Targeting the human papillomavirus for prevention of cervical cancer

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TARGETING THE HUMAN PAPILLOMAVIRUS FOR PREVENTION OF CERVICAL CANCER

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CONTENTS

SUMMARY

Different types of human papillomavirus (HPV) vary in the extent they cause precursor lesions (CIN) and cancer. There are limited long-term efficacy data on HPV testing in primary screening

Among 72 cervical cancers in Mozambique, HPV 16 and 18 were the most frequent HPV types (69% of cases). Comparing 108 cervical cancers cases and 517 matched controls nested within a population-based cohort in Taiwan, HPV 16 seropositivity implied a 6-fold increased cancer risk. In a cohort of 5696 women in Sweden, HPV types 16, 31 and 33 conveyed the highest risks for future high-grade CIN (CIN 2+), attributing to 33.1%, 18.3% and 7.7% of CIN 2+ cases, respectively. In a pooled analysis of seven European longitudinal studies of HPV-based cervical screening, the cumulative incidence rate of CIN grade 3 or worse (CIN 3+) was higher after 3 years among women with normal cytology than among women with a negative HPV test after 6 years. Finally, in a randomized cervical cancer screening trial in Sweden, adding testing for HPV persistence resulted in a 51% (95% CI: 13-102) increase of CIN 2+ at prevalent screening, which was followed by a reduction of 42% (95% CI: 4-76) of CIN 2+ at incident screening.

In conclusion, HPV-based cervical cancer screening protects against future CIN 2+, and the long-term protective effect should enable extended screening intervals to 6 years. Albeit HPV 16 is the most important carcinogenic HPV type all over the world, different "high-risk" HPV types convey distinctly different risks for CIN 2+, which should be considered in design of screening tests and vaccines.

SAMMANFATTNING PÅ SVENSKA

Varje år insjuknar ungefär 450 000 kvinnor i livmoderhalscancer (cervixcancer) i världen. Som en följd av screening för livmoderhalscancer, där kvinnor testar sig regelbundet för cellförändringar, har insjuknandet i livmoderhalscancer minskat. Det cellprov som används i screeningen lyckas dock inte upptäcka alla kvinnor som har ökad risk för framtida cancer. Humant papillomvirus (HPV) är främsta orsaken till livmoderhalscancer. Det är en mycket vanlig sexuellt överförbar infektion som vanligen läker ut av sig själv, medan ett fåtal kvinnor får en kvarstående infektion som kan ge cancer. Att testa för om en kvinna är infekterad med HPV skulle kunna användas som ett komplement till, eller istället för, det cellprov som används idag. Sedan ett år tillbaka finns det ett förebyggande vaccin som skyddar mot vissa typer av HPV. Det finns många olika typer av HPV och alla ökar inte risken för cancer. För att avgöra vilka HPV-typer som det vore värdefullt att testa för i hälsokontroller och/eller inkludera i framtida vacciner är det viktigt att undersöka vilka som är de viktigaste cancerorsakande HPV-typerna.

De tre första vetenskapliga studierna som är en del av denna avhandling har utförts för att undersöka vilka HPV-typer som orsakar livmoderhalscancer och dess förstadier. Två studier har utförts för att undersöka långtidseffekten av att inkludera ett HPV-test i hälsokontroll för livmoderhalscancer.

I studie I, har vi funnit att HPV-typerna 16 och 18 är det typer som är vanligast förekommande i livmoderhalscancrar i Moçambique. I studie II, har vi funnit att HPV 16 är det viktigaste cancerorsakande HPV-viruset i Taiwan. Risken för livmoderhalscancer föreföll vara mindre om man varit infekterad med både HPV-typ 6 och 16 jämfört med om man varit infekterad med HPV-typ 16 endast. I studie III fann vi att risken för allvarliga cellförändringar beror på vilken HPV-typ som kvinnan är infekterad med. HPV-typerna 16, 31 och 33 är de typer som är de viktigaste orsakerna till allvarliga cellförändringar i Sverige. I studie IV och V fann vi att vid användning av HPV-testning i screening skulle intervallen mellan varje screening kunna förlängas från dagens rekommenderade 3 år till 6 år med samma skydd mot allvarliga cellförändringar samt att risken för framtida allvarliga cellförändringar minskar. Resultaten talar för att användning av HPV-testning skulle kunna förbättra screeningen mot livmoderhalscancer.

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Long-term efficacy of HPV testing in primary cervical cancer screening among middle-aged women: Randomized controlled trial.
Manuscript

LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ASCUS	Atypical squamous cells of undetermined significance
ASC-H	Atypical squamous cells "cannot exclude HSIL"
CI	Confidence intervals
CIN	Cervical intraepithelial neoplasia
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
HC	Hybrid Capture
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HSIL	High-grade squamous intraepithelial lesion
HSV	Herpes simplex virus
IARC	International Agency for Research on Cancer
Ig	Immunoglobin
LSIL	Low-grade squamous intraepithelial lesion
MIF	Microimmunofluorescence
OC	Oral contraceptives
ORFs	Open reading frames
PCR	Polymerase chain reaction
PVN	Predictive value negative
PVP	Predictive value positive
RNA	Ribonucleic acid
VLP	Virus-like particle

HUMAN PAPILLOMAVIRUS

History

The cottontail rabbit papillomavirus was linked to cancer already in 1933[1]. Papillomas induced by the virus were shown to undergo malignant progression[2]. A more detailed molecular biological analysis of papillomavirus was not possible until the late 70s when the papillomaviruses could be cloned in bacteria[2]. Molecular techniques expanded in the 70s and were incorporated into epidemiological research[3]. Subsequently it was possible to establish human papillomavirus (HPV) as a major etiological factor in the development of cervical cancer[4-6].

Structure

Papillomavirus is a circular non-enveloped double stranded DNA virus that comprises about 8000 basepairs[2]. The capsid forms an icosahedral structure of 72 capsomers that measures 55 nm in diameter[2]. Papillomavirus encodes about ten open reading frames (ORFs)[2]. The genome can be divided into three regions: the long control region (LCR), the region of early genes (E1-E7) which express proteins that regulate the virus life cycle, and the region of late genes (L1 and L2) that express structural proteins that form the virus capsid[2]. There are no ORFs in the LCR but it contains several control elements that regulate HPV DNA replication and gene expression[2].

Classification

Papillomaviruses constitute a separate independent virus family, the Papillomaviridae[7]. Papillomaviruses evolve very slowly and are widespread in higher vertebrates[8,9]. The virus classification is based on

DNA sequence homology of the L1 gene, the most highly conserved viral gene[7]. A distinct papillomavirus type is defined by 71-89% homology of the L1 gene[7]. Differences of 2-10% constitute a subtype and less than 2% a variant[7]. Papillomavirus types that share 60-70% homology are classified as being part of the same species, while types with less than 60% homology are classified as being part of different genera[7] (Figure 1). The taxonomic classification is related to both tropism and viral properties[7]. For example, HPV types that belong to alpha papillomavirus species 7 (HPV types 18, 39, 45, 59, 60 and 70) and 9 (HPV types 16, 31, 33, 35, 52, 58 and 67) are found in malignant mucosal lesions in humans[7]. A classification often seen in the literature is "low-risk" and "high-risk" HPV types. This is an epidemiological classification based on the HPV type-specific association with cervical cancer[10].

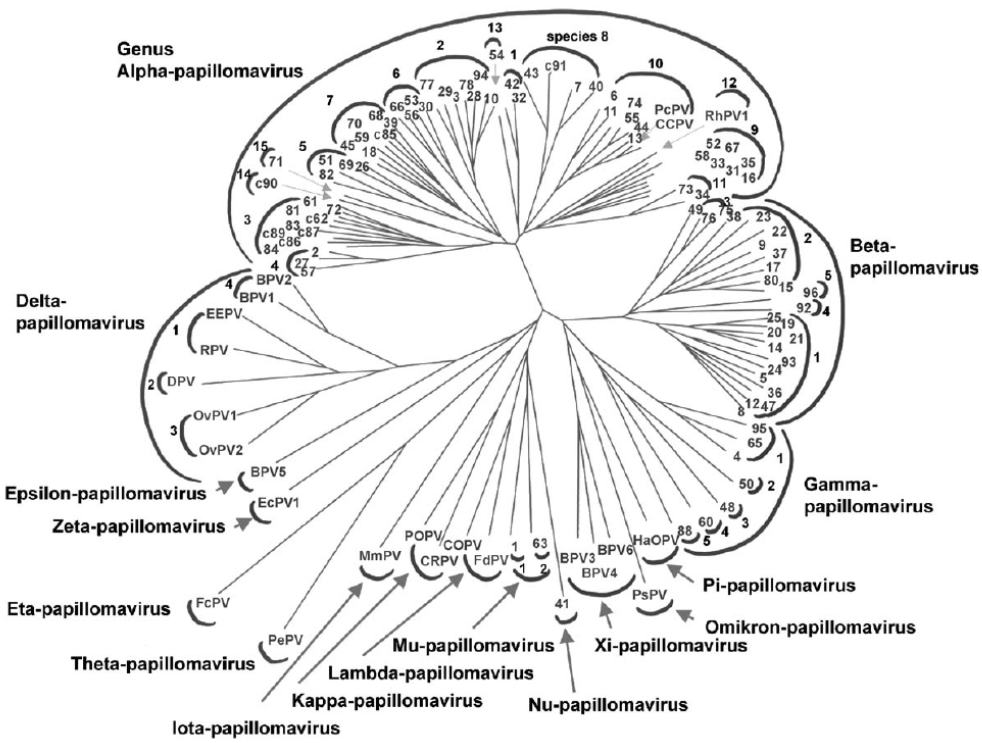

Figure 1. Phylogenetic tree of Papillomaviruses. Reprinted from de Villiers et al[7] with permission from Elsevier.

Viral life-cycle and Oncogenesis

Papillomavirus has a specific tropism for epithelial cells and the viral life cycle is closely linked to the normal epithelial differentiation[11]. The virus infects cells in the basal layer[11]. It has not been established which cell surface receptor is essential for infection, but both integrin α4β6 and heparan sulfate have been proposed to be important surface receptors for infection[12,13]. There is no viraemic phase and the virus maintains itself in episomal form except in cancer cells where it is often integrated into the host genome[11,14]. Viral particles are released from the cell in the upper layers of the epithelium through a nonlytic process[11].

The oncogenic potential of papillomavirus is linked to its ability to overcome growth arrest and maintain a replication-competent cell[12]. The E6 and E7 viral proteins are important for malignant transformation and have immortalization capacity in human cell lines[15]. The E6 protein forms a complex with and can functionally inactivate the tumor suppressor protein p53 (Figure 2)[16]. It has been shown that p53 levels in cells infected with high risk HPV types are lower than in uninfected cells[17]. The E6 protein can also activate telomerase[18]. The E7 protein promotes proteolysis of pRB and thereby activates the transcriptional factor, E2F, permitting S-phase entry in the cell-cycle[16]. It has been reported that E6 proteins encoded by low-risk HPV types 6 and 11 bind pRB with lower efficiency than E6 proteins encoded by high-risk types HPV 16 and 18[16]. The E7 protein can also interact with p53 and inactivate the cyclin-dependent kinase inhibitor p21[16] (Figure 2). The E5 protein is the major transforming protein for bovine papillomavirus, but has for human papillomaviruses only been shown to have weak transformation abilities in cell culture that might have an additive effect on E6 and E7 transformation capacity[18].

