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OUTCOMES OF CERVICAL HUMAN PAPILLOMAVIRUS (HPV) INFECTIONS AMONG MOTHERS IN THE FINNISH FAMILY HPV STUDY

by

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To my wonderful husband Jaakko
and children
Linnea, Lennart and Linus

ABSTRACT

Karolina Louvanto

Outcomes of cervical human papillomavirus (HPV) infections among mothers in the Finnish Family HPV Study.

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To understand the natural history of cervical human papillomavirus (HPV)-infections, more information is needed on their genotype-specific prevalence, acquisition, clearance, persistence and progression.

This thesis is part of the prospective Finnish Family HPV study. 329 pregnant women (mean age 25.5 years) were recruited during the third trimester of pregnancy and were followed up for 6 years. The outcomes of cervical HPV infections were evaluated among all the mothers participating in the study. Generalized estimating equation (GEE)-models and Poisson regression were used to estimate the risk factors of type-specific acquisition, clearance, persistence and progression of Species 7 and 9 HPV-genotypes.

Independent protective factors against incident infections were higher number of life-time sexual partners, initiation of oral contraceptive use after age 20 years and becoming pregnant during FU. Older age and negative oral HR-HPV DNA status at baseline were associated with increased clearance, whereas higher number of current sexual partners decreased the probability of clearance. Early onset of smoking, practicing oral sex and older age increased the risk of type-specific persistence, while key predictors of CIN/SIL were persistent HR-HPV, abnormal Pap smear and new sexual partners.

HPV16, together with multiple-type infections were the most frequent incident genotypes, most likely to remain persistent and least likely to clear. Collectively, LR-HPV types showed shorter incidence and clearance times than HR-HPV types. In multivariate models, different predictors were associated with these main viral outcomes, and there is some tentative evidence to suggest that oral mucosa might play a role in controlling some of these outcomes.

Keywords: Human papillomavirus, uterine cervix, HPV genotype, high-risk, low-risk, mothers, prevalence, incidence, clearance, persistence, progression, CIN, risk factors, GEE-model

TIIVISTELMÄ

Karolina Louvanto
Kohdunkaulan papilloomavirus (HPV) -infektiot ja niiden taudinkulku suomalaiseen HPV-perhetutkimukseen osallistuneilla nuorilla äideillä.
Suupatologian ja -radiologian osasto, Hammaslääketieteen laitos, MediCity Tutkimuslaboratorio, Lääketieteellinen tiedekunta, Turun yliopisto; Naistenklinikka, Turun yliopistollinen keskussairaala, Lääketieteellinen tiedekunta, Turun yliopisto; Syöpätautien klinikka, Turun yliopistollinen keskussairaala; ja Valtakunnallinen kliininen tutkijakoulu, VKTK.

Ymmärtääksemme paremmin ihmisen papilloomavirus (HPV)-infektion luonnollista taudinkulkua tarvitsemme lisää tietoa eri HPV-genotyyppien yleisyydestä, uusien infektioiden ilmaantuvuudesta, niiden paranemisista tai muuttumisista kroonisiksi infektioiksi ja etenemisistä kohdunkaulan syövän esiasteiksi (CIN).

Tämä väitöskirjatyö on osa monivuotista seurantatutkimusta, jossa selvitetään HPV-infektioiden dynamiikkaa 329:llä suomalaisperheen vanhemmilla ja näiden lapsilla. Tässä työssä keskityttiin 329 tutkimukseen osallistuneihin äitien (keski-ikä 25.5 vuotta) kohdunsuun HPV infektioihin, joiden seuranta aika oli kuusi vuotta. Yleistettyä estimointiyhtälöä, GEE-menetelmää (Generalized Estimating Equations) ja Poisson regressioanalyysiä käyttäen tunnistettiin tekijöitä joka edesauttoivat infektoitumista ryhmän 7 ja 9 kuuluvilla HPV tyypeillä, tai ennustivat infektion paranemista tai kroonistumista.

Lukuisat seksi-partnerit, ehkäisypillereiden aloitusikä yli 20-vuotiaana ja uusi raskaus seurannan aikana suojasivat uudelta HPV-infektiolta, infektion paranemista ennustivat korkeampi ikä ja se, ettei suusta todettu korkean riskin HPV-tyyppien aiheuttamaa infektiota, kun taas lukuisat tämän hetkiset seksi-partnerit ennustivat huonompaa HPV-infektioiden paranemista. HPV–infektion koronistumisen oli todennäköisempään niillä naisilla, jotka olivat aloittaneet nuorena tupakoinnin, harrastivat suuseksiä ja olivat iältään vanhempia. HPV-infektion etenemistä syövän esiaste muutoksiin asti lisäsi alkutilanteessa todettu korkean riskin HPV-tyyppien aiheuttama infektio ja näiden kroonistuminen seurannan aikana, sekä aikaisemmin todettu muutos papa-irtosolunäyteissä että uusi seksi-partneri.

Täten yhteenvetona voidaan todeta, HPV16 ja useat eri HPV tyypit yhdessä olivat tässä tutkimuksessa naisten yleisimmät uusien HPV-infektioiden aiheuttajat, ja nämä infektiot myös yleisimmin kroonistuvat ja paranivat hitaimmin. Matalan riskin (LR)- HPV-tyyppien aiheuttamat infektiot ilmenivät nopeammin, mutta myös paranivat nopeammin verrattuna korkean riskin (HR)-HPV-tyyppeihin. Useita altistavia tekijöitä tunnistettiin, mutta uutena havaintona oli suun HPV-infektion mahdollinen vaikutus genitaalialueen HPV-infektion taudinkulkuun.

Avainsanat: ihmisen papilloomavirus, HPV DNA, HPV-genotyypit, nainen, esiintyvyys, ilmaantuvuus, parantuminen, persistointi, taudin eteneminen, korkean riskin HPV, matalan riskin HPV, suun HPV-infektio

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ABBREVIATIONS

ASCUS	atypical squamous cells of undetermined significance
ASC-H	atypical squamous cells suggesting HSIL
CC	cervical cancer
CI	confidence interval
CIN	cervical intraepithelial neoplasia
CR	clearance rate
DNA	deoxyribonucleic acid
FU	follow-up
GEE	generalized estimating equation
GP	general primer
HIV	human immunodeficiency virus
HPV	Human papillomavirus
HR	high-risk
HSIL	high-grade intraepithelial lesion
IARC	International Agency for Research on Cancer
IR	incidence rate
IRR	incidence rate ratio
LCR	long control region
LR	low-risk
LSIL	low-grade intraepithelial lesion
MFI	median fluorescence intensity
NIS	New Independent States of the former Soviet Union
OC	oral contraceptive
OR	odds ratio
ORF	open reading frame
PA	population averaged
Pap smear	Papanicolaou smear
PCR	polymerase chain reaction
RR	risk ratio
SD	standard deviation
CIN/SIL	name of the group of progressors combined of women who either developed a biopsy-confirmed CIN lesion or presented with a incident ASC-H cytology (IV)
STD	sexually transmitted disease
TBS	the Bethesda System
TZ	transformation zone
VLP	virus-like particle
wmr	women months at risk

LIST OF ORIGINAL PUBLICATIONS

This study is based on the following publications referred to in the text by the Roman numerals I-IV.

I. **Louvanto K**, Rintala M, Syrjänen K, Grénman S, Syrjänen S. Incident genital infections with high- and low-risk human papillomavirus (HPV) infections among mothers in the prospective Finnish Family HPV Study. Submitted

II. **Louvanto K**, Rintala M, Syrjänen K, Grénman S, Syrjänen S. Genotype-specific clearance of genital human papillomavirus infections among mothers in the Finnish Family HPV Study. J Clin Microbiol. 2010;48(8):2665-71.

III. **Louvanto K**, Rintala M, Syrjänen K, Grénman S, Syrjänen S. Genotype-specific persistence of genital human papillomavirus (HPV) infections in women followed for 6 years in the Finnish Family HPV Study. J Infect Dis. 2010;202(3):436-44.

IV. **Louvanto K**, Syrjänen K, Rintala M, Grénman S, Syrjänen S. Human papillomavirus and predictors of cervical intraepithelial neoplasia among young mothers in a prospective follow-up study. Acta Obstet Gynecol Scand. 2011;90(2):167-73.

1. INTRODUCTION

Human papillomavirus (HPV) is recognized as the main causal factor of cervical cancer (CC) and its precursors called CIN (cervical intraepithelial neoplasia). Mucosal HPVs are also involved in a substantial proportion of other anogenital neoplasms and also implicated in etiology of several non-genital cancers, most notably in the head and neck region. In addition, HPVs can also cause benign tumours like papillomas and genital warts as well as asymptomatic infections.

Over 160 different HPV genotypes have been recognized and some 40 of those infect the female genital tract. HPV genotypes are classified as high-risk (HR) and low-risk (LR) -types according to their clinical behaviour. Worldwide, the eight most common HR-HPV types found in CC are all included either in species 7 (HPV18, 45) or species 9 (HPV16, 31, 33, 35, 52, 58).

Genital HPV infection is traditionally considered to be a sexually transmitted disease (STD), with the prevalence peaking at the age of 18-25 years. The majority of HPV infections in young women are transient and up to 80-90% of these women will clear their infection. The transit time of a genital HPV infection is estimated to be 12-15 months for HR-HPV types and about half that for LR-HPV types. HPV infections that fail to clear spontaneously remain persistent. These persistent infections are considered to be the main risk factor and causal link for CIN and CC. These precursors of CC are separated as low grade lesions (CIN 1) and high grade lesions (CIN 2-3).

Factors increasing the risk of HR-HPV infections, CIN and CC are linked with high-risk sexual behaviour of both sexes, including early initiation of sexual relationships, high number of sexual partners, prolonged use of oral contraceptives (OC), high parity and other STDs. Other implicated risk factors include tobacco smoking, nutritional deficiencies, immunosuppression and (possibly) some genetic factors.

The Finnish Family HPV Study is a long-term prospective cohort study, originally designed to model the dynamics of HPV infections within regular families, including a mother, a father and their newborn baby. At baseline, 329 Finnish families, including 329 mothers, 131 fathers and 331 infants were enrolled in this cohort study between 1998 and 2002. The original 3-year follow-up was extended to cover six years, completed by 161 mothers and 44 fathers between 2006 and 2008.

The original studies included in this thesis focused on cervical HPV infections of the mothers included in this cohort, evaluating their outcome and its predictors in a longitudinal setting. During this 6-year follow-up, incidence, clearance and persistence were analyzed at the genotype level calculating the type-specific times and rates for these events. Women who developed an incident CIN during the follow-up were analyzed in a separate study.

2. REVIEW OF THE LITERATURE

2.1 HUMAN PAPILLOMAVIRUS

2.1.1. STRUCTURE

Human papillomavirus (HPV) is a small non-enveloped DNA virus that is approximately 55nm in diameter (Williams et al., 1961). The HPV genome consists of approximately 8000 base-pairs in a double-stranded DNA molecule enclosed in an icosahedral protein capsid composed of 72 capsomers (de Villiers et al., 2004). The HPV genome contains eight open reading frames (ORFs) that can be divided into three functional regions: 1) non-coding regulatory region, termed as upstream regulatory region (URR) or long control region (LCR), which modulates viral DNA replication and gene transcription; 2) an early (E) region, which harbours the early genes (E1, E2, E4-7) which code for proteins involved in viral genome persistence and replication, viral transcription and regulation of cell proliferation; and 3) a late (L) region which is composed of two genes, L1 and L2, which code for the major and minor capsid proteins (McMurray et al., 2001; Howley and Lowy, 2007). The HPV genome organisation is shown in **Figure 1.**

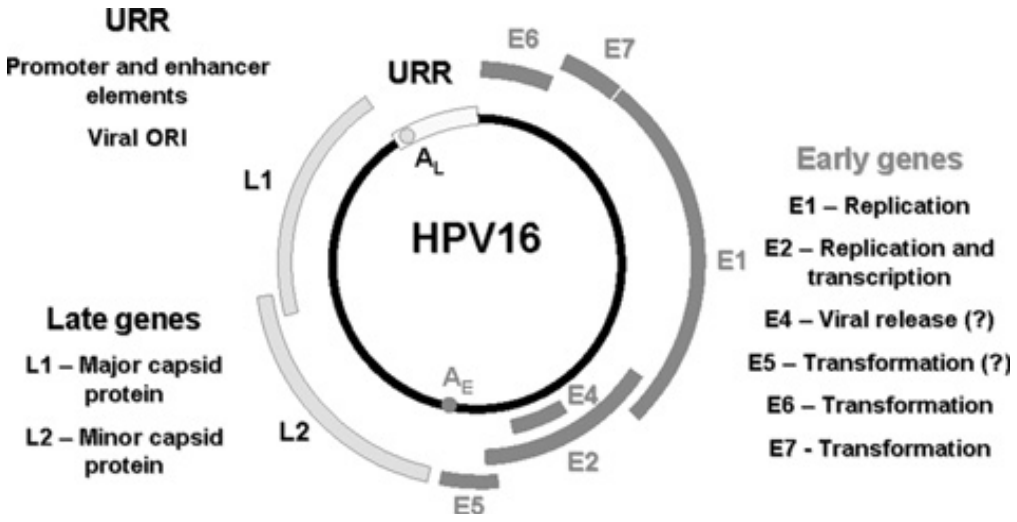


Figure 1. Genome organisation of HPV16. Adapted from Stanley et al., 2007 with permission from Portland Press.

2.1.2 CLASSIFICATION

Papillomaviruses are strictly host-specific. To date, 120 different HPV types have been classified (Bernard et al., 2010). They are classified as genotypes and each type is numbered in order of their discovery. HPVs belong to the Papillomaviridae family which is divided into genera, species, types, subtypes and variants. The taxonomy of HPV is based on comparison of the nucleotide sequences and homology of the L1 ORF. If the DNA sequences of the L1 genes differ over 10% from the closest known HPV type, it is recognized as a new type. A subtype is defined with a 2-10% difference in the DNA sequences. Less than 2% is defined as an intra-type variant (de Villiers et al., 2004)

HPVs are grouped according to the type of epithelia they infect. At present, there are about 40 HPVs infecting the mucosal sites of the body, including the ano-genital tract of both genders. All HPV genotypes infecting the genital tract belong to the alpha-papillomavirus genus which includes 15 species and 58 HPV genotypes. All genotypes are also classified according to their clinical behaviour (i.e. association with malignancy) into high-risk (HR)-types, low-risk (LR)-types and probable HR-types as listed in **Table 1**.

Table 1. Classification of HPV types by their malignant potential (Muñoz et al., 2003; de Villiers et al., 2004)

	Species	**Types**
HR-types	5	51, 82
(n=15)	6	56,
	7	18, 39, 45, 59, 68
	9	16, 31, 33, 35, 52,58
	11	73
Probable HR-types	5	26
(n=3)	6	53, 66
LR-types	1	42
(n=12)	3	61,72,81, CP6108(cand89)
	7	70
	8	40, 43
	10	6, 11, 44
	13	54
Undetermined risk	3	83
(n=3)	4	57
	11	34

2.2 HPV INFECTIONS

2.2.1 HOST EPITHELIA

HPVs are considered to be strictly epitheliotropic and thus confined to either skin or different mucosal epithelial linings. In addition to the anogenital tract, HPVs also infect oral mucosa, urinary tract, larynx, trachea, sinonasal mucosa, oesophagus, tonsils and conjunctiva (Syrjänen and Syrjänen, 2000).

