al. 2001, Munoz et al. 2004, Nielsen et al. 2009). This is probably due to the micro-wounds easily caused in the immature genital tract, and in most populations the incidences of genital HPV infections peak soon after sexual activity (Munoz et al. 2004, Nielsen et al. 2009, Luovanto et al. 2011, Banura et al. 2011).

Number of sexual partners The association between the number of sexual partners and HPV acquisition has been commonly reported (Carter et al. 2000, Rousseau et al. 2003, Nielsen et al. 2009, Banura et al. 2010, Luovanto et al. 2011a) and verified in a number of meta-analyses (de Sanjose et al. 2007, Bruni et al. 2010). Different individuals harbour different HPV types with different levels of viral load hence increased frequency of exposure to multiple sexual partners increases the risk of acquiring HPV infections. It is not clear whether the transmission of multiple HPV infections is simultaneous or serial. There is some evidence on concomitant acquisition of more than one type from several sexual partners (Rousseau et al. 2003).

The male factor Studies from male cohorts show that HPV is highly prevalent in uncircumcised men and also men with multiple sexual partners (Castellsague et al. 1997, 2002, Banura et al. 2011).
Conversely, circumcision is associated with reduced risk of acquisition of STIs, especially HIV and HPV, both in men and in their sexual partners (Serwadda et al. 2010, Wawer et al. 2011). Irrespective of marital status men are regarded as reservoirs and vectors for HPV infections provided they have had many life-time sexual partners (Castellsague et al. 1997). The prevalence of STDs such as HIV and HPV has been reported to be high among women with cross-generation male sexual partners and those in polygamous marriage (Kelly et al. 2003). Condom use, however, offers partial protection since HPV can be transmitted by the contact of the uncovered skin (Winer et al. 2006).

2.2   Identification of HPV

Because HPV infection can last for a long time in its nonproductive/asymptomatic stage, it is difficult for an individual to know if she/he is infected until the clinical symptoms occur, often from precancerous/cancerous lesions (Stanley 2007). So far the, available knowledge on hrHPVs has made it possible to detect the viral infection at early stage to prevent progression of the persistent infection to invasive cervical cancer (ICC). Several direct and indirect methods have been developed to identify HPV.

Direct tests for the presence of HPV DNA or protein(s)
The following methods are based on the detection of nucleic acids (HPV DNA and HPV RNA) or the detection of HPV proteins in the infected tissues by specific antibodies.

HPV DNA detection
Over the years several techniques have been developed to detect HPV infections using nucleic acid hybridization. These include; Southern blot, dot blot, in situ hybridization, hybrid capture II and polymerase chain reaction (PCR). The last two are currently the most widely used methods for the detection of HPV DNA.

Polymerase chain reaction is widely used due to its high sensitivity and specificity in HPV detection. It is a selective DNA-target amplification assay that is capable of exponential and reproductive increase in the HPV sequences present in the biological specimens (Brink et al. 2007). Theoretically, it can produce up to a billion copies from a single-stranded DNA molecule in 30 cycles of amplification. There are variations in the sensitivity and specificity of PCR depending on the site and type of collected samples, their storage and transportation, procedures of DNA extraction, primer sets, size of the PCR product, the PCR reaction conditions and performance of the DNA polymerase used in the reaction, as well as the spectrum of HPV types amplified, and the ability to detect multiple types (Brink et al. 2007). Most HPV-PCR assays utilize consensus primers (such as GP5/6 and/or its modified extended version GP5+/6+, the MY09/11 pair of degenerate primers and its modified version PGMY09/11 and SPF10 system), directed at the conserved parts of the L1 gene and this allows detection of all mucosal HPV types (Karlsen et al. 1996). Amplification with each of these primers will result in different size amplification products (amplicons), and different sensitivities in the detection of certain HPV genotypes. (Manos et al. 1989, de Roda Husman et al. 1995, Jacobs et al. 1997, Kleter et al. 1998, Gravitt et al. 2000, Kornegay et al. 2001). The efficiency of amplification with these validated primers as well as comparisons of their performance has been widely reported (Gheit et al. 2006, Galan-Sanchez et al. 2009, Barcellos et al. 2011). Al-

though there is general agreement in performance, the different HPV-typing assays have good agreement in infections with single types but poor agreement in infections with multiple HPV types (Brink et al. 2007, Sabol et al. 2008, Roberts et al. 2011).

Multiplex HPV genotyping is bead-based quantitative and high-throughput DNA-hybridization method that uses the Luminex suspension array technique to simultaneously detect and genotype up to 100 HPV types (Schmitt et al. 2006). This method is based on the amplification of a sequence of HPV DNA (in the L1 gene) identified by the consensus primers GP5+/6+ and the subsequent detection of the amplicons with type-specific oligonucleotide probes coupled with fluorescence-labelled polystyrene beads with a diameter of 5.6 μm that are internally dyed with various ratios of two spectrally distinct fluorophores, creating an array of 100 different bead sets with specific absorption spectra (Louvanto et al. 2010a, b). It has a comparable principle to that of Multiplex HPV serology.

The Hybrid Capture II assay (Digene) is commercially available. It is a nucleic acid hybridization assay with signal amplification that utilizes microplate chemiluminescent detection. The assay is highly sensitive and can detect DNA for 13 high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) and five low-risk (6, 11, 42, 43, 44) HPV types (Clavel et al. 1998, Chaudhary et al. 2010). Despite its good performance as a triage test it is not a sensitive or type-specific assay.

HPV RNA detection

The detection of hrHPV type E6/E7 is strongly associated with the progression of HPV-associated neoplasia because the proteins induce malignant transformation (Halfon et al. 2010). These tests are commercially available and detect E6/E7 mRNAs of 14 hrHPV types (Castle et al. 2007, Tropé et al. 2009), but have not yet been approved for diagnostic screening by the FDA.

The limitations of the nucleic acid tests are that they differ in sensitivity and some tests may overestimate the prevalence/incidence of hrHPV types due to high sensitivity. Also, contamination can easily occur during the PCR process. On the other hand, detecting only current infections may underestimate the HPV exposure. Since a HPV DNA test is suggested to be included as a primary screening test in the package of CC screening/treatment programmes, WHO is working on the protocol for the international standardization (IS) of HPV DNA tests in their reference labs (Labnet (WHO HPV- Labnet Newsletter 6. 2010, Wilkinson et al. 2010).

Tests for the detection of HPV-induced antibodies

HPV serology is essential for both epidemiological studies and for monitoring of HPV vaccine imunogenicity but not for diagnostic purposes due to the absence of antibody response in a proportion of genital HPV infections. It measures antibodies against specific HPV types stemming from past or present infections or derived from HPV vaccination. Several methods have been used to detect HPV antibodies in the past but the sensitivity and specificity of the tests were poor, largely due to the suboptimal methodology. This affected the progress in HPV serology work for a decade (Dillner et al. 1996).

The discovery that capsid proteins of HPV produced in insect cells self-assemble into virus-like particles (VLPs) from the L1 protein (Kirnbauer et al. 1994; Carter et al. 1995) provided an invaluable tool for HPV serology and for the development of prophylactic HPV vaccines (Lowy and Schiller 2006). VLPs have features that include the display of type-specific common surface-exposed neutralizing epitopes and are suitable for use in enzyme immunoassays (Schiller et al. 1996, Kirnbauer et al. 1994, Dillner 1999,).

The use of VLPs led to fast progress in seroepidemiological HPV studies (Lehtinen et al. 1996, Dillner et al. 1997, Mork et al. 2001, Lehtinen et al. 2006). There is good evidence that HPV antibodies are type specific; e.g. against HPV6/16/18 capsids (Carter et al. 2000). Pre-adsorption experiments showed that sera reacting with multiple capsids contained multiple type-specific antibodies rather than cross-reactive antibodies. These studies also showed a strong correlation shown between seropositivity to a specific HPV type and detection of the same type HPV DNA (Kirnbauer et al. 1994, Carter et al. 2000). Because HPV antibodies are stable over five years (af Geijersstam et al. 1998) and they are suitable immunological markers for cumulative exposure (past and current) to HPV infections.

The methods successfully used in HPV serology are VLP-based ELISA, pseudo-virion-based neutralisation assay, Luminex assays, and Multiplex serology assays.

Enzyme linked immunosorbent assay (ELISA) based on VLPs is the most commonly used method in HPV seroepidemiology and HPV vaccine studies. In ELISA, a known amount (concentration) of type-specific HPV VLP (antigen) is affixed to the surface of a microtitre plate, and then a known standardized amount of serum with unknown amount of antibodies is applied. Known amounts of secondary antibody linked to an enzyme are then added, and in the final step a standard substrate is added for the enzyme to convert to a detectable signal (colour change). The reaction is stopped and read with a microplate reader and the colour change is quantified.

Due to the conformational integrity of the antigenic VLP epitopes this method is type-specific (Dessy et al. 2008). It detects total immunologloblin G (IgG) or IgA but does not distinguish between neutralizing and non-neutralising antibodies (Dessy et al. 2008). Its sensitivity ranges between 50 and 70% and specificity 95%-99% (Dillner et al. 1996, Kjellberg et al. 1999). VLP-based ELISAs have been used in a number of epidemiological studies (Lehtinen et al. 2006, Simen-Kapeu et al. 2008, Kaasila et al. 2009, Merikukka et al. 2011).

Multiplex serology based on glutathione S-transferase fusion-protein capture on fluorescent beads, detects antibodies against several specific HPV types simultaneously. It measures total IgG concentrations and does not distinguish between neutralizing and non-neutralizing antibodies. It is more sensitive than direct standard ELISA it can detect HPV16 E6 antibodies (Waterboer et al. 2005, Micheal et al. 2008, Clifford et al. 2007, Dondog et al. 2008). This method has also been used in a number of seroepidemiological studies (Waterboer et al. 2005; Waterboer et al. 2006; Syrjänen et al. 2009, Michael et al. 2010)

The interpretation of HPV serology results depends on individual in-house controls. For the comparability and reliability of serological data an international standardisation, particularly in terms of antibody units is required. WHO in collaboration with the National Institute for Biological Standards and Control (NIBSC), established the first IS for anti-HPV 16 serum (product no.05/134) (Ferguson et al. 2006) . This will serve as the primary biological standard for antibodies to HPV16 and may be used in the immunoassays utilizing VLPs and neutralizing assays utilizing pseudovirions (PsVs). The IS for other HPV types, e.g. HPV18 are in preparation (WHO HPV-Labnet Newsletter 06, 2010).

Other indirect methods for diagnosing HPV infection

The effects of the virus on mucosal cells have been used for indirect diagnosis of HPV infections.

The Pap test, the gold standard for detecting abnormal cell changes in the uterine cervix due to HPV was developed by Dr G. Papanicolaou in the 1920s (Carmichael 1973). Cells are collected from the outer opening of the cervix and the endocervix (transformation zone) using a speculum and /or brush and fixed on a glass followed by examination under a microscope to look for abnormalities. The test aims to detect potentially pre-cancerous changes, which could be removed. It is the most widely used test in screening for CC worldwide. In America, the Pap test has reduced the incidence of CC from 40/100,000 to 8/100,000 women. In Finland, Sweden, British Columbia and Canada, the incidence of CC has decreased up to 70-80% (Hakama 1982, Nieminen et al. 1995, Hristova and Hakama 1997, Nieminen et al. 1999). It

has been observed that the introduction of the Pap test in a population which has never been screened for CC (like Uganda) may reduce the risk by 60-90% among women over 25 years of age (IARC 2005). Although the Pap test is a gold standard, if done once it has a sensitivity ranging from 50-80%. Hence repeated sampling following an organized programme is crucial.

Liquid-based cytology (LBC) is a modification of Pap smear cytology, applying plastic sampling devices (Karnon et al. 2004). The cells are suspended in the collection medium and a thin layer of cells is fixed on the slide. LBC has been widely accepted as the primary tool in CC screening. (Nieminen et al. 2007, Arbyn et al. 2008, Gibbs and Marten 2011, Anttila et al. 2011). Liquid-based cervical cytology has been reported to markedly reduce the numbers of unsatisfactory slides (Beerman et al. 2009), but the sensitivity and specificity for the detection of high-grade cervical intraepithelial neoplasia is very close to that of conventional cytology test (Arbyn et al. 2008).

In visual inspection after application of acetic acid (VIA) or Lugol's iodine (VILI) the cervix is examined with naked eyes or with a hand–held magnifying device. The reporting depends on the colour changes. VIA was one of the first CC screening tests. It was introduced by Schiller in the 1930s (Schiller 1938). Although Schiller's test had poor specificity and was almost entirely replaced with the advent of cervical cytology, it was revived to be used in developing countries. In India, a single screen with VIA achieved a reduction in the CC incidence by 25% and CC mortality by 35% (Sankaranarayanan et al. 2007). Several other developing countries have started using the VIA in combination with other tests such as the Pap smear test for CC screening (Muwonge et al. 2010). VIA is cheap and easy to perform, it gives immediate results, has high sensitivity (96.7%), but contributes to high numbers of false positives due to low specificity (36.4%) compared to conventional cytology (Goel et al. 2004).

2.3   Epidemiology

2.3.1   Occurrence of genital HPV infections

The life-time risk of HPV infection is 80% (Syrjanen et al. 1990). The age-specific HPV prevalence peaks at younger ages (< 30 years) worldwide, then a consistent age-related decline in HPV prevalence and plateau between 35- 40 years, and in the Americas, Africa and Europe, a clear second peak at 45 years has been documented (Herrero et al. 2005, de Sanjose et al. 2007, Smith et al. 2008, Bruni et al. 2010). In prospective studies, the first peak is reported shortly after sexual debut for most

women, and is generally attributed to higher levels of sexual activity with multiple partners or partners with risky sexual networks, immature mucosal epithelial layers and to low viral immunity (Kjaer et al. 2000, Carter et al. 2000, Viscidi et al. 2004, Auvinen et al. 2005, Banura et al. 2011, Weaver et al. 2011). While the overall, country-specific HPV prevalence values are more or less comparable, the age-specific prevalences are higher in developing countries than in the developed countries (Smith et al. 2008, Bruni et al. 2010).

The age-specific genital HPV DNA prevalence among women under 25 years was reportedly 20% in Africa, America, and Latin America while in Europe and Asia it was 15% in a review article (Smith et al. 2008). However, within regions country-specific variations were observed. In Africa the prevalence ranges between 2% and 60% with the highest prevalence found in the East African countries, Kenya, Mozambique, Tanzania and Uganda (Smith et al. 2008). In Uganda the prevalence of HPV among women aged under 25 years, ranges between 23.7% and 67.1% in HIV negatives and 41.6% -75% in HIV positives (Banura et al. 2011). Whereas in Finland the HPV prevalence among female university students was 33% and out of these 82% had hrHPV types (Auvinen et al. 2005). Among women aged 25-29 years in a population-based cervical cancer screening programme HPV prevalence was 24.1 % (Leinonen et al. 2008).

The prevalences of genital HPV DNA depend on the sample collection method and site and on the test used, as some are more sensitive than others, and the study population, which is why age is important factor in reporting HPV prevalence because different age groups are engaged in different risky sexual behaviours. It is well known that 90% of infected women clear their viruses within two years (Carter et al. 2000, Banura et al. 2010, Luovanto et al. 2010b). Hence the DNA prevalence may be underreported in some cases.