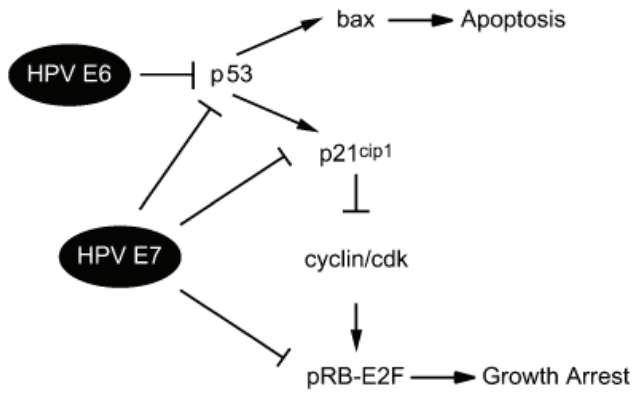

Figure 2. Oncogenic mechanisms of the E6 and E7 proteins. Reprinted from Munger et al[16] with permission from Elsevier.

Detection methods

DNA detection

Detection methods of HPV DNA can be divided into direct probe methods, signal amplification and target amplification[19]. Southern Blot and in situ hybridization are direct probe methods based on a probe that binds to target DNA. The disadvantages of these methods are low sensitivity and the need for large amounts of purified DNA[19]. Hybrid Capture 2 (HC2), which has been approved by the US Food and Drug Administration for clinical use in cervical cancer screening, is a signal amplification method[20]. It is an immunoassay which is based on RNA probes that are directed against HPV DNA. RNA-DNA hybrids are then detected using antibodies that are labeled with a conjugate that leads to a luminescence signal[20]. There are two cocktails of RNA probes, one containing high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and the other low-risk types (HPV 6, 11, 42, 43 and 44)[20]. Polymerase chain reaction (PCR) is a target amplification method which is based on exponential amplification of target DNA using DNA sequence specific primers[19]. The DNA is amplified in cycles which make

this method highly sensitive. To detect HPV DNA there are HPV type-specific primers that target E6 and E7 genes and several "general primers" (e.g. GP5+/6+, MY09/11, PGMY09/11)[21-23] that target the L1 gene of a range of HPV types. The specific HPV type can then be detected by type-specific hybridization techniques or direct sequencing[19]. With real-time PCR it is possible to quantify the viral load[20]. A semi-quantification of the viral-load is the strength of the signal obtained with HC2[20]. HC2 and PCR techniques are the most widely used methods to detect HPV DNA in HPV epidemiological research. The disadvantages of HC2 compared to PCR techniques are that HC2 does not provide specific information regarding which HPV type is present in the specimen and the DNA detection limit is lower[20]. However, the agreement of HC2 and PCR has been found to be substantial when used as screening tests[24,25]. The general agreement between different PCR systems is good, but MY09/11 has been found to detect more multiple infections than GP5+/6+ and there also appears to be some differences regarding sensitivity of detection for some HPV types[26].

Antibody detection

Serum antibodies against HPV are a marker of the cumulative exposure to HPV infection[27]. Human IgG antibodies against virus-like particles (VLPs) that consist of the structural proteins of HPV can be detected e.g. using enzyme-linked immunosorbent assay (ELISA)[28,29]. The specificity of the assay is high (>98%) and there is very little cross-reactivity with the exception of HPV 6 and 11[30]. The sensitivity is limited as only about 50% of women having had an HPV infection seroconvert[28,30,31].

Immunity

Cell-mediated Immunity

It is thought that the cell-mediated immune response is important for clearance of HPV infection[14]. HIV positive women take longer to clear HPV infections[32]. Large infiltrates of CD4+ and CD8+ cells can be found in regressing warts[33] and cell-mediated responses to E7 peptides of HPV 16 have been correlated with regression of cervical intraepithelial lesions and resolution of viral infection[34]. An interesting observation is that women with antibodies against both HPV 6 and HPV 16 are at lower

risk of future cervical cancer compared to women with antibodies against HPV 16 only[35-37]. Since there is no cross-neutralization *in vitro* between HPV 6 and 16 antibodies, it has been suggested that the observed antagonism may be explained by cell-mediated cross- protection[37].

Humoral Immunity

In prospective follow-up studies with repeated testing most women seroconverted with IgG antibodies 6-12 months after incident infection[38,39]. Seroconversion is more common when there are high viral loads and persistence of the infection[39,40]. HPV antibodies appear to be stable over time, even after more than a decade of follow-up[41,42]. There have been conflicting results regarding duration of natural immunity after HPV infection. In a study by Ho et al. of female university students in the US, persistent HPV 16 antibody response was associated with a reduced risk of subsequent infection with HPV-16 or HPV-16 phylogenetically related types during 3 years of follow-up[43]. However, in a large cohort of more than 7000 women in Costa Rica HPV 16, 18 or 31 serological status at baseline did not influence the risk of having a positive HPV DNA test with the homologous type 5-7 years later[44].

Epidemiology of genital HPV in women

Prevalence

Genital HPV is the most common sexually transmitted infection in humans[45]. In population-based studies of cervical samples the HPV prevalence varies from 2-40% depending on the geographical location, cytology and age[46-51]. Even though the HPV prevalence increases with cervical lesion severity[52] the prevalence is high also among women with normal cytology[47,49]. The prevalence of genital HPV is largely age dependent with a peak in the early 20s followed by a gradual decline[47,51,53]. However some studies have found a bimodal U-shaped age-specific prevalence curve with an increase among women older than 50 years of age[49,53]. It is debated if this is due to a mere stochastic effect, a cohort effect, re-activation of latent infections or newly acquired infections[54].

It is somewhat difficult to compare the HPV type-specific prevalence levels between different studies since different inclusion

criteria of study subjects as well as different detection methods have been used. However, HPV 16 is the most frequently found HPV type in cervical samples in most studies[46-49]. With the reservation that some parts of the world have not been extensively studied, there appears to be some heterogeneity in the geographical HPV type distribution once HPV 16 is excluded. HPV 35 has been reported to be relatively more common in Africa[46,55] while HPV types 52 and 58 are more common in East Asia than in other populations[56]. Infections with multiple types are fairly common as several population-based studies have reported that more than 30% of HPV infected women are infected with more than one HPV type[47,49].

Time trends of HPV prevalence have been assessed in sera from maternity cohorts in the Nordic countries. In a study of pregnant women in Sweden there was a significant increase in HPV 16 seroprevalence from 16% in 1969 to 22% in 1983 and 21% in 1989[57]. In a study from the Finnish maternity cohort the seroprevalence of HPV 16 increased from 17% in 1983-85 to 24% in 1995-97, while the seroprevalence of HPV 6 and 11 was stable at 9-12% throughout the study period[58].

Incidence

The incidence of genital HPV infection is high in young women. In cohorts of female university students in the US and Canada followed with repeated cervical sampling the cumulative incidence of HPV infection was 40-60% among women that were HPV negative at baseline during 3 years of follow-up[59-61]. In a cohort of initially HPV negative women aged 15-19 years in England, the cumulative risk of HPV infection was 44% after 3 years and 60% after 5 years[62].

Risk Factors for acquisition of HPV infection

HPV prevalence and incidence is strongly associated with sexual behavior. The prevalence peak in young women coincides with sexual debut. Studies of adolescents and young women in Sweden and Denmark have found that detection of HPV DNA or HPV antibodies are extremely rare among women without coital experience[63-65]. In the Danish cohort it was found that only women who became sexually active during follow-up became HPV 16 DNA and/or HPV 16 seropositive[64]. Several studies have found that increasing numbers of lifetime sexual partners and numbers of partners during the last year are associated with HPV

infection[49,61,66,67]. Concordance of the same HPV type among partners is also higher than would be expected due to chance[68].

Conflicting results have been obtained on the issue of whether condoms protect against genital HPV infection[69]. No association was found in a pooled analysis of population-based cross-sectional studies performed by IARC[70], but in a recently published prospective study there was a 70% reduction of HPV incidence among women whose partners used condoms all the time compared to women whose partners used condoms less than 5% of the time[71].

HPV prevalence is higher in immunocompromised hosts such as renal transplant recipients and HIV positive subjects[72]. HPV prevalence and incidence have been reported to be associated with both CD4+ count and HIV RNA level[73]. Other factors that have been reported to increase the risk for HPV infection are smoking and oral contraceptives[61,74]. However, these factors tend to covariate with sexual risk-taking behavior and other studies have failed to find an association[70,75].

Clearance/Persistence of HPV infection

Several cohort studies have reported that most women with an incident HPV infection will have cleared their infection within approximately one year[59,76,77]. In a study of university students in the US, where persistence was defined as detection of the same HPV type in two consecutive samples 6 months apart, and clearance was defined as not detecting the same HPV type in the following sample, 70% of the women had cleared their infection after 12 months and by 24 months only 9% remained infected with the same type[59]. Several studies have found HPV 16 to be more persistent than other HPV types[77,78]. However, studies of clearance and persistence of HPV infections are somewhat difficult to interpret since the sampling intervals differ between studies and reactivation of HPV infections has been reported[73]. Risk factors that have been reported to be associated with HPV persistence except for HPV type are increasing age[59,79], HIV infection[32], lack of condom usage[80,81] and history of *Chlamydia trachomatis* infection[81].

HPV in men

A recent review of studies of genital HPV in men, reported that HPV DNA prevalence varied from 1.3-72.9% depending on the population, the sampling technique and the anatomical site where the sample was collected[82]. The HPV 16 seroprevalence varied from 3-48%[82] and it has been reported that men are less likely to mount a detectable humoral response to HPV[83]. In a cross-sectional study of military conscripts in Sweden, 8% had HPV DNA positive urethra samples and 5% had HPV DNA positive urine samples[84]. A prospective study performed on military male conscripts aged 18-29 years in Denmark, where samples were taken from the penis, reported that 33.8% were HPV DNA positive at baseline and 13.8% had acquired a new infection at the second examination 6-8 months later[85]. The numbers of sex-partners during follow-up was the most important risk factor for incident HPV infection[85]. A reduced risk of prevalent HPV infection has been reported among circumcised men in a pooled analysis of partners to women taking part in seven case-control studies of cervical cancer[86].

HPV associated diseases

There is a strong association between HPV types from species 7 and 9 of the alpha genus and cervical cancer (See section about natural history of cervical cancer)[10,87]. Other ano-genital cancers that are associated with high risk HPV types and foremost HPV 16 are carcinoma of the vulva, in particular of basaloid histology[88], penile cancer[89] and anal cancer[90]. Benign genital warts are associated with low-risk HPV types, primarily HPV 6 and 11[91]. HPV 11 is also strongly linked to development of recurrent respiratory papillomatosis[92]. Apart from being associated with common warts of the skin, an association between HPV and skin cancer has been found among patients with epidermodysplasia verruciformis[93]. However, there is a high prevalence of various HPV types in healthy skin[94,95] and epidemiological studies have been inconclusive regarding the causal link between HPV and skin tumors[93]. A recently published large seroepidemiological study found that antibodies against HPV types of the beta species are associated with squamous cell carcinoma but not with basal cell carcinoma of the skin[96]. Case-control studies have reported

conflicting results of the association between HPV DNA detection and head and neck cancers, possibly due to different sampling techniques[97-99]. However, seroepidemiological studies have consistently reported an increased risk of head and neck cancers among individuals with antibodies against HPV 16[98,100,101].

NATURAL HISTORY OF CERVICAL CANCER

Cervical cancer incidence

Cervical cancer is the second most common cancer among women worldwide with an estimated incidence of 493 000 cases and 246 000 deaths in the year 2002[102]. In Sweden approximately 450 women are diagnosed with cervical cancer each year[103]. Over 80% of cancers occur in developing countries with particularly high incidences in parts of Latin America, Sub-Saharan Africa and South-East Asia[102]. The differences in incidence and mortality rates reflect existence of screening, exposure to risk factors, accessibility to health care and report frequency (Table 1)[102]. The cumulative risk for women to develop cervical cancer up until 65 years of age has been estimated to be approximately 1.5% in developing countries and 0.8% in developed countries[102]. The incidence of cervical cancer begins to rise at age 20-29 years and peaks around 45-49 years of age[104]. Cervical cancers can be divided into squamous cell carcinomas, adenocarcinomas and a small miscellaneous group including adeno-squamous carcinoma and neuro-endocrine tumors[105]. Squamous cell carcinomas account for approximately 80% and adenocarcinomas for 20% of invasive cancers in screened populations, while the other types are extremely rare[106].