The initiation of HPV infection necessitates viral access to the cells at the basal layer, which is thought to require a break in the stratified epithelium, such as a mild abrasion, micro-trauma or immature squamous epithelium (Egawa, 2003, Culp et al., 2006). In the skin, such basal cells are abundant within the hair follicle and are considered as the site of virus entry (Egawa, 2003, Doorbar, 2005). The best known site of entry, however, is the transformation zone (TZ) which is the junction where two different types of epithelium join together. These can be found in the uterine cervix, anal canal, upper respiratory tract, and bronchus (Syrjänen and Syrjänen, 2000). The TZ of the uterine cervix consists of columnar epithelium and squomous epithelium. The columnar epithelium undergoes replacement by squamous epithelium through a process called squamous metaplasia including a sequence of reserve cell hyperplasia, immature squamous metaplasia and mature squamous metaplasia with the formation of a new squamocolumnar junction. Due to the continuous metaplasia the TZ is particularly susceptible to oncogenic stimulation.

2.2.2 VIRAL LIFECYCLE

The replication of HPV is dependent upon complete keratinocyte differentiation. Following the access of viral particles to the basal layer keratinocytes, high level expression of viral proteins and viral assembly occur only in the upper layers of the squamous epithelia (Doorbar, 2005). After infecting the cells at low copy number, viral DNA replication amplifies the viral copy number approximately 50 to 100 copies/cell. These infected cells leave the basal layer and enter into the proliferation compartment of the epithelium. A sophisticated transcriptional cascade then occurs as the dividing keratinocytes become increasingly differentiated in the upper layers of the epithelium as shown in **Figure 2**. When the keratinocyte reaches the superficial layer and dies, viral genomes are repackaged into capsids and shed from the cell (Doorbar, 2005; Stanley, 2006).

It has been estimated that the time from infection to virus release takes at least three weeks. This is the time required for the keratinocyte to undergo complete differentiation and desquamation. In humans, the time from infection to appearance of HPV induced lesions can vary from weeks to months (Doorbar, 2007). This HPV infectious cycle is effectively evaded from the immune system because there is no retention of HPV antigens until the infected cell reaches the epithelial surface (Stanley, 2006; Wang, 2007).

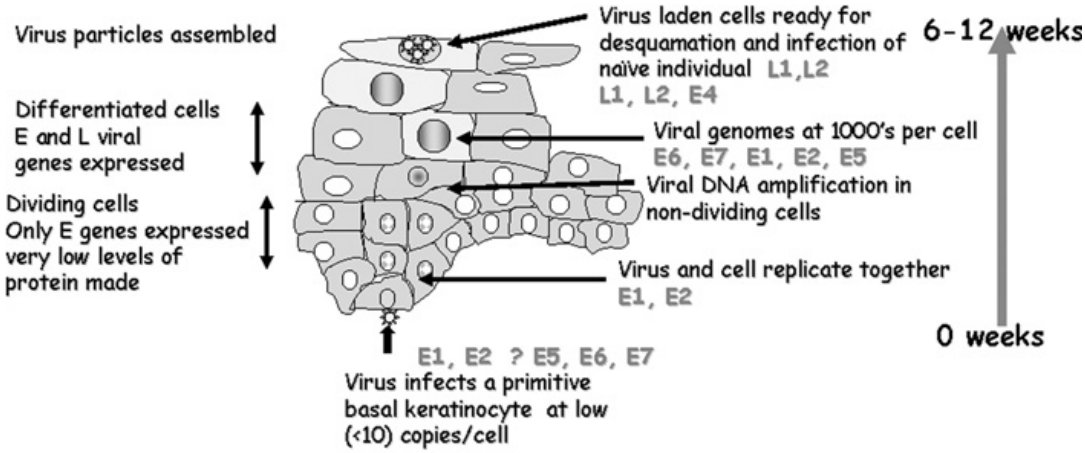


Figure 2. Infectious cycle of HPV. Adapted from Stanley et al., 2007 with permission from Portland Press.

2.2.3 HOST IMMUNE RESPONSE

HPV has developed complex mechanisms to escape the host immune surveillance which is built into its natural life-cycle. Primary infection occurs in the basal cells of the stratified epithelium where viral genomes are maintained only at very low levels. Viral proteins are also very weakly expressed. Increased protein expression only occurs as keratinocytes migrate through the upper layers of the epithelium where the adaptive immune system has limited access. Finally, as the newly assembled viral particles are released by natural shedding, there is no cell lysis involved, which thereby prevents dentritic cell activation, pro-inflammatory cytokine liberation, and antigen presentation by Langerhans cells in the proximal layers of the epithelium (Tindle, 2002; Kupper and Fuhlbrigge, 2004; Stanley, 2006; Lehoux et al., 2009).

2.2.4 TRANSMISSION

HPV is considered to be an STD, usually transmitted through sexual intercourse by genital-genital contact (Tchernev et al., 2009) However, because the virus is also detected in virgins (Xi et al., 2002), and children (Syrjänen and Puranen, 2000; Kojima et al., 2003; Rintala et al., 2005; Syrjänen., 2010), the possibility of alternative modes of transmission have also been studied. Accordingly, transmission of HPV might also occur 1) vertically by infecting the newborn in the birth canal, which may lead to laryngeal papillomatosis (Hajek et al., 1956; Kashima et al., 1987), or 2) horizontally via saliva and hands (Rintala et al., 2005b). HPV DNA has been found in the amniotic fluid, cord blood cells and placenta all implicating potential trans-placental transmission (Sedlacek et al., 1989; Chaterjee et al., 1998; Syrjänen and Puranen, 2000, Sarkola et al., 2009). On the other hand, HPV transmission by blood is considered impossible because HPV infection does not seem to produce viraemia.

2.2.5 MANIFESTATIONS

Clinical infection
An HPV infection produced by the LR types (most commonly HPV types 6 and 11) induces proliferation of the squamous epithelia leading to benign tumors, such as warts, papillomas and condylomas. These clinical lesions are a result of a productive HPV infection (with expression of all its proteins) in the maturing epithelial cells. This leads to morphological changes in the infected epithelium, including cellular proliferation (epithelial acantosis) and degenerative changes in the nuclei and cytoplasm (koilocytosis) (Koss and Durfee, 1956; Meisels, 1976; Purola and Savia, 1977). This productive infection is usually followed by spontaneous regression and virus clearance or maintenance of the viral genome as latent episomes in the basal cells (Doorbar, 2007).

Subclinical infection
Subclinical HPV infections are defined as lesions which are only visible under a colposcope and in histological specimens that demonstrate only minor epithelial changes that are not consistent with characteristic clinical HPV lesions (Syrjänen and Syrjänen, 2000).

Latent infection
HPV infection is considered to be latent when the virus can only be detected by sensitive molecular methods in an otherwise normal epithelium without any cytological, morphological or colposcopic alterations.

Malignant transformation
HPV infections by the HR types are associated with premalignant lesions and cancer, in which the most frequent genotypes represent species 7 and 9 (Bosch et al., 2008). In addition to cancer and its precursors, these HR types are detected in women with no or only mild cytological abnormalities (Clifford et al., 2005; Woodman et al., 2001; Richardson et al., 2003). In most cases, however, HPV infections will regress within 2 years (Holowaty et al., 1999; Wang et al., 2009), but in some cases, the infection remains persistent for years and even decades, which eventually leads to the development of cervical cancer (CC).

The mechanisms of progression towards CC are not fully understood but the crucial event is probably the uncontrolled expression of viral transforming proteins E6 and E7 that occur following integration of the viral genome into the host cell chromosome. Integrated HPV DNA is found in 100% and 80% of HPV 18- and HPV 16-positive CCs, respectively (Cullen et al., 1991; Pirami et al., 1997; Melsheimer et al., 2004; Cheung et al., 2008; Saunier et al., 2008). Integration of HPV DNA into the host genome is a critical event in carcinogenesis, but controversy exists whether it is an early or a late event (Klaes et al., 1999; Tonon et al., 2001; Peitsaro et al., 2002; Arias-Pulido et al., 2006; Kulmala et al., 2006). A consistent feature of HPV integration is the loss of the viral E2 gene (Choo et al., 1987; Kalantari et al., 1998).

Although HPV integration is a crucial event in malignant transformation, some cases of CC contain HPV as an episomal form, which suggests that mechanisms other than viral integration are present, such as promoter methalylation or direct mutation of E2 (Kalantari et al., 2004; Turan et al., 2006; Turan et al., 2007).

2.3 DETECTION OF HPV INFECTION

HPV cannot be cultured, and the detection methods of HPV are divided into 1) morphological methods; 2) HPV DNA detection, 3) HPV RNA detection, and 4) Serology.

2.3.1 MORPHOLOGICAL METHODS

Visual examination (VIA, VILI)
Visual inspection of the cervix at physical examination, using either acetic acid (VIA) or Lugol's iodine (VILI) is to visualize the cervical lesions to make them visible to the "naked eye".

Colposcopy
The colposcope provides a magnified visual impression of the labia, vagina and the cervix (vagina) and TZ. Application of 5% acetic acid solution results in acetowhite staining of the abnormal areas in the epithelium. Women are referred for colposcopy after detection of an abnormal Pap test, usually ASCUS or dyskaryosis. Colposcopy is a descriptive diagnostic tool suggesting an abnormality, and directed punch biopsies are necessary to confirm the findings using light microscopy.

Pap smear cytology
Cervicovaginal cytology is the time-honoured diagnostic method used in screening for CC precursor lesions. This diagnostic tool is known as the Papanicolaou (Pap) test or simply Pap smear. Exfoliated cells from the vagina and uterine cervix are collected with a wooden spatula and a small brush (cytobrush), followed by fixation of the smear onto a glass slide. To classify the abnormalities in the Pap smear, different classification systems are in use as shown in **Table 2.** The 2001 Bethesda system (TBS 2001) is currently the most widely used classification (Solomon et al., 2002).

The widespread use of Pap smear cytology for screening has reduced the incidence and mortality of CC in many countries, albeit the rates still vary depending on the level of implementation (Sankila et al., 2001; Peto et al., 2004; Anttila et al 2004). In countries where organised screening programmes have been active for a long time, e.g. in Finland, Sweden, British Columbia and Canada, the incidence of CC has decreased up to 70-80% (Hakama, 1982; Nieminen et al., 1995; Nieminen et al., 1999; Hristova and Hakama, 1997). Failures to reduce CC incidence and mortality especially in developing countries have been ascribed to non-availability or low quality of screening, the low sensitivity of the conventional Pap smear or low quality of colposcopy, treatment failures and lack of follow-up practices. The key to all successful screening is the high coverage and attendance rates among the total female population (Hakama, 1982; Anttila et al., 1999)

Liquid-based cytology (LBC)
Liquid-based cytology (LBC) is a modification of Pap smear cytology, where the sample is collected from the cervix in the same way as with Pap smear cytology, but only plastic sampling devices may be used (Karnon et al., 2004). LBC has been widely accepted as the primary tool in CC screening. The cervical sample in this method involves making a suspension of the cells, which is then used to produce a thin layer of cells on the cytological slide. At present there are several commercial LBC tests available, of which the ThinPrep (Cytyc, Boxborough MA, USA) and the SurePath system (TriPath Imaging Inc., Burlington, NC, USA) are the most used (Arbyn et al., 2008).

Table 2. Classification of the CC and its precursor lesions in cytology and histology.

Papanicolaou Classification	**BSCC Terminology**	**Bethesda System**	**Dysplasia-Carcinoma in situ**	**Cervical intraepithelial neoplasia (CIN)**
Class 1	Negative for malignant cells	Negative for intraepithelial lesions or malignancy	Normal histology	Normal histology
Class 2	Minor cellular abnormalities considered benign	Reactive cellular changes and infections	Inflammation, regeneration, erosion, etc	Inflammation, regeneration, erosion, etc
	- Inflamatory atypia - Squamous atypia - Koilocytotic atypia	Atypical squamous cells (ASC) - of undetermined significance (ASC-US) - cannot exclude HSIL (ASC-H)	Metaplasia or other benign abnormality	Metaplasia or other benign abnormality
Class 3	Mild dyskaryosis	Low Grade Squamous Intraepithelial Lesion (LSIL)	Mild Dysplasia	CIN 1
	Moderate dyskaryosis	High Grade Squamous Intraepithelial Lesion (HSIL)	Moderate Dysplasia	CIN 2
	Severe dyskaryosis		Severe Dysplasia	CIN 3
Class 4	Carcinoma in situ		Carcinoma in situ	
Class 5	Invasive carcinoma	Invasive carcinoma	Invasive carcinoma	Invasive carcinoma

Histopathology

The histopathological examination is the gold standard in the diagnosis of CIN lesions and CC. CIN lesions are classified into three grades according to their severity. The basic histological criteria include: epithelial differentiation indicated as loss of polarity, nuclear atypia, and abnormal mitotic figures (**Table 2).** Representative examples of CIN 1, 2 and 3 are shown in **Figure 3.**

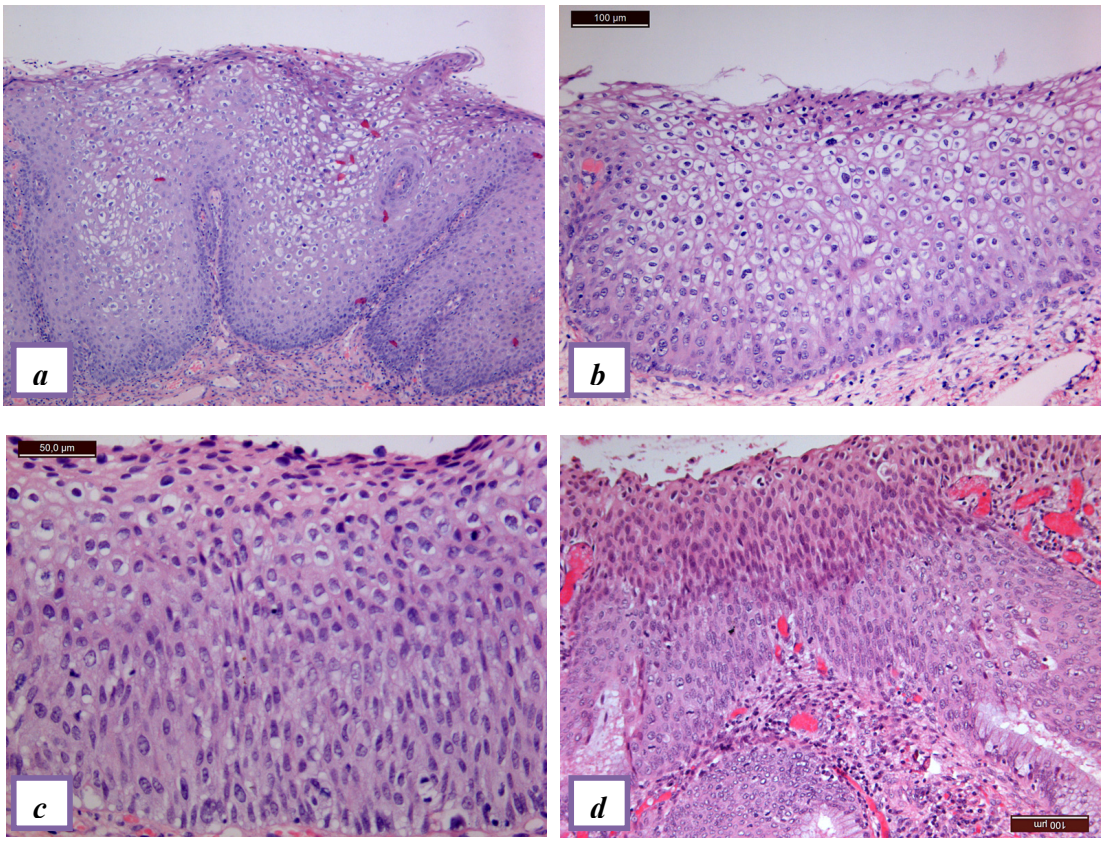


Figure 3. Representative histopathologic examples of a) Non-CIN, a flat condyloma without CIN (HE, original magnification x 50); b) CIN 1, a typical flat HPV lesion with mild dysplasia (HE, original magnification x 100); c) CIN 2, a flat HPV lesion with signs of moderate dysplasia (HE, original magnification x 100) and d) CIN 3, a full-thickness lesion with penetration into glandular openings (HE, original magnification x 50)

2.3.2 HPV DNA DETECTION

Nucleic acid hybridization

Following rapid technological development, several types of hybridisation methods have become available for HPV testing since the early 1980's. All nucleic acid hybridisation methods are based on HPV DNA or RNA detection, in which a probe sequence is bound to a complementary sequence in the sample. The most common methods used in HPV testing include the following: Southern transfer hybridization (STH), dot blot hybridization (DB) and in situ hybridization (ISH). In routine HPV testing, all these have been mostly replaced by PCR-based techniques.