2.3.2 Seroepidemiology of HPV

HPV DNA data has been widely used to describe the epidemiology of HPV, however, there are some limitations related to HPV DNA detection. HPV infections are transient, and the time and site of sample collection are crucial. HPV serology is therefore an important epidemiological tool for the detection of past and current HPV infections and for estimating HPV-associated neoplasia risk. The natural history of HPV antibodies is well studied (Ho et al. 1998, Porras et al. 2010). Up to 70% of HPV infected individuals seroconvert to a detectable level (Ho et al. 1998, Kjellberg et al. 1999, Carter et al. 2000) and antibodies once detected last for more than 5 years (Carter et al. 1996, af Geijersstan et al. 1998). Seroconversion takes from some 8 months to years (Ho et al. 2002), however type-specific studies indi-

cate that lrHPV types seroconvert faster than hrHPVs (Carter et al. 1996, 2000, Syrjanen et al. 2009). HPV antibody positivity is strongly associated with lifetime number of sexual partners (Dillner at al. 1996, Viscidi et al. 1997, Lehtinen et al. 2006, Porras et al. 2010), which suggests that sexual contact is still the main route of HPV transmission. Transmission through other routes is very low (Sarkola et al. 2008a, 2008b)

HPV serology identifies both past and current infections (cumulative incidences) which makes it a suitable immunological tool to monitor HPV prevalence and incidence in the population and it is used to predict the future burden of CC (Lehtinen et al. 2006, Kaasila et al. 2009). Seroepidemiological studies on the occurrence of common HPV types in populations are well documented in most developed countries (Dillner et al. 1996, Carter et al. 2000, Lehtinen et al. 2001, 2006, Ho et al. 2002, 2004, Simen-Kapeu et al. 2008, Kaasila et al. 2009, Syrjanen et al. 2009, Merikukka et al. 2011). Serological studies have extensively documented the natural history of HPV16, 18 and 6 (Carter et al. 1996, 2000, Ho et al. 2002, Syrjanen et al. 2009). Variations in seroprevalences have been reported to range between 1.6 and 44% for HPV16, and 4.2% to 35.6% for HPV18. The determinants of HPV seropositivity included age, level of education, use of oral contraceptives; number of lifetime sexual partners (Ho et al. 2004, Wang et al. 2004, Lehtinen et al. 2006, Syrjanen et al. 2009, Porras et al. 2010). Unfortunately, data are still limited in Sub-Saharan Africa, especially in countries where HIV and other tropical infections are endemic. For instance, in a review of Ugandan HPV studies only two out of the 22 HPV studies documented till 2010 reported HPV antibodies (Banura et al. 2011).

2.3.3 Multiple HPV infections

The proportion of women harbouring multiple (at least two) HPV infections among those infected with HPV ranges between 20 and 50% (Franco et al. 1999, Liaw et al. 2001, Nielsen et al. 2008, Pista et al. 2011). The recent increase in multiple HPV infections is basically due to improved technology such as polymerase chain reaction (PCR)-based methods using L1 modified general primers (MY11/GP6+ and GP5+/GP6+) in combination with type-specific (TS) E7 primers (Huang et al. 2004, Schmitt et al. 2006, Mejlhede et al. 2009, Schmitt et al. 2010). Multiple HPV infections are strongly associated with young age, below 30 years, increased number of sexual partners, uncirmucised partner and HIV infections (Clifford et al. 2005, Nielsen et al. 2008, Pista et al. 2010, Banura et al. 2011). In a meta-analysis, 3.2% of women with normal cytology (20% of those HPV positive) had multiple HPV infections (Bruni et al. 2010). Multiple HPV infections are on the increase in Africa, Europe and Oceania and declining in Asia and North America (Smith et al. 2007, de

Sanjose et al. 2010). These differences could be due to different study populations, number of studies conducted on each continent, methods of HPV detection and other factors.

Seropositivity to multiple HPV types has been frequently reported (Carter et al. 2000, Lehtinen et al. 2006, Kaasila et al. 2009). The possibility of some hrHPV types having a competitive advantage over other types has been suggested in population-based longitudinal studies (Merikukka et al. 2011). Our current seroepidemiology study focuses on some of those issues among Finnish and Ugandan women.

2.4 Immune system and genital HPV infections

2.4.1 Innate immunity and HPV infection

Innate immunity is included in the basic resistance to infectious agents that an individual encounters. Innate immunity provides the first line of defensive host response and eliminates most of the microorganisms encountered by a healthy individual. HPV cannot penetrate the basal membrane or infect stromal or other underlying cells without a micro-wound (Stanley 2010a). On the other hand, the virus has receptors that can infect only mucosal cells. Thus, the line of protection against HPV infection is the intact mucosa and skin whose basal cells are the innermost cells targeted by HPV.

In the primary foci HPV remains exclusively intraepithelial and its replication takes place in a differentiation-dependent manner in the keratinocytes. HPV is not cytolytic and no immediate inflammation accompanies viral infection and replication. This impairs the efficacy of the innate immune response. Antiviral and antiproliferation responses, however, take place, whereby keratinocytes secrete various cytokines (e.g. tumour growth factor beta (TGF- β), tumour necrosis factor alpha (TNF- α), and interferon alpha and beta (IFN-α and -β)) which regulate HPV infected cells (Stadnyk 1994, Wang et al. 1999).

2.4.2 Adaptive immunity and HPV infection

Adaptive immunity is initiated by the recognition of a foreign antigen and represents the response of the immune system to this recognition. It is antigen specific, diverse, comprises immunologic memory, and distinguishes self from non-self antigens. The immune system fights the HPV invasion in: cell mediated immune response and humoral immune response. In 90% of HPV infected subjects, the response is suc-

cessful as judged by clearance of the virus (Bulkmans et al. 2007, Stanley 2010a). The interaction of cytokines/chemokines liberated during cellular immune reaction regulates the immune control of lesions associated with HPV.

2.4.3 Cell-mediated immunity (CMI) and HPV infections

Cell-mediated immunity is based on T lymphocytes of two types: T helper cells and cytotoxic T cells. When a T helper cell interacts with a microbial antigen-MHC II molecule complex present on an antigen presenting cell (APC), it becomes activated and begins to secrete cytokines. In general, depending on the activated T helper cell type Th1 or Th2, these cytokines activate cytotoxic T cells or B cells, in addition, various phagocytic cells are activated.

When a cytotoxic T cell interacts with the microbial antigen-MHC I complex, it is activated under the influence of cytokines produced by already activated T helper cells. Then the activated cytotoxic T cells differentiate into cytolytic T lymphocytes (CTLs). The CTLs kill infected cells displaying an antigen-MHC I molecule. Together, T helper cells and CTLs are modulators and effectors of the cell-mediated immune response.

HPV does not enter the bloodstream, and the viral antigens expressed/released on the mucosal surfaces elicit the immune response (Stanley 2007). The cervical epithelium contains a variety of cells which, after coming into contact with the HPV antigens, are able to present fragments, mostly short peptides derived from cleaved viral proteins on the cell surface (Gonçalves and Donadi 2004). Antigen presenting cells (APCs), such as dendritic cells (Langerhans cells) and macrophages, activate the T-helper type-1 cells, which in turn activate the CTLs to destroy the virus-infected cells. T-helper type-2 cells activate B cells, which produce antibodies and activate the complement mechanism. Several chemokines and cytokines which stimulate or suppress the pathways are released by the Th2 cells.

In natural HPV infection, cell-mediated immunity works hand in hand with the innate immunity to clear the virus-infected cells from pre-cancerous lesions usually within 18 months of acquisition of the infection (Frazer et al. 2009). Evidence of effective cell-mediated immunity has been found in 90% of HPV infected individuals who clear the virus within 18 months (Scott et al. 1999). Persistent infections with oncogenic HPV types, however, tend to suppress the cell-mediated immune response. On the contrary, in immune-suppressed individuals there is an increased prevalence of persistent, and in the long run also multiple HPV infections (Clifford et al. 2006, Banura et al. 2011).

Evidence from immune-suppressed individuals such as HIV positives, those with inherited immune deficiencies, renal transplant recipients, and patients on chemotherapy shows that T helper CD4 cells play the key role in controlling HPV infections and related neoplastic progression (Clifford et al. 2006). Clinical studies have reported cutaneous and genital warts among immuno-suppressed individuals (Palefsky 2006, Lutzner 1985, Leigh et al. 1995). In meta-analyses the prevalence of genital HPV infections in HIV-positive women has been consistently reported more than twice as often as in HIV-negative women (Cu-Uvin et al. 1999, Palefsky et al. 1999). Prospective studies have shown incident HPV infections and HPV persistence doubling among women with CD4 cell count less than 200 cells/uL compared to women with more than 500 cells/uL (Ahdieh et al. 2001, Strickler et al. 2005). Poor clearance of HPV infections among HIV infected individuals has been reported (Banura et al. 2010). Co-infections with multiple HPV types were found in 20% to 50% of HIV-infected women (Liaw et al. 2001, Clifford et al. 2005, 2006).

Although multiple oncogenic HPV types are common among HIV positives, the association between markers of HIV disease status (such as viral load and CD4 lymphocyte count) and HPV infections is inconsistent. Some studies suggest that HPV prevalences were higher in the late stages of HIV disease (Ahdieh et al. 2001, Strickler et al. 2005), whereas other studies suggest that the occurrence of HPV infections and SIL are influenced by the HIV status independently of CD4 counts (Vernon 1999, Moscicki et al. 2000).

2.4.4   Humoral response and HPV infections

The humoral response is based on antibodies produced by antigen-activated and T helper cell stimulated B cells. Antibodies bind to the antigens and facilitate their elimination, e.g by forming clusters or rough cross linking of antigen molecules, which are then ingested by phagocytic cells. Antibody binding to a microorganism may activate the complement system to lyse the microorganism. Most importantly, antibody binding to virions prevents subsequent viral binding to host cells.

In natural HPV infections antibody production is a very slow process, which may take several months to years and up to 50% of infected individuals may never sero-convert (af Geijersstam et al. 1998, Carter et al. 1996, 2000, Syrjänen et al. 2009). The transient nature of HPV infection may not allow enough time for the HPV antibodies to develop (Carter et al. 2000). Antibody levels produced in natural HPV infections are generally low, probably due to the immune evasion strategies of the virus (Stanley 2010). Continuous HPV exposure has been reported as a risk factor for HPV seropositivity (Carter et al. 1996, 2000, Porras et al. 2010) which has been considered to be a marker of persistent HPV infection (Dillner et al. 1999). HPV

antibodies, once detected, remain stable for at least 5-10 years mainly for the hrHPV types (af Geijersstam et al. 1998, Kjellberg et al. 1999, Carter et al. 2000). This makes HPV antibodies a good immunological marker of cumulative HPV incidence (past and/or present infections) for epidemiological studies.

Follow-up studies have reported that antibodies to lrHPV types rise faster to a detectable level than antibodies to hrHPV types (Carter et al. 2000, Ho et al. 2004). While seroreversion for lrHPV types may occur faster than for hrHPV types (Carter et al. 2000), total antibody decay is rare for both lr and hr HPV types (af Geijersstam et al. 1998, Laukkanen et al. 2003, Kaasila et al. 2009, Syrjänen et al. 2009). Unlike other infections, where antibodies seem protective against re-infections, antibodies induced in natural HPV infections may not be protective against new infections (Ho et al. 2004) due to low antibody titres. In HPV vaccinated individuals, high antibody titres are easily transduced to mucosa (Petaja et al. 2010) where neutralization and blocking of the viruses take place.

HPV seropositivity is a risk indicator for seroconversion to other different HPV types (Carter et al. 2000, Ho et al. 2004, Kaasila et al. 2009, Merikukka et al. 2011) most probably due to underlying risk behaviour (Dillner et al. 1996). Some studies have reported persistent HPV antibodies to be protective against new infections of the same or different HPV types (Ho et al. 2002, Viscidi et al. 2004, Malik et al. 2009), however, seroconvertants have remained susceptible to subsequent reinfection with the same HPV type (Schwarz and Leo 2008, Viscidi et al. 2005). Identification of a threshold level for the protective level of natural HPV infection induced antibodies, and for how long this kind of antibody response can offer protection warrant further studies.

2.4.5 Seroconversion

A successful immune response is characterized by seroconversion, i.e. production of antibodies against a specific antigen. HPV seroconversion takes several weeks to years depending on the individual immune status (Carter et al. 2000, Ho et al. 2004, Syrjanen et al. 2009). Approximately 50% of HPV16 DNA positive women seroconvert (Kjellberg et al. 1999, Carter et al. 2000, Syrjänen et al. 2009). Seroconversion to HPV16 and 18 has been reported to require persistent HPV DNA positivity, whereas for HPV6 seroconversion, transient HPV DNA positivity alone was enough (Carter et al. 2000). The risk of seroconversion for hrHPV types was associated with young age at sexual debut, increased life-time number of sexual partners, seropositivity for any type at baseline, and HPV DNA positivity at baseline (Carter et al. 2000, Lehtinen et al. 2006, Kaasila et al. 2009, Syrjänen et al. 2009).

2.4.6 Maturation of the HPV antibodies

The functionality of the immune response induced antibodies in natural HPV infections may vary. In general, neutralizing IgG HPV antibodies are considered to have high avidity (Wang and Hildesheim 2003), and the maturation antibody avidity correlates with the ability to neutralize the infection (Nair et al. 2009). Low antibody avidity has been associated with poor protection against several viruses in immunosuppressed individuals (Nair et al. 2009)

Maturation of IgG avidity and associated protection depends on the interplay between T-helper cells and B cells (Stavnezer et al. 1996, Gulbranson-Judge et al. 1997, Bachmann et al. 1997, Nakanishi et al. 2009). The fact that high avidity antibodies are usually detectable six months post primary infection has been successfully used in the distinction of acute, recent and past infections with e.g. cytomegalovirus, Epstein-Barr virus, measles, parvovirus, rubella and toxoplasmosis) (Hedman et al. 1988, 1989, Söderlund et al. 1995, Aalto et al. 1998, Paunio et al. 2003). Sometimes the low-avidity antibodies can persist for months or years post primary infection among the immuno suppressed individuals (Bodeus et al. 1998, Lazzarotto et al. 1998).

Under normal circumstances, T-helper cells recognize antigen and induce antigen-primed B cells to produce high affinity antibodies. Therefore in a situation where T helper cells are deprived or not presented with the antigen by APCs, maturation of avidity may take a long time as observed in HIV and organ transplant patients (Brinkman et al. 2003, Nair et al. 2009). In natural infections the slow maturation of antibody avidity may correlate with impaired protection against the infection, and may increase the likelihood of multiple HPV infections with HPV types that have shown poor viral clearance (Banura et al. 2010, Luovanto et al. 2010).

The reasons why HPV antibodies induced in natural HPV infections do not remain protective against subsequent infections with the same HPV types are not clear. Equally unclear is why some individuals acquire multiple HPV infections despite the presence of at least partially cross-reactive antibodies (Carter et al. 2000, Syrjanen et al. 2009, Kaasila et al. 2009, Parros et al. 2010). Some studies suggest that inadequate avidity of the antibodies may play a role here (Bachmann et al. 1997, Wang and Hildesheim 2003).