	Incidence rates	Mortality rates
Tanzania	68.6	55.6
Mozambique	33.6	27.2
Brazil	23.4	10.2
Syria	2.0	1.0
United Kingdom	8.3	3.1
Sweden	8.2	3.1

Table 1. Estimates of age-standardized incidence and mortality rates, per 100 000/year in 2002 (GLOBOCAN 2002)[107]

Pre-invasive lesions

Stratified squamous epithelium of the vagina and ectocervix meet the columnar epithelium of the uterine cavity at the squamocolumnar junction[108]. At this site of the cervix columnar epithelium transforms into squamous epithelium through metaplasia and is then called the transformation zone[109]. This is the site of origin of most pre-invasive and invasive cervical squamous cell neoplasias[108].

The "golden standard" for diagnosis of cervical lesions is histopathology. In most of Europe squamous lesions are classified as cervical intraepithelial neoplasia (CIN) 1, 2 or 3 representing increased severity of lesion[110]. Grading is dependent on the level of differentiation measured as the proportion of the thickness of the epithelium that shows cytoplasmic maturation as well as nuclear abnormalities[110]. In the US, lesions are classified according to the Bethesda system where low-grade squamous intraepithelial lesion (LSIL) is equivalent to CIN 1 and high-grade squamous intraepithelial lesions (HSIL) includes both CIN 2 and 3[111]. Despite standardized criteria it has been shown that there is substantial inter-observer variability in interpretation of CIN especially regarding CIN 1 lesions[112]. The histological classification of pre-malignant cervical glandular lesions is less clear but the World Health Organization classifies lesions as glandular dysplasia and adenocarcinoma *in situ*[113].

Data from cross-sectional studies suggest a long precancerous state before development of invasive cervical cancer since women with cervical cancer tend to be more than 10 years older, on average, than women with CIN 3[109]. Estimates of progression and regression rates of different histological stages are highly uncertain, primarily due to misclassification of lesions and treatment of CIN lesions. However, in a review of the literature by Ostor, it was found that the approximate likelihood of CIN 1 progressing to CIN 3 is 10% and progression to cancer 1%[114]. The corresponding approximations for CIN 2 are 20% and 5% respectively[114]. The likelihood of CIN 3 progressing to cancer is more than 12%[114]. Progression and regression rates can also be estimated from mathematical models of the natural history of cervical carcinogenesis where the model outputs are calibrated to population-based data. In a natural history model by Myers et al, the progression and regression rates were age dependent, with increased progression rates and decreased regression

rates with increasing age[115]. The probability of progression from LSIL to HSIL ranged from 0.1-0.3/72 months for women aged 15-34 years, and 0.3-0.5/72 months for women aged ≥35 years[115]. The base case probability of progression from HSIL to invasive cancer was estimated to be 0.4/120 months[115].

Risk factors

Methodological issues in assessing risk factors for cervical cancer

The association between exposure and outcome is best assessed using prospective studies since this gives a clear indication of the temporal order of events. However, it has been difficult to assess the natural history and risk factors associated with cervical cancer in cohort studies for primarily three reasons: 1) cervical cancer is a rare disease and therefore very large and expensive cohort studies would be needed if cervical cancers was to be the measured endpoint, 2) it is unethical to not treat women with pre-invasive lesions, and 3) the induction period of cervical cancer is long, often extending 10-15 years. Therefore most epidemiological studies that have investigated risk factors for cervical cancer have been case-control studies. However, case-control studies are prone to several biases, notably selection bias, reverse causality bias and differential sampling bias. Nested case-control studies performed within population-based cohorts or biobanks are cost-effective and minimise reverse causality biases. An alternative approach has been to use pre-invasive lesions, which is a surrogate for cervical cancer, as the endpoint in cohort studies. It has been proposed that CIN 3 lesions are a more appropriate surrogate for cervical cancer than CIN 2 lesions since the progression and regression rates differs between these two histological stages[45].

Human papillomavirus

There are four separate lines of evidence associating HPV infection with cervical cancer[116]. (I) Large case series have found that HPV DNA is present in over 99% of carcinoma *in situ* and cervical cancer lesions[117,118]. A meta-analysis of 8550 squamous cell carcinomas (also including unspecified cancers) and 1508 adeno- and adenosquamous-carcinomas found that HPV 16 was the predominant type in squamous cancer in all

regions with a prevalence ranging from 45.9% in Asia to 62.6% in North America[119]. HPV 18 was the most common type in adeno- and adenosquamous-carcinomas and the second most common type in squamous cervical cancer in all regions[119]. The distribution of less prevalent HPV types in squamous cell carcinomas varied between regions; HPV 33 and 31 in Europe and North America, HPV 45 in Africa and HPV 58 and 52 in Asia[119]. (II) In case-control studies the odds ratios for cervical cancer associated with presence of HPV DNA are very high. A study that pooled type-specific HPV DNA data from 1918 women with invasive cervical cancers and 1928 controls from 11 separate IARC case-control studies found that fifteen types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) conveyed odds ratios of 50 or more using women negative for any HPV type as reference[10]. Therefore they suggested that these 15 types should be classified as high-risk types. (III) A strong association has been found between HPV infection and nal Article">17</ref-type><contributors><authors><author>Parkin, D. M.</vical cancer in prospective studies. In a matched case-control study nested within a population-based cervical cancer screening programme in Sweden pap smears taken several years previous of development of cervical cancer were HPV tested[87]. Women with cervical cancer were 15 times more likely to have had a previous pap smear that was positive for HPV DNA and there was perfect concordance between the HPV type detected in the pap smear and the type detected in the subsequent cervical cancer. In nested case-control studies in Nordic countries it has also been found that the presence of antibodies against HPV many years before the development of cervical cancer infer an increased risk of future cervical cancer even after adjustment for other potential risk factors[120]. Several prospective cohort studies that have used pre-invasive cervical lesions as endpoints have found that several HPV types convey an increased risk for future pre-invasive lesions with HPV 16 consistently inferring a very high risk [51,78,121-124]. In a US cohort of 20 514 women with normal, equivocal or mildly abnormal cytology at baseline, it was found that infection with HPV 16 and HPV 18 conferred a 10-year cumulative risk of future CIN 3+ of 17.2% and 13.6% respectively[122]. It has also been shown in prospective studies that there is a strong association between persistent HPV infections, in particular HPV 16 and 18, and future development of cervical neoplasia[125,126]. (IV) Laboratory studies have shown that HPV have transformation capacity in

have shown that HPV have transformation capacity in model systems (See section about viral life-cycle and oncogenesis of HPV).

Cross-sectional data has modelled the time between HPV infection and development of CIN 3 to be 7-15 years with HPV infections occurring in the late teens or early 20s and CIN 3 diagnoses peaking around 25-30 years of age[109]. However, recent prospective studies have shown that high-grade lesions (CIN 2 and 3) can develop within months after incident infection[62,127]. It has been reported that in women with high-risk HPV infections, mild cytological abnormalities or mild histological abnormalities compared to normal cytology or histology, do not influence the risk of future high-grade lesions[122,128]. Therefore it has been suggested that instead of emphasising progression through distinct stages of low, to moderate to high-grade intraepithelial lesions, it makes increasing sense to consider HPV infection as a single broad transition state between normal and pre-cancer with viral genotype and persistence being the most important viral characteristics[109,129] (Figure 3).

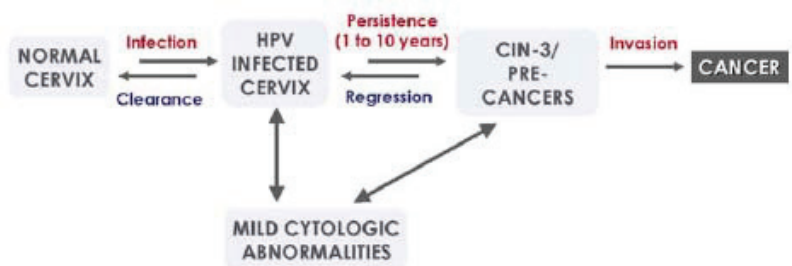

Figure 3. Natural history of cervical cancer. Reprinted from Moscicki et al[109] with permission from Elsevier.

Other sexually transmitted infections

It has been difficult to assess to what extent other sexually transmitted infections are causally associated with cervical cancer or if it is merely confounded by HPV. Antibodies against *Chlamydia trachomatis* and *C. trachomatis* DNA has in most studies been found to be associated with cervical cancer independently of HPV infection[130-132]. In a large prospective seroepidemiological nested case-control study in the Nordic

countries it was found that antibodies against *C. trachomatis* were associated with a two-fold increase in risk of invasive squamous cervical cancer after adjustment for antibodies for HPV and cotinine (an indicator for tobacco smoking)[130]. Proposed mechanisms for *C. trachomatis* in cervical carcinogenesis have been a local inflammation which induces genetic damage[133], inhibition of cell apoptosis[134] and interaction with clearance of HPV infection[81]. Several studies have failed to find an association between *C. trachomatis* and adenocarcinoma[130,131,135].

Results regarding the association between Herpes simplex virus type 2 (HSV-2) and cervical cancer is more inconclusive. Some case-control studies have reported an increased cervical cancer risk for women with antibodies against HSV-2[136,137]. However, in a prospective nested case-control study that also included a meta-analysis of longitudinal studies no association between HSV-2 and cervical cancer or cervical neoplasia was found after adjustment for HPV infection or among HPV seropositive women[138].

Smoking

Both case-control studies and cohort studies have consistently found smoking to be associated with pre-invasive lesions and cervical cancer even after adjustment for HPV infection or stratification to HPV positive women only[79,139-142]. The increased risk among smokers compared to non-smokers has been approximately 2-3 fold in most studies and some studies have found the effect to be dose-dependent[140-143]. A proposed mechanism is the exposure to genotoxic compounds as nitrosamine 4-(methylnitrosamino)-1-(3-pyridol)-1-butanone which has been found in high concentrations in cervical mucous among smokers[144].

Immunocompromised hosts

An increased risk for ano-genital cancers is observed following organ transplantation[145,146] and cervical cancer is classified as an AIDS defining disease among HIV positive women[147]. The incidence of CIN is higher among HIV positive women compared to HIV negative women[148]. It has also been found that incident CIN among women is negatively associated with the CD4+ cell count[148,149]. One study has reported the increased risk of CIN among HIV positives to be primarily explained by an augmented risk of persistent HPV infection[149].

Multiparity

Multiparity has been found to be associated with cervical cancer and pre-invasive lesions in most case-control studies[141,150,151]. In a pooled analysis of more than 11 000 invasive cervical cancer cases and 33 000 controls there was a linear trend of increasing cancer risk according to numbers of full-term pregnancies[151]. The risk almost doubled among women with >7 full-term pregnancies compared to 1-2 pregnancies and the results were similar when the analyses where restricted to HPV positive women[151]. However, in two prospective studies development of future CIN 3 or worse (CIN 3+) was not associated with parity in analyses restricted to HPV positive women[143,152].