Polymerase chain reaction (PCR)

PCR-based methods are commonly used highly sensitive and specific methods for HPV detection. PCR is a selective target amplification assay capable of exponential and reproducible increase of the HPV sequences present in biological specimens (Garland and Tabrizi, 2006). It can theoretically produce one billion copies from a single-stranded DNA molecule after 30 cycles of amplification.

When performing PCR, care must be taken to avoid false-positive results, which may be derived from cross-contaminating specimens or reagents with the PCR products of previous rounds. Several procedures are available to avoid this problem while using the PCR protocols for HPV detection (Iftner and Villa, 2003). Therefore, the sensitivity and specificity of PCR techniques can vary depending for instance on the primer set, size of the PCR product, reaction conditions, performance of the DNA polymerase used in the reaction, as well as the spectrum of HPV types amplified and the ability to detect multiple types (Brink et al., 2007).

Most PCR assays utilize consensus primers, directed to a conserved L1 gene, and hence are able to amplify most of the mucosal HPV types. Consensus primers described include the single pair GP5/6 (Van den Brule et al., 1990; De Roda Husman et al., 1995) and its modified extended version GP5+/6+ (Jacobs et al., 1997), the MY09/11 pair of degenerate primers (Manos et al., 1989) and its modified version PGMY09/11 (Gravitt et al., 2000) and SPF10 system (Kleter et al., 1998). Amplification with each of these primers will result in different size amplification products (amplicons) and this can result in different sensitivity in detection of certain HPV genotypes (Kornegay et al., 2001).

The nested-PCR is a variation of the PCR method which includes an additional round of PCR amplification using specific internal primers. The inner and outer primers target the same region. The nested PCR is a valuable tool for detection of HPV in samples containing a low-copy number of HPV DNA or samples with limited number of cells.

Multiplex HPV genotyping

Multiplex HPV genotyping (MPG) is a recent simple bead-based high-throughput hybridization method based on Luminex suspension array technology (Schmitt et al., 2006), which allows simultaneous detection and genotyping of up to 100 HPV types. MPG is based on the amplification of HPV DNA by the consensus primers GP5+/6+ and the subsequent detection of the products with type-specific oligonucleotide probes coupled to fluorescence-labelled polystyrene beads, which create a suspension array with unique absorption spectra. This allows up to 100 different targets to be measured simultaneously in a single reaction. The schematic overview of the Luminex assay is presented in **Figure 4**.

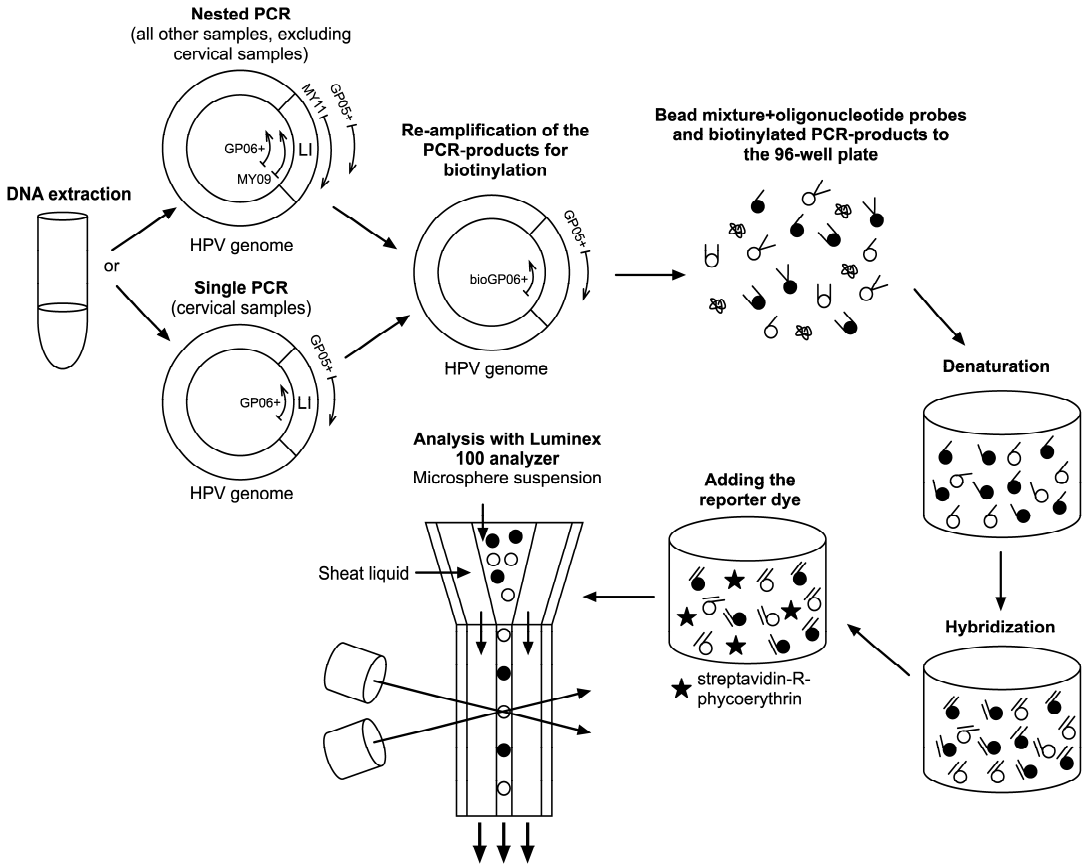


Figure 4. Schematic overview of HPV genotyping by bead-based multiplex HPV genotyping (Luminex) assay and PCRs used in the Finnish Family HPV Study (**II**).

2.3.3 HPV RNA DETECTION

There are commercially available HPV RNA tests to detect HPV mRNA transcripts coding for E6/E7 and thereby the presence of oncogene activity (Castle et al., 2007; Tropé et al., 2009). A nucleic acid sequence-based amplification method detecting E6/E7 transcripts of the five most common HR-HPV types in cervical carcinoma (types 16, 18, 31, 33 and 45) is commercially available from two companies (same kit): the PreTect HPV-Proofer (Norchip AS, Klokkarstua, Norway) and the NucliSENS EasyQ HPV test (BioMérieux S.A., Marcy l'Etoile, France). The Gen-Probe APTIMA® HPV Assay detects HPV E6/E7 mRNA of 14 HR-HPV types (Castle et al., 2007); claimed to be more specific than HPV DNA tests, these HPV RNA assays might be useful biomarkers for progressive disease (Halfon et al., 2010).

2.3.4 SEROLOGY

Until now, serology has played no role in the diagnosis of HPV infections. Serological techniques measure specific natural antibodies against different HPV types (Carter et al., 1996). Interest in serology has increased considerably during recent years when prophylactic HPV vaccines have

become available worldwide. HPV antibodies can be detected only in half of those exposed to HPV. Therefore, antibody testing is unreliable for the diagnosis of current or past HPV infection of individual persons. Serology is considered to measure a past HPV exposure, and there are a variety of different technical modifications available. The most commonly used method is enzyme linked immunosorbent assay (ELISA) using virus-like particles (VPLs) as antigens.

2.4 NATURAL HISTORY OF HPV INFECTION

2.4.1 PREVALENCE

HPV is the most common STD worldwide. Global estimates of the population prevalence of HPV infections in women range from 2% to 44% (Bosch and de Sanjose, 2003; Baseman and Koutsky, 2005; Herrero et al., 2005). This wide variation in estimates is largely explained by differences in the age range of the populations studied as well as on the sensitivity of DNA assays used for HPV detection. Earlier, it was estimated that in healthy women, the life-time risk of contracting a clinical HPV infection is around 80% (Syrjänen et al., 1990). Worldwide, the crude prevalence of HPV among women with normal cytology is 10% (de Sanjosé et al., 2007; Bosch et al., 2008), with the highest prevalence being recorded in Africa (31%) and the lowest prevalence (6.2%) in southeastern Asia, followed by southern Europe (6.8%) (de Sanjosé et al., 2007).

Age
In practically all populations, the prevalence of genital HPV infections is highest among young women <25 years of age, increasing rapidly in adolescence following their sexual debut (Kjaer et al., 2001, de Sanjosé et al., 2007). This peak is followed by an age-related decline in prevalence until age 50-55, followed by a second peak in older women (post-menopause) (de Sanjosé et al., 2007; Syrjänen et al., 2008). This secondary peak is often explained by reactivation of a latent infection or incident new infection perhaps from a new sexual partner. (Syrjänen et al., 2008).

Type-specific HPV prevalence
Worldwide, HPV16 is the most common genotype in women with normal cytology, with a point prevalence of 2.6% (Bosch et al., 2008). HPV16 is followed by HPV18 in Europe, and Central and South America, whereas in Africa, HPV18 is the third most common type after HPV16 and HPV52. In Asia and Northern America, HPV18 is the fourth most frequent type (Bosch et al., 2008). Co-infections with multiple HPV types are observed more frequently among young women (Molano et al., 2002; Rousseau et al., 2003), encountered in 20% to 50% of all women infected with HPV (Liaw et al., 2001; Clifford et al., 2005). A recent study shows that multiple infections occur more often than would be expected by chance (Vaccarella et al., 2010).

According to their type-specific prevalence, the eight most common HPV-types found in women with CC are all included either in species 7 (HPV18, 45) or species 9 (HPV16, 31, 33, 35, 52, 58) (Bosch et al., 2008). Among these women, the point prevalence of HPV16 is 54.4%, and together with HPV18, these two genotypes contribute approximately 70% of all CC cases (Bosch et al., 2008).

2.4.2 INCIDENCE

The incidence of HPV is similar to the age trends of HPV prevalence, and the highest incidence is found among sexually active young women (Franco et al., 1999; Winer et al, 2003; Goodman et al., 2008) and has been shown to decrease with increasing age (Goodman et al., 2008). In the New Independent States of the former Soviet Union (NIS) cohort study, the incidence rates for HR-HPV were clearly age-dependent, being highest among younger ages, but exceeded by the clearance rates already at the age of 30 years onwards (Syrjänen et al., 2005).

Type-specific incidence
Among young women, incident infections with HR-HPV types, especially HPV16 (Franco et al., 1999; Guiliano et al., 2002; Muñoz et al., 2004; Winer et al., 2003; Oh et al., 2008; Goodman et al., 2008; Fukuchi et al., 2009) seem to be more common than those of LR-HPV types (Guiliano et al., 2002; Richardson et al., 2003). The incidence of HPV16, which is the most common type in incident infections, ranges from 10.4 % 15.5% (Winer et al., 2003, Muñoz et al., 2004). In other studies, however, HPV52 (Goodman et al., 2008), HPV58 (Fukuchi et al., 2009) or HPV51 and HPV 52 (Banura et al., 2010) have been the most common HPV types, followed by HPV16.

Incidence rates have varied between studies, most of which have focused on adolescents or young women (Trottier et al., 2009). When the incidence rate is recorded according to 1000 person-months (=women months at risk, wmr), the rate for any HPV type falls between 5.2/1000 wmr to 29.4/1000 wmr (Ho et al., 1998, Franco et al., 1999; Woodman et al., 2001; Ahdieh et al., 2001; Guiliano et al., 2002; Winer et al., 2003; Richardson et al., 2003; Muñoz et al., 2004; Trottier et al., 2008). LR-HPV types seem to have a lower incidence rate as compared to HR types, varying from 1.7 to 12.4/1000 wmr and 4.2 to 14.0/1000 wmr, respectively (Franco et al., 1999; Richardson et al., 2003; Muñoz et al., 2004; Trottier et al., 2008). For HPV16 alone, the incidence rate varies between 0.8 to 5.9/1000 wmr (Franco et al., 1999; Woodman et al., 2001; Ahdied et al., 2001; Guiliano et al., 2002; Winer et al., 2003; Richardson et al., 2003; Muñoz et al., 2004, Trottier et al., 2008)

Risk-factors for incident infections

Age
Young age has been regarded as a prime determinant of incident HPV infections (Muñoz et al., 2004; Fukuchi et al., 2009; Velicer et al., 2009), which displays a dramatic drop with increasing age (Syrjänen et al., 2005; Goodman et al., 2008)

Sexual behaviour
The risk type sexual behaviour is closely related to incident HPV infections. A new sexual partner has been accosiated to increase the risk of an incident infection (Moscicki et al., 2001; Guiliano et al., 2002; Winer et al., 2003; Sellors et al., 2003, Muñoz et al., 2004; Velicer et al., 2009). Some studies have reported that the number of life-time sexual partners is related to the risk of incident HR-HPV infections (Sellors et al., 2003; Goodman et al., 2008; Fucuchi et al., 2009, Velicer et al., 2009). A study of young female students showed that knowing the partner for less than 8 months before sexual intercourse increased this risk (Winer et al., 2003). The same study also stated that reporting a new sexual partner who had one or more (or an unknown number of) prior female partners was also a predictor of incident infections (Winer et al., 2003).