2.4.7 Immune evasion strategies

In the infected cell HPV may go unrecognized by the immune system due to its evasion strategies, which include being restricted to mucosal surfaces, not being cytolytic, sequential downregulation of cellular signatory proteins for immune response (MHC-1 antigens and cytokines) and low level of viral protein expression.

Occurrence of multiple HPV types among
Finnish and Ugandan women

Viral DNA replication and virion assembly occur in cells already programmed to death, so there is no danger signal to alert the immune system (Stanley 2010b). The Langerhans cells are also depleted from HPV16 infected mucosal surfaces (Lehtinen et al. 1993). Furthermore, interferons (IFN), which are key to antiviral defence are actively suppressed with the E6 and E7 proteins of the hrHPV, in turn inhibiting the IFN-gamma receptor signalling pathway and activation of immune response genes (Doorbar. 2005, Pett et al. 2006, Kanodia et al. 2007, Ghittoni et al. 2010).

HPV escapes from the immune surveillance through different mechanisms: using the host-cell machinery for replication, maintaining a small number of viral copies or expressing very low numbers of viral proteins in the basal cells of the stratified epithelium. To reduce the recognition of infected cells by antigen-presenting cells, HPV downregulates the expression of cell-surface signalling molecules that keratinocytes express to limit the activation of cytotoxic T cells which kill the infected cells (Scott et al. 2001, Einstein et al. 2009). For instance, viral E7 protein also downregulates TLR9 (Hasan et al. 2007). Although the lack of MHC class I antigens may activate the natural killer cells (NK) only a few NKs patrol the upper epithelial layers, and the prospects for persistently infected cells to evade the host's immune surveillance are good.

There is experimental evidence that prior natural infection may interfere with vaccine-induced immunity at the level of clonal B-cell selection (McHeyzer-Williams 2005). This could also be true in the case of subsequent infections with the same or closely related HPV types. The T-helper cell support for immunity is influenced and perturbed by age, persistence of the antigen and the immune response to natural infection at other anatomical sites (Fazilleau et al. 2007, Brink et al. 2008).

2.5 Natural history of genital HPV infections

2.5.1 Incident HPV infections

Human papillomavirus is the most common sexually transmitted infection with an 80% lifetime risk of infection (Syrjänen et al. 1990). The incubation period of genital HPV infection to the first manifestations of the infection is 3-4 weeks (Stanley 2010). HPV infections clear spontaneously within 4 to 9 months (lrHPV) and 12 to 18 months) in 90% of immunocompetent individuals (Figure 3). In the remaining 10% the infection persists and may progress via various stages to genital warts (lrHPV) or CC (hrHPV) (Bosch et al. 2008). The peak period of HPV acquisition is soon after the first sexual intercourse (Kjaer et al. 2001, de Sanjose et al. 2007, Bruni et al. 2010) between the ages of 18 to 22 years (Smith et al. 2008), but the hrHPV infection incidences remain high and multiple infections occur in young adult women aged <30 years (Lehtinen et al. 2006, Nielsen et al. 2008, Smith et al. 2008, Kaasila et al. 2009, Bruni et al. 2010). The incidences, however, decrease and prevalences/seroprevalences level out after 25-30 years of age (Clifford et al. 2005, de Sanjose et al. 2007, Smith et al. 2008, Bruni et al. 2010, Li et al. 2011)

A secondary peak among >45 years has been observed in a few countries (Munoz et al. 2004, de Sanjosé et al. 2007, Bruni et al. 2010, Liu et al. 2011). The reasons for the second peak are unclear, but after the age of 45 years women may be susceptible to persistent HPV infections like young women due to hormonal imbalances which lead to cervical ectopy.

2.5.2 Persistent lrHPV infections

Persistent lrHPV types 6 and 11 induce proliferation of squamous epithelia to benign tumours (external genital warts, also known as condyloma accuminata). The lrHPV types are common and peak in young women aged 15-24 years. Disease burden of benign warts associated with lrHPV is very common and there are differences in the geographical distribution of incidence rates (Kliewer et al. 2009, Kraut et al. 2010). A study in the Scandinavian countries showed that HPV6 and 11, which are responsible for genital warts, are the most common sexually transmitted infections with a 10% risk of lifetime acquisition (Kjaer et al. 2007). In West Africa (Burkina Faso) the prevalence of HPV6 and HPV11 was 6% and 4% respectively, and the genital warts were primarily associated with HPV6 (Low et al. 2010).

2.5.3 Persistent hrHPV infections

Persistent hrHPV infections precede the development of squamous intraepithelial lesions and cervical neoplasia (Ho et al. 2011, Matsumoto et al. 2011). HPV positivity for any hrHPV types on at least two occasions within a minimum of six months increases the risk of developing lesions compared to repeated negative test (Liaw et al. 1999, Kjaer et al. 2002, Ho et al. 2004, Auvinen et al. 2007, Syrjänen et al. 2009b). The risk appears to be higher for single type-specific persistence than persistence of multiple hrHPV types (Kjaer et al. 2002, Luovanto et al. 2010a) but the differences in PCR sensitivity have not been fully accounted for. HPV16 dominates persistent infections followed by HPV types 18, 31, 33 and 45. The risk factors affecting the likelihood of HPV persistence are: pre-existing HPV infection, multiple HPV infections, HIV status, smoking, age and genetical factors (Tam et al. 2010, Banura et al. 2010, Luovanto et al. 2011b). The period of persistence is associated with the production of low virion copy numbers, disruption of the maturation of the epithelium and abnormal cellular changes (Schiffman et al. 2005). The progression rate to squamous intraepithelial lesion (SIL) ranges between 20% and >70%. About 70% of LSILs regress spontaneously, while about 6% of LSIL progress to HSIL or worse. In about 10% to 20% of women with HSIL the lesion progresses to ICC (Campion et al. 1986, Bosch et al. 2008) in a period of 20 years (Figure 3). However, there are cases of aggressive carcinomas mainly caused by persistent HPV18 that may occur in young women (Ostör 1993).
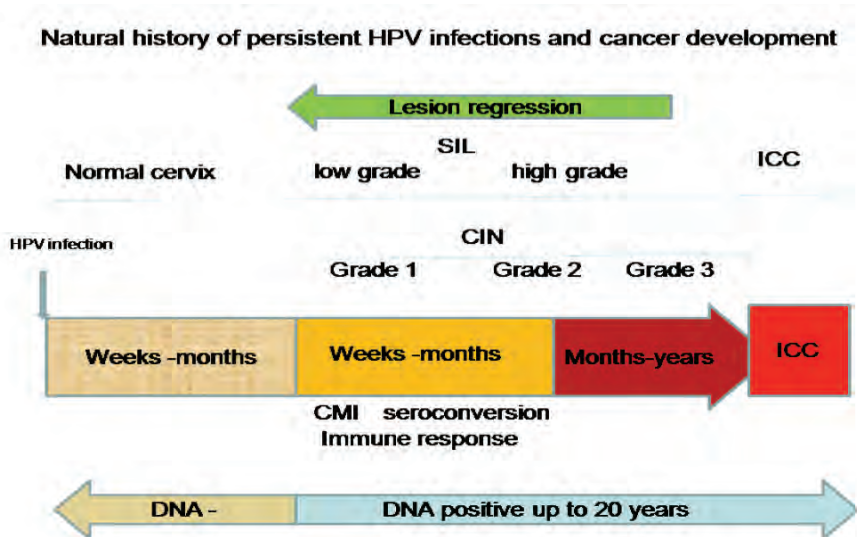

Figure 3. Natural history of persistent HPV infections and disease progression.

The morphological changes of the persistently hrHPV infected cell are characterized microscopically and graded from normal cytology to ICC according to the severity of tissue damage (Figure 3). Different systems use different terminologies (Solomon et al. 2002) for the precancerous abnormalities as summarized below (Table1).

Table 1. Terminologies used to describe cervical precancerous abnormalities by different systems

Pap classification	1	2	3	4	5
Dysplasia	Normal	Inflammation, metaplasia	Mild , Moderate, severe	Carcinoma in situ	ICC
CIN	Normal	-Inflammation -metaplasia cellular atypia	CIN1, CIN2, CIN3	CIN3	ICC
Bethesda	-Negative for malignancy - reactive cellular changes and infections	ASC or ASCUS	LSIL or HSIL	HSIL	ICC

The terminologies used above can be summarized as follows: Dysplasia or Neoplasia indicate a change in the cells on the surface of the cervix. They do not necessarily signal cancer, but a repeat Pap test is necessary. Atypical Squamous Cells of Undetermined Significance (ASCUS) indicates mild cellular changes, which makes it difficult to distinguish between benign tissue and neoplasia, a repeat Pap test in 6 to 12 months is recommended. Low-Grade Squamous Intraepithelial Lesion (LSIL): indicates potentially precancerous change, follow-up with either a Pap test in 6 to 12 months or colposcopy is necessary. High-Grade Squamous Intraepithelial Lesions (HSIL), also known as moderate or severe dysplasia or carcinoma in situ (CIS), requires surgical treatment to remove the lesions if the severity is confirmed by biopsy. Squamous cell carcinoma: a cancerous lesion requires treatment by radical hysterectomy, radiation therapy and chemotherapy.

2.5.4 Cofactors of persistent HPV infections

Young women involved in risky taking sexual behaviour from an early age onwards, exposes them to several sexual partners who are likely to be HPV infected (Burchell et al. 2006, Wellings et al. 2006, Luovanto et al. 2011a). This increases the risk because young women have a large cervical transformation zone (TZ) which makes them more susceptible to acquiring cervical HPV infections than older women. Similar observations have been made for HIV acquisition among adolescent women (Moss et al. 1991). Moreover, inadequate immune response/poor virus clearance at a young age may increase susceptibility to the acquisition and persistence of viral infections (Cuschieri et al. 2004, Moscicki et al. 2001, Malik et al. 2009, Banura et al. 2010).

Prolonged use of contraceptives has been associated with increased risk of HPV acquisition (Smith et al. 2003, Nielsen et al. 2009, Kaushic et al. 2011). Oral contraceptives are known to promote cervical ectopy and to increase susceptibility to HPV acquisition, also after adjusting for smoking and risky taking sexual behaviour (Winer et al. 2003). A recent article by Kaushic et al. (2011) suggests that changes in the microenvironment of the female tract due to hormonal, progestrogen based contraceptives especially increase the risk of acquiring STIs. These biological phenomena are highly relevant since contraceptive use is associated with unprotected sex, often with several partners.

Host genetic factors have been reported to be associated with the establishment of persistent HPV infections and the development of CIN (Segat et al. 2009, Castro et al. 2009). But a lot is still unknown about genetic susceptibility to HPV infections and their sequelae. Furthermore, it is not clear if genetics is associated with all HPV infected people not seroconverting. In other persistent viral infections, genetics does not seem to be associated with antibody response, even if specific immune genes (e.g. IL-10) are associated with their severity and reactivation (Kuparinen et al. 2011, Hurme et al. 2003). More research on the genetic determinants of HPV infections is needed.

Impaired cellular immunity is associated with increased incidence and prevalence of multiple HPV infections reported in renal transplant patients and HIV positive individuals (Viscidi et al. 2004, Banura et al. 2008a, Banura et al. 2010). CD4 T helper cells are known to play a role in the control of HPV at the mucosae, hence HIV depleted T helper cells give more room for the establishment of HPV infection (Clifford et al. 2006). Even after the introduction of anti-retroviral therapy, the

prevalence and incidence. i.e., acquisition, of multiple HPV infections remains high in the HIV infected although cervical lesions regress (Palefsky et al. 2009).

Other sexually transmitted infections with *Chlamydia trachomatis*, and herpes simplex virus 2 (HSV-2) are known to cause mucosal inflammation and immune suppression, which facilitates the establishment of HPV infections. *C.trachomatis* may also be an indepent co-factor of CIN3 development (Lehtinen et al. 2011). Some studies also suggest that HPV increases the risk of HIV infection (Smith-McCune et al. 2010, Auvert et al. 2010). On the other hand, the associations of different STI occurrences may be all due to risky taking sexual behaviour, and the nature/mechanisms of possible interactions between any two given STIs are unclear (Herfs et al. 2010).

Smoking, the association between smoking and HPV carcinogenesis has been established in a number of studies (Szarewski et al. 1996, Plummer et al. 2003, Simen-Kapeu et al. 2009). Smoking impairs antibody response to genital HPV infections (Simen-Kapeu et al. 2008). While HPV IgG antibodies protect against incident HPV infection and its progression to CIN (Malik et al. 2009), impaired IgG antibody response has been suggested to increase the risk of reinfection and persistence of the virus (Ho et al. 1995, Carter et al. 2000, Viscidi et al. 2004). Immunosuppressive effects of smoking on both innate and adaptive immunity have been described (Sopori et al. 2002).

2.6 Cervical Cancer

Cervical cancer is the third most common incident cancer among women worldwide (Ferlay et al. 2010, Arbyn et al. 2011) with an overall age-standardised incidence rate (ASR) of 15.3 per 100,000 women albeit with a wide range of 0.4 to 56.3 (Figure 4) (Globocan 2008, Parkin et al. 2010, Arbyn et al. 2011). Globocan 2008 (Global Cancer Statistics), showed that out of the 180 countries included in the programme, CC ranks first in 42 countries (Globocan 2008 Arbyn et al. 2011). In 2008, there were 530,232 new CC cases and 275,008 deaths due to CC (Globocan 2008, Feray et al. 2008). The majority of new cases, 453,531 (85.5%) and 242,077 (88.0%) deaths due to CC occur in developing countries (Lehtinen et al. 2006, Sankaranarayanan et al. 2006, Jamel et al. 2011, Arbyn et al. 2011).

Before the introduction of organised population based screening programmes the incidence of CC in the developed countries of Europe, North America and Australia, was as high as the current situation in the developing countries especially Sub Saharan Africa. Following the implementations of these programmes the incidences have been low and the survival rates are quite high in developed countries, especially Nordic countries and North America (Bergstron et al. 1999, Anttila et al. 1999,

Smith et al. 2000, Petterson et al. 2011, Arbyn et al. 2011) and some parts of Asia (Sankaranarayanan et al. 2008). In other parts of the world devoid of screening programmes the incidence of and mortality from CC are increasing, for instance, in East and Central Africa, Central and Southern America, South-Central Asia, and Melanesia (Arbyn et al. 2011). In the last 10-15 years, however, some European countries have reported increasing incidences of cervical cancer in fertile-aged women (Anttila et al. 1999, Arbyn et al. 2011).