Oral contraceptives

Case-control studies have yielded conflicting results regarding the association between oral contraceptives (OC) and cervical cancer[152-155]. In a systematic review that included more than 12 000 invasive cervical cancer and carcinoma *in situ* cases the risk increased with increasing duration of use of OC from 1.1 among women who used OC less than 5 years to 2.2 among women who used OC more than 10 years[155]. The results were similar in analyses restricted to HPV positive women and after adjustment for other potential confounders. Contrary to these findings, two prospective studies failed to find an association between OC and CIN 3+ in analyses restricted to HPV positive women[143,152]. One of the studies however, found an increased CIN 3+ risk among HPV positive women with current use of injectable hormonal contraceptives[152]. A proposed mechanism for oral contraceptives in cervical carcinogenesis has been that 16-α hydroxyesterone enhances HPV gene activity[156].

Genetic susceptibility

Hereditary components are important in cervical carcinogenesis. Familial clustering of cervical cancer among biological but not adoptive relatives to women with cervical cancer has been observed[157]. It has been reported that the familial risk of cervical cancer or carcinoma *in situ* is approximately doubled for mothers with affected daughters and vice versa[158]. It has also been reported that the risk of cervical cancer is higher for women with a mother and/or sister with cervical tumors than for women with an affected grandmother and/or aunt[159]. The most extensively studied genes in relation to cervical cancer have been Human

Leukocyte Antigen complex (HLA) which is involved in antigen presentation. The most consistent findings have been that HLA-DRB1*13 and/or HLA-DQB1*0603 are associated with an approximately 50% reduction in risk of cervical cancer[160]. In a nested case-control study of cervical cancer in Sweden, HLA class II haplotypes DR15 and DQ6 were associated with an increased risk of cancer[161].

CERVICAL CANCER PREVENTION

Principles of prevention of cervical cancer

Prevention of cancer can be performed in three principally different ways. Primary prevention is the prevention of disease from occurring, i.e. prophylactic vaccination against HPV[162]. Secondary prevention attempts to reduce morbidity in pre-symptomatic subjects with established disease by its early detection and treatment, i.e. screening programs to detect CIN and cervical cancer[162]. Tertiary prevention is implemented on patients with a view of cure, palliation, rehabilitation, or prevention of recurrence or complications[162]. Only primary and secondary prevention of cervical cancer will be considered here.

Preventive measures can be evaluated in terms of efficacy, defined as the extent to which an intervention produces a beneficial result under ideal conditions, or effectiveness, defined as the extent to which a specific intervention, when deployed in the field in routine circumstances, does what it is intended to do for a specified population[163]. To date there are limited data available on the effectiveness of HPV-based screening or prophylactic HPV vaccination. The efficacy of preventive measures is best assessed in randomized controlled trials which ensure high internal validity[164]. The natural history of cervical cancer makes it somewhat difficult to evaluate the efficacy of primary and secondary prevention. First, as described previously, it is difficult to use cervical cancer as the endpoint in studies since it is a rare event with a long induction period and when pre-invasive lesions are detected it is unethical to leave them untreated. Second, the infectious nature of HPV makes individual-based studies inappropriate since these studies can not assess indirect effects of preventive measures, i.e. that vaccination or screening of one individual reduces the risk for other individuals to acquire infection. This is of particular importance in evaluating vaccine efficacy (but also screening) and can best be assessed in large cluster randomized-controlled trials[165].

Primary prevention of cervical cancer

HPV vaccines

Two commercial prophylactic vaccines, from Merck (Gardasil®) and GlaxoSmithKline (Cervarix), have been developed against HPV. Gardasil® was approved by the Food and Drug Association (FDA) in the US and the European Medicine Agency in 2006 to be used in young women and in Europe also in men[166]. These vaccines are based on virus like particles (VLPs) consisting of the L1 capsid protein[33]. Cervarix is a bivalent vaccine consisting of HPV 16 and 18 VLPs and Gardasil® is a tetravalent vaccine consisting of HPV 6, 11, 16 and 18 VLPs[167,168]. The reason for inclusion of HPV 6 and 11 is that these HPV types are associated with *condyloma acuminata* and laryngeal papillomatosis[169]. Both vaccines have shown very promising results in prevention against HPV infections and HPV associated disease with very little adverse events[166]. The vaccines are highly immunogenic with IgG antibody level responses manifold higher than after natural infection and the antibodies persist for at least 4 to 5 years[167,168]. The reason for the high antibody titers after vaccination might be that the vaccines are delivered intramuscularly, thereby mimicking a viraemia which does not occur in natural infection[33]. Results from phase II randomized controlled trials for both vaccines have shown 90% efficacy or more against persistent HPV infections with the vaccine types, and 100% efficacy against CIN lesions associated with the vaccine types during 4-5 years of follow-up[167,168]. The results from a phase III trial of the tetravalent vaccine with 3 years of follow-up have recently been published[170]. It was reported that the vaccine prevented 98% of HPV 16/18-related high-grade cervical lesions among women who had no virologic evidence of infection with HPV 16 or HPV 18 through 1 month after the third dose (month 7)[170]. In the intention-to-treat analysis when also women with previous infections were included, the vaccine prevented 44% of HPV 16/18-related high-grade cervical lesions[170]. It is probable that the protective effect of the vaccines are attributed to serum neutralizing antibodies since only half of the women immunized with HPV 11 VLPs and even less of women immunized with HPV 16 VLPs have detectable antibodies in cervicovaginal secretion[33,171]. Cross-protection against phylogentically related HPV types 31 and 45 has been demonstrated after vaccination with the bivalent vaccine[167]. This might be explained by the higher antibody levels obtained after vaccine

immunization compared to that of natural infection for which very little humoral cross-protection appears to be present.

The vaccines have shown very promising results in Phase II and III trials, but there are many questions that need to be addressed by policy makers on how to administer vaccination against HPV[169]. The enrolment criteria of the clinical trials restricted the numbers of lifetime partners and past history of cervical abnormalities and included only women aged 15-25 years[167,168]. Thus, the high efficacy found in these trials applies to primarily unexposed women. In the general population of women having been sexually active for some time, efficacy is likely to be much lower. Importantly there has been no evidence of protection for future disease among vaccinated women who entered the studies with current or past HPV infection with the vaccine types[169]. More data regarding duration of protection, efficacy in already infected individuals and cross-protection against non-vaccine types will come from ongoing phase III trials[165].

Individual-based trials can not assess the indirect effect of vaccines, i.e. herd immunity which is the population-level consequence of acquired immunity among some individuals that can reduce the risk of acquiring infection among susceptible individuals[172]. To assess this issue and to evaluate the effectiveness of HPV vaccines, large cluster randomized phase IV trials are currently being planned[165]. These trials are planned to be randomized in such a way that they will give information regarding the effect of catch-up vaccination and vaccination of both genders compared to females only[165].

The population attributable proportions of cervical cancers caused by each HPV type form the basis for decisions regarding which HPV types should be included in vaccines. The population attributable proportion is defined as the proportion of cases within a population that would not have occurred in the absence of exposure[173]. In a study by Munoz et al. the HPV type-specific prevalence in cervical cancers was assessed worldwide to estimate how much each HPV type attribute to the cervical cancer burden[174]. It was estimated that a vaccine directed against HPV 16 and 18 could potentially prevent 71% of cervical cancers worldwide and a vaccine that included the 8 HPV types that are most prevalent in cervical cancers could prevent 87% of cancers. However, these estimates were based only on the prevalence of each HPV type in cervical cancers which is different from the population attributable proportion[175]. Nevertheless if the relative risks are high (as with many

HPV types) the population attributable proportion is approximately the proportion of cases exposed[173]. Further studies are needed that correctly assess how much each HPV type attributes to pre-invasive lesions and cervical cancers.

Mathematical models have predicted that if the vaccination coverage is high, vaccinating both boys and girls will have little benefit on the incidence of cervical cancer compared to vaccinating girls only but it will substantially increase the costs of vaccination programs[176,177]. Since the vaccines appear to only protect women who are not already infected it would seem reasonable to vaccinate before sexual debut[169]. In a recent modeling study by French et al. it was reported that with the assumption of 70% coverage, the long-term benefits of vaccination are greater if vaccination occurs before sexual debut, and vaccinating both males and females have a greater impact when vaccination occurs before the age of 15 compared to later[178]. A 3 year catch-up vaccination was found to prevent 7.1-13.5 % of cervical cancers during the next 40 years depending on the age of vaccination[178]. Cervical cancer is a major health problem in developing countries[102]. The prophylactic HPV vaccine could be of great benefit for these countries but a major obstacle is the high price of the vaccines, a three-dose series of Gardasil® is estimated to cost $ 360 in the US[179]. For countries with a gross domestic product of less than $ 1000 per capita, the per-dose cost may need to be as low as $ 1 to $ 2 to make vaccination both cost-effective and affordable[179]. It is therefore likely that developing countries will have to rely on financial support from e.g. the GAVI Alliance, to be able to introduce HPV vaccination[179].

Based on evidence from HPV vaccine studies and data on age-specific sexual behavior data from the USA, the American Cancer Society have developed the following guidelines on HPV vaccination[169]:

-Routine vaccination is recommended for females of 11-12 years
-Vaccination is also recommended for girls 13-18 years to catch up missed vaccination
-There is currently insufficient data to recommend for or against universal vaccination of women 19-26 years. Vaccination of these women has to be based on an informed discussion between the woman and her health care provider which takes into account previous numbers of partners (i.e. previous risk of HPV exposure)

-HPV vaccination is currently not recommended for women over 26 years of age or for males.
-Cervical cancer screening should continue in both vaccinated and unvaccinated women

Secondary prevention of cervical cancer

Assessment of screening tests

The parameters defining the validity of a screening test are its sensitivity, specificity, predictive value positive (PVP) and predictive value negative (PVN)[180]. The sensitivity is the probability that a test correctly classifies people with disease as positive and the specificity is the probability that a test correctly classifies people without disease as negative[164]. The predictive value positive (PVP), which is a measure of the extent of procedures induced by screening that were actually necessary, is defined as the proportion of people with a positive test that has the disease[164]. The predictive value negative (PVN) is defined as the proportion of people with a negative test that does not have the disease[164]. A low PVP is commonly the result of poor specificity and will yield a high number of false-positives[164]. However when a disease is rare - as in most situations where screening is implemented - a low PVP is also a result of low disease prevalence[180]. This is the reason for targeted screening of populations where the prevalence of disease is high[164]. A low PVN is more likely to result from poor sensitivity than poor specificity[164]. Another important feature of a screening test is its reliability which is the test capacity to give the same results on repeated testing[164].

Verification bias occurs when the probability of disease verification via the gold standard is dependent on the screening test result[181]. This has been a methodological problem in many studies that have assessed the efficacy of cervical cancer screening tests since only women with positive screening tests have been referred to colposcopy[181]. Verification bias will result in overestimation of the absolute sensitivity and underestimation of the absolute specificity[182]. Other biases that might overestimate the effect of screening are lead-time bias and length bias. Lead-time bias is the spurious increase in longevity associated with screening which results from earlier detection of disease that gives the impression of longer survival time[180]. Length bias results from the detection of slow-

developing disease due to screening which will result in the impression of increased survival time for screen-detected cases[183]. Another potential problem of screening is overdiagnosis of lesions that are detected at screening but which would not have surfaced clinically in the lifetime of the individual[184].