Condom usage
The use of condoms has in many studies failed to show a protective effect against new HPV infections (Ho et al., 1998; Winer et al., 2003). In fact, a recent study reported that condom use of male partners increased the risk of incident infections (Goodman et al., 2008), while another study reported an infrequent use of condoms increased this risk (Fukuchi et al., 2009).

Sexually transmitted diseases
Of the other STDs, a history of herpex simplex virus has been shown to increase the risk (Moscicki et al., 2001, Fukuchi et al., 2009). Also a history of other STDs, such as chlamydia, gonorrhoea, T. vaginalis or syphilis seems to be associated with an increased risk (Syrjänen et al., 2005; Fukuchi et al., 2009, Velicer et al., 2009). Similarly, the association between HIV infections and higher incidence of HPV infection is well known (Safaeian et al., 2008; Auvert et al., 2010).

Smoking
Smoking has been shown to be an independent predictor of incident HPV infections (Winer et al., 2003; Sellors et al., 2003, Oh et al., 2008, Sarian et al., 2009), while other studies have failed to find such a direct association (Muñoz et al., 2004; Goodman et al., 2008).

Oral Contraceptives
The current use of OC has been shown to increase the risk of incident HPV (Winer et al., 2003, Sellors et al., 2003), whereas in other studies, it has displayed a protective effect (Moscicki et al., 2001) or no association at all (Wheeler et al., 1993; Burk et al., 1996). It is interesting that one study reported the past users of OCs to be protected, and HPV incidence decreased with years of OC use (Goodman et al., 2008).

Other risk-factors
There are also some other co-factors that have been reported to increase the risk of new HPV infections, including *pregnancy* during the FU (Muñoz et al., 2004); *marital status* other than first marriage (Velicer et al., 2009); history of *vulvar warts* (Moscicki et al., 2001) and current *alcohol drinking* (Goodman et al., 2008).

2.4.3 CLEARANCE

Genital HPV infections are transient in most cases (Ho et al., 1998; Franco et al., 1999). Most studies on HPV clearance have addressed HR-HPV types collectively, and/or have compared clearance between HR- and LR-HPV types (Franco et al., 1999, Giuliano et al., 2002; Muñoz et al., 2004; Plummer et al., 2007; Rodriquez et al., 2008; Rosa et al., 2008; Sellors et al., 2003; Syrjänen et al., 2005, Syrjänen et al 2005b). Previous data suggest that HR-HPV infections usually clear more slowly than LR-HPV infections (Goodman et al., 2008; Trottier et al., 2008; Giuliano et al., 2002) and the likelihood of an infection not clearing increases in parallel with their duration (Ho et al., 1998; Plummer et al., 2007). The median duration of HPV infection for any HPV types has been within the range of 4 to 20 months (Ho et al., 1998; Richardson et al., 2003; Schiffman et al. 2003; Trottier and Franco, 2006). Clearance rates have are variable, and between 40 to 90% of all infected women have been shown to clear their infection within 1 year (Schiffman et al., 2003; Trottier and Franco, 2006).

Type-specific clearance
It was not until recently that data on HPV clearance at the genotype level was available (Goodman et al., 2008; Lai et al., 2008; Safaeian et al., 2008; Trottier et al., 2008). The results suggest that HR-

types do, indeed, clear more slowly as compared to LR types. HPV16 seems to have the longest clearance times (Bulkmans et al., 2007), while in some other studies, the longest clearance time has been recorded for HPV31 and HPV35 (Safaeian et al., 2008) or HPV70 (Goodman et al., 2008), followed by HPV16. The clearance time for HPV16 has varied from 11 months to 22 months (Trottier and Burchell, 2009; Goodman et al., 2008)

As to the clearance rates (CR), LR-types seem to have higher CRs as compared to HR types. In published studies, the lowest CRs have been recorded for HPV31, 35 and 16 (Muñoz et al., 2004; Richardson et al., 2003; Safaeian et al., 2008).

Factors accosiated to clearance

Age
Most studies have shown clearance to increase with age (Goodman et al., 2008; Safaeian et al., 2008), albeit contradictory reports exist as well (Lai et al., 2008). In some studies, HR-HPV clearance has been constant over the age groups (Syrjänen et al., 2005; Franco et al., 1999). In one study, LR-HPV types were shown to have longer duration among younger women (<35 years) than in older women, whereas the woman's age did not affect the duration of HR-HPV infections (Franco et al., 1999).

STDs
Co-infection with C. trachomatis has been shown to increase clearance (Rosa et al., 2008), whereas HIV-positivity decreases HPV clearance (Strickler et al., 2005: Safaeian et al., 2008)

Other risk-factors
Other co-factors associated with increased clearance are: ever use of *oral contraceptives* (Molano et al., 2003); history of previous *Pap smear* screening (Rosa et al., 2008); *black race* (Rosa et al., 2008); *daily consumption of vegetables* (Richardson et al., 2003); women having a *high number of life-time partners* (>2) (Safaeian et al., 2008); women with *higher education* (Safaeian et al., 2008); and regular *condom use* were all shown to increase the rate of LR-HPV clearance (Richardson et al., 2003). The risk factors associated with decreased clearance have been: high *parity* (Molano et al., 2003; Goodman et al., 2008) and the *use of tampons,* but this was only reported for HR-HPV types (Richardson et al., 2003).

2.4.4 PERSISTENCE

HPV infections are considered to be transient in the vast majority of cases, and eventually almost up to 90% will clear the infection within a relatively short time. On the other hand, a persistent HPV infection especially with HR types has been considered to be the principal risk factor of CC and its precursors (Walboomers et al., 1999; zur Hausen, 2000).

Definition of persistence
The definition of HPV persistence has varied between investigators as recently reviewed by Koshiol and co-workers (Koshiol et al., 2008). The most common definition is having two or three (or more) HPV DNA-positive tests during follow-up (Liaw et al., 1999; Woodman et al., 2001; Kjaer et al., 2002; Schiffman and Castle, 2005, Cuschieri et al., 2004). Other studies have evaluated persistence using duration to clearance (Giuliano et al., 2002; Molano et al., 2003; Moscicki et al., 2004) or counted the proportion of HPV-positive visits (Ahdieh et al., 2001). Consecutive positivity has been a requirement in most of these studies, but some studies have also accepted intervening HPV-

negative visits to be included in the persistent category (Ahdied et al., 2001; Schlecht et al., 2001; Moscicki et al., 2004).

Type-specific persistence
Because of their intimate link with CC, HR-HPV infections have been studied more intensely than LR-infections, of which only a few longitudinal studies are available (Richardson et al., 2003; Herrero et al., 2005). As part of multiple-type infections, LR types can also be associated with CC or high-grade CIN (Gargiulo et al., 2007), but it is not clear what their impact is in the etiology of CIN lesions (Bosch et al., 2002; Castle et al., 2009).

Of all HR-HPV types, HPV16 has been regarded as the most persistent type (Richardson et al., 2003, Liaw et al., 2001; Schiffman and Castle, 2005; Kulmala et al., 2007; Rodriguez et al., 2008). Some authors have shown multiple-type HPV infections to prolong the time of persistence of HR-infections (Trottier and Franco, 2006; Ho et al., 1998), whereas others have failed to show such an increase (Liaw et al., 2001; Rousseau et al., 2001; Molano et al., 2003; Cuschieri et al., 2004; Kulmala et al., 2007).

Risk factors for persistence
Co-factors that are linked to HR-HPV persistence include: *older age* (Castle et al., 2005; Nielsen et al., 2008); *young age at first sexual intercourse* (Rosa et al., 2008); *smoking*; long-term use of *oral contraceptives*; *high parity*; number of *sex partners* (Rosa et al., 2008); and exposure to other STDs, e.g. C. trachomatis, HSV2 and HIV (Castellsague et al., 2002; Nielsen et al., 2008).

2.4.5 PROGRESSION

Persistent infection with any HR-HPV type increases the risk of progression (Koshiol et al., 2008). The majority of all HPV infections will regress within two years (Holowaty et al., 1999; Wang et al., 2009), but sometimes cytological changes suggest rapid progression within three to four months of infection (Woodman et al., 2001; Syrjänen et al., 2004; Winer et al., 2005).

Both LR- and HR-HPV can cause LSIL, but most HSIL lesions are caused by an HR-HPV type. Altogether, HPV16 and HPV18 contribute approximately 70% of all CC cases (Bosch et al., 2008). It has been estimated that only 10-20% of persistent HPV infections are associated with incident CIN (Ho et al., 1995; Schiffman et al., 2007). The time from HPV exposure to CIN2+ may vary considerably, although most of the cases will occur within three years of viral persistence (Castle et al., 2005; Winer et al., 2005).

Risk factors
Several HPV covariates seem to be involved in the progression of HPV infections. Of these cofactors, current and past *smoking* consistently increases the risk of CIN and CC (Sarian et al., 2009; Simen-Kapeu et al., 2008; Plummer et al., 2003). Other potential cofactors include *oral contraceptives* (Moreno et al., 2002); *high parity* (Hildesheim et al., 2001; Muñoz et al., 2002); and *number of sexual partners* (Castellsagué et al, 2004; Deacon et al., 2000). The role of *genetic and immunological factors* has emerged only recently (Wang et al., 2010).

Cervical cancer

Cervical cancer (CC) is the second most common cancer among women worldwide and the seventh most common cancer overall (Denny, 2008). Up to 80% of CC cases are detected in developing countries (Parkin et al., 2005). The incidence of CC rises after the age of 20 to 29 years and has peak incidence in women older than 55 years (Curado et al., 2007). In Finland, incidence rates have significantly decreased over the past three decades due to the well organised national screening program (Finnish Cancer Registry, 2002; van Ballegooijen et al., 2000). However, since 1992 the incidence rate of CC in Finland has shown a steady increase particularly among younger women (Anttila et al., 1999), due to the main reason being decreased attending to the national screening program (Laukkanen et al., 2003; Syrjänen, 2010).

2.4.6 PREVENTION

Until now, the most important means to prevent CC has been by organised screening, based on detection of CC precursors by the Pap test. This approach belongs to the category of secondary prevention, however, which can never overcome the efficacy of primary prevention. For the first time ever for human cancer, the latter is now available through prophylactic HPV vaccines. There are two prophylactic HPV vaccines currently available: a bivalent Cervarix® (GlaxoSmithKline; London, UK), which targets HPV16 and HPV18, and a quadrivalent Gardasil® (Merck & Co.; NJ, USA), which targets HPV6, 11, 16 and 18. Both vaccines are subunit vaccines consisting only of the L1 protein assembled into macro-molecular structures known as virus-like particles (VLPs). HPV L1 VLPs are conformationally correct empty capsids that are morphologically and antigenically almost identical to the native virus particles but they do not contain any DNA and therefore are not infectious (Stanley, 2008). Both vaccines have been shown to be highly effective against the HPV lesions caused by the vaccine HPV types (Harper, 2008; Paavonen et al., 2009). These VLP-based vaccines are prophylactic and not therapeutic and as such do not have any efficacy against existing HPV infections or clinical HPV lesions (Hildesheim et al., 2007).

3. AIMS OF THE PRESENT STUDY

The Finnish Family HPV Study was originally designed to evaluate the dynamics of HPV infections within regular Finnish families. This collection of work consists of studies evaluating genital (cervical) HPV infections of the mothers included in this cohort, prospectively followed up for six years. The general objective was to assess the natural history (outcome) of these infections and their predictors using univariate- and multivariate statistical techniques.

The specific aims were:

1. To establish the genotype-specific point prevalence of genital HPV infections at different time points during the 6-year follow-up of these women.

2. To disclose the incident HPV infections in a sub-cohort of baseline HPV-negative women and calculate the actuarial and crude incidence times and incidence rates at genotype level, as well as to assess their predictive factors in univariate and multivariate Poisson regression.

3. To focus on HPV clearance in a sub-cohort of HPV-positive women and estimate the actuarial and crude genotype-specific clearance times and clearance rates as well as the predictors of these events in univariate and multivariate Poisson regression model.

4. To gain further insights into the determinants of genotype-specific persistence of HR-HPV infections by analysing the predisposing factors of persistence in univariate and multivariate GEE models.

5. To estimate the times and rates of incident CIN lesions detected among baseline negative women during the 6-year follow-up and to assess the role of persistent HR-HPV infections and other predictors of this progression in univariate and multivariate Poisson regression.

4. MATERIALS AND METHODS

4.1. THE FINNISH FAMILY HPV STUDY

The Finnish Family HPV Study is a prospective cohort study conducted at the Department of Obstetrics and Gynecology, Turku University Hospital (TUH) and at the Institute of Dentistry, Faculty of Medicine, University of Turku. The study was designed to evaluate the dynamics of HPV infections in mothers, fathers and their newborn infants. A total of 329 women, 131 men and 331 children were recruited to this study between 1998 and 2002. An extended six-years follow-up of 161 women and 44 men was performed between 2006 and 2008. The Joint Commission on Ethics of Turku University and TUH has approved the study protocol and its amendments (#2/1998 and #2/2006).

Mothers
The subjects in this cohort comprised mothers-to-be, with the mean age of 25.5 years, who were recruited at a minimum of 36 weeks of pregnancy and followed-up for up to 6 years after the delivery (median 62.4; range 1.6-94.5 months). The number of women examined at each follow-up visit and the design of the study are shown in **Figure 5** which also gives the mean follow-up times**.**

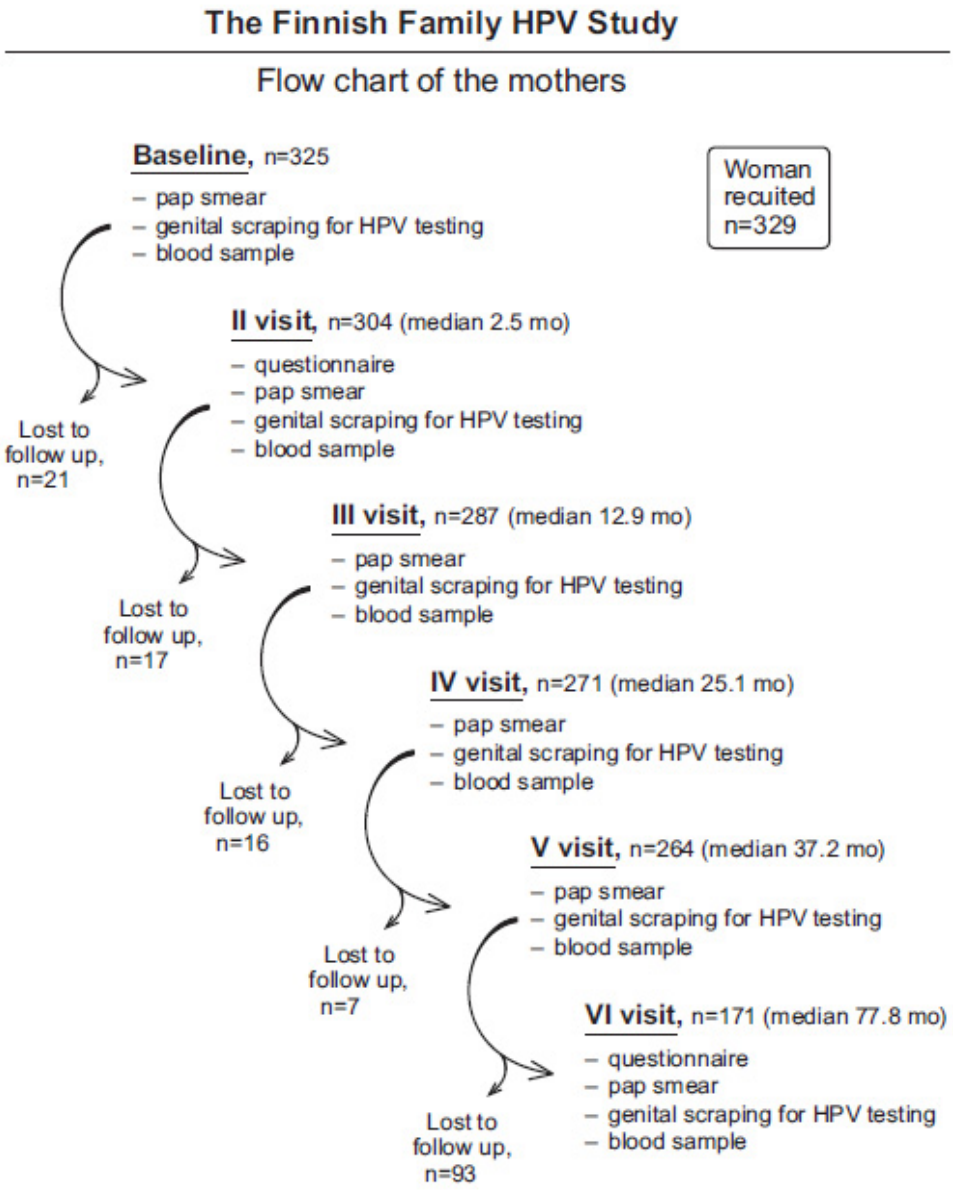


Figure 5. Flow chart of the mothers included in the Finnish Family HPV study. The follow-up visits were completed between 1998 and 2008.