Figure 4. Global view of the age-standardized incidence rates (ASIRs) of cervical cancer within each country as estimated by GLOBOCAN 2008. (In the legend, the numbers in parentheses indicate the number of countries in each range of ASIRs).
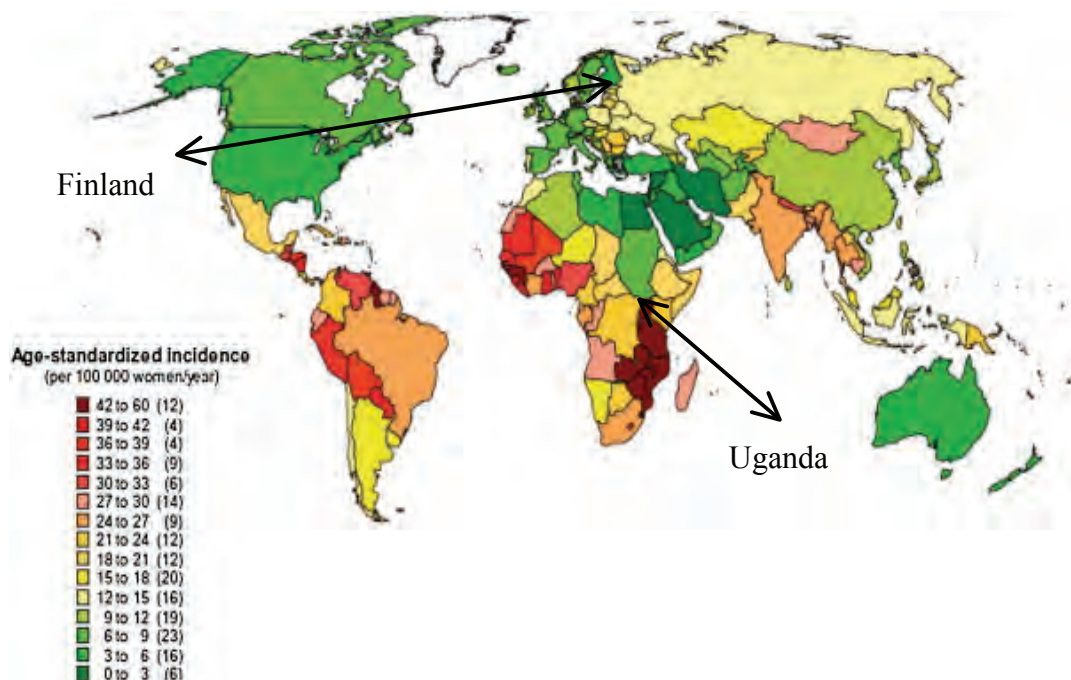

Figure 4. (Source: Prev Med 2011; 53 Suppl 1:S12-21)

2.6.1 Burden of CC in developed countries (Finland)

Following the introduction of cervical screening, CC incidence and mortality rates significantly declined by 80% in developed countries (IARC 2005). This is due to organised cytological screening programmes (Anttila et al.1999, Anttila et al. 2000, IARC 2005, Lazcano-Ponce et al. 2008, van der Aa et al. 2008, Pettersson et al.

2011). In 2008, the number of new cases was 54,517, 5,213 and 151 for Europe, Northern Europe and Finland, respectively and 24,874, 2,141, and 63 deaths respectively. The highest overall ASRs (14.5 per 100,000 women) are found in Eastern Europe and the lowest ASRs (6 per 100,000 women) in Western Europe. In recent decades the incidence of CC has, however, increased in a number of European countries: Sweden (1960s), Iceland (1980s), Finland, UK, Slovenia (1990s), and Bulgaria, Romania, Lithuania, Latvia (2000s) (Sigurdsson 1994, Bergström et al. 1999, Anttila et al. 1999, Arbyn et al. 2011, Ferlay et al. 2011). The increase of CC incidence in fertile aged women has been due to low attendance of young women for cytological screening (Finnish Cancer Registry 2010). A similar increase was also observed in the UK among women born before 1996 and aged 20-29 years of age (Foley et al. 2011). The major factors for this increase were adoption of risky taking sexual behaviour followed by a specific increase of HPV16 infection incidence (Laukkanen et al. 2003, Nikula et al. 2007).

Furthermore, despite the early success in reducing squamous carcinomas (SCC) in the developed world, especially in the Nordic countries (Figure 5), the incidence of adenocarcinomas (AC) is on the increase (Bray et al. 2005, Bulk et al. 2005, Pettersson et al. 2011).

2.6.2 Burden of CC in Africa (Uganda)

In Sub-Saharan Africa, cervical cancer accounts for 22.2% of all cancers in women and is the leading cause of cancer death in women (Parkin et al. 2003). About 60-75% of women in Sub-Saharan Africa who develop CC live in the rural areas and mortality is very high not only due to lack of access (financial and geographical) to health facilities but also to lack of knowledge of the disease both among health care workers and the female population (Parkin et al., 2002, Mutyaba et al. 2006, 2007, Urasa and Darj 2011). According to Globocan 2008, in the Sub-Saharan region, the highest ASR of CC is in East Africa (34.5 per 100,000 women), followed by Western, Southern, and Middle Africa with 33.7, 26.8, and 23 per 100,000 women respectively. By country Guinea has the highest and Egypt the lowest overall ASR and mortality rates of CC (Arbyn et al. 2011).

Countries like Zimbabwe (Parkin et al. 1994) and Uganda (Wabinga et al. 2000, Parkin et al. 2003) have shown an increased incidence of CC over time, but no such increase has been observed in Nigeria and South Africa (Parkin et al. 2003). This increase may not be due to HIV, which is highly endemic in Eastern and Southern Africa as there have been no changes in the incidence of CC in Zimbabwe during the AIDS period (Parkin et al. 2003). Also in Uganda, the increase in the incidence

of CC began before the onset of the AIDS epidemic (Wabinga et al. 2000). Thus, the role of HIV on CC remains unknown.

In Uganda, one of the countries with the highest age-standardized CC incidence (Parkin et al. 2005, Arbyn et al. 2011), it is estimated that 3,577 women are diagnosed and 2,464 women die from CC every year (www.who.int/hpvcenter/en/ Accessed 20 April 2011). The incidence rates of CC have tripled between the 1950s and the 2000s (Table 2). According to the Ugandan Cancer Registry, which was established in 1954, CC incidence has been increasing due to cases reported/attending hospital following opportunistic screening. Perhaps the numbers will increase further if population based screening programme is set up.

Table 2. Age-standardized incidence rates of cervical cancer in Uganda

Period	1950s	1960-1966	1967-1971	1991-1995	1996-2001	2002-2006
ASR per 100,000	12.6	17.7	22.5	38.1	44.9	52.4

(Wabinga et al. 2000, Parkin et al. 2010)

The age-specific incidence rates show an increase in CC by age from the 1950s. After the 1990s the peak is in menopausal women, followed by a plateau. This may be a birth cohort effect due to the social disruption during the wars of 1970 and the 1980s which favoured the spread of STIs (Wabinga et al. 2000). The mean age at CC diagnosis is 43 years in all periods and the annual increase in ASR is 3% (Parkin et al. 2010). Screening of CC in Uganda is opportunistic and data on CC incidence in young age groups is lacking. The increase was assumed to be due to the HIV AIDS epidemic. In contrast to studies from developed countries reporting increased risk of CC among HIV positives (Biggar et al. 2007) studies in the East Africa, however, report marginal risk in HIV and others report no association between HIV and CC (Odida et al. 2011). On the other hand, African countries have reported a high risk of pre-neoplasia among HIV positives (La Ruche et al. 1998, Leroy et al. 1999, Moodley et al. 2006).

In Uganda the prevalence of HIV in general population is 6.5% (Todd et al. 2009). Among the HIV positives, 57% are women, and out of those women 76% are under 30 years of age. HIV alone may not account for the high CC incidence and mortality in the country (Wabinga et al. 2000, Odida et al. 2011). Among the young women

(<30 years) risky taking sexual behaviour is on the increase. In addition, cross-generation and polygamous marriages and child abuse have contributed to increased rates of HPV, HIV infections, and unwanted pregnancies, which are risk factors for cervical cancer (Parkin et al. 2002, Parkin et al. 2010). Thus the incidence of CC is likely to increase in Uganda.

In contrast to a number of European countries, Uganda has no population-based cervical screening programmes and little is known about the causative agent and the disease among health care workers and women (Mutyaba et al. 2007). Therefore women seek medication mainly at the terminal stage of CC (Wabinga et al. 2003), and survival from CC is poor.

2.7 HPV and Cancer

2.7.1 History

The link between cervical cancer and sexually transmitted infections was proposed by Domenico Antonio Rizoni-Stern in 1842. He noted that CC was less common among nuns than married women or widows (Rigoni-Stern 1842). Several studies later reported that the sexual behaviour of the male partner determines a woman's risk for CC (Rotkin et al. 1973, Briton et al. 1989). Further work indicated that widows of men with penile cancer had increased mortality from CC (Smith et al. 1980) and that circumcision of the partner reduced the risk of developing the disease (Castellsague et al. 2002). All these studies indicated a sexually transmitted agent linked to CC. For nearly two decades most studies concentrated on the herpes simplex virus type 2 (HSV-2) until a prospective study and lack of consistent findings on the virus from CC tissue refuted the suspicion in the middle of 1980's (Vonka et al. 1984, Roizman et al. 1985). The causal association was flawed by residual confounding and homology between proliferating cell nuclear antigens and herpes virus proteins (Lehtinen et al. 2002).

As early as in 1976 Harald zur Hausen hypothesized the causal relationship between CC and human papillomavirus, and in the early 1980s his research group in Heidelberg detected HPV16 and HPV18 in the CC tissues (zur Hausen et al. 1976, Durst et al. 1983, Schwarz et al. 1985). This opened the door to rapid development of HPV virology, and following the discovery of virus-like particles (Kirnbauer et al. 1992), to prophylactic vaccine development.

Persistent infection with hrHPV types is the necessary cause of cervical cancer (Figure 5) (zur Hausen. 1996, Walboomers et al. 1999, Bosch et al. 2002, Munoz et al. 2003, de Sanjose et al. 2010). The prevalence of hrHPV in cervical cancer cases

ranges between 89.9 and 99% (Clifford et al. 2006, Li et al. 2011) with hrHPV infections accounting for 91% (de Sanjose et al. 2010). There is an increase in the overall HPV infections from 85.6% to 92.9% and a reduction in single HPV infection from 82.1% to 76.8% in CC reported in studies published 1990-2010 (Li et al. 2011) and during the same period an increase was also reported in the proportions of HPV16 from 51.8 to 60.0%.
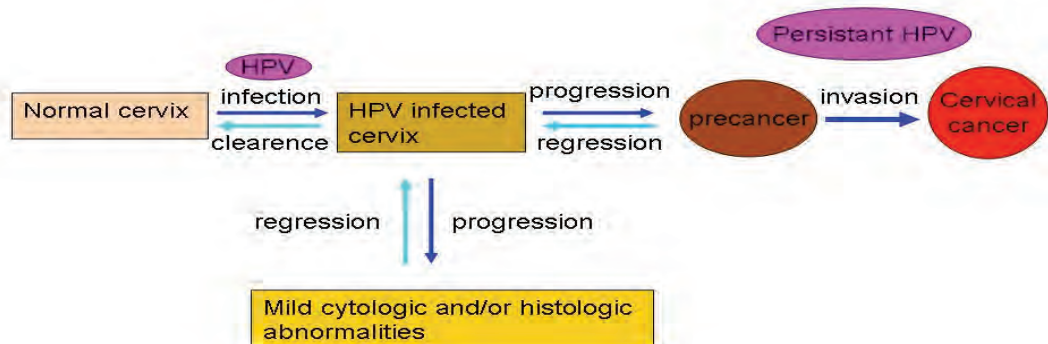

Figure 5. Natural history of HPV cervical cancer development

2.7.2 HPV types related to CC

Meta-analyses of studies on the occurrence of HPV types in CC published between 1990 and 2010 showed that the predominant types in descending order are HPV16, 18, 58, 33, 45, 31, 52, 35, 59, 39, 51 and 56 (Smith et al. 2007, Li et al. 2011). The same types were also observed in an IARC multicentrec cervical cancer study of 3,085 invasive cervical cancer samples tested uniformly (Munoz et al. 2004), and in a large multi-centre study (Bosch et al. 2008). In all parts of the world HPV 16 is the most commonly identified type in CC tissues followed by HPV18. There are, however, geographical differences in the third position; HPV45 is the third most common type in Africa, North America, West/Central Asia, and Oceania, whereas HPV31 comes third in Europe and South/Central America, and HPV58 in Eastern

Asia (Li et al. 2011). Another study which analysed all the 22,661 paraffin-embedded samples from 10,575 CC women from all over the world, reported the commonest hrHPV types to be HPV16, 18, 31, 33, 35, 45, 52 and 58 (de Sanjose et al. 2010). HPV16 alone was found in 60.6%, and HPV18 in 10.4% of CC cases. HPV16, 18, and 45 were found in 94% of cervical adenocarcinomas and have been associated with young age (de Sanjose et al. 2010).

2.7.3 Multiple HPV infections and CC

Several studies have shown an association between multiple persistent hrHPV infections and increased risk of CIN and CC (Cushieri et al. 2004, Herrero et al. 2005, Trottier et al. 2006, Odida et al. 2008, Munagala et al. 2009, Chaturvedi et al. 2011, Pista et al. 2011, Odida et al. 2011). The proportion of infections with multiple HPV types in ICC ranges 4-40% (Bosch et al. 2002, Trottier et al. 2006, Munagala et al. 2009, de Sanjose et al. 2010, Pista et al. 2011). In CIN2+ multiple infections with HPV16, 18, 31, 33 and 51 are more common than single hrHPV infections (Pista et al. 2011). A meta-analysis of studies published from 1990 to 2010 showed an increase in the prevalence of multiple HPV infections from 4.0% to 15.7% (Smith et al. 2007, de Sanjose et al. 2010, Li et al. 2011). Geographical differences in the occurrence of multiple HPV infections among squamous cervical cancers (SCC) were reported with the highest prevalence found in Africa (12.4%), America (9.4), Australia (7.8), Asia (7.8%), and Europe (6.2) (Munoz et al. 2004, Clifford et al. 2003, 2006, Smith et al. 2007, Li et al. 2011).

Multiple hrHPV infections are associated with HPV viral persistence (Ho et al. 1998, Banura et al. 2011,) faster progression to CIN (Bachtiary et al. 2002), and poor response to tumour treatment (Munagala et al. 2009). On the other hand, some studies have reported very low prevalence of multiple HPV infections associated with CIN3 lesions or ICC (van Door et al. 2001, Okolo et al. 2010, Odida et al. 2011), and others have reported no difference in the severity of disease progression between single and multiple HPV infections (Bosch et al. 2002, Herrero et al. 2000).

2.7.4 HPV seroepidemiology and cervical cancer

Most meta-analyses report on HPV DNA based data in different parts of the world; however, with a high rate of acquisition and clearance of the virus, it is difficult to assess the total lifetime number of HPV types one is exposed to. This is feasible with HPV serology due to the stability of antibodies for more than five years. Ecological studies correlating HPV DNA prevalence with CC risk should be interpreted with caution as in the case of Denmark and Greenland. It had been observed that, women in Greenland had a low HPV DNA prevalence but a higher risk of CC com-

pared to those in Denmark. However, when HPV serology was done, it was proved that although women in Greenland had low levels of current infections detected, their cumulative HPV exposure (indicated by antibodies) was higher than among women in mainland Denmark (Nonnenmarcher et al. 1996).