Cytology screening

For a disease to be suitable for screening, it has to have a preclinical phase during which it is undiagnosed but detectable, early treatment must offer advantage over late treatment, and a screening test with high validity must exist[164]. The natural history of cervical cancer makes it suitable for screening. In 1941 G. Papanicolaou published a paper in which he described the diagnostic value of cytological smears in cervical cancers[185]. The pap smear has been used in organized screening programs for cervical cancer since the 1960s in most Nordic countries and in many other European countries since the 1980s[186]. The approach is based on cytological identification of cells of cancer or its precursors and subsequent diagnostic confirmation by histology[187]. The effectiveness of cytology-based cervical cancer screening programs has not been evaluated in randomized control trials but before-and-after analyses have estimated a 20-80% reduction of cervical cancer incidence due to screening even after birth cohort effects have been taken into account[188-190]. Studies have also reported a substantial preventive effect of cytology screening on cervical cancer mortality[191,192]. The effect of cervical cancer screening has almost exclusively been restricted to squamous cell carcinomas whereas there has been a moderate to no effect on the incidence of adenocarcinoma[188,193]. A major issue is to what extent opportunistic screening reduces cervical cancer incidence compared to organized screening. The organization of the screening program is related to the population coverage, which is an important determinant of the potential effect of screening. Furthermore, organized screening ensures that the correct age groups are targeted. It has been observed that there was a larger decrease in cervical cancer incidence following the introduction of screening in Finland, Sweden, Iceland and Denmark, which all introduced organized screening programs in the 1960s, compared to Norway which had opportunistic screening until 1995[194]. The importance of organized screening has also been reported from England and Wales[191]. Despite the existence of opportunistic screening, there was an increase in cervical

cancer death rates among women aged 20-34 years, from 0.7 per 100 000 women in 1963-67 to 2.2 per 100 000 in 1983-87[191]. Since the national screening program was implemented in 1988 this trend has been reversed[191]. However, the observed reduction is most likely not a true estimate of the effect of screening, since the preceding rise in mortality was most likely explained by an increase in HPV prevalence[191]. The increase in background risk makes it difficult to correctly estimate the effect of screening. In an age and birth cohort model it was estimated that the organized screening reduces the risk of death from cervical cancer by more than 80%[191]. In Norway there has been an approximately 20% reduction of cervical cancer incidence since the implementation of the organized screening program in 1995[195].

Cytological classification systems differ between countries. In Sweden squamous cells are classified as: benign, atypia, HPV infection (koilocytosis), CIN 1, CIN 2, CIN 3 and squamous cell carcinoma. Glandular cells are classified as glandular cell atypia or adenocarcinoma/adenocarcinoma *in situ*. According to the Bethesda System used in the US, koilocytotic atypia and CIN 1 is combined into a single category of low-grade squamous intraepithelial lesions (LSIL) and CIN 2 and 3 are combined into high-grade squamous intraepithelial lesions (HSIL)[196]. Initially the Bethesda system also included the category atypical squamous cells of undetermined significance (ASCUS) which recently has been divided into two subcategories of atypical squamous cells of "undetermined significance" (ASC-US) and atypical squamous cells "cannot exclude HSIL" (ASC-H)[196]. Different classification systems makes it somewhat difficult to compare results from screening studies performed in different countries and it has been reported that the expert cytopathologists participating in major studies of the role of HPV in cervical screening in Sweden, the UK and the US classify normal, equivocal and squamous intraepithelial lesion slides differently, the most notable feature being that smears classified as ASCUS in the US and UK are commonly classified as normal in Sweden[197].

Follow-up of women with abnormal cytology differs between countries and even between regions. In Sweden, some regions refer all women with atypia or worse to colposcopy while other regions refer only women with CIN 2 and CIN 3 and women with atypia or CIN 1 are invited back after 4-6 months for a new cytology. Excision treatment is usually performed on women with histologically verified CIN 2 and 3

while expectant management without treatment is preferred for women with CIN 1 since the majority of these lesions will resolve spontaneously[198].

Cervical cancer remains a leading cause of death and morbidity despite the existence of organized screening programs[103]. In a study that examined the screening history of cervical cancer cases in Sweden, it was reported that 61% had a former pap smear registered and among cases younger than 60 years of age only 16% had never had a pap smear taken[199]. Among women with invasive cancer in the UK it was reported that 47% had an adequate screening history[200]. Cytological screening has several limitations. First, it has poor sensitivity to detect pre-cancerous cervical lesions and cancer. In a meta-analysis of studies without verification bias the sensitivity of conventional cytology to detect histologically verified high-grade lesions (CIN 2 and 3) ranged from 44 to 99% (threshold for cytology: low-grade squamous intraepithelial lesions) and the specificity ranged from 91 to 99%[201]. Due to the limited sensitivity of cytology the test is repeated annually in the US and every 3-5 years in most European countries[186]. The limited sensitivity of cytology is caused both by sampling errors, i.e. that abnormal cells are not collected or are not transferred to the pap slide, and detection errors, i.e. that abnormal cells on the pap slide are missed or misinterpreted by the reader[201]. Second, the reproducibility is inadequate with substantial inter-observer interpretative variability[112]. Third, cytology screening is labor intensive[198]. New technologies, such as liquid-based cytology (exfoliate collected from the cervix with a spatula is stirred into a pot containing a preservative and then in the laboratory the cells are aspirated onto a filter and stained on a glass slide) and automated computer-based technology to read slides have been developed to improve the efficacy of cytological screening[198]. Results regarding the superiority of liquid-based compared to conventional cytology have been inconclusive[202-204].

HPV testing in primary screening

A substantial number of studies using both Hybrid Capture 2 (HC2) and PCR methods have been conducted to evaluate the performance of HPV testing in primary screening[182]. A pooled analysis of 60 000 women included in cross-sectional studies performed in North America and Europe showed that HPV tests (HC2 and PCR) have substantially better sensitivity but a reduced specificity to detect histologically verified CIN 2

or worse (CIN 2+) compared to cytology (cytological cut-off: ASCUS)[205]. The sensitivity of HPV testing was 96.1% with no indication of heterogeneity between studies[205]. The overall sensitivity of cytology was 53.0% but varied substantially between studies which reflects the subjective nature of cytology sampling and interpretation[205]. The specificity of cytology was 96.3% compared to 90.7% for HPV testing[205]. The specificity increased with age for both tests which reflects the natural history of HPV infection with a higher rate of transient infections among young women[205]. A meta-analysis that estimated the relative sensitivity and specificity of HC2 and cytology in primary screening found that HC2 is 23% (95% CI: 13-23%) more sensitive than cytology (cytological cut-off: ASCUS in all but two studies where it was LSIL) at detecting CIN 2+ while the specificity of HC2 was 6% (95% CI: 4-8%) lower than for cytology[182]. The relative sensitivity and specificity are not affected by verification bias since the proportional effects are similar among women with HPV negative tests and normal cytology[205]. Several randomized controlled trials of HPV testing in primary screening are ongoing[50,203,204,206-209] but only cross-sectional data has been published so far. These trials have consistently reported a higher sensitivity of HPV-based compared to cytology-based screening at detecting CIN lesions[203,204,208].

A few cohort studies have published results on the long-term predictive value of HPV tests in primary screening. In a US cohort of 20 810 women that was followed for 10 years, women with normal cytology and negative HPV tests (HC2) at baseline had a cumulative incidence of CIN 3+ of 0.16% (95% CI: 0.08-0.24) after 45 months and 0.79% (95% CI: 0.54-1.04) after 122 months compared to 0.52% (95% CI: 0.39-0.66) and 1.38% (95% CI: 1.10-1.67) for women with normal cytology only[210]. Very high long-term PVN of double negative test results have also been reported from Dutch, German and French cohort studies[211-213]. HC2 has been accepted as an adjunct to cytology in primary screening for women over 30 years of age in the US, as a consequence of the higher sensitivity of HPV testing than cytology in primary screening and the very high long-term PVN of combined testing[214].

A concern of HPV testing is the lower specificity and PVP compared to cytology, which results in a large proportion of false-positive women that will be referred to unnecessary procedures. In the US cohort study described above, the PVP to detect CIN 3+ was 4.40% (95% CI:

3.44-5.36) for HC2, compared to 9.63% (95% CI: 7.21-12.05) for cytology, during 45 months of follow-up[210]. Several screening options have been proposed to increase the PVP of HPV testing. As most HPV infections are transient among young women restricting HPV-based screening to older women would reduce the numbers of false-positives[205]. Referral to colposcopy could be restricted to women with persistent HPV infections[207] or to women with high viral loads[215]. Since different high-risk HPV types infer different risks and attribute unequally to future high-grade lesions, screening algorithms could also be based on HPV type-specific screening tests[121,122,124]. Commercial HPV tests also exist that detect the mRNA from the oncogenes E6/E7 from five HPV types[216].

There are limited data on how to manage women with positive HPV tests and normal cytology. In a study by Cuzick et al., women aged 30-60 years of age with positive HPV tests, borderline cytology or both were randomized to immediate colposcopy or repeat cytology and HPV test 6 and 12 months later[217]. A similar number of CIN 2+ lesions were found among women randomized to immediate colposcopy as were found among women randomized to repeated testing[217]. No CIN 2+ lesion was diagnosed among women who became HPV negative at 6-12 months or among women with an initial negative HPV test and borderline or mild cytology[217]. Thus the authors concluded that it is safe to monitor women with positive HPV tests and negative or borderline cytology with yearly HPV tests and cytology [217]. This algorithm would reduce the numbers of colposcopies induced by HPV-based screening.

Another concern of HPV-based screening programs is that the increased sensitivity of HPV tests results from overdiagnosis of lesions that would regress spontaneously. This can best be evaluated in prospective randomized controlled trials that assess if HPV-based screening leads to a reduced incidence of high-grade lesions in subsequent screening rounds compared to cytology-based screening.

The cost-effectiveness of introducing HPV tests in primary cervical cancer screening has been assessed in the UK, Italy, Netherlands and France[218]. Two strategies, one in which HPV tests were used as triage of women with equivocal cytology, and one in which HPV tests were performed in combination with cytology in women above 30 years of age, were compared to current screening policies[218]. It was found that both screening alternatives incorporating HPV tests, using 3- or 5-year screening intervals, would provide greater benefit (life-time risk of cervical cancer and life expectancy) than the

currently used screening strategies[218]. Both screening strategies were more cost-effective than screening with cytology alone if the sensitivity of cytology was below 90%[218]. In a cost-effectiveness model of cytological screening in high-income countries it was found that intensifying screening to intervals shorter than every 2-3 years would result in modest increase in benefits (life-years-gained) but a considerable increase in costs[219]. Strategies that use screening tests with higher sensitivity than cytology (e.g. HPV test) without increasing the screening intervals offer little incremental benefit but increases cost[220].

HPV testing as triage

Since ASCUS and LSIL have low interpretive reproducibility, especially from an international standpoint[197], it is somewhat difficult to compare studies that have investigated HPV tests as triage for women with ASCUS and LSIL cytology. In a meta-analysis, HPV triage of women with ASCUS was shown to be significantly more sensitive in detecting CIN 2+ lesions compared to repeated cytology (cut-off: ASCUS) with similar specificity[221]. However, the inter-study variation in sensitivity and specificity of HPV testing was large, in particular due to different HPV testing assays[221]. There was also substantial heterogeneity in sensitivity and specificity of repeated cytology[221]. In the ASCUS-LSIL Triage Study, including more than 5 060 women with ASCUS and LSIL in the US, women were randomized to 1) immediate colposcopy, 2) triage with HPV test (HC2) and if positive referral to colposcopy, and 3) cytological follow-up[222]. All women were continuously followed every 6 months and all had a colposcopy at exit after two years of follow-up. It was found that HPV triage was more sensitive to detect CIN 3 than either immediate colposcopy or cytological follow-up (cytological cut-off: HSIL) while only referring about 50% of the women to colposcopy[222]. Serial cytology, at the ASCUS threshold, would have required two visits to achieve similar sensitivity as one HPV test but would have referred more women to colposcopy[222]. The HPV triage arm for LSIL was closed early since over 80% of LSIL were HPV positive[223]. After two years of follow-up it was concluded that that the best way to manage women with LSIL, is immediate colposcopy since triage with cytology would result in too high referral rates if ASCUS was used as cut-off and higher cut-off would result in unacceptably low sensitivity[223]. As a consequence of these results, HPV triage of ASCUS has been introduced in some countries.