4.2 DEMOGRAPHIC DATA AND SAMPLE COLLECTION

4.2.1 DEMOGRAPHIC DATA

A structural questionnaire for recording demographic data and potential risk factors was recorded at baseline, and repeated at 36-month and 6-year FU-visits. Demographic data included the parents' social status, obstetric and gynaecological history and risk factors for HPV infections. Selected data were used for risk assessments.

4.2.2 SAMPLES

Cervical scrapings
The cervical scrapings for HPV testing were taken at the baseline and at 2-, 12-, 24-, 36-month and 6-year visits (counted from the study entry). Sampling was done from the uterine cervix with a cytobrush cervix (Cytobrush®, MedScand, Malmö, Sweden) using a sampling media of 0.05M phosphate-buffered saline with 100µg gentamycin. The samples were immediately frozen at -20°C and stored at -70°C.

Oral scrapings
Oral scrapings were taken from the buccal mucosa of both cheeks and from the upper and lower vestibular area by using a small brush (Cytobrush®). The brush was placed in 80% ethanol, frozen at -20°C and stored at -70°C (Rintala et al 2005). Only the baseline DNA-data was used in the present study for statistical analyses.

4.2.3 PAP SMEARS

A routine Pap smear was taken from all women at baseline, and at 12-, 24-, 36-month, and 6-year by using conventional three-sample technique with a wooden spatula and a cytobrush (Medscand, Malmö, Sweden). The slides were fixed with a preservative (Spray-cyte; Sparks, Maryland, USA). The Pap smears were analyzed according to the Bethesda System (National Cancer Institute Workshop, 1988). This analysis included quality of the smear, infections, cytological changes and their location with a descriptive diagnosis. The samples were also classified by Papanicolaou classification (class I-V) and degree of background inflammation (1-3).

Histological examination
Following two abnormal (ASCUS+) Pap smears taken at 6-12-mo intervals, women were referred for colposcopy and directed punch biopsies to confirm the cytological findings. All biopsies were fixed in formalin, embedded in paraffin and processed for hematoxylin-eosine stained sections following routine procedures. Biopsies were originally diagnosed as CIN at the Department of Pathology, TUH, and all CIN diagnoses (n=10) were confirmed by an independent reviewer (KS). All women were treated by conisation using LLETZ.

4.3 HPV DNA TESTING

4.3.1 DNA ISOLATION

HPV DNA was extracted from the genital scrapings with the high salt method of Miller and co-workers (1988). Samples were lysed in lysis buffer (10mM Tris, 400mM NaCl, 100mM EDTA, 1% SDS) and digested overnight at 37°C with proteinase K (10µg/ml). After digestion, proteins were precipitated with saturated NaCl and ethanol. DNA was dissolved in 50µl water, mixed for 15-30 minutes and stored at -20°C.

4.3.2 PCR

HPV-testing was done with PCR using GP05+/GP06+ primers for cervical scrapings. Nested PCR with MY09/MY11 and GP05+/GP06+-primers was used for oral scrapings (Snijder et al., 1990). The PCR was done in a 25µl reaction mixture using Amplitaq Gold DNA polymerase (Perkin Elmer, NJ, USA). The amplification programs of these different primers are shown in **Table 3**. The sensitivity of the PCR method was approximately 20 copies of HPV.

Detection of high-risk HPV types
PCR products were run in 2.0% agar gels (DNA agar, MBI, Derventway Delta, Canada), transferred to a nylon membrane (GeneScreen Plus; PerkinElmer, Boston, MA, USA) and hybridized with digoxigenin-labelled (DIG Oligonucleotide 3'-End Labeling Kit; Roche Diagnostics GmbH, Penzberg, Germany) HR-HPV oligoprobe cocktail (HPV-types 16,18,31,33,35,39,45,51,52,54,56 and 58) to determine whether the sample was HR-HPV+ or HR-HPV- (Anttila et al 1999). After hybridization, the positive spots on the films were graded according to the signal intensity as weak, moderate or strong.

Table 3. The PCR protocols for different primers

	PCR primers					
	My 09/11		**GP05+/06+**		**Multimetrix,Primer Set I (GP05+/GP06+)**	
PCR stages	Time (min)	T (C°)	Time (min)	T (C°)	Time (min)	T (C°)
Hot start	10:00	95	10:00	95	15:00	94
Denaturation	0:30	95	1:00	94	0:20	94
Annealing	0:50	55	2:00	40	0:30	38
Elongation	1:00	72	1:30	72	1:20	71
Final extension	7:00	72	7:00	72	4:00	71
	for ever	4	for ever	4	for ever	4
Cycles	30		40		40	

4.4 HPV GENOTYPING

For HPV genotyping, the earlier PCR product available was now re-amplified for biotinylation with GP05+ and bio-GP06+-primers (**Table 3**). HPV-genotyping was done with multiplex-HPV-genotyping kit (Multimetrix, Progen Biotechnik GmbH, Heidelberg, Germany). The kit identifies the following 24 LR- and HR-HPV-genotypes:LR-HPV: 6, 11, 42, 43, 44, 70; HR-HPV: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82 (Schmitt et al 2006). The provider's protocol was followed except only half of the volumes were used. However, in the final step, the same volume was used as in the protocol (100µl) for reading the hybrids with Luminex LX-100-analyzer (Bio-Plex 200 System, Bio-Rad Laboratories, Hercules, USA). The medium fluorescence intensity (MFI) of at least 100 beads was computed for each bead set in the sample. The cut-off value for each run and HPV-type was 1,5x background MFI (negative control) + 5 MFI. The Schematic overview of HPV genotyping is shown in **Figure 4** (page 20).

All HPV16-positive samples were retested using the original sample for nested PCR with MY09/MY11 primers and GP05+ and bioGP06+-primers, and in-house bead-based assay only for HPV type 16 as described by Schmitt and co-workers (Schmitt et al 2006). This test was to exclude potential contamination with HPV16 during previous testing.

4.5 DEFINING THE OUTCOMES OF HPV INFECTION

First level outcomes (I-III)
The outcomes of the HPV infections are presented in **Figure 6**. At the first level, the genotype-specific outcome of HPV infection in each woman was assessed by comparing the viral events at each FU visit to the baseline HPV status. Six different main outcomes were identified as shown in **Figure 6**. Of these 1) always negative and 2) incident HPV are clear-cut outcomes; 3) genotype-specific persistence denotes any case with two (or more) consecutive FU samples positive for the same individual genotype as a single infection or as a part of multiple-type infection; 4) non-genotype specific persistence includes all cases with two (or more) consecutive samples positive for different HPV genotypes; 5) fluctuation is a pattern where consecutive samples are intermittently HPV+ and HPV- with different HPV genotypes, without any two consecutive samples positive for the same or different viral genotype. 6) In this primary categorization, virus clearance included only the baseline HPV+ cases that cleared the infection by the last FU visit.

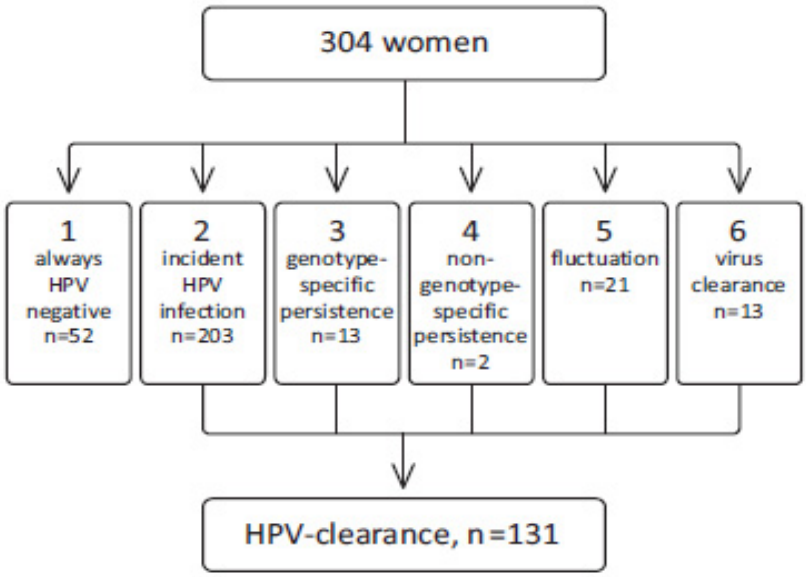


Figure 6. The first level outcomes **(I-III**) and outcome of type specific clearance (**II)**

Type-specific incident infection (I)
The study on incident HPV infections focused on those 203 women (category 2), who developed an incident HPV-infection during the FU as shown in **Figure 6**.

Type-specific clearance (II)
The study on type-specific clearance focused on the women who tested HPV-positive at least once during the FU and thus accumulated time at risk for HPV clearance (category 2-6) as shown in **Figure 6**. Clearance was defined as an event (at any FU visit) when a previously HPV-positive test turned out to be negative and remained HPV-negative until the end the last visit. The outcome of those women who acquired another positive HPV test following a negative result was classified as fluctuation, and as such excluded from this analysis.

Type-specific persistence (III)
Genotype-specific persistence denotes any case with two (or more) consecutive FU samples positive for the same individual genotype as a single infection or as a part of a multiple-type infection. Because in many cases, HPV infections in individual women would fit into more than one outcome pattern, a second-level approach was defined to maximally exploit the data of the different outcome patterns for viral persistence. Accordingly, patterns 2, 3, 4, 5 and 6 were further stratified into two persistent-outcome categories (2 and 3) as follows. The schematic overview of these outcomes is presented in **Figure 7**. Among category 1 (incident infections) all cases demonstrating genotype- and non-genotype-specific persistence of this incident infection were included, as defined exactly by the same criteria as above. This resulted in new categories 2 and 3, with 71 and 106 cases, respectively. Similarly, the original categories 3 and 4 were further stratified into genotype-specific and non-genotype specific persistence, whenever they were different or additional to these

same categories in the main assessment. By definition, category 5 (fluctuation) does not include any viral persistence, but, importantly, the cases testing HPV-negative at the last FU visit were graded clearance in this secondary categorization to indicate that these infections were transient in nature (n=36). The same was true for category 2 cases if the incident infection cleared without persistence. Of the last category 6, all cases fulfilling the above defined criteria of type-specific or non-type specific persistence were also taken into account, to be included as such in this secondary classification. This leaves only 9 cases for category 6 in the new classification. The exact times (months) of persistence for each individual case of persistent lesion (n=177) were calculated. These primary and secondary approaches were combined to define the persistent cases as type-specific and non-type-specific persistors. The individual genotypes responsible for either type-specific or non-type-specific persistence at each FU visit were recorded, and the same was done for individual HPV species, using phylogenetic HPV classification (de Villiers et al., 2004). As the ultimate step, identification was made on the basis of whether or not the species that persisted was, indeed, the same as detected at the baseline visit or at the FU-visit when the patient first tested HPV-positive. This enables computing species-specific (recorded at genotype level) persistence and its predictors, as explained below.

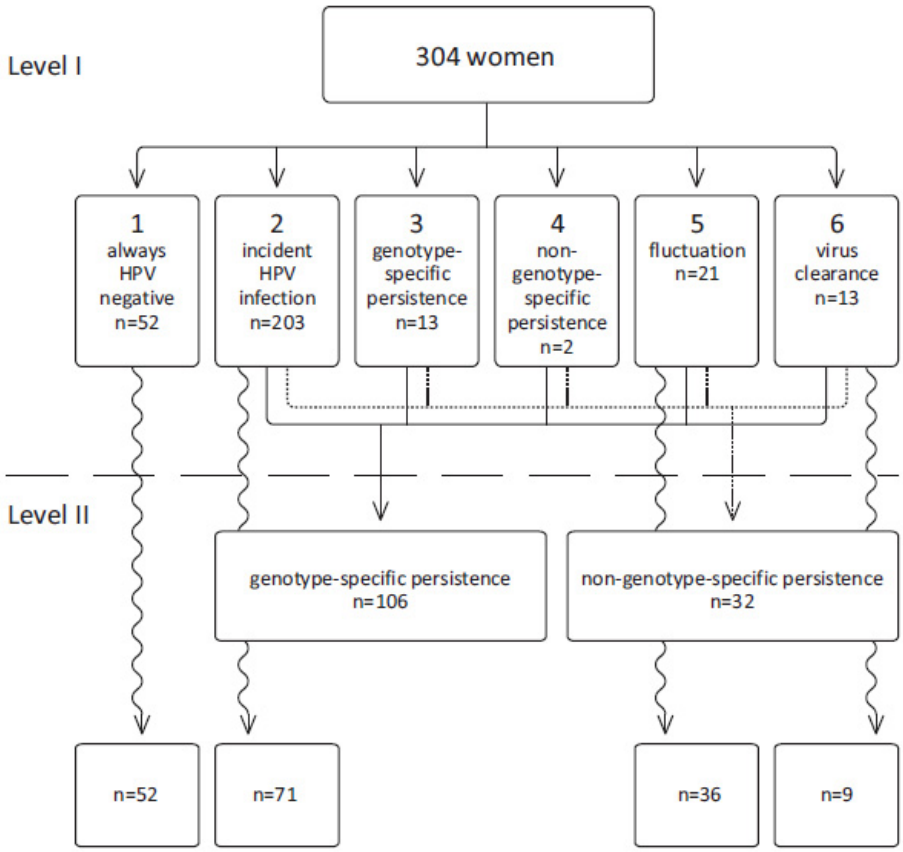


Figure 7. First and subsequent outcomes of HPV infections (**III**).