Several prospective studies have suggested that seropositivity for HPV16 increases the risk for future development of cervical cancer (Carter et al. 1996, Lehtinen et al. 1996; Dillner et al. 1997, Björge et al. 1997, Dahlström et al. 2011). Some studies showed an association between seropositivity to HPV18 and 33 and invasive cervical cancer (Dillner et al. 1997, Wang et al. 1997). Others suggested that seropositivity to both HPV types 16 and 18 increases the risk for CC development (Dillner et al. 1997). The risk disappears if individuals are seropositive for both HPV16 and HPV6 (Luostarinen et al. 1999). The order of acquiring infections with different HPV types appears to be important, since only individuals seroconverted for HPV6 before HPV16 seroconversion had negligible risk of CIN3+ (Luostarinen et al. 2011). On the other hand, some studies suggested that HPV antibodies are protective against disease development (Lehtinen et al. 1993, Ho et al. 2002). In a follow-up study there was no risk of progression to CIN3+ among women who seroconverted compared to non-seroconverters (Carter et al. 2000, Syrjänen et al. 2009a).

Because of the high vaccine efficacy in HPV vaccine clinical trials WHO has recommended mass HPV vaccination in developing countries (Villa et al. 2006, Paavonen et al. 2007, 2009). However, there are no HPV vaccine clinical data available from Sub-Saharan Africa. One study from an HIV cohort in the USA (Levin et al. 2010) showed lower poorly induced antibodies among HIV infected compared to HIV negatives. HPV DNA data show type difference among HIV positive and HIV negative groups (Clifford et al. 2005) and geographical HPV type variation has been reported, whereby in East African countries HPV16 was second to HPV52 among women with normal cytology (de Sanjose et al. 2007, Bruni et al. 2011, Banura et al. 2011). However, it is unclear how the HPV types may differ among the HIV positive and HIV negative groups.

The reduced risk of developing cervical squamous intraepithelial lesions in women with a history of anogenital warts was first suggested by Evans et al. (1992) to be due to antagonism between HPV types. Since then antagonistic interaction has been confirmed in a longitudinal setting among women seropositive for both lrHPV6/11 and hrHPV16 antibodies with no or very low risk of ICC (Luostarinen et al. 1999, Dahlström et al. 2011). In serial sample analysis the antagonistic interaction is found only if HPV6 infection precedes HPV16 infection (Lehtinen et al. 2011). Antagonistic interactions were also detected for combinations of HPV16 and HPV18 and of HPV16 and HPV33 (Luostarinen et al. 1999).

The interaction between HPV types is pivotal in understanding HPV-induced carcinogenesis and predicting the likely effects of different preventive measures. For instance, it is not known why CC incidence is high in Sub-Saharan Africa, where lrHPV6 is highly prevalent. Could there be other mechanisms that affect the establishment of persistent HPV16 infection since it is not very common in general population (Bruni et al. 2010, Banura et al. 2011) but most common in the CC patients (Smith et al. 2007, Banura et al. 2011, Li et al. 2011, Odida et al. 2011) in this region?

2.7.5 HPV and non-cervical anogenital cancers (Anal, penile, vulva)

HPV has also been widely reported in non-cervical anogenital cancers, the incidences of which are on the increase in the most developed regions, apart from penile cancer, which is common in Africa (de Sanjose et al. 2007). Current data indicate that hrHPV infection is associated with cancers (Table 3); 90%-93% of anal cancers, 12%-63% of oropharyngeal cancers, 36%-40% of penile cancers, 40%-64% of vaginal cancers, and 40%-51% of vulvar cancers (De Vuyst et al. 2009, Chaturvedi 2010). The commonest HPV types responsible for these cancers are HPV16 and HPV18, and account for 93%, 91%, 88%, and 73% of anal, vulvar, vaginal and penile cancers respectively (Smith et al. 2004). HPV16 alone was found in 73.7% and 68.4%, of invasive anal cancer, and anal HSIL tissues, and HPV18 was 7.6% and 7.0% respectively (Brooke et al. 2009).

Table 3. Burden of HPV related cancers.

site	[1]Annual numbers of cases worldwide.		[1]Proportion of cancers attributable to HPV infections	[2]Proportion of HPV-positive cancers attributable to HPV16/18
	Total	women		
Anus	27, 360 - 28,272	14,310 - 14,787	90-93	93
Oropharynx	6,252 - 32,823	1,152 - 6,048	12-63	89-95
Penis	9,468 - 10,520	NA	36-40	63-87
Vagina	16,000 - 25,600	16,000 - 25,600	40-64	80-88
Vulva	16,000 - 25,600	16,000 - 25,600	40-51	80-86
Cervix	492,800	492,800	100	70-76

Adapted from Chaturvedi (2010) J Adolesc Health. 46:S20-6. [1]Gillison et al. 2008, Parkin and Bray 2006, [2]Gillison et al. 2008

2.8 Prophylactic HPV Vaccine

The primary mechanism of action for prophylactic viral vaccines is induction of humoral response (Breitburd et al. 1995). There are two licenced prophylactic HPV vaccines Cervarix™ (HPV16/18) and Gardasil™ (HPV6/11/16/18). These vaccines consist of virus-like particles (VLPs) that are produced by expressing the L1 gene of HPV 6/11/16/18 in yeast and of HPV16/18 in insect cells. The VLPs were highly immunogenic in the phase II studies (Villa et al. 2005, Harper et al. 2004). Aluminum derivatives in the adjuvant enhance the immune response. The HPV 6/11/16/18 vaccine contains aluminiumhydroxy-diphoshosulphate, and the HPV-16/18 vaccine formulated with the AS04 Adjuvant System, comprises aluminium hydroxside supplemented with the monophosphoryl lipid AS04. The monophosphoryl lipid AS04 vaccine further increases the production of antibodies and specific memory B cells compared to the aluminum hydroxide adjuvanted vaccine (Giannini et al. 2006, Garcon et al. 2011).

Following vaccination a robust antibody production takes place and memory B cells are induced to maintain circulating antibody levels. Most importantly, memory B cells help the immune system to swiftly generate high levels of antibodies when

challenged. Unlike other common viruses, HPV infections are local; being in the mucosa the virus tends to evade the immune system. Therefore generation of neutralizing antibodies is important to restrict the infection within the host and transmission from the host (Stanley 2010a). Even though vaccine-induced HPV antibody levels are very high in all ages, HPV immune responses are age-dependent, the age group below 15 years showing the highest levels (Perez et al. 2008, Petäjä et al. 2009, 2010). Factors such as smoking do not affect the vaccine-induced antibody levels (Kapeu et al. 2009). The effect of HIV on the HPV vaccine response is still unclear. High and sustained antibody responses have been observed up to 10 years after vaccination for both HPV16 and 18 with the bivalent vaccine and for at least 8.5 years with the quadrivalent vaccine (Harper et al. 2008, Frazer 2010).

Although cross-protection against other HPV types has been demonstrated following HPV vaccination, the correlates and duration of protection are still unclear (Garcon et al. 2011, Wheeler et al. 2009, 2012). The vaccine efficacy against CIN3 associated with any HPV type is more than 93% among baseline HPV naïve women (Lehtinen et al. 2012). Efficacy was about 45% including women with baseline HPV infections (Lehtinen et al. 2012). Despite the promising HPV vaccine results from developed countries, HPV vaccine data is still unavailable from developing countries. A pilot study that evaluated the feasibility of HPV vaccine introduction, conducted in India, Peru, Uganda and Vietnam by PATH, (PATH 2010). Although, WHO recommends mass HPV vaccination basing on the results from developed countries, developing countries may consider implementing it only provided that resources are secured like in Rwanda the pioneering African country to implement mass HPV vaccination (Lancet 2011). Such programmes are of great importance if sustained. However, for such a big national health investment, effectiveness/impact data is required to help in planning for the best implementation strategies. Due to differences in environmental factors, there is an urgent need also for clinical trials to evaluate the effect of factors such as HIV, malaria, helminths infections which are endemic in Sub-Saharan countries on the immunogenicity and efficacy of the HPV vaccine.

3 AIMS

The aim of this study was to use HPV antibodies as markers of cumulative HPV exposure (cumulative incidence) to study the occurrence of multiple HPV serotypes and to assess associated risk factors among Finnish and Ugandan women.

The specific aims were:

1. To determine the distribution of HPV seroprevalence and the occurrence of multiple HPV types among Finnish and Ugandan women
2. To evaluate risk factors of seropositivity for multiple HPV types among Ugandan women
3. To determine the risk of seroconversion to multiple HPV types among Finnish women
4. To evaluate the role of HPV16 antibody avidity in the acquisition of multiple HPV types.

4 MATERIALS AND METHODS

4.1 Study sites and cohorts

The study participants were from two geographically different sites; Finland in Northern Europe and Uganda in East Africa.

4.1.1 Finnish Maternity Cohort (FMC)

The Finnish Maternity Cohort (FMC) is one of the largest serum biobanks in the world with more than 1.8 million serum samples collected from about 760,000 pregnant women in their first trimester (gestation period 8-12 weeks). The FMC biobank was established in 1983 with the aim of screening for congenital infections, and currently screens for HIV, hepatitis B virus and *Treponema pallidum* infections (Koskela et al. 2000). Since 2001, all women consent for their samples to be used in health related research performed by the National Institute for Health & Welfare (THL). For the samples collected before 2001 a law was passed by the Parliament of Finland for their use in the above context (Pukkala et al. 2007).

At the THL Oulu laboratories all serum samples for screening obtained from the municipal antenatal clinics are processed and stored at -25^{0}C. The FMC covers more than 98% of all Finnish pregnant women. With appropriate ethics review board permissions, exposure data obtained from laboratory analyses of these samples can be linked to other registries (such as the Finnish Cancer Registry and available demographic and maternity data) by using the Finnish unique personal identity number (PID).

4.1.2 Kuopio Cohort (KC)

Kuopio is the capital of Eastern Finland. The KC was established from 1981 to 1998 at the University of Eastern Finland (formerlUniversity of Kuopio) Hospital. Participants were 532 women with   koilocytotic cells with or without dyskaryotic abnormal Pap test from the routine cervical cancer-screening programme. A comprehensive gynaecological examination including repeated Pap test, colposcopy and a directed punch biopsy were done at recruitment and a questionnaire completed for other risk factors. Blood samples were taken at six-month intervals for up to 10 years (Kataja et al. 1992; Syrjänen et al. 1985).

4.1.3 Ugandan pregnant women

A cross-sectional study was conducted among pregnant women attending antenatal clinics in three hospitals in central Uganda: Naguru Teenage Health and Information Center, Entebbe and Nsambya Hospitals. Entebbe Hospital is a district referral hospital in a semi-urban setting about 40 km from Kampala (the capital city). It serves women within a distance of about 80 km including the islands in Lake Victoria. Nsambya Hospital is a tertiary, private (nonprofit) referral hospital in the suburbs of Kampala. It serves women of all income levels in private and public clinics within a distance of about 40 km. Naguru Teenage Health and Information Center is a municipal health centre, about 5 km from Kampala city centre, Uganda, it serves women from nearby suburbs and especially teenage mothers.

In Uganda all pregnant women are recommended to visit antenatal clinics for pregnancy related examination and for congenital screening of HIV and *Treponema pallidum* and to be vaccinated against tetanus. During the antenatal visit health talks are conducted, HIV pre-counselling in small groups (5 women) is offered, demographic data is collected, blood samples are donated to the laboratory and HIV post-counselling per individual on delivery of blood test results. HIV and syphilis results are released on the same day and those positive for syphilis are treated and HIV positives are referred to other units for management (Mpairwe et al. 2003, Banura et al. 2008a, 2010).

We conducted our study in collaboration with the Prevention of Mother to Child HIV Transmission Programme in antenatal clinics. The first group comprised women who participated in a follow-up study of young primiparous pregnant women at the Naguru Teenage Health and Information Center (Banura et al. 2008b) between May and November 2004. The second group of pregnant women was enrolled between October and December 2008 at the Entebbe and Nsambya hospitals during their first antenatal visit. Information about HPV was included in the routine health information given by trained midwives and nurses to pregnant women. A questionnaire to collect risk factors for HPV infections was completed after women consented in writing to participate in an STI serology study.

4.1.4 Ethical clearance

Ethical approvals for the respective studies were obtained from the ethics review boards at the National Institute for Health and Welfare, Finland; Uganda Virus Research Institute, Entebbe, Uganda; St Raphael of St Francis Hospital Nsambya, Kampala, Uganda and The Uganda National Council of Science and Technology. All women consented individually to participate in the STI serology study.

4.2 Study populations and data collected

The following describe the four studies conducted as part of this thesis, which are subcohort of the above-mentioned cohorts.

Paper I

A cross-sectional study was conducted among the Finnish and Ugandan pregnant women as follows: A stratified random sub-cohort of 3,251 Finnish pregnant women with a mean age of 22 years (range 14-28 years, standard deviation (SD 3) who donated serum samples between 1995 and 2003 was selected from the FMC (Kaasila et al. 2009). A total of 2,053 Ugandan pregnant women with a mean age of 23 years (range 14-48 years, SD 5) were enrolled. Altogether 471 Finnish and 69 Ugandan women were excluded due to inadequate volume of serum sample. A total of 2,780 Finnish and 1,984 Ugandan women were included in the HPV antibody analysis against 7 HPV types 6/11/16/18/31/33/45 using VLP-based ELISA.

Paper II

Out of the 1,984 serum samples used in Paper I from the Ugandan pregnant women, all 1,943 serum samples with adequate volume were analysed for antibodies to eight oncogenic HPV types (16/18/31/33/35/45/52/58) using multiplex serology assay at the German Cancer Research Center (DKFZ) in Germany.

Paper III

Four hundred and eighty (480) women from the Kuopio cohort with abnormal Pap tests were followed up at 6-month intervals for an average of 60.3 months (Kataja et al. 1992; Syrjänen et al. 1985, Simen-Kapeu et al. 2008), but only 437 women had at least two serum samples and were included in this HPV seroconversion study. Antibodies to seven HPV types 6/11/16/18/31/33/45 were analysed using VLP-based ELISA.

Paper IV

Three hundred and sixty five (365) pregnant women (both Finnish and Ugandan) were selected by simple random method from 994 HPV16 antibody-positive women identified from Paper I and included in a study on HPV16 antibody avidity with a modified VLP-based ELISA.

4.3 Laboratory analysis

4.3.1 Measuring HPV antibodies using VLP-based ELISA (Papers I and III)

Serum IgG antibodies against seven specific HPV types 6/11/16/18/31/33/45 were analysed using virus-like particle (VLP) based Enzyme-linked Immunosorbent Assay (ELISA) as described previously (Dillner et al. 1996, Dessy et al. 2008, Kaasila et al. 2009). VLPs were generously donated by Dr. Kathrin Jansen (HPV6/11/16,

Merck Research Laboratories, Philadelphia, PA, USA), Dr. Reinhard Kirnabauer, University of Vienna, Austria (HPV types 31/33) and Dr. Francis Dessy (HPV types 18/33/45, GlaxoSmithKline Biologicals, Rixensart, Belgium). Sera and controls were diluted 1:30 in assay diluents. Fifty µl of the diluted samples were added in singlicates on the 96-well plate. Assay drift was monitored by adding blank (assay diluent), negative, positive and low-positive (1:100) controls in duplicate per plate. The negative control was serum samples pooled from adolescent virgins as indicated in previous work (Dillner et al. 1996, Lehtinen et al. 2006, Simen-Kapeu et al. 2008), and later a new negative control, a pool of sera seronegative for all the seven HPV types 6/11/16/18/31/33/45 was applied. The positive controls were pooled from samples seropositive for all seven types.