HPV testing in post-treatment follow-up

Women who have been treated for CIN remain at an elevated risk for subsequent invasive cervical cancer for up to 20 years and therefore continuous frequent follow-up of these women is needed[224]. In a meta-analysis it was found that the treatment failure rate over 2 years was on average 10%[182]. It was concluded that post-treatment follow-up with HPV testing picks up residual disease quicker and with higher sensitivity and similar specificity compared to cytology but that there is insufficient long-term data to present detailed evidence-based follow-up algorithms[182].

Proposed HPV-based screening algorithm

How should HPV tests be integrated into current screening programs? Basic principals suggest that the most obvious screening algorithm would be to perform screening with the more sensitive test first (HPV test) and follow this with the more specific test for those who test positive initially (triage with cytology)[225]. This algorithm is currently being evaluated in a Finnish randomized control trial[208]. This screening algorithm is appealing for several reasons: 1) if HPV vaccines are introduced in the organized vaccination program HPV-based screening would be an appropriate way to examine breakthrough HPV infections and type-replacement of HPV types not included in the vaccine, 2) with HPV vaccination it is probable that a greater proportion of abnormal cytologies will be HPV negative, thereby reducing the PVP of cytology 3) the current use of HPV test in triage of women with ASCUS would not be needed, 4) the reliability of HPV tests is higher than for cytology (less inter-sampling, inter-laboratory and inter-observer variability), 5) the labor intensive work of cytological reading could be focused on slides from women at high risk, 6) an extended screening interval could be introduced for those women with a negative HPV test. A possible HPV-based screening algorithm is shown in figure 4. The algorithm is based on data from published studies and the two manuscripts on primary screening included in this thesis (Paper IV and V).

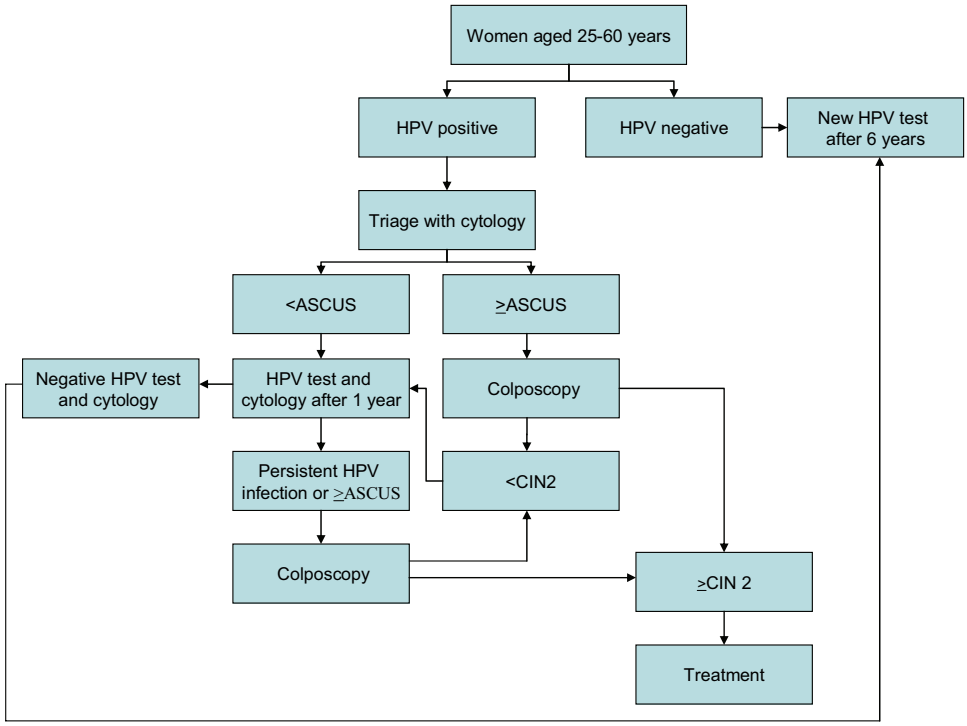

Figure 4. Possible HPV-based screening algorithm for women aged 25-60 years (HPV test used as primary test and cytology used as triage). Adapted from Cuzick et al[225].

Screening in developing countries

Most developing countries do not have a functioning screening program due to poverty, lack of resources and infrastructure, and disenfranchisement of women[226]. Therefore, screen-and-treat options have been evaluated for screening in developing countries. In a randomized clinical trial in South Africa, 6 555 women aged 35-65 years who had never been screened underwent a gynecological examination which included visual inspection with acetic acid (VIA) by nurses and HC2 test[227]. Women were then randomized to three strategies: 1) treatment with cryotherapy if positive HPV test, 2) treatment with cryotherapy if positive VIA, or 3) delayed evaluation (no

treatment)[227]. Women treated with cryotherapy after a positive VIA or a positive HPV test had a significantly reduced risk of high-grade lesions at follow-up after 6 and 12 months compared to the untreated control group[227]. A large cluster-randomized controlled trial of screening with VIA, cytology or HPV test has been initiated in India to assess the impact on cervical cancer incidence and mortality[228]. In cost-effectiveness analysis it has been reported that screening of women in developing countries with HPV tests or VIA once in their life time at age 35 could reduce the lifetime cervical cancer risk with 26-35% at a cost of 10-467 international dollars per year of life saved[229].

AIMS OF THE INVESTIGATION

Paper I To investigate the prevalence of different genotypes of HPV in cervical cancers in Mozambique.

Paper II To investigate the cervical cancer risk of past HPV and *Chlamydia trachomatis* exposures, and in particular the effect of the interaction between different HPV types.

Paper III To investigate the HPV type-specific risk of future high-grade cervical intraepithelial neoplasia.

Paper IV To investigate the long-term CIN 3+ predictive values of HPV tests and cytology in primary cervical cancer screening in a pooled cohort from seven primary European screening studies.

Paper V To investigate the long-term efficacy of combining cytology with testing for HPV persistence compared to cytology only in primary cervical cancer screening of middle-aged women in Sweden.

MATERIALS & METHODS

Epidemiological design

Three studies evaluate the HPV type-specific risk of cervical cancer or pre-invasive lesions. Paper I is a case-series of tumor biopsies from cervical cancers. Paper II and III are prospective population-based studies which have the advantages that temporal associations can be established between exposure and outcome, and results are generalizable. Two studies are designed to evaluate the long-term efficacy of HPV testing in primary cervical cancer screening. Paper IV is a pooled analysis of seven prospective European primary screening studies. Paper V is a randomized control trial (RCT), which is the preferred method to evaluate screening programs since potential confounding factors will tend to be distributed equally between the intervention and control arm[164].

Laboratory Methods

HPV DNA testing

In Paper I and III-V cervical samples were analyzed using a general HPV primer GP5+/6+-mediated PCR-enzyme immunoassay (PCR-EIA) consisting of a pool of digoxigenin-labeled HPV type-specific oligonucleotide probes of 14 high-risk HPV types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68)[21,230]. Human β-globin was amplified simultaneously in the PCR-EIA assay to test for sample DNA quality. Known β-globin and HPV DNA positive samples were used as positive controls, and water was used as a negative control. All HPV positive samples were typed by reverse dot blot hybridization (RDBH) using HPV type-specific plasmids corresponding to the different HPV types tested for in the PCR-EIA[48]. PCR-EIA-positive samples negative in RDBH were cloned and sequenced. Samples were considered HPV positive only if successfully typed by RDBH or by DNA sequencing. In paper I, samples that were negative in the PCR-EIA were further analysed using 1) type-specific PCR for HPV 16 and 18, 2) general HPV primer MY09/11

followed by HPV typing by sequencing, and 3) multiply primed rolling-circle amplification[231] followed by GP5+/6+ PCR and RDBH. In Paper IV, HC1, HC2 and SHARP-PCR were also used to analyze cervical samples depending on the study site[126,212,232,233].

HPV antibody testing
In Paper II, ELISA was used to detect IgG antibodies against HPV 6, 16 and 18[29]. Type-specific VLPs were coated onto ELISA plates. Human antibodies against VLPs were detected using a two-step ELISA with monoclonal antibodies against human IgG and a goat anti-mouse IgG horseradish peroxidase conjugate. A seropositive reference serum was used on each plate as a positive control and reference serum. All samples were first screened at a 1/30 dilution where samples above pre-assigned cut-off levels[63] were selected for confirmatory analysis that were performed using a sample titration series (1/10, 1/31.6, 1/100). The cut-off levels in the confirmatory analyses were pre-assigned[234] as described in detail in Paper II.

C. trachomatis antibody testing
Chlamydia-specific lgG antibodies were detected using microimmunofluorescence[130,235]. For *C. trachomatis* serovars D-K were used. *C. pneumoniae* served as control antigen. All samples that were positive for titers ≥16 were further analysed in a titration series (16, 32, 64, 128 and 256). Titers of ≥64 were considered positive for both *C. trachomatis* and *C. pneumonia.*

Ethics

All studies have been approved by ethical committees in the countries where the studies were conducted.

Paper I

From June 2002 to April 2003, tumour biopsies and serum samples were collected from all women with suspected cervical cancer at the Department of Gynaecology at the Central Hospital of Maputo, Mozambique, who consented to participate. Two biopsies were taken from each tumour, one was formalin-fixed and used for histological

diagnosis, and the other biopsy was frozen for HPV DNA detection. Human immunodeficiency virus (HIV) testing was performed on serum samples using Axsym (Abbot), followed by confirmation using RIBA (Chiron). Biopsies and serum samples were successfully obtained from 88/94 patients. Fourteen patients were excluded because invasive cervical cancer could not be confirmed and two samples were negative in the specimen adequacy test (β-globin PCR). One patient had adenosquamous carcinoma and the others squamous cell carcinomas. The prevalence of each HPV type was calculated with 95% confidence intervals.

Paper II

A nested case-control study was performed within a community-based cervical neoplasia screening project in Taiwan. A population of 41280 women aged 30-64 years were invited. Two health examinations were performed in 1991-93 and 1993-95, where 13595 women had at least one health examination. Baseline serum or plasma was collected from all study subjects. A questionnaire was administered where demographic characteristics, lifestyle habits, Pap smear history and reproductive and sexual history were recorded. Women who had never been married, had a history of cervical neoplasia or had been hysterectomized were not eligible. Through linkage with the national cancer registry, the national death certification system and the catastrophic illness registry, 60 women with invasive cervical cancer and 54 women with carcinoma *in situ* were found during follow-up until December 31, 2000. Cases were grouped into prevalent cases (72), incident cases (35), and unscreened cases (7). Prevalent cases were defined as women who developed cancer within one year after the date of enrolment. Incident cases were defined as women who developed cancer or carcinoma *in situ* more than one year after enrolment. Women who were diagnosed with cervical cancer during follow-up, but who did not receive a Pap smear at enrolment, were referred to as unscreened cases. Controls were individually matched and selected at random from women who did not have cervical neoplasia in any of the three registries at the time when the matched case was diagnosed. The matching criteria were gender, age, area of residence, type of sample (serum or plasma) and date of enrolment (+/- two months). Up to six controls per case were selected. In total 519 eligible controls

were matched to 108 cases in the analyses (eligible controls were not found for 6 cases). IgG antibodies against HPV 6, 16, 18 and *C. trachomatis* were analyzed in serum and plasma samples. Odds ratios (ORs) and 95% CIs were estimated in conditional logistic regression models. The analyses of interaction between different HPV types were estimated using both an additive and multiplicative model[164].