4.5.1 ACTUARIAL AND CRUDE INCIDENCE TIMES AND RATES (I)

Times (months) for incident infections were calculated from baseline visit to the first incident event separately for actuarial and crude times. To calculate the former, all baseline HPV-negative women (n=252) were included in Kaplan-Meier analysis, with cases being censored at the first incident event or at the end of the FU (those who did not develop an incident event). To calculate crude incidence times, only those 203 women with an incident event were included. Both actuarial and crude incidence rates (IR) were calculated, and expressed per 1000 women months at risk (wmr). To get genotype-specific actuarial IRs, the number of incident events for each individual genotype (and species) was divided by the total wmr (6,212 months for all baseline HPV-negative women), including also the women with no incident event. To calculate crude IRs, only the women with incident events were included and the number of incident events for each individual genotype (or species) was divided by the wmr of those women only. To compare the individual IRs, rate ratio statistics (STATA) were used, with test-specific 95% confidence intervals (95%CI).

4.5.2 ACTUARIAL AND CRUDE CLEARANCE TIMES AND RATES (II)

The interval times (months) to the first clearance event were calculated from the first HPV-positive visit (baseline or any FU visit) to the first clearance event, separately for both actuarial and crude times. To calculate the actuarial clearance time both women who showed a clearance event and those who did not (n=252) were included in Kaplan-Meier analysis. To calculate the crude clearance times, only those 131 women with a clearance event were included. Both actuarial and crude clearance rates were also calculated, and expressed as events per 1000 women months at risk (wmr). To obtain genotype-specific actuarial clearance rates, the number of clearance events for each individual genotypes and species was divided by the total wmr (i.e. 9.146 months accumulated by all 252 HPV-positive women), thus including also the women with no clearance event. To calculate crude clearance rates, only the women with clearance events were included and the number of clearance events for each individual genotype (or species) was divided by the wmr accumulated by those women only. To compare the individual clearance rates, RR (rate ratio) statistics (STATA) were used, with test-specific 95% confidence intervals (95%CI).

4.5.3 PROGRESSION TO CIN (IV)

Of the 329 mothers enrolled in this study, ten developed an incident CIN. In addition, cytological progression to ASC-H in Pap smear was detected in four women, not histologically confirmed. With these women, two separate endpoints of progression were used: i) lesions progressed to biopsy-confirmed CIN, and ii) a combined category with progression confirmed by either biopsy or by repeated cytology. Actuarial and crude times (months) to the first progression event were calculated from the baseline visit to the point when progression was confirmed. To calculate the actuarial progression time, all women at risk (n=308) were included in Kaplan-Meier analysis. In calculating the crude progression times, only those women who had progression events were included. Similarly, actuarial and crude incident rates (IR) for CIN were calculated and expressed as events per 1000 women months at risk (wmr). For actuarial IR, the number of progression events, 10 for CIN and 14 for CIN/SIL was divided by the total wmr, i.e., 17,049 wmr accumulated by all 308 women at risk. In crude IRs, only the women with progression events were included, the number of progression events being divided by wmr accumulated by those 10 and 14 women only.

4.6 STATICAL ANALYSES

Statistical analyses were run using SPSS® (SPSS, Inc., Chicago, USA) and STATA (Stata Corp., College Station, TX, USA) software packages (PASW Statistics for Windows, version 18.0.1 and STATA/SE 11.0). Frequency tables were analyzed using the χ^2-test, with the likelihood ratio (LR) or Fisher's exact test for categorical variables. Differences in the means of continuous variables were analyzed using non-parametric (Mann-Whitney or Kruskal-Wallis) tests for two- and multiple independent samples, respectively.

4.6.1 GEE-MODELING (III)

To analyze the predictors of genotype-specific HPV-persistence, a species-specific persistence approach was used to avoid stratifying the cases into individual types with single or few cases only. Furthermore, of interest was only the persistence of the key HR-HPV-types i.e. those of Species 7 (HPV-types 18,39,45,59,68,70,85) and Species 9 (HPV-types 16,31,33,35,52,58,67).

In this analysis, a generalized estimating equation (GEE) modeling was used, clustered according to the womens IDs and run in univariate and multivariate mode (Diggle et al., 1994, Hardin and Hilbe, 2003). GEE adjusts for the serial correlation within subjects (women) due to the longitudinal nature (FU visits) of the data by modeling the covariance structure within subjects. The dependent variable was binomial (persistence: yes/no), and hence the logit link function was used. An independent working correlation structure with a robust variance estimator (for 95%CI) to account for within-subject correlation was selected as the best-fitted covariance pattern, using the Quasi-likelihood Information Criterion (QIC) (Hardin and Hilbe, 2003). Because HPV-persistence depends on the time between the subsequent samples, a time variable (FU visit) was included as a covariate in these GEE-models. In the univariate GEE models, we first tested all covariates recorded at baseline (including serological data) and previously implicated as potential risk factors of HPV infections in our cohort (Rintala et al 2005., Syrjänen et al., 2009). In the final multivariate GEE-model, only the variables that were significant in the univariate model were entered, adjusted for age (continuous variable recorded at each FU visit). All statistical tests performed were two-sided and declared significant at p-value <0.05 level.

4.6.2 POISSON REGRESSION (I, II, IV)

To analyze the predictors of incident HPV-infections and HPV clearance, a species-specific incident infection and clearance approach was used to avoid stratifying the material into individual genotypes with single or a few cases only. Furthermore, we were only interested in incident or clearance of the HPV-infections by the key HR-HPV-types, i.e., those of Species 7 and Species 9.

Poisson regression analysis was used for panel data, clustered by mother-ID and run (in univariate and multivariate-mode) using a population-averaged (PA) model (Diggle et al 1994, Hardin and Hilbe., 2003). FU visit was the time variable in the panel settings, and incident HPV-infection or HPV clearance (count variable) as the dependent variable, with the Poisson log link function. The independent within-group correlation structure for the PA model, with a robust variance estimator (of 95%CI) to account for within-subject correlation was the best-fitted covariance pattern, defined by QIC (quasi-likelihood information criterion) (Hardin and Hilbe., 2003). With these options, Poisson regression for panel data is similar to the PA GEE model, both giving identical results.

In univariate Poisson, all covariates recorded at the baseline questionnaire as well as some selected variables from the FU questionnaire (e.g. a new partner) were first tested, previously implicated as potential risk factors of HPV in this cohort (Rintala et al., 2005, Syrjänen et al., 2009). In the final multivariate model, only variables that were significant (or borderline significant) in univariate Poisson were entered and were adjusted for age at study entry. All statistical tests were two-sided and declared significant at p-value <0.05 level.

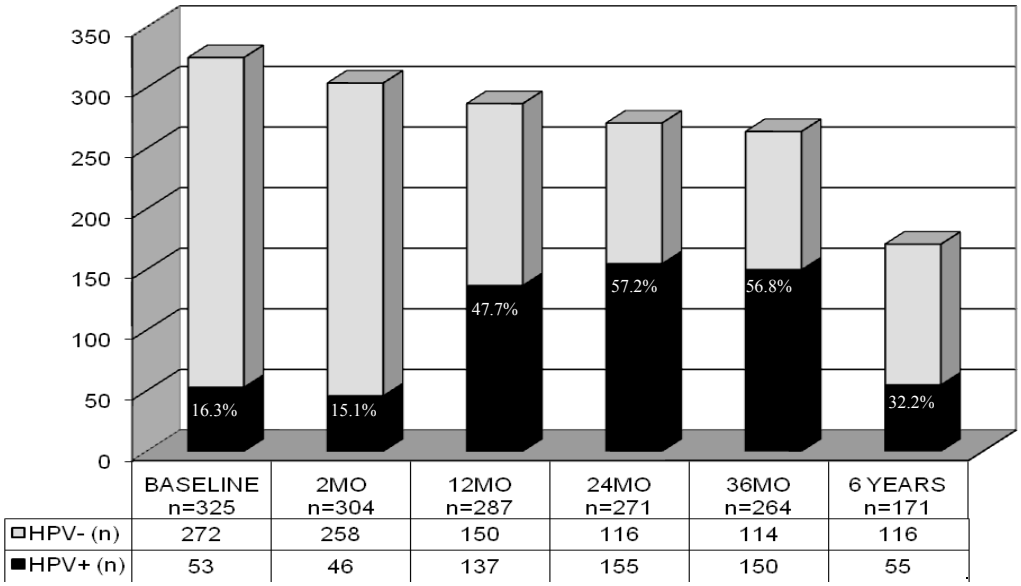
To estimate the predictors of progression, Poisson regression was used as described above but only the cone biopsy-confirmed histological CIN endpoint (n=10) was used; the FU visit was the time variable in the panel settings, and the incident CIN (count variable) as the dependent variable, with the Poisson log link function.

5. RESULTS

5.1 PREVALENCE OF HPV (III)

5.1.1 TYPE-SPECIFIC PREVALENCE

The type-specific point prevalence was evaluated at each six follow-up visits separately; baseline, 2-month, 6-month, 12-month, 24-month, 36-month and 6-years. At all time points, the single most frequent genotype was HPV 16 followed by multiple-type infections. The HPV overall prevalence at each FU point is shown in **Figure 8**.



	BASELINE n=325	2MO n=304	12MO n=287	24MO n=271	36MO n=264	6 YEARS n=171
HPV- (n)	272	258	150	116	114	116
HPV+ (n)	53	46	137	155	150	55

Figure 8. Prevalence of HPV during the FU

Baseline
At baseline, 16.3% (n=53) of the women tested HPV-positive. The single most frequent genotype was HPV 16 20.8% (n=11), followed by HPV 45 5.7% (n=3), HPV43 3.8% (n=2), HPV58 3.8% (n=2) and HPV70 3.8% (n=2). Multiple-type infections comprised 45.3% (n=24) of all HPV-positive cases, and HPV16 was detected in 83.3 % (n=20) of these multiple-type infections (**Figure 9**).

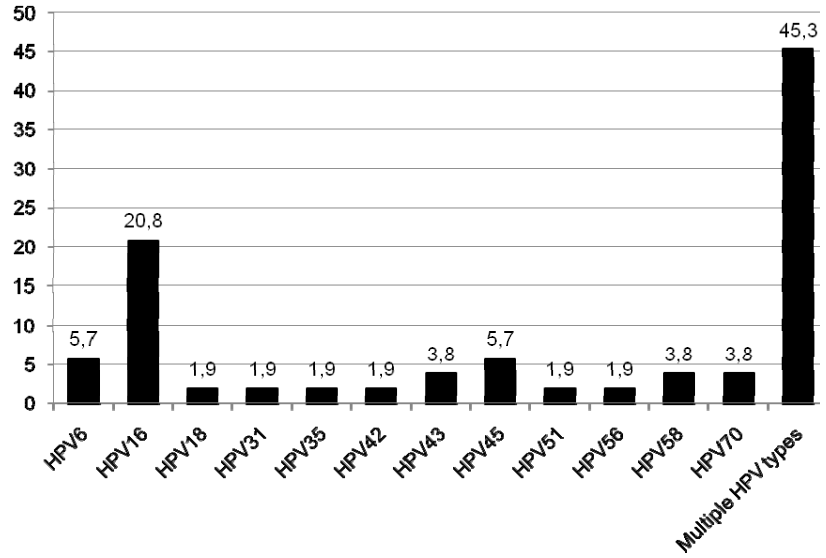


Figure 9. HPV genotype prevalence (%) among the baseline positive samples

II visit

At the second visit (median 2.5 months), the total HPV prevalence remained practically unchanged, 15.1%, HPV 16 being the most common individual type, 30.4% (n=14). Next in frequency were HPV 56 and HPV 70, both with 8.7% prevalence (n=4). Multiple-type infections decreased to 26.1% (n=12). HPV 16 was detected in 50% (n=6) of the multiple-type infections (**Figure 10**).

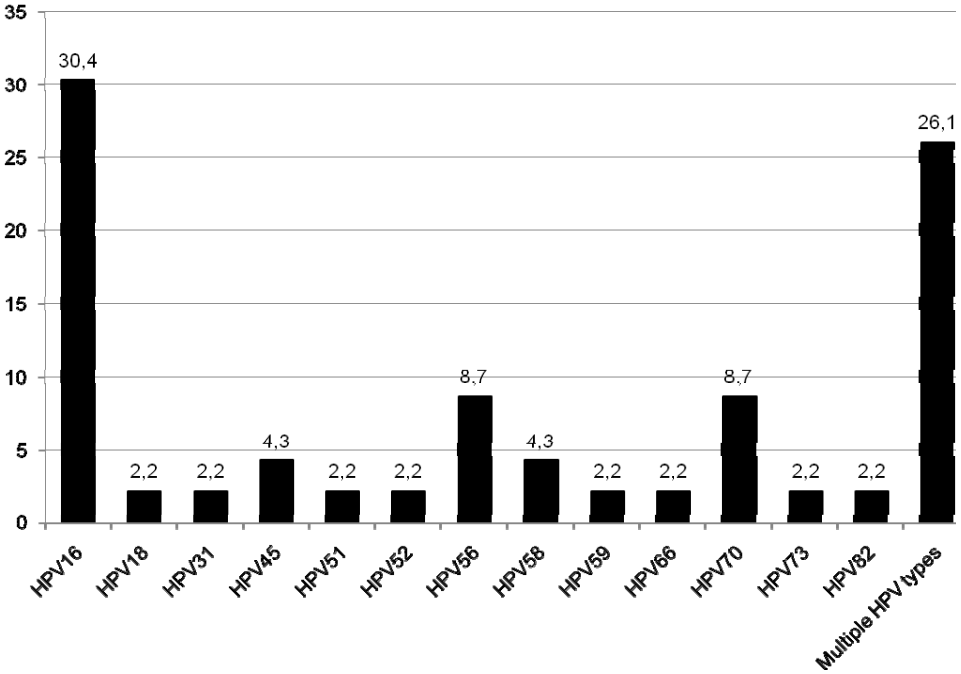


Figure 10. 2-month type-specific HPV prevalence (%)

III visit

At the third visit (median 12.9 months), HPV prevalence increased substantially to 47.7% (n=137). At this point, HPV16 represents 32,1% (n=68) of the HPV-positive cases, and multiple types equal 32.1% (n=44), of which HPV 16 was detected in 65.9% (n=29) (**Figure 11**).