4.3.2 Multiplex serology (Paper II)

We analysed the Ugandan sera to include other HPV types commonly reported in Africa. Antibodies against eight different oncogenic HPV types were analysed simultaneously (Waterboer et al. 2005). Briefly, bead sets (3,000 beads per set per well) carrying different antigens were mixed. Equal volumes of diluted serum and mixed beads were mixed into 96-well plates. The beads were washed off after one hour's incubation at room temperature. Secondary antibody and conjugate were added, with incubation and washing steps in between. Reporter fluorescence of the beads was determined with a Luminex analyser and expressed as median fluorescence intensity (MFI) of at least 100 beads per set per well. Cut-offs for seropositivity of specific hrHPV types 16/18/31/33/35/45/52/58 were 422, 394, 712, 515, 552, 368, 547, 592 MFI respectively (Dondog et al. 2008).

4.3.3 Modified ELISA for HPV16 IgG antibody avidity measurements (Paper IV)

The procedures for HPV VLP-based ELISA were followed with modifications. First we optimized for the molarity of urea between the ranges of 4M to 8M and the best curve was obtained with 6M of urea (unpublished). For the rest of the sample analysis we used 6M urea. Serum samples were serially diluted: 1:1, 1:4, 1:16, 1:64, 1:256 in phosphate-buffered saline (PBS) with 10% fetal bovine serum (blocking buffer, BB). Fifty µl of diluted samples were added to wells A-D (1:4, 1:16, 1:64, 1:256) and wells E-H (1:1, 1:4, 1:16, 1:64). Blank (assay diluent), low- and high-avidity controls were added to columns 1, 2 and 3, and incubated overnight at 4°C. Wells A-D were washed three times with 200µl of PBS/0.05% Tween 20 (PBS+T), and wells E-H were washed with 6M urea after we optimized for assay. (Promega, Biofellows, Finland) in PBS. Each wash was for 5 min. Avidity index (AI) and cut-off of low avidity were calculated as described previously (Hedman et al. 1989, Herne et al. 1997, Namujju et al. 2011).

4.3.4   Other serology measurements (Papers I-IV)

HIV-serology

In Uganda, a serial testing algorithm was applied to detect HIV antibodies: All serum samples were screened using the Abbott rapid kit (Abbott Laboratories, Abbott Park, IL, USA) and positive samples were re-analysed on Unigold rapid kit (Unigold Trinity Biotech PLC, Bray, Ireland) and in case of discordant samples a tie breaker, Murex HIV-1.2.0 ELISA (Murex Biotech, Ltd.,Dartford, UK) was used to confirm the results, (Mpairwe et al. 2005, Banura et al. 2008). All Ugandan samples were re-analyzed in Oulu, Finland, using the Abbott Combo test (Abbott Ireland, Diagnostic Division, Sligo, Ireland). The Finnish samples were analyzed with the Abbott Combo test.

***Chlamydia trachomatis* serology**

IgG antibodies to *C. trachomatis* were analysed using a commercial kit (Anilabsystems, Helsinki, Finland) as described (Dillner et al. 1996).

Cotinine

Cotinine is a metabolite (byproduct) of nicotine as it is processed by the human body. It is a biomarker for nicotine exposure. Cotinine measurement was done using a commercial qualitative immunoassay (OraSure Technologies, Bethlehem, Pennsylvania) following the manufacturer's instructions. Results above the cut-off level (>20ng/mL) indicated a current smoker (Simen-Kapeu et al. 2008)

4.4   Statistical methods

Statistical analyses for Papers I, II, & IV were carried out using Stata 8 (Stata Corp., College Station, Texas, USA) and for Paper III SPSS16.0 for Windows was used (SPSS Inc., Chicago, IL, USA). Two-sided p<0.05 was considered to be statistically significant. Descriptive statistics such as mean, standard deviations and proportions were calculated for the variables.

A logistic regression model was used to calculate the relative risk (RR), estimated as crude and adjusted odds ratios (OR) with 95% confidence interval (CI) for seropositivity to multiple HPV types. We defined overall seropositivity as antibody positivity to at least one of the HPV types analysed in that study, and multiple HPV types as positivity to at least two of the HPV types analysed.

Paper I

Being seropositive for multiple HPV types was the main outcome measurement. Univariate logistic regression was used to estimate the relative risk (OR) with 95% CI of being seropositive for at least two HPV types among seropositive women for a specific type as compared to seronegative women for that particular type. Adjustments were made for age, smoking and HIV.

Paper II

The main outcome measurement was seropositivity for multiple hrHPV types. The univariate, logistic regression model was used to estimate the relative risk (OR) with 95% CI of being seropositive for multiple hrHPV types among Ugandan pregnant women according to different variables (age, age at sexual debut, number of lifetime sexual partners, parity, education, smoking, HIV, and *C. trachomatis*). Multivariate and stepwise (eliminating one variable at a time) multivariate logistic regression models were used to assess the independent and stand-alone independent risk factors respectively. We also estimated the risk (OR) with 95% CI of being seropositive for multiple HPV types among women seropositive for HPV16 or HPV18 as compared to seronegative women for that particular type by HIV status. We further estimated the risk of being seropositive for double HPV types among seropositive women for a specific HPV type compared to seronegative women for that particular type by HIV status. Adjustment was made for age and *C. trachomatis* seropositivity.

Paper III

The proportions of seroconversions were calculated among women who were antibody negative for a specific HPV type at baseline but seropositive for that particular HPV type at the follow-up sample. We used the univariate logistic regression model to estimate the relative risk (OR) with 95% CI of seroconversion for another HPV type among baseline seropositive women for a specific HPV type compared to seronegative women for that particular type. We further stratified the HPV types as low-risk (lr) or high-risk (hr), and estimated the RR with 95% CI of seroconversion to hrHPV types among lrHPV seropositives as compared to seronegatives.

Paper IV

As HPV16 is one of the most common hrHPV types, we assessed the association (OR with 95% CI) of low-avidity HPV16 antibodies with seropositivity for multiple HPV types compared to being seropositive for HPV16 only among HPV16 seropositive women using the univariate model. Adjustment was made for age, *C. trachomatis* and HIV seropositivity, and smoking.

5 RESULTS

Paper I -Occurrence of seropositivity to multiple HPV types among Finnish and Ugandan women

The seroprevalences of specific HPV types, HIV and *C. trachomatis* antibodies, and smoking among both Finnish and Ugandan pregnant women are summarized below for Papers I, II and III (Table 4).

Table 4. A summary of HPV seroprevalence (%) based on HPV VLP ELISA and multiplex HPV serology measurements, and other STIs among Finnish and Ugandan women in Papers I, II, III.

	VLP-based ELISA				Multiplex serology		
	Pregnant women (**Paper I**)		*Kuopio –Finnish women with abnormal Pap tests* (**Paper III**)		*Ugandan pregnant women* (N=1943) (**Paper II**)		
	Finnish women (FMC) **(N=2784)**	**Ugandan** women **(N=1964)**	Baseline sample **(N=437)**	Follow-up sample	sero-prevalence	Among HIV+	Among HIV-
HPV6	12	30	33	32	na	na	na
HPV11	11	26	20	18	na	na	na
HPV16	21	21	21	19	15	22	14*
HPV18	14	8	11	10	16	17	16
HPV31	12	16	15	12	13	12	13
HPV33	11	23	16	15	9	11	9
HPV35	na	na	na	na	7	4	7
HPV45	5	11	27	31	13	19	12*
HPV52	na	na	na	na	8	16	8*
HPV58	na	na	na	na	5	3	5
C. trachomatis	25	27	28	30	27		
HIV	0	7	na	na	7		
	26	3	36	35	3		

* Types significantly different by HIV status in Paper II only, na= not applicable

Seroprevalence

The overall seroprevalence (for at least one) and for multiple HPV types (at least two) of the HPV types 6/11/16/18/31/33/45) were low among Finnish (44% (1,222) and 22% (619) respectively) compared to Ugandan women (57% (1 118) and 30% (588) respectively) (p <0.001). For the Finnish women, age-specific overall HPV seroprevalence peaked in the age-group 20-24 years, while the seropositivity rate, i.e. seroprevalence for multiple HPV types increased with age up to the age group 25-29 years. Whereas for the Ugandan women both prevalences peaked in < 20-year olds and declined thereafter with increasing age (Paper I). Seropositivity for multiple HPV types among HPV positive women was common in both Finnish (51%) and Ugandan (53%) women. In Finland 26%, 12% and 13% of the seropositive women had antibodies for two, three and four or more HPV types respectively, whereas in Uganda 30%, 12%, 9% had similar antibodies respectively. The HPV16 seroprevalences were similar in both countries (21%). Among the Finnish women seropositivity was most common for HPV types 16, 18, 31 and 33, whereas among the Ugandan women the corresponding HPV types were 33, 16, 31 and 45 (Table 4).
Ugandan HIV-positive women had higher HPV seroprevalences both overall (74%, 104 of 140) and for multiple HPV types (53%, 74 of 140) than HIV-negative women (56%, 1,014 of 1,842 and 28% 514 of 1,824) for overall and multiple HPV types respectively.

Risk of being seropositive for multiple HPV types

Overall, seropositive women compared to women seronegative for any of the specific HPV types (6/11/16/18/31/33/45), had an increased risk of being seropositive for a second HPV type with the OR ranging between 1.8 and 12.2 among Finnish women and 1.7 to 5.3 for Ugandan women. Compared to Ugandan women (HPV45$_U$) (lower left half in Table 5), Finnish women who were seropositive for HPV45 (HPV45$_F$) (upper right half in Table 5) had a significantly higher risk estimate of double seropositivity for HPV types 16, 18, 31 and 33. There was no significant differences for the phylogenetically related HPV31/33 between Finnish and Ugandan women despite the increased risk estimates for double seropositivity among the HPV16 seropositives (Table 5).

Table 5. Odds ratios with 95% confidence intervals of being seropositive to another HPV type if seropositive to a defined HPV type among pregnant Finnish (upper righr half, n=2,780) or Ugandan (lower left half, n=1,964) women, with HPV-seronegative women as the reference group.

HPV antibody status	Risk of double HPV antibody positivity					
	6/11 OR(95%CI)	16 OR(95%CI)	18 OR(95%CI)	31 OR(95%CI)	33 OR(95%CI)	45 OR(95%CI)
6/11						
Positive	na	2.4 (1.6–3.7)	3.1 (1.9–4.9)	3.4 (1.9–5.8	2.2 (1.3–3.7)	1.8 (0.8–4.1)
16						
Positive	1.9 (1.5–2.5)	na	6.7 (4.3–10.5)	4.7 (2.8–7.9)	7.0 (4.3–11.4)	6.5 (3.2–13.3)
18						
Positive	2.0 (1.3–3.0)	5.3 (3.8–7.5)	na	5.2 (3.0–9.0)	6.9 (4.1–11.7)	10.9 (5.3–23)
31						
Positive	2.2 (1.6–2.9)	3.8 (2.9–5.0)	3.1 (2.2–4.4)	na	4.5 (2.5–8.2)	6.1 (2.8–13.4)
33						
Positive	3.0 (2.3–3.8)	4.6 (3.6–5.8)	3.3 (2.3–4.6)	3.5 (2.7–4.5)	na	12.2 (5.8–26)
45						
Positive	1.7 (1.2–2.4)	2.0 (1.4–2.7)	3.4 (2.3–5.0)	2.2 (1.6–3.0)	3.3 (2.4–4.5)	na

Adjusted for age, smoking (Finland and Uganda) and HIV positivity (Uganda), A significant difference = when the confidence intervals of estimates for risk of double seropositivity of particular HPV types between the two populations do not overlap.

Paper II- Risk of seropositivity for multiple hrHPV types among Ugandan HIV-positive and HIV- negative women

Determinants of seropositivity for multiple hrHPV types

We observed that age (30 years and above) and education (tertiary level) had a protective effect against being seropositive for multiple HPV types. Conversely, age at sexual debut, number of sexual partners and smoking had no effect, but parity (>5), C.*trachomatis* and HIV antibody positivity increased the risk of being seropositive for multiple HPV types in a univariate model. Again, in the multivariate model all factors remained significant except that the risk associated with C.*trachomatis* seropositivity disappeared (Table 6). In the stepwise model (analysed by elimination of the variables one at a time), only HIV remained the stand-alone, independent risk factor for seropositivity to multiple hrHPV types.

Risk of seropositivity for multiple hrHPV types by vaccine types

We observed that women seropositive for vaccine type HPV16 or HPV18 compared to women seronegative for those particular types had an increased risk of being seropositive for multiple hrHPV types. The differences, however, were not statistically significantly different by HIV status (HPV16/HIV+; OR 11.8, 95% CI 4.5-31.5 vs HPV16/HIV-; OR 21.8, 95%CI 15.4-30.8 and HPV18/HIV+; OR 58.1, 95% CI 13.9-242 vs HPV18/HIV-; OR 44.8, 95% CI 31.1-64.7). Furthermore, although seropositivity for a specific hrHPV type compared to seronegativity increased the risk of being seropositive for another hrHPV type, no statistical differences were observed in the risk estimates between HIV-positive and HIV-negative women.

Table 6. Univariate and multivariate estimation of the relative risk (RR) of seropositivity for multiple high-risk human papillomavirus (HPV) types among pregnant Ugandan women (N=1,209)

Characteristic			hrHPV antibody		Univariate multiple hrHPV types RR (95% CI)	Multivariate multiple hrHPV types RR (95%CI)
	number (n)	percentage	positive	(%)		
Age						
<20	150	12 %	38	(25)	1	
20-24	425	35 %	83	(20)	0.7 (0.5-1.1)	0.7 (0.4-1.1)
25-29	385	32 %	86	(22)	0.8 (0.5-1.3)	0.8 (0.5-1.3)
>=30	249	21 %	42	(17)	**0.6 (0.4-1.0)**[*]	**0.5 (0.3-0.8)**[*]
Age at sexual debut						
<15	120	10 %	29	(24)	1	
15-20	903	75 %	185	(20)	0.8 (0.5-1.3)	1.0 (0.6-1.7)
>20	186	15 %	35	(19)	0.7 (0.4-1.3)	1.1 (0.5-2.2)
Life-time partners						
1	314	26 %	64	(20)	1	
2	377	31 %	76	(20)	1.0 (0.7-1.4)	1.1 (0.7-1.6)
3	238	20 %	52	(22)	1.1 (0.7-1.6)	1.0 (0.6-1.6)
>3	280	23 %	57	(20)	1.0 (0.7-1.5)	1.0 (0.6-1.6)
Pregnancies						
<5	970	80 %	192	(20)	1	
≥5	239	20 %	57	(24)	1.3 (0.9-1.8)	**1.6 (1.0-2.5)**[*]
Education[a]						
<=Primary	352	33 %	92	(26)	1	
Secondary	445	42 %	91	(20)	0.7 (0.5-1.0)	0.8 (0.6-1.2)
Tertiary	266	25 %	43	(16)	**0.5 (0.4-0.8)**[*]	**0.6 (0.4-1.0)**
Smoking[b]						
no	1 103	96 %	228	(21)	1	
yes	47	4 %	10	(21)	1.0(0.5-2.0)	0.9 (0.4-1.9)
HIV						
negative	1 114	92 %	297	(27)	1	
positive	54	8 %	35	(37)	**1.8 (1.1-2.8)**[*]	**1.7 (1.0-2.8)**[*,†]
Chlamydia[c]						
negative	875	73 %	164	(19)	1	
positive	322	27 %	81	(25)	**1.5 (1.1-2.0)**[*]	1.1 (0.8-1.6)

[*] = statistically significant risk factor for multiple hrHPV seropositivity, [†] = statistically significant, independent risk factor for multiple hrHPV seropositivity [a]n=1063, [b]n=1150, [c]n=1197

Comparison of Results from HPV VLP ELISA and multiplex serology assays for Ugandan women

Antibodies against HPV 11/16/18/31/33/45 were analysed using two techniques: VLP based HPV ELISA (Paper I) and multiplex serology (Paper II) in two laboratories, Oulu (Finland) and Heidelberg (Germany). The results showed observed agreement above 70% but the kappa values were very poor (Table 7).