Paper III and V

A population-based RCT was started in Sweden in 1997 with the main purpose of evaluating the effect of HPV testing in primary cervical cancer screening. Women aged 32-38 years in five regions in Sweden (Gothenburg, Malmö, Stockholm, Umeå and Uppsala) who took part in organized cervical cancer screening were invited to take part in the study. Following informed consent, 12527 women were enrolled and randomized either to the intervention arm - action on HPV tests (6257 women), or to the control arm - no action on HPV tests (6270 women). All women had a cervical brush sample taken at baseline that was used for routine cytological screening and then frozen in 1 ml of 0.9 % NaCl for future HPV DNA analysis. Referral to colposcopy was based on routine clinical management. Furthermore, HPV-positive women in the intervention arm who did not have an abnormal enrolment smear in cytology and pathology registries were invited for a second HPV test and cytology on average 19 months later and if persistently positive invited to colposcopy. A matched number of women from the control arm were also invited for an HPV test, cytology and colposcopy to avoid ascertainment bias. All women were followed by registry linkages with both the regional cytology and pathology registries in the enrolling regions, as well as with the national cervical screening registry, to detect development of CIN 2+. All women with an abnormal histopathological diagnosis as well as all women invited for colposcopy within the study protocol had their specimens re-evaluated by a single expert pathologist who was blinded to the HPV status of the women. Analyses were based on re-evaluated diagnoses but if the specimens could not be located in the pathology archives, the original diagnoses were retained.

For paper III, a population-based cohort was formed from 6257 women in the intervention arm as well as 409 women randomly selected

from the control arm that had HPV tests performed on their baseline samples. The analyses were restricted to 5696 women with adequate DNA samples and who had cytological or histological samples registered during follow-up. Women were censored at their last testing date or when they were diagnosed with CIN 2+. In total 148 women developed CIN 2+ during a mean follow-up time of 4.1 years. The HPV type-specific risks of CIN 2+ were assessed as absolute cumulative risks, unadjusted and adjusted relative rates, and population attributable proportions. Type-specific differences in relative rates were evaluated by comparing the beta coefficients of each individual HPV type with HPV 16 in a multivariate regression model.

For paper V, follow-up data during a mean follow-up time of 4.1 years, from the 12 527 women included in the RCT, was used to evaluate the long-term efficacy of screening with combined cytology and testing for HPV persistence (intervention arm) compared to cytology only (control arm). The analyses were restricted to women with cytological or histological samples registered during follow-up and women were censored at their last testing date or when they were diagnosed with high-grade lesions. The primary outcome of the trial was the incidence of CIN 2+ lesions found by incident screening (screening performed after the enrollment screening). Secondary outcomes were the incidence of CIN 2+ lesions found at prevalent screening (enrollment screening and associated follow-up) and the long-term predictive values for CIN 2+ of the two different intervention strategies. In addition, the outcome results were reported stratified by CIN 2 and CIN 3+ lesions as endpoints.

Paper IV

A joint cohort, of 24295 women was formed from seven primary HPV screening studies in six European countries (Denmark, Germany, France, the UK, Spain and Sweden)[81,126,212,232,233,236], and was followed for up to 6 years to define the CIN 3+ predictive value of HPV testing. Only women with adequate cytology and HPV test at baseline and with at least one cytological or histological test during follow-up were included. Histologically confirmed CIN 3+ was the endpoint. In all studies abnormal cytology was regarded as the equivalent of ASCUS or worse. HPV tests were performed using HC2 in four studies, GP5+/6+ PCR in

two studies and SHARP-PCR, HC 1 and HC 2 in one study. Women were followed by linkage to national and regional registries that record cytological and histological results in Sweden, Denmark and the UK. In Germany, Spain and France women were followed with repeated cytological testing. In the Swedish, the UK, the Hannover, and French studies, a sub-sample of women with normal cytology and negative HPV tests were referred to colposcopy. Four studies mandated extra testing and/or colposcopy after baseline HPV-positivity (Sweden, Hannover, UK and France). Women were censored at their last registered testing date. However, if a biopsy was taken the women were censored at the first biopsy date. If a CIN 3+ diagnosis was found later, the women were censored at the date of the CIN 3+ diagnoses even if there were previous biopsies. Data from the seven studies were pooled and to correct for heterogeneity among the studies, multi-level techniques by bootstrap analysis were used. The cumulative incidence rates (CIR) were calculated according to cytological and HPV status at baseline. Predictive values, sensitivity and specificity were calculated for cytology alone, HPV test alone and cytology and HPV test combined (at least one of the two positive) stratified by age groups (<35years, 45-49 years and >49 years) and a test for trend was performed. The heterogeneity between the studies was assessed.

RESULTS & DISCUSSION

HPV type prevalence in cervical cancers in Mozambique (Paper I)

We report that HPV 16 and 18 are the most frequent HPV infections associated with cervical cancer in Mozambique which is in concordance with the overall world distribution of HPV types in cervical cancers[119]. Together HPV 16 and 18 were present in 69% of cancers. The overall HPV prevalence in cervical cancers was 97% (70/72). Detection of HPV DNA from more than one HPV type was common (33% of women were infected with two or more HPV types). Single infections with either HPV 16 or 18 were present in 36% of cervical cancers. HIV positive women were not more likely to be infected with multiple HPV infections.

The reason for conducting the study was that a previous population-based study in Mozambique had reported an unusual type distribution where HPV 16 was infrequent and HPV 35 the dominant type both among women with and without CIN[55]. In our study HPV 35 was present in 19% of tumors. Meta-analyses have found that the HPV type distribution differs between women with and without invasive cancer[237]. The HPV type-specific prevalence among women without cancer may therefore not accurately reflect which HPV types cause invasive cervical cancer in a population. We believe that the HPV type distribution in tumours from women with symptomatic invasive cancers is more likely to be a representative sample of HPV types that are associated with cervical cancer. In tumour samples with multiple HPV infections it is difficult to assess to what extent each type contribute to the development of the tumour. To further evaluate the relative importance of different HPV types for the development of cervical cancer we have expanded the study to also include hospital-based controls which will enable us to adjust for multiple infections.

Risk of cervical cancer associated with past exposure to HPV and *C. trachomatis* (Paper II)

This nested case-control study confirms previous findings that past exposure to HPV 16 is strongly associated with cervical cancer, and women with past exposure to HPV 6 and 16 appear to have a decreased risk of developing cervical cancer compared to women with past exposure to HPV 16 only.

Antibodies against HPV 16 implied a six-fold increased risk for cervical cancer and the risk was similar for invasive cancer and carcinoma *in situ*. HPV 6 was overall not associated with cervical cancer but was associated with cervical cancer among incident cases. Antibodies against HPV 18 conferred an increased risk neither in the overall analyses nor in sub-group analyses. A significant association between cervical cancer and *C. trachomatis* was observed among incident cases but not among prevalent cases or when all subjects were included.

Surprisingly we did not find an association between antibodies against HPV 18 and cervical cancer which has been observed in previous studies[120,238]. A possible explanation is that previous studies have included a substantial proportion of adenocarcinomas, which are in particular associated with HPV 18[120], while our study included only squamous cell carcinomas. Incident and prevalent cases were analyzed separately, because prospective data are more informative on a possible etiologic role of association. That both *C. trachomatis*, HPV 6 and HPV 16 were associated with cervical cancer among incident cases might be a mere chance finding (due to sub-group analysis) but possibly the more reliable prospective analyses had an increased ability to detect a cofactor role of sexual high risk taking behaviour. Past exposure to *C. trachomatis* has been associated with cervical cancer in other studies both in analyses that have adjusted for HPV infection and in stratified analyses where only HPV DNA positive cases and controls have been included[130,131]. In the present study there was an association only among incident cases which might have several explanations. Plasma samples were more common among prevalent cases than among incident cases and there was a suggested cross-reactivity between *C. trachomatis* and *C. pneumoniae* in plasma samples (especially among controls) which was not observed among serum samples. Therefore it is possible that the lack of association between *C.*

trachomatis and cervical cancer among prevalent cases is explained by misclassification of exposure status. It is also known that *C. trachomatis* antibodies decline over time[239], which would make earlier, prediagnostic measurements more accurate. Finally, it is also possible that *C. trachomatis* only has an effect many years before cancer and that non-causative *C. trachomatis* exposures occurring close to diagnosis (too short lag time for an effect) will dilute associations in studies of prevalent cases.

A significant antagonism was detected between HPV 6 and 16 (p-value: 0. 025), but not between HPV 6 and 18 (p-value: 0.94), or HPV 16 and 18 (p-value: 0.20), in a multiplicative model of interaction. In the additive model of interaction there was a tendency of an antagonistic effect between HPV 6 and 16 and between HPV 16 and 18 but not between HPV 6 and 18. Theses findings are in concordance with previous studies that have demonstrated not only an antagonistic effect between HPV 6 and 16, but also reported a tendency of antagonistic interaction between HPV 16 and 18[36,37]. However, future larger studies are needed to investigate the interaction of HPV types. In these studies it would be desirable to test for a broad range of HPV types to be able to adjust for potential confounding, since infections with HPV are associated with both exposures to other HPV types as well as cervical cancer[240]. There is no cross-neutralization *in vitro* between HPV 6 and 16 antibodies and therefore it has been suggested that the antagonism is explained by cell-mediated cross-protection[37]. Since it is believed that the cell-mediated immune response is important for clearance of HPV infection[14], an informative study design to investigate whether past exposure to one HPV type influences clearance of other HPV types, would be to test a cohort of HPV DNA positive women for antibodies against different HPV types and assess if this predicts clearance of HPV infections.

HPV type-specific risk of future high-grade lesions (PAPER III)

In this population-based cohort study we report that HPV 16, 31 and 33 conveyed the highest risks for and attributed to most CIN 2+ lesions among Swedish middle-aged women. Several HPV types previously classified as "high-risk" types conveyed significantly lower risk for future CIN 2+ compared to HPV 16.

The HPV type-specific cumulative absolute risk of CIN 2+ during a mean follow-up time of 4.1 years, ranged from 0% for HPV 59 to almost 50% for HPV 33. Very high cumulative absolute risks (>25%) of CIN 2+ were also observed for women positive for HPV 16, 18, 31 and 58. The cumulative absolute risks for CIN 3+ were highest for HPV 16, 31, 33 and 58. The relative rates of CIN 2+ for each HPV type were assessed using women negative for the corresponding HPV type as the reference group. After adjustment for concomitant infections with other HPV types, the type-specific relative rates segregated into three groups. HPV16, 31 and 33 had very high relative rates (exceeding 10), with all other types having significantly lower risks than HPV16. HPV types 18, 39, 51, 52, 56 and 58 conferred significantly elevated risks for CIN 2+, with relative risks in the range 3-7-fold. We were not able to detect any excess risk for CIN 2+ associated with HPV 35, 45, 59 and 66. Population attributable proportions were based on the type-specific relative rates adjusted for all other HPV types. HPV 16 attributed to 33.1%, followed by HPV 31 (18.3%), HPV 33 (7.7%) and HPV 18 (5.7%). HPV 35, 39, 45, 56, 59 and 66 each contributed to 2.0% or less of CIN 2+ in the population. HPV 16 and 18 together attributed to 39.0% of CIN 2+ and the four types (HPV 16, 18, 31 and 33) that contributed most individually, jointly attributed to 64.0% of CIN 2+.

Our findings are in line with a previous study from Costa Rica that have found HPV 16 to be uniquely carcinogenic among high-risk HPV types[78], and a prospective study in Portland that found HPV 16 and 18 to convey substantially higher risk for future CIN 3+ than other high-risk HPV types[122]. Except for HPV 16 we found that also HPV 31 and 33 conveyed rate ratios of future CIN 2+ above 10, which are similar to a Dutch cohort study, where these three types were associated with the highest risk for future CIN 2+[121].