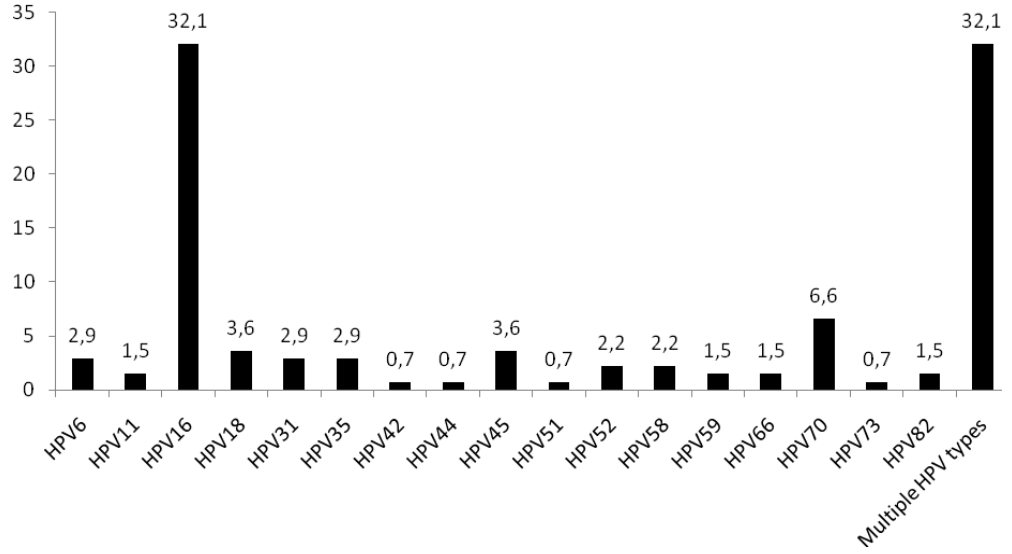


Figure 11. 12-month type-specific HPV prevalence (%)

IV visit

At the fourth visit (median 25.1 months), HPV prevalence had further increased up to 57.2%, i.e. by 10% from the last visit. Much of this increase was due to HPV16, representing 43.9% (n=68) of all HPV-positive cases, as well as to multiple-type infections with 38.1% (n=59) point prevalence, of which HPV16 was involved in 76.3% (n=45) (**Figure 12**).

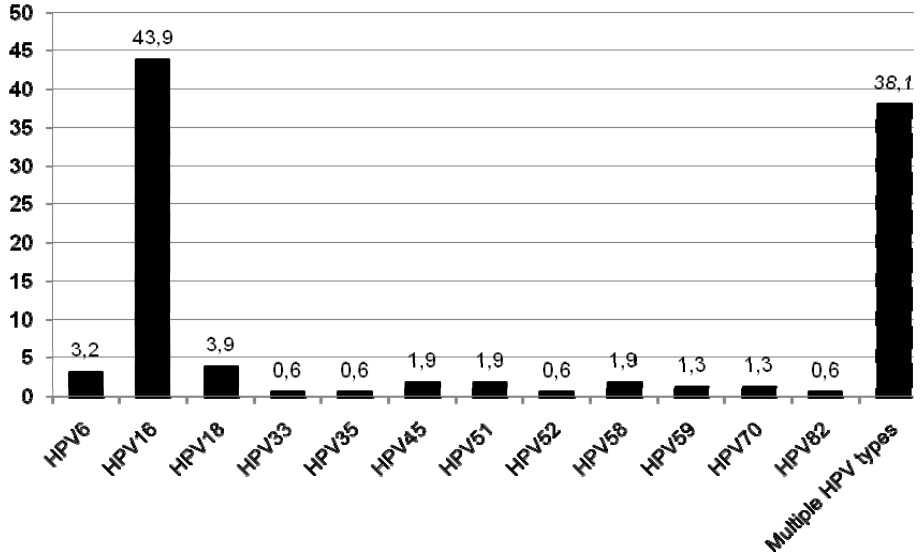


Figure 12. 24-month Type-specific HPV prevalence (%)

V visit

At the fifth visit (median 37.2 months), HPV prevalence had leveled-off (56.8%), with slight additional increase of HPV 16 (63.3%; n=95), in contrast to the multiple-type infections which had decreased to 21.3% (n=32), of which HPV 16 was present in 87.5% (n=28) of the cases(**Figure 13**).

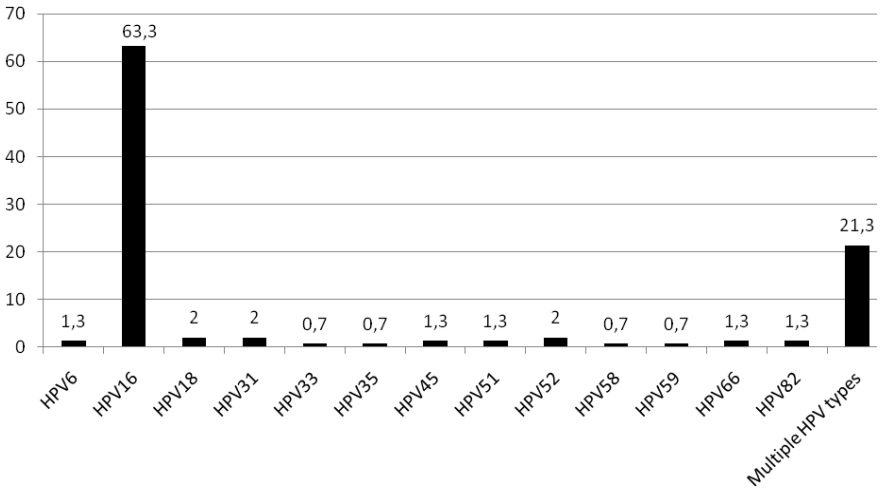


Figure 13. 36-month type-specific HPV prevalence (%).

VI visit

At the last FU visit (median 77.8 months), several women were lost to the follow-up since the previous visit, which led to somewhat decreased HPV prevalence (32.2%; n=55). Also at this 6-year visit, HPV 16 was the most dominant type, representing 63.3% (n=35) of all HPV-positive cases, followed by multiple-type infections, with 23.6% (n=13) prevalence, where HPV 16 was detected in 76.9% (n=10) of the cases (**Figure 14**).

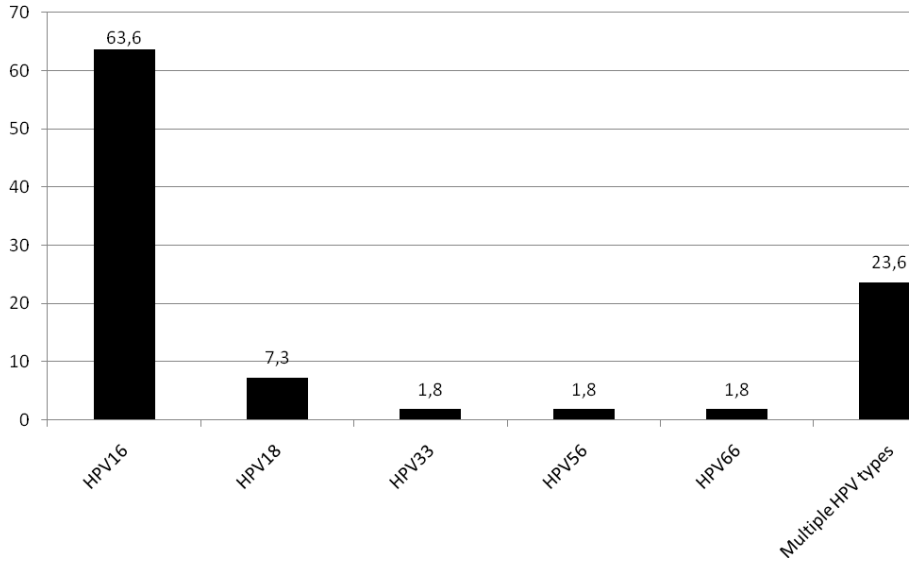


Figure 14. The 6-year type-specific HPV prevalence (%)

5.1.2 SPECIES-SPECIFIC PREVALENCE

Of the HPV species detected, the most dominant one at baseline was species 9 which covered 28.3% (n=15) of the cases. The second most common was species 7 which consisted of 11.3% (n=6) of the cases. The trend observed during the follow-up was an increase in species 9 and decrease in species 7 prevalence, with the peak for species 9 being reached at the 36mo visit, 69.3% (n=104) (**Figure 15**).

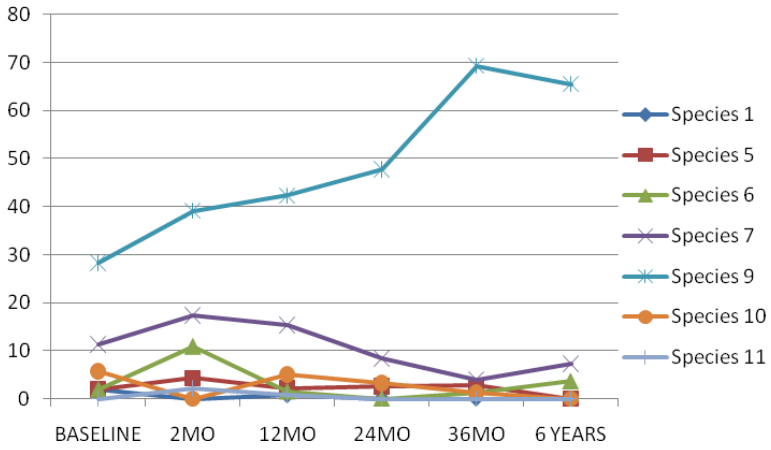


Figure 15. Species specific prevalence during the FU

5.2 INCIDENT HPV INFECTIONS (I)

Of the 252 baseline HPV-negative mothers enrolled in the cohort, 203 experienced an incident event during the mean FU time of 58.6±25.2 (SD) months (median 64.3; range 6-94.5).

Species-specific incident infections
Species 9 was the most dominant species covering 54.7% (n=111) of all incident infections followed by species 7 and species 10, with 10.8% (n=22) and 3.9% (n=8) frequency, respectively.

Type-specific incident infections
HPV16 caused most of these incident infections (47.8%, n=97), followed by HPV18 (3.9%, n=8), HPV70 (3.0%, n=6), HPV6 and HPV45 (both 2.5%, n=5). Multiple-type infections were the second most common cause of incident events, with 25.1% (n=51) frequency. HPV16 was detected in 70.8% (n=36) of all multiple-type infections as well.

5.2.1 INCIDENCE TIMES

Species-specific actuarial and crude incidence times
Species 9 was the most dominant species with an actuarial mean time of 27.5 months (95% CI 23.3-31.7) and crude mean time of 22.6 months (95% CI1 9.7-25.4). The second and third most common species were species 7 and species 10, with 10.8% (n=22) and 3.9% (n=8) frequency, respectively.

Actuarial mean times for species 7, 9 and 10 were almoust identical, 27.5, 27.5 and 27.0 months, respectively. Species 6 and 9 had the longest crude incidence times, 25.0 and 22.6 months, respectively.

The cumulative incidence of species 7 and 9 was compared with that of species 10 in univariate survival (Kaplan-Meier) analysis (see Figure 3, in original publication I). Despite a lower cumulative incidence rate for species 10 (benign types), the difference was not statistically significant when compared to that of species 7 and 9 (log-rank test; p=0.892).

Type-specific actuarial incidence times
The longest actuarial time (75 months) was associated with a single case of HPV 33. Of the types with more than one case, HPV 31 and HPV 45 had actuarial times of 34.5 months and 32.8 months, respectively, while the actuarial times for incident HPV 6, 16, 18, 35, 56, and 58 varied between 21.5-29 months.

Type-specific crude incidence times
Apart from the single case of HPV 33, HPV 56 showed the second longest crude time of 42.4 months, followed by HPV 16, 18 and 31, with 23.1 months, 20.6 months and 20.1 months, respectively. The rest of the genotypes had crude mean times for incident infection between 6.3 and 17 months.

5.2.2 INCIDENCE RATES

Actuarial incidence rates
Of the single genotypes, HPV 16 had by far the highest actuarial IR, 15.6/1000 wmr (95% CI 12.5-18.6), followed by multiple-type infections, with 8.2/1000 wmr (95% CI 5.96-10.45). Actuarial IR for HPV18 was markedly lower, 1.3/1000 wmr (95% CI 0.39-2.17). Due to the dominant role of HPV 16, species 9 showed the highest IR of 17.9/1000 wmr (95% CI 14.6-21.2), far exceeding that (3.4/1000 wmr) of species 7.

Crude incidence rates
The highest crude IR was ascribed to HPV 70, accumulating incident events at a rate of 157.8/1000 wmr. This was followed by HPV 73, HPV 66, HPV 82 and HPV 52, with IRs of 133.3/1000 wmr, 133.1/1000 wmr, 107.1/1000 wmr and 107.1/1000 wmr, respectively. The crude IRs of HPV 16 and HPV 18 were far lower, and almoust identical to each other, 43.3/1000 wmr and 43.4/1000wmr, respectively. Due to this wide variation among individual types included in different species, the crude IRs between the HPV-species showed much less variation, i.e. from 40/1000 wmr to 91/1000 wmr, when species 11 (HPV 73 alone) was excluded.

5.2.3 PREDICTORS OF INCIDENT HPV INFECTION

The predictors of genotype-specific incident infections during the follow-up were analyzed for species 7 and 9 using Poisson regression. In univariate Poisson regression, seven variables were significant predictors: 1) mother being seroconverted to HR-HPV during the FU, 2) <13years of age at onset of sexual activity, 3) 1-2 lifetime sexual partners 4) initiation of oral contraceptives (OC) at an older age (age>20 years, protective), 5) age at initiation of smoking (age 10-13 years, protective), 6) pregnancy at FU visit (pregnancy protective) and 7) ≥2 sexual partners during the FU.

When all significant and borderline significant variables were entered in the multivariate Poisson PA model, together with the mother's age, three variables retained their significance as independent factors with a lower risk for incident species 7 and 9 infection during the FU: 1) >2 life-time sexual partners (IRR=0.91; 95% CI 0.84-0.98; p=0.014), 2) late (>20 years) initiation of OCs (IRR=1.11; 95% CI 1.01-1.21; p=0.017), and 3) pregnancy at the FU visit (IRR=0.32; 95% CI 0.17-0.61, p=0.0001)(**Figure 16**).

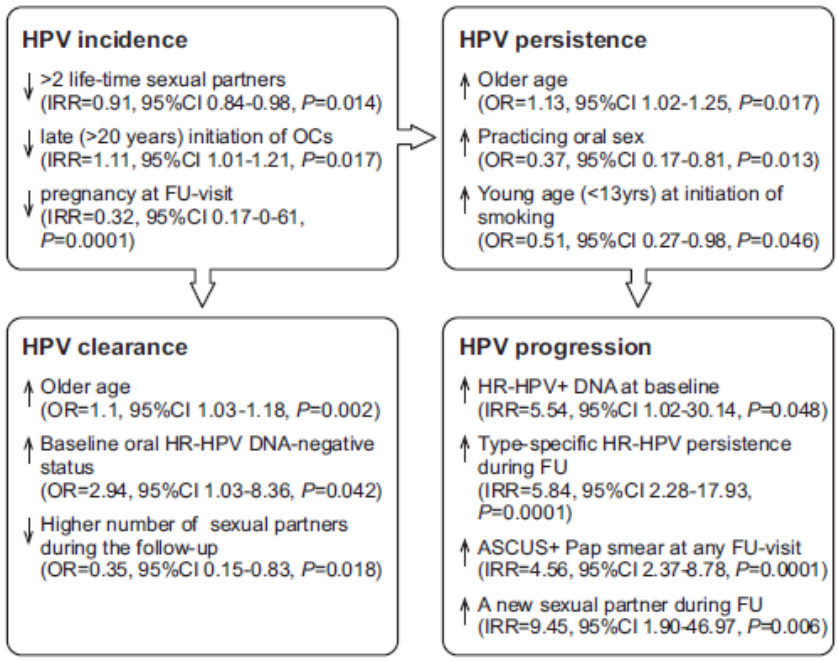


Figure 16. Predictors of incident and persistent HR-HPV infections, virus clearance and disease progression to CIN.