Table 7. Comparison between conventional HPV VLP based ELISA and Multiplex serology methods in the analysis of HPV IgG antibodies.

HPV type	Total number* n=1948		Observed agreement (%)	Expected agreement (%)	kappa	95% Confidence interval	Z
	Positive on ELISA	Positive on multiplex					
HPV11	420	344	71.3	58.4	0.31	0.26-0.37	11.32
HPV16	406	285	77.4	70.6	0.23	0.18-0.28	10.35
HPV18	153	316	78.4	78.4	0.18	0.12-0.23	8.49
HPV31	304	246	75.7	75.7	0.18	0.13-0.24	8.01
HPV33	440	184	72.2	72.2	0.20	0.15-0.25	10.09
HPV45	205	251	79.3	79.3	0.11	0.05-0.16	4.76

* = excludes HPV11 (n=1304)

Occurrence of multiple HPV types among Finnish and Ugandan women

Paper III - Risk of seroconversion to multiple HPV types in a follow-up study

Proportions of seroconversion among baseline seropositives

We observed no protection against seroconversion to another HPV type among baseline seropositive women. The proportions of seroconversions among women for at least one other HPV type (multiple HPV types) among baseline HPV seropositives to any of the HPV types 6, 11, 16, 18, 31, 33, and 45, were generally high, ranging from 73% to 98% as reported in the descending order among baseline seropositive women for hrHPV types: 98% (HPV18), 90% (HPV16), 78% (HPV45), 77% (HPV33) and 73% (HPV31) and for the lrHPV types were 95% (HPV11) and 93% (HPV6).

Risk of seroconversion among baseline HPV16 or HPV18 seropositives

We observed that among the HPV16 or HPV18 baseline seropositive women the risk of seroconversion was statistically significantly increased for the phylogenetically related HPV types HPV31 and HPV33 (HPV16/HPV31: OR 2.5, 95CI 1.5-4.3; HPV16/HPV33: OR 3.4, 95%CI 2.0-5.8; HPV18/HPV31: OR 6.2, 95%CI 3.2-12.0; HPV18/HPV33: OR 8.6, 95%CI 4.2-17.0. On the other hand, the risk of seroconversion to HPV18 among the baseline HPV16 seropositive women was 5.5 fold (95% CI 2.8-10.0) and for seroconversion to HPV16 among the HPV18 baseline seropositives was 7.4 fold (3.8-14) (Table 8).

We further noted that when multiple types were stratified into low and high risk types, seropositivity to multiple lrHPV (6/11) types at baseline compared to seronegativity increased the risk of seroconversion to multiple hrHPV types (16/18/31/33/45) (OR 2.3, 95% CI 1.1-4.7).

Table 8. Risk of HPV seropositive compared to seronegative (reference group) women at baseline to seroconvert to another HPV type during follow-up study.

First sample HPV antibody status	Risk of seronconversion						
	6 OR (95%CI)	11 OR (95%CI)	16 OR (95%CI)	18 OR (95%CI)	31 OR (95%CI)	33 OR (95%CI)	45 OR (95%CI)
6							
Positive	-	6.5 (3.9–11)	2.2 (1.3–3.6)	2.4 (1.3–3.5)	1.5 (0.9–2.5)	1.7 (1.1–2.7)	0.8 (0.5–1.4)
11							
Positive	8.2 (4.7–14)	-	2.6 (1.5–4.7)	1.8 (0.9–3.7)	2.6 (1.5–4.5)	2.7 (1.6–4.7)	0.8 (0.4–1.5)
16							
Positive	2.4 (1.5–4.0)	2.0 (1.1–3.5)	-	5.5 (2.8–10)	2.5 (1.5–4.3)	3.4 (2.0–5.8)	0.7 (0.4–1.3)
18							
Positive	1.7 (0.9–3.3)	1.9 (0.9–3.9)	7.4 (3.8–14)	-	6.2 (3.2–12)	8.6 (4.2–17)	1.4 (0.6–3.0)
31							
Positive	1.1 (0.6–2.1)	1.0 (0.5–2.2)	0.8 (0.3–1.8)	0.7 (0.2–2.1)	-	4.3 (2.3–7.9)	2.5 (1.4–4.4)
33							
Positive	1.3 (0.7–2.4)	1.0 (0.5–2.1)	0.7 (0.3–1.6)	0.7 (0.3–2.1)	4.3 (2.2–8.2)	-	5.9 (3.4–10)
45							
Positive	0.8 (0.5–1.5)	1.2 (0.6–2.2)	0.9 (0.5–1.7)	0.9 (0.4–2.2)	2.7 (1.5–5.8)	4.9 (2.8–8.5)	-

Adjusted for *Chlamydia trachomatis* antibodies

Paper IV- Association of low-avidity HPV16 antibodies and seropositivity for multiple HPV types

The overall proportion of women with low avidity HPV16 antibodies was 18% (68/365). The proportions of women with low avidity HPV16 antibodies who were also HPV6/11, HPV31/33 or HPV18/45 positive were 33.8%, 10.8% and 14.3% respectively (Table 9). After stratification by country, the proportion of low-avidity HPV16 antibodies was significantly lower among Finnish (15%) than Ugandan

(26%) women (p<0.0001). The age-, *C. trachomatis* seropositivity and smoking-adjusted risk for being seropositive to lrHPVtypes 6/11 among women with low-avidity HPV16 antibodies was significantly increased among Ugandan women (OR 2.21, 95%CI 1.01-8.40) compared to Finnish women (OR 0.80, 95%CI 0.94-6.82) (Table 9).

Table 9. Association (adjusted odds ratio, OR, with 95% confidence interval, CI) of low avidity HPV16 antibodies with increased risk of being seropositive for other HPV types among Finnish and Ugandan women.

Population	Seropositivity	Total	Number with low Ab-avidity	OR[1] (95% CI)	OR[2] (95% CI)	OR[3] (95% CI)
Finland (n=248)	HPV16 only	113	19	1	1	1
	HPV16+6 or 11	36	8	1.1 (0.4-2.8)	1.9 (0.6-6.1)	0.8 (0.1-6.8)
	HPV16+31 or 33	59	5	0.3 (0.1-1.0)	0.3 (0.1-1.3)	0.8 (0.0-1.0)
	HPV16+18 or 45	40	6	0.7 (0.2-1.8)	0.5 (0.1-2.0)	0.3 (0.2-3.8)
Uganda (n=117)	HPV16 only	45	9	1	1	1
	HPV16+6 or 11	32	15	3.5 (1.3-9.7)	3.0 (1.1-8.5)	2.9 (1.0*-8.4)
	HPV16+31 or 33	24	4	0.8 (0.2-2.9)	0.7 (0.2-2.5)	0.6 (0.2-2.3)
	HPV16+18 or 45	16	2	0.6 (0.1-3.0)	0.5 (0.1-2.9)	0.5 (1.0-2.8)

Cutoff-30% = using low-avidity controls mean plus 3 standard deviations

OR[1]=adjusted for age; OR[2]=adjusted for age, *Chlamydia trachomatis, HIV*

OR[3]=adjusted for age, *C. trachomatis, HIV* and smoking; *1.0 = lower 95% confidence limit 1.01

6 DISCUSSION

HPV is the most common sexually transmitted infectious agent with a high transmission probability of about 0.6 per act and a lifetime expectance for acquisition of 80%. Fortunately, HPVinfection is transient in 90% of immunocompetent women (Kjellberg et al. 1999, Ho et al. 2004, Barnabas et al. 2006, Syrjanen et al. 2009). HPV prevalence is age-dependent, peaking after sexual debut below 25 years of age, thereafter leveling out and decreasing slightly for the next 35 to 40 years (Kjaer et al. 2001, Viscidi et al 2004, Leinonen et al. 2008, Bosch et al. 2008, Syrjanen et al. 2009). A second peak at about 45 years of age has been reported in some regions (Castellsaque et al. 2009, Munoz et al. 2009, Bruli et al. 2010).

In the developing countries HPV types 51, 52, and 58 have been reported to be the most common followed by HPV16 among women with normal cytology (Clifford et al. 2005, Bruni et al. 2010, Banura et al. 2011). In the developed countries the most common types are HPV16, 18, 45, 31 (de Sanjose et al. 2007, Smith et al. 2008, Bruni et al. 2010). In the ICC, however, the most common HPV types in all regions are HPV16, 18, 45, 31, 33, 52, 58, and 35 (de Sanjose et al. 2010, Li et al. 2011). Among HIV positive ICC cases the commonest types are HPV16, HPV18 and HPV45 (Clifford et al. 2006, Odida et al. 2011).

Multiple HPV infections have been reported more in young sexually active women and among HIV-positive individuals, and are heavily dependent on HPV acquisition and persistence rates (Banura et al. 2010, 2011, Luovanto et al. 2010). Notable proportions of ICC cases are also positive for more than one HPV type (Mejlhede et al. 2009, Li et al. 2010, Pista et al. 2010, Odida et al. 2011). HPV serology identifies both current and past infections with approximately equal sensitivity. Thus, single and/or multiple HPV infections can be studied assessing trends of HPV infections in unvaccinated populations.

The present seroepidemiological study on the occurrence of multiple type HPV infections compares HPV epidemiology in Finland and Uganda. The choice of HPV types (lrHPV 6, 11 and hrHPV 16, 18, 31, 33, 35, 45, 51, 58) in our study is based on the protective effect documented from the current HPV vaccines (Brown et al. 2009, Wheeler et al. 2009, 2011), and also reflects their dominant role in HPV-associated disease burden in the two countries and large also in regions of the world.

6.1 Study strengths and limitations

Representativeness of the study populations and sub-samples

The women enrolled in our study were pregnant, which represent sexually active women with mean ages of 22 and 23 years for Finnish and Ugandan women respectively. These cohorts are similar to those of other studies reporting a multiple HPV DNA prevalence peak in <30 year-old women (Clifford et al. 2004, Smith et al. 2008, Nielsen et al. 2008, Luovanto et al. 2011). Cohorts of pregnant women have been used to report sexually transmitted infections (such as HIV, syphilis, hepatitis B, etc) in sexually active populations (Koskela et al. 2000, Lehtinen et al. 2006, Mpairwe et al. 2005), and probably represent women with normal distribution of cytological findings (Sarkola et al. 2009). Also, the slightly increased HPV DNA prevalence observed during pregnancy due to associated immune suppression is counterbalanced by the high rate of HPV clearance after delivery (Banura et al. 2008, Sarkola et al. 2009).

Our large sample sizes allowed comparison of variables with ample ($\geq$80%) power and standard (5%) significance level for the identification of odds ratios of < 0.44 and >1.82 for independent variables. Even if our study population was young, we expect to have covered all the types which matter in vaccination strategies given the currently available HPV vaccines. The geographical locations of the study populations made it possible to assess HPV epidemiology for HPV vaccinology for both developed and developing countries.

In spite of moderate sensitivity of 50 to 70% the laboratory methods applied represented the most recent techniques, and the sensitivity reflects the fact of antibody response not existing in a number of HPV DNA positive/transiently infected individuals. The specificity of the serological methods was considered high (>98%) and they have been reported to be type-specific (Dillner et al. 1996, Waterboer et al. 2005). This gave us an opportunity to study HPV type distributions and cumulative incidences of both single and multiple HPV types in young populations. Caution is warrented when comparing serological studies using different methods. A standard protocol for HPV serology studies is required for comparability of results from different regions. Lack of comparability may be one of the reasons for geographical differences in HPV seroepidemiological studies.

On the other hand, the cross-sectional nature of our two studies (Papers I-II) made it impossible to define the order of acquiring infections with different HPV types. In the prospective study (Paper III) we assessed the order but the exact time from HPV

acquisition and sample collection was still not known. We also lacked HPV DNA and IgM data to estimate current HPV infections, and CD4 counts were also not available to assess the immunological competence of the HIV participants.

6.2 Seroprevalence and HPV type distribution

We demonstrated that seropositivity to any of the HPV types 6/11/16/18/31/33/45 and multiple HPV types were common among both Finnish and Ugandan women (Paper I) similarly to previous studies (Laukkanen et al. 2003, Lehtinen et al. 2006, Kaasila et al. 2009). The prevalence peak in women under 25 years of age in our study is in line with earlier HPV DNA and seroepidemiology studies (Carter et al. 2000, Lehtinen et al. 2006, Syrjanen et al. 2009, Porras et al. 2010). The high prevalence of HPV among young age groups has been suggested to be a result of increased risky taking sexual behaviour among young people worldwide. In Finland, seroprevalence to specific HPV types is on the increase among young women (Laukkanen et al. 2003, Lehtinen et al. 2006, Kaasila et al. 2009, Syrjanen et al. 2009, Merikukka et al. 2011). In Uganda, Botswana, Kenya and South Africa similar observations have been reported from both HPV DNA and/or serology studies (De Vuyst et al. 2010, Banura et al. 2011, Firnhaber et al. 2011, Marais et al. 2008).

In our study the seroprevalence for multiple HPV types was lower among Finnish women than Ugandan women. This may have been due to the prevalence of HIV infection among the Ugandan women, which was 7%, compared to zero among the Finnish women. HIV is associated with increased HPV acquisition/persistence (Clifford et al. 2005, Marais et al. 2008, Banura et al. 2010, 2011) hence increased multiple HPV prevalence. This has also been reflected in geographical differences reported from the meta-analyses of HPV DNA studies in young women. The highest prevalences of both single and multiple HPV infections were reported in African countries (Clifford et al. 2004, de Sanjose et al. 2008, Smith et al. 2008, Bruni et al. 2010). Within Africa, the prevalences were highest in East Africa (including Uganda) where HIV is endemic. In Ugandan women the multiple HPV prevalence was higher among HIV positive HIV negative women in line with other studies (Viscidi et al. 2004, Mbulawa et al. 2010, Banura et al. 2011).