Risk classification of genital HPV types has hitherto foremost been based on case-control studies[10]. In a pooled analysis of 11 case-control studies performed by IARC, 15 HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) were classified as high-risk types and HPV 26, 53 and 66 as probably high-risk types[10]. After adjustment for other HPV types, we found no elevated risk for future CIN 2+ among women infected with HPV 35, 45, 59 and 66. It is possible that our failure to detect an association with disease for these HPV types may be attributable to limited statistical power. Also it has been shown that the

HPV type distribution differ between high-grade lesions and cervical cancer[237]. However, prospective studies confer important methodological advantages to establish causal relationships. Case-control studies are sensitive to reverse causality bias as well as differential sampling bias which might affect HPV test performance. Furthermore it is often difficult to obtain population-representative controls. Therefore we believe that more robust risk classification of HPV types can be obtained from prospective population-based studies.

The population attributable proportions were based on HPV type-specific relative rates adjusted for concomitant HPV infections, i.e. the rates for a specific HPV type compared to the rate among women negative for that HPV type (not compared to women negative for all HPV types). This choice of reference group is more appropriate for studying attributable proportions since the elimination of one HPV type does not imply that the population would become negative for all HPV types. Our results indicate that a prophylactic HPV vaccine directed against HPV 16 and 18 could potentially reduce the burden of CIN 2+ by 39% among middle-aged Swedish women. It is noteworthy that HPV 31 and 33, which are not included in current prophylactic vaccines, both contributed to more CIN 2+ than HPV 18. A vaccine directed against HPV 16, 18, 31 and 33 could potentially reduce the burden of CIN 2+ by almost 65%. Importantly, population attributable proportions are not portable from one population to another since they depend not only on the relative risk but also on the fraction of the population exposed and somewhat on the distribution of confounders in the population[173]. Hence, it is important to study to what extent different HPV types attribute to pre-invasive lesions and cervical cancers in different populations.

HPV testing in primary cervical cancer screening (Paper IV & V)

We present data from two studies on the long-term efficacy of HPV testing in primary cervical cancer screening. The results indicate that the screening intervals could safely be extended to 6 years for women with a negative HPV test, and HPV-based primary screening among middle-aged women results in protection against CIN 2+ and CIN 3+ in subsequent screening.

In the pooled analysis of seven European primary HPV screening studies it was found that the 6-year cumulative incidence rate (CIR) of CIN 3+ was 51 % for women with baseline abnormal cytology and positive HPV test (cyt+/HPV+), 14 % for women with normal cytology and positive HPV test (cyt-/HPV+), 3 % for women with abnormal cytology and negative HPV test (cyt+/HPV-), and 0.3 % for women with normal cytology and negative HPV test (cyt-/HPV-). A normal cytology alone had higher 3-year CIR of CIN 3+ than the 6-year CIR of CIN 3+ for double negative or for HPV-negative women. Moreover, there was very little difference in the CIR for CIN 3+ between double negative and HPV-negative women. The CIR for CIN 3+ among HPV-positive women was lower than for women with abnormal cytology, but was increasing continuously and gradually approached the CIR of cytology-positive women. Both cytology and the HPV test had higher specificity for women above 35 years of age, but did not improve any further among women above 49 years of age. The CIR of CIN 3+ was not significantly different between the seven studies among cyt-/HPV-, cyt-/HPV+ or cyt+/HPV- women. However, there was a significant heterogeneity among cyt+/HPV+ women ($p<0.001$).

The Swedish population-based RCT of HPV testing in primary cervical cancer screening was nested within the organized screening program, a similar number of random double-blinded procedures were performed in the control arm and women were followed with comprehensive registry based follow-up, all to ensure high internal validity and generalizability. In total 139 women in the intervention arm and 119 women in the control arm were diagnosed with CIN 2+ during follow-up. The discrepancy was primarily a difference in detection of CIN 2 lesions rather than CIN 3+ lesions. At incident screening there was a 42% (95% CI: 4-76) reduction of CIN 2+ lesions and a 47% (95% CI: 2-71) reduction of CIN 3+ lesions in the intervention arm compared to the control arm. This was preceded by a 51% (95% CI: 13-102) increase of CIN 2+ lesions in the intervention arm compared to the control arm at prevalent screening. There was also a tendency that more CIN 3+ lesions were detected at prevalent screening in the intervention arm compared to the control arm [31% (95% CI:-12-87)]. There were 92% (95% CI: 13-302) more CIN 2 lesions detected at prevalent screening in the intervention arm compared to the control arm but this was not followed by a reduction of CIN 2 lesions in the intervention arm

at incident screening. The screening history was assessed among women that developed high-grade cervical lesions. Women with persistent HPV type-specific infections, who were not diagnosed with CIN 2+ at the study colposcopy, remained at high risk for future CIN 2+ (and CIN 3+) during several years subsequent to the colposcopy. The 6-year PVN of combined testing with HPV test and cytology was 99.42% (95% CI: 98.91-99.7) compared to 98.31% (95% CI: 97.53-98.85) for cytology only. The PVP of cytology only was higher than for combined testing but the difference appeared to attenuate as time of follow-up increased.

The uniformly low CIR among cyt-/HPV- women in the joint European study suggests that double negativity tests confer a long-lasting protective effect that is robust, considering that the participating studies used different types of HPV tests, in different settings, in different age groups. Also there seemed to be little gain in the long-term PVN for women with double negative test results compared to HPV-negative women. In the Swedish RCT the 6-year PVN was significantly higher with combined testing with HPV test and cytology compared to cytology only. This is the first RCT that have assessed long term predictive values of HPV testing within an organized screening program and therefore the results can be directly applied to an HPV-based cervical cancer screening program. Although data from the Swedish trial was included in the European study, the data and the analysis differed considerably between the studies. The Swedish data provided to the European study included only women with HPV test results, lesions in the European study were based on the regional pathology lab diagnosis while in the RCT it was based on re-reviewed diagnoses, and the analysis in the randomized control trial was based on comparison of intervention and no intervention on HPV test results. The results from these two studies confirm findings from previous cohort studies that have reported a low risk of future CIN 3+ for cyt-/HPV- women[210,211,213]. Together these studies indicate that the screening intervals could safely be lengthened to 6 years among women with a negative HPV test.

The increased sensitivity of HPV tests compared to cytology may result from overdiagnosis of regressive lesions, and observational cohort studies do not provide information on the effect of an intervention (i.e. a program consisting both of testing and action on the test results). A European Union working group has recommended that results from RCT of HPV-based cervical screening should be available before adopting

HPV testing as a primary screening tool. Several such trials have been started but so far only cross-sectional data has been published[50,203,204,206-209]. We found that screening with combined cytology and testing for HPV persistence resulted in an increased incidence rate of CIN 2+ at prevalent screening followed by a reduced incidence rate of CIN 2+ and CIN 3+ at incident screening. Since HPV infection precedes cytological abnormality it is likely that this is explained by gain in lead time (the amount of time by which the disease is detected earlier due to screening[164]). The stratified analysis of CIN 2 lesions indicated that there might be some overdiagnosis of CIN 2 lesions due to HPV-based screening. First, there was a significant increase of CIN 2 lesions at prevalent screening in the intervention arm which was not followed by a reduction of CIN 2 lesions at incident screening. Second, the discrepancy in lesions detected in the intervention arm compared with the control arm during the whole follow-up period was primarily caused by more diagnoses of CIN 2 in the intervention arm. However these results were based on a limited number of observations and the imprecision of the estimates were large. Furthermore, a limitation to our study is that our study followed the women only for on average 4 years. Although this time span covered the organized 3-year screening round, some women with abnormal cytologies are followed with repeat cytology for several years, and therefore to fully evaluate the effect of over-diagnosis in the subsequent screening round a longer follow-up is needed.

Since HPV tests have lower specificity than cytology in primary screening[205] a concern has been the low PVP (large proportion of false-positives) induced by HPV testing. However, results from both the European study and the Swedish RCT indicate that the discrepancy in the PVP of cytology-based compared to HPV-based screening appear to attenuate as time of follow-up increases. This implies that the problem of HPV-based screening resulting in increased false-positives with women unnecessarily referred to clinical procedures is attenuated in evaluations with longer follow-up.

Few studies have investigated how to monitor women with normal cytology and positive HPV tests[217]. In the Swedish RCT, women with persistent type-specific HPV infections were at continuous high risk of CIN 2+ (and CIN 3+) for several years after the study colposcopy in spite of the fact that "blind" biopsies were taken in case of normal colposcopical

findings. Therefore continuous active follow-up is warranted among these women.

Verification bias may overestimate the performance of screening tests when only women with a positive screening test are referred to colposcopy[181]. However, it is rare to diagnose CIN 2+ by colposcopy among women with negative HPV test and normal cytology[241] and the relative efficacy of screening strategies evaluated in the Swedish RCT would not be affected by verification bias since the proportional effects are similar in the two arms. Therefore it is unlikely that verification bias notably affected our results.

CONCLUSIONS

Primary prevention

Results from phase II and III trials regarding the efficacy of prophylactic HPV vaccines show great promise. However current vaccines include only HPV 6, 11, 16 and 18 and studies are needed to evaluate to what extent each HPV type attribute to cervical cancer burden. Ideally these data should be based on population-based prospective studies which have high internal and external validity. In this thesis, data have been presented that indicate that a prophylactic vaccine directed against HPV 16 and 18 could prevent approximately 40% of CIN 2+ among middle-aged women in Sweden while a vaccine with protection against HPV 16, 18, 31 and 33 would prevent almost 65% of CIN 2+. Several HPV types currently classified as high-risk types attributed to 2.0 % or less of CIN 2+ and inclusion of these types in HPV vaccines therefore seems less motivated, as the theoretical possibility exists that inclusion of too many HPV types in second generation HPV vaccines might impair the response against the most important HPV types. Since population attributable proportions are not transferable from one population to another it is important to conduct further studies to investigate to what extent different HPV types attribute to pre-invasive lesions and cervical cancer in different populations. It has also been reported that HPV 16 and 18 are the most prevalent HPV types in cervical cancers in Mozambique. Current prophylactic HPV vaccines that include these two types are therefore likely to have a substantial impact on cancer incidence in Mozambique.

Finally, data were presented that indicate that HPV 6 and 16 interact in the development of cervical cancer. It is important to continue to study whether interaction of different HPV types reflects biologically meaningful interferences since it could influence the effect of multivalent prophylactic HPV vaccines.

Secondary prevention

HPV tests in combination with cytology in primary cervical cancer screening have so far only been accepted in the US. Results from RCTs are awaited in Europe before a decision is made regarding use of HPV testing in primary screening. In this thesis data have been presented from a joint European study and a Swedish RCT that indicate that screening intervals could safely be extended to 6 years within an HPV-based screening program. In the Swedish RCT it was found that HPV-based screening results in protection against CIN 2+ and CIN 3+ in subsequent screening. This indicates that HPV-based programs result in gain of lead time rather than overdiagnosis of regressive lesions. The specificity and PVP of HPV tests are lower than for cytology which might induce extra cost and anxiety for women if screening is performed with HPV tests. However, both studies indicate that the difference between the PVP of HPV tests and cytology attenuates as follow-up increases. Finally it was also found that women with persistent HPV infections remain at continuous high risk for future CIN 2+ also after gynecological assessment, even when the clinical management included "blind" biopsies in case of normal colposcopical findings. The results from these two studies indicate that HPV-based screening programs are feasible. However, further studies are needed in particular regarding cost-effectiveness, how to optimize the clinical management, treatment and follow-up of women who test positive for HPV, and to what extent HPV-based screening results in overdiagnosis of CIN 2 lesions.

In Paper III we also report that different so-called "high-risk" HPV types convey significantly different risks for and attribute differently to CIN 2+. HC2, which is a commercial HPV test, does not distinguish between different high-risk HPV types. Our findings indicate that the efficacy of HPV-based primary screening could be improved using HPV type-specific assays as they will provide better information on CIN 2+ risks which is likely to be important in designing appropriate algorithms for clinical management, treatment and follow-up.

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