The mean ages at sexual debut for both groups are very similar: 16.2 years among Ugandan women (Slaymaker et al. 2009) and 16.6 years among Finnish women (Haavo-Mannila et al. 2001). Ugandan women may engage in risky sexual activities which expose them to high-risk partners at a younger age than do Finnish women. Also, more common unprotected sex among Ugandan women might explain the increase in multiple infection rates (Neema et al. 2004). In our second study (Paper II), however, there was no significant association between seropositivity to multiple

hrHPV and age at sexual debut. This concurs with earlier studies suggesting that young age at sexual debut, although a risk factor for HPV DNA positivity, is not a stand-alone risk factor for HPV seropositivity but increases the frequency of HPV exposure among young people (Aral et al. 1999, 2008, Kahn et al. 2002, Collins et al. 2002). This, in turn, could lead to seroconversion (Carter et al. 2000, Ho et al. 2002, Syrjanen et al. 2009, Porras et al. 2010).

Among the Ugandan women, risk factors of seropositivity for multiple hrHPV types were young age (<30 years), low levels of education (< secondary level), parity (> 5) and HIV positivity, while number of lifetime sexual partners, age at debut, smoking had no effect (Paper II). This suggests that young women in Uganda face the risk of HPV acquisition(s) due to cross-age generation marriages/relationships, and polygamous marriages as reported previously (Uganda Bureau of Statistics. 2001, Munoz et al. 2002, Wellings et al. 2006, Munoz et al. 2009) or coerced sex (Neema et al. 2004), which increase their risk of acquiring of multiple HPV infections from the risk-taking sexual network of their partners compared to the Finnish women with multiple partners (Auvinen et al. 2005, Louvanto et al. 2011).

The HPV seropositivity rates among Finnish women may have been affected by smoking, which is more prevalent in Finnish (26%) than in Ugandan (3%) women. Smoking is associated with impaired antibody response in natural HPV infections (Simen-Kapeu et al. 2008, Syrjanen et al. 2009). Highly prevalent *C. trachomatis* seropositivity (26%) was not associated with seropositivity for multiple HPV types, although it is a proxy for increased risky taking sexual behaviour (Samoff et al. 2005), and has been associated with HPV DNA positivity (Luovanto et al. 2011).

6.3   Risk of co-occurrence of HPV types

Our results showed that if a woman was seropositive for any of the specific HPV types (6/11/16/18/31/33/45), her risk estimate of being seropositive for another HPV type was significantly increased compared to a seronegative woman for that particular type. Our results agree with previous work that showed HPV seropositivity as a risk factor for multiple HPV types (Carter et al. 2000, Lehtinen et al. 2006, Kaasila et al. 2009). The reasons why HPV antibodies induced in natural HPV infections are not cross-protective are still unclear but there is speculation that the antibody titres are not high enough (Stanley et al. 2010, Safaeian et al. 2010, Paaso et al. 2011). The slow process of seroconversion may also contribute at young ages and high incidence peaks. Possibly the HPV viral load among sexually active women is higher than what cross-reactive antibodies could neutralize (Viscidi et al. 1997, Wang & Hildesheim 2001). Some follow-up studies have suggested that higher HPV

antibody levels are protective against incident infections of both related and unrelated type (Ho et al. 2002, Malik et al. 2009, Paaso et al. 2011).

We further observed that Finnish women had a significantly increased risk of double seropositivity involving HPV18 and 45 compared to Ugandan women. HPV45 and HPV18 are responsible for adenocarcinoma diseases. The observed increased risk among Finnish women could be an effect of long time screening that may have changed the HPV ecological niche in Finland to favour the alpha 7 HPV species because adenocarcinoma and pre-adenocarcinomatous lesions, which are poorly detected by current techniques, have been on the increase in countries with organized cytology screening programmes (Bray et al. 2005, Bulk et al. 2005, Pettersson et al. 2011). In Finland, a possible competitive advantage has been associated with HPV type 33 but not HPV18 or HPV45 (Merikukka et al. 2011). Our observation needs to be investigated further in order to predict how the prevalance reduction or elimination of HPV6/11/16/18 included in the current vaccines following mass HPV vaccination will affect the occurrence of other genital HPV types.

HPV 33 was the most common type among Ugandan women and in Ugandan ICC cases, and has previously been detected at a rate of 87.5% in multiple HPV infections. Its interaction with other HPV types, however, is not well understood. HPV16 as a single infection or in combination with other types increases the risk of CIN and CC development (de Sanjose et al. 2010). The increased risk of multiple seropositivity for oncogenic types (both vaccine and non-vaccine related) among HPV16 and HPV33 seropositives should be monitored.

Compared to East Africa HPV16 has been more common in Europe (De Vuyst et al. 2009, Bruni et al. 2010, Banura et al. 2011) probably because the European HPV16/18 strains persist longer than African strains. In East Africa HPV52 is most common in cytologically normal populations (Banura et al. 2011), however, HPV16/18 predominate in cervical tissue samples (de Vuyst et al. 2011, Odida et al. 2011) obtained from women with cytological abnormality due to persistent HPV infections.

6.4 Risk factors for seropositivity to multiple hrHPV types

We observed young age, low education level, parity, HIV and *C. trachomatis* antibody positivity to be risk factors for seropositivity for multiple HPV types. This has been observed earlier in HPV DNA studies and serological studies. Our further analysis, however, indicated that HIV was the only independent, stand-alone risk factor.

Multiple HPV infections have been reported in earlier studies to be more frequent among HIV positives than HIV negatives (Study I, Viscid et al. 2004, Clifford et al. 2006, Banura et al. 2011). HIV has also been reported to be a stand-alone risk factor for multiple HPV infection (Banura et al. 2008). This is in line with with our results that HIV was the only independent, stand-alone risk for antibody positivity to multiple HPV types. HIV is associated with HPV persistence and poor clearance of the virus (Banura et al. 2010) probably due to reduced levels of CD4 cells. On the other hand, the persistence of HPV has been reported to increase the risk of seroconversion (Carter et al. 2000, Viscidi et al. 2004, Syrjanen et al. 2009). Furthermore, Uganda is one of the countries with the youngest population (http://www.africomnet.org/communication-resources/highlights/1182-uganda-has-worlds-youngest-population.html) and highest frequencies of teenage pregnancies. This suggests that risky taking sexual behaviour, and also unprotected sex (pregnancy) exposes young women to other STIs such as HIV and *C. trachomatis,* which were risk factors for seropositivity to multiple HPV types in our study. These factors have previously been reported to increase the risk of multiple HPV acquistion and persistence (Banura et al. 2010).

6.5 Risk of seroconversion for multiple HPV types in a longitudinal study

The results of our prospective follow up study indicated that women who at baseline were HPV seropositive for any of the HPV types 6/11/16/18/31/33/45 had an increased risk of seroconverting to another HPV type. Seropositivity to any of the above mentioned HPV types increased the estimated risk for being seropositive for another type. This fits the observations that natural HPV-infection derived antibodies do not protect against incident HPV infections (Viscidi et al. 2004). This has been suggested to be due to the low levels of antibodies induced in natural HPV infections compared the antibodies induced by the HPV vaccines which are logs higher than antibodies induced in natural infections (Olsson et al. 2008, Petäjä at al. 2009). Antibodies induced by HPV vaccine are readily transduced to the mucosa to offer protection (Petaja et al. 2010). Some studies, however, have suggested that

even if antibodies induced in natural HPV infections do not prevent new infections, they protect against disease (Carter et al. 2000). This was observed in the follow-up study, which showed that women who failed to seroconvert were at increased risk of precancerous lesions compared to seroconverted women (Carter et al. 2000, Viscidi et al. 2005, Syrjanen et al. 2009). On the other hand, a study by Ho et al. (2002) suggested that sustained high levels of antibodies to HPV16 were 50% protective against subsequent challenge by related types. A recent study in Finland has also suggested that former antibodies may protect against incident HPV infections with the same type (Paaso et al. 2011), but the level of antibodies that may offer protection is not known.

The risk of seroconversion was observed for the alpha 9 phylogenetically related types 31 and 33 among women seropositive for either HPV16 or HPV 18. This has also been observed in a recent study by Merikukka et al. (2011) which showed a consistent excess risk of seroconversion for HPV33 among the baseline HPV16- and HPV18-seropositive women irrespective of age or presence or absence of antibodies to other HPV types. In our study, seropositivity for HPV31 or 33 was more common in individuals positive for HPV16, 18 and 45 antibodies (Papers I and II). Infections with HPV31 or HPV33 have been reported to persist for a long time (> 46 months), which may predispose to the suggested co-occurrence of HPV infections (Luovanto et al. 2010). These non-vaccine types need to be monitored because the cross-protection to HPV31, 33 and 45 from the licenced HPV vaccines as reported in the earlier studies (Brown et al. 2009, Wheeler et al. 2009, 2011) may not last long.

Seropositivity to multiple lrHPV types 6/11 at baseline increased the risk of sero-conversion to multiple hrHPV types 16/18/31/33/45 but the reverse was not true. This may be explained by observations where seroconversion following infections with lrHPV types was faster than that following hrHPV infection. Moreover, lrHPV type antibodies were detectable for a shorter time than the hrHPV types antibodies (Carter et al. 2000). Seropositivity for multiple HPV types is also strongly associated with HPV16 seropositivity (Carter et al. 2000, Porras et al. 2010). HPV16 has been found in 50% - 87.5% of multiple HPV infections (de Ona et al. 2010, Luovanto et al. 2010). Both HPV16 and multiple hrHPV infections clear very slowly (Banura et al. 2010, Luovanto et al. 2010), which allows more time for seroconversion than in infections with lrHPV types. Carter et al (1996) showed that persistence of HPV16 DNA was associated with seroconversion and SIL development among young women.

6.6 Low-avidity HPV16 antibodies associated with seropositivity for multiple lrHPV types 6/11 but not multiple hrHPV16/18/31/33/45.

Low-avidity HPV16 antibodies were found in 15% of Finnish and 26% of Ugandan HPV16 seropositive women, and was associated with an increased risk of being seropositive for HPV6/11 but not for the hrHPV types 18/31/33/45. The risk was significantly increased among (non-smoking) Ugandan women but was probably confounded by smoking among Finnish women.

Low antibody avidity indicates the immaturity of B-cells; therefore, it is widely recognized as a marker for acute infections (Hedman et al. 1989). The maturation of B cells depends on T-helper cell function (Stavnezer J. 1996, O'Rourke et al. 1997). In our study, the low avidity of HPV16 antibodies may therefore be due to recent HPV16 infection, genetic factors or immune suppression. Firstly, lrHPV6/11 antibodies are detectable almost simultaneously with HPV6/11 DNA, whereas the appearance of antibodies to the high-risk HPV types takes a longer time. Sexual risky taking behaviour may have predisposed the study subjects (pregnant women) to a number of different low-risk and high-risk HPV types at about the same time but the antibody production is slower for hrHPV types.

The association between low-avidity HPV16 antibodies and susceptibility to HPV types 6/11 was more obvious among Ugandan women compared to Finnish women. This may indicate an acute HPV16 infection among Ugandan pregnant women but IgM/DNA data to confirm this is lacking. Among the Ugandan women the HIV proportion was 9% (Paper IV) but had no effect on the HPV16 antibody avidity after stratifying by HIV status among Ugandan women. This was in line with earlier studies, which have demonstrated that antibody avidity in general is not affected by HIV infections (Bodeus et al. 1998, Lazzarotto et al. 1998). One reason why the possible effect of HIV status on avidity of HPV16 antibodies was not seen in our study could be low number of cases, further studies with large numbers of subjects need to be considered. In contrast to natural infections, vaccination of immuno-suppressed individuals shows very slow maturations of antibody avidity compared to immuno-competent individuals (Brinkman et al. 2003, Nair et al. 2009). This is because avidity maturation is T-cell dependent, and the destruction of the activated helper T-cells by HIV, delays the maturation process in the germinal centres of the secondary lymphoid tissue. However, low antibody avidity may sometimes persist for a longer time after primary infections (Bodeus et al. 1998, Lazzarotto et al. 1998) probably due to the genetic factors of individuals.

On the other hand, smoking, which is strongly associated with impaired HPV-antibody production (Simen-Kapeu et al. 2008), possibly also had an effect on HPV-antibody avidity as the observed association (Paper IV) disappeared after adjusting for smoking among Finnish women.

Women who are HPV16 seropositive at baseline have an increased risk estimate of seroconversion for other hrHPV types (Kaasila et al. 2009, Paper III). We found no association between low-avidity HPV16 antibodies and seropositivity for other hrHPV types, i.e., susceptibility to acquiring either phylogenetically related or unrelated hrHPVs. Thus the increased risk estimates of having multiple hrHPV infections once seropositivite for HPV16, may not be explained by specific (neutralizing) antibody avidity or related immunological maturation of antibody producing cells.

Longitudinal studies are needed to establish whether low-avidity HPV16 antibodies persist for a longer period, and whether or not this plays a role in susceptibility to multiple HPV infections and progression to malignancy.

7 CONCLUSION

The current work was undertaken to study the occurrence of common HPV types among the Finnish and Uganda female populations and to determine the risk of occurrence of HPV antibodies to infections with multiple HPV types

1. Antibodies to multiple HPV types were very common among both Finnish and Ugandan women, but were most common in Ugandan women. HPV seropositive women (positive for any of the HPV types 6/11/16/18/31/33/45) compared to seronegative women for that particular type had an increased risk of being seropositive for other HPV type. The most common hrHPV antibodies among Finnish women were against HPV16 and HPV18 and the least common were against HPV45, whereas among the Ugandan women HPV33, HPV16, and HPV31 antibodies were most common, and HPV18 antibodies least common.

2. The risk factors of seropositivity for multiple HPV types among Ugandan women were; young age, low level of education, parity, and HIV positivity, but not the number of lifetime sexual partners. HIV positivity was the only independent, stand-alone risk factor for seropositivity for multiple HPV types, but the relative risks of co-occurrence of antibodies to the hrHPV types were not significantly different in HIV-positive and HIV-negative women.

3. HPV antibodies induced in natural HPV infections are not cross-protective against other HPV infections. On the contrary, the risk of seroconversion to another HPV type was increased among seropositive women, who at baseline had antibodies to any HPV type. After stratification by HPV risk type, there was a statistically significant increased risk of seroconversion to hrHPV types among baseline lrHPV seropositive women compared to seronegative women.

4. Among Ugandan women, the risk for being HPV6/11-antibody positive among women with low-avidity HPV16 antibodies was increased compared to those with high-avidity HPV16 antibodies. No comparable excess risk estimates were observed for hrHPV types.

5. This was the first study to compare the risk of co-occurrence of HPV types between Finland and Uganda. It clearly shows that although the seroprevalences are higher among Ugandan women, probably due to HIV and other immunological and environmental factors, Finnish women too have high hrHPV seroprevalences. The reduction of CC incidence and mortality among Finnish women is probably due to population-based screening for cervical cancer in Finland, the means for which are lacking in Uganda. Mass HPV vaccination

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will be most effective in the fight against cervical cancer by significantly reducing the causative agent (HPV) in a low developing country like Uganda.

8 ACKNOWLEDGEMENTS

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