5.3 HPV CLEARANCE (II)

Of the 329 women enrolled in this study, 252 tested HPV+ at some point. Of these 131 experienced a clearance event during the FU of 58.8±25.1 (SD) months (median 65.0, range 6-95).

Species-specific clearance
Of the individual HPV species, species 8 cleared the infection in 100% (n=2). Species 5, 10 and 7 showed clearance in 80% (n=4/5), 77.8% (n=7/9) and 67.9% (n=19/28) of the cases, respectively. For species 9, clearance was only 49.2% (n=61/124) which is similar to the rare species 6 and 11, 40% (n=2/5) and 50% (n=1/2), respectively.

Type-specific clearance
By the end of the FU, 100% of HPV 11, 43 and 51 infections cleared, as did 83.3% of HPV 6 infections (n=5/6). For most of the other genotypes, clearance was less frequent, detected in approximately 50% of the cases. This applies to HPV 16, of which 50.5% (n=54/107) cleared as well as to multiple-type infections, among which the clearance frequency was even less; 46.1% (n=35/76) of the cases.

5.3.1 CLEARANCE TIMES

Species-specific actuarial and crude clearance times
Of the individual HPV species, species 8 cleared the infection in 100% of the cases with both actuarial and crude time to clearance being 24.8 months. The actuarial times to clearance were most prolonged for species 6 (46.2 months), followed by species 9 with clearance time of 37.4 months. Both the actuarial and crude clearance times were shortest for species 11, only 11.7 and 10.4 months, respectively. The most prolonged crude times to clearance were recorded for species 9 and 8, 37.4 and 24.3 months, respectively.

The cumulative clearance of species 7 and 9 was compared with species 10 in univariate survival (Kaplan-Meier) analysis (see Figure 4 in original publication II). Species 10 (benign types) showed a significantly more rapid and complete clearance as compared with species 7 and 9, which also markedly deviated from each other, species 9 showing the least cumulative clearance (log-rank test; p=0.013). When HPV 16 alone was compared with all the other genotypes in a similar Kaplan-Meier analysis, the difference was also significant (p=0.015), HPV 16 clearance being almost 20% less than that of all other genotypes (data not shown in the Figures).

Type-specific actuarial clearance times
The longest actuarial times to clearance were found for single cases of HPV 33 and HPV 44, which did not show clearance. This was followed by HPV 66, 35, and 31 with actuarial times of 48.1 months, 45.6 months and 45.5 months, respectively. On the other hand, the shortest actuarial times were recorded for HPV types 73, 11 and 59; 11.8 months, 12.4 months and 12.8 months, respectively. Most of the remaining types (HPV 16, 18, 52, 58 and 70) had actuarial times ranging between 30.7-36.9 months, whereas multiple-type infections had a somewhat longer clearance time, 41.1 months.

Type-specific crude clearance times
Crude times only take into account the cases that cleared their infections and are therefore shorter than the actuarial times. The longest crude time of 49.5 months was recorded for HPV 52. This was followed by HPV 16, 31,43,51,56 and 70 with a crude time between 22.1-28.5 months. The shortest crude time of 10.4 months was shown by HPV 73, which was somewhat shorter than that for the remaining genotypes, ranging between 11.9-14.8 months. For multiple-type infections, the crude time to clearance was 21.7 months.

5.3.2 CLEARANCE RATES

Actuarial clearance rates
The actuarial CR reflects the rate (per 1000 wmr) at which the individual genotypes or species accumulate clearance events among all HPV-positive women. Of the single genotypes, the most frequent genotype HPV 16 also showed by far the highest CR, 5.9/1000 wmr (95% CI 4.3-7.4),

followed by multiple-type combinations that cleared at a rate of 3.8/1000 wmr (95% CI 2.5-5.1). Actuarial CR for HPV 18 was markedly lower, 0.65/1000 wmr (95% CI 0.1-1.2). Due to the dominant role of HPV 16, species 9 showed the highest CR of 6.7/1000 wmr (95% CI 5.0-8.3), far exceeding that (2.0/1000 wmr) of species 7. This is logical because since the CR is likely to correlate with the detection rate of a specific HPV type.

Crude clearance rates
When counted exclusively for cases with a clearance event, the crude CRs reflect the rate (per 1000 wmr) at which individual genotypes accumulate these events (once demonstrated as doing so), being a robust measure to compare different HPV genotypes and HPV species. Of all genotypes, HPV 66 and 82 cleared most rapidly, both accumulating clearance events at a rate of 83.2/1000 wmr, which far exceeded the speed of HPV 6 and HPV 59, with crude CRs of 67.4/1000 wmr and 76.9/1000 wmr, respectively. The crude CR of HPV 16 was far lower, 45.2/1000 wmr. Due to this relatively wide variation among the individual genotypes included in different species, the crude CRs between HPV species showed much less variation, species 10 and 11 having the highest crude CRs 70.9/1000 wmr and 96.1/1000 wmr, respectively, while the remaining species cleared more slowly, CRs ranging between 40.8/1000 wmr to 59.3/1000 wmr.

5.3.2 PREDICTORS OF HPV CLEARANCE

The predictors of species 7 and 9 genotype-specific clearance during the FU were analyzed using the Poisson regression PA model, a clearance event at any FU visit being used as the dependent variable. In the univariate Poisson, two variables were significant predictors of these clearance events: age (clearance is more common with increasing age) and the number of sexual partners during the FU (women with 0 partners (recorded at last visit) all cleared).

When all significant and borderline significant variables of univariate Poisson were entered in the multivariate Poisson model together with the mother's age, three variables retained their significance as independent predictors of species 7 and 9 clearance: 1) age (clearance is more common with increasing age)(p=0.002), 2) baseline oral HR-HPV DNA status (being HR-HPV negative increases the probability of clearance)(IRR=2.94, p=0.042), and 3) the number of sexual partners during the FU (all women with >2 partners failed to clear)(IRR=0.35; 95% CI 0.15-0.83)(p=0.018).

5.4 PERSISTENT HPV INFECTION (III)

Persistence was defined as type-specific persistence i.e. any woman testing positive for the same HPV-genotype in two (or more) subsequent samples during the FU. The time of persistence was calculated for the single genotypes and their respective species.

5.4.1 PERSISTENCE TIMES

Species-specific persistence
The persistence time of species 9 was significantly longer than for all others; 24.1 months (range 2.4-88.8, n=90). The persistence time of species 7 was only 13.4 months (range 1.6-34.1), while the other species had persistence times between 12.4-19.1 months.

Type-specific persistence
Of the individual genotypes, the longest persistence time was recorded for HPV 35 (38.7 months), but there was only a single case. This was followed by HPV 58 with a mean of 32.1 months (n=4, range 12.0-88.8 months). For HPV 16, which was the most common type, the persistence time was 23.9 months (n=80; range 2.4-78.8 months). The time of persistence for multiple-type infections was close to that of HPV 16; 20.9mo (n=7; range 11.2-33.9). For all other HR- and LR-HPV types, the persistence time was much shorter, e.g. for HPV 6 it was only 14.1 months (n=3; range 12.2-15.5) (**Figure 17**).

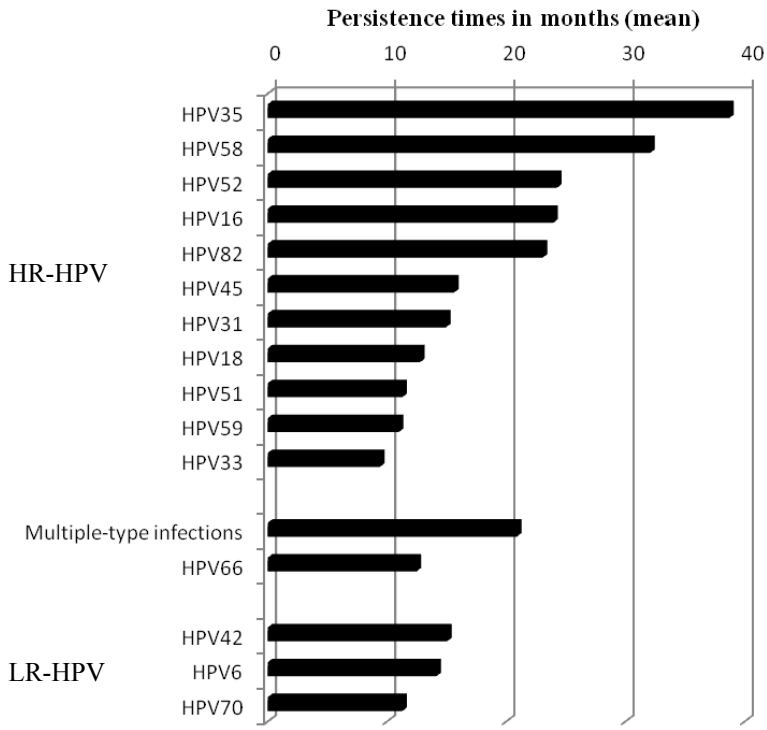


Figure 17. Type-specific persistence times

5.4.2 PREDICTORS OF PERSISTENCE

The predictors of species-specific persistence of HPV-genotypes during the follow-up were analysed for species 7 and 9 using GEE-modeling. In the univariate GEE-model, three variables were significant predictors of species 7 and 9 HPV-persistence: early onset of 1) sexual activity, 2) initiation of OC usage, and 3) smoking.

When all significant and borderline significant univariates were entered in the multivariate (adjusted) GEE-model together with the mother's age, three variables became independent predictors of persistence Species 7 and 9 HPV-infections: 1) age (persistence more common in older women), 2) oral sex increased the risk (non-practicing being protective with OR=0.37), and 3) early onset of smoking (10-13 years as reference; OR=0.51; p=0.046).

5.5 PROGRESSION TO CIN (IV)

Of the 329 women enrolled, all were baseline negative for cytological abnormalities (ASCUS cut-off) or CIN. The mean follow-up time was 54.9 months (SD=27.3; median= 62.4; range 2-94.5), and by the study endpoint, 10 women (3.2%) had developed a biopsy-confirmed CIN lesion; CIN1 (n=2), CIN2 (n=3), CIN3 (n=5). In addition, four women presented with an incident ASC-H cytology and were included in the combined CIN/SIL group of progressors (n=14), representing 4.5% of the women of the total cohort.

5.5.1 INCIDENCE TIMES OF PROGRESSION

The actuarial times to incident CIN and CIN/SIL were the same, 55.3 months (95% CI 52.3-58.4). Crude time, however, was longer for CIN; 74.5 months (95% CI 58.7-90.3) than for CIN/SIL, 66.3 months (95% CI 52.3-80.2), because of a shorter time needed to develop incident ASC-H.

Also the crude times from the detection of incident HR-HPV infection until incident CIN could be calculated for four women; 70.3 months (95% CI 50.9-89.7). For seven women in the CIN/SIL-group, time from incident HR-HPV to the combined endpoint was shorter, only 47.8 months (95%CI 17.7-77.9).

Univariate (Kaplan-Meier) survival analysis was performed to illustrate the cumulative incidence of CIN and CIN/SIL events, stratified by the baseline HR-HPV DNA status (see Figure 1 and 2 in original publication IV). The cumulative incidence of both CIN and CIN/SIL outcomes was significantly higher among baseline HR-HPV-positive than –negative subjects; the log-rank (Mantel-Cox) test was p=0.0001 and p=0.003, respectively.

5.5.2 INCIDENT RATES OF PROGRESSION

Taking into account all persons at risk, the actuarial IR reflects the rate (per 1000wmr) at which these women at risk accumulated incident CIN and CIN/SIL events. The actuarial IR for CIN was 0.59/1000 wmr (95% CI 0.22-0.95) and that for CIN/SIL was 0.82/1000 wmr (95% CI 0.39-1.25). Calculated only for persons with confirmed progression events, the crude IRs reflect the rate (per 1000 wmr) at which individual women accumulated such an event: 3.4/1000 wmr for CIN and 15.1/1000 wmr for CIN/SIL.

5.5.3 PREDICTORS OF PROGRESSION

The progression event documented at any FU visit (panel data) was used as the dependent variable in the Poisson regression analysis for risk factors. In univariate Poisson, four variables were significantly associated with progression events: 1) cervical HR-HPV DNA + at baseline 2) type-specific HR-HPV persistence, 3) ASCUS+ Pap smear at any FU visit and 4) a new sexual partner during the study period.

When all significant and borderline significant univariate predictors were entered in the multivariate Poisson-model (adjusted for mother's age at study entry), all four variables retained their significance also as independent predictors of incident CIN.

6. DISCUSSION

6.1 HPV PREVALENCE

HPV prevalence among the mothers in the Finnish Family HPV study varied at each FU visit, the lowest prevalence of 16.3% and 15.1% was recorded at the baseline and 2-month FU visits, respectively and the highest 57.2% at the 24-month FU visit. At enrollment, all women were pregnant in their third trimester. Interestingly, there was an almost 3-fold increase in HPV prevalence from the baseline to the 12 month visit from 16.3% to 47.7%. This corroborates with the recent data from this same cohort, where a clear association was disclosed between HPV carriage (point prevalence) and timing of two pregnancies (index pregnancy and 2nd pregnancy) (Sarkola et al., 2009). There was a significantly increased carriage of HR-HPV in both cervical and oral mucosa during the inter-pregnancy period as compared with the index pregnancy or the 2nd pregnancy. This suggests that hormonal or immunological factors during the post-partum period may be protective against HPV infections. The observed increase in HPV prevalence at the 12-month FU visit might be explained by new exposure during this inter-pregnancy period, which is supported also by the serological data from these women, showing a clear peak between the baseline visit and the 12-month FU visit (Syrjänen et al., 2009).

Type-specific prevalence
The point prevalences of 24 different HPV-genotypes were analyzed in six consequent FU visits during a 6-year period. HPV 16 was the most common HPV-genotype at baseline and during the entire follow-up. This is in line with most of the other studies on female genital HPV infections (Kulmala et al., 2007; Nielsen et al., 2008; Stevens et al., 2009; Ivanson et al., 2009; Chen et al., 2009; Ralston Howe et al., 2009; Menegazzi et al., 2009; Uuskűla et al., 2010). In this study, HPV16 was followed in decreasing order of frequency by HPV 6, 45, 43, 58 and 70. In a few studies, HPV 58 has been reported as the second most prevalent genotype after HPV16 (Herrero et al., 2005; Trottier et al., 2008; Chen et al.,2009; Brandão et al., 2009; Ralston Howe et al., 2009;Conesa-Zamora et al., 2009). Geographically, HPV 58 is regarded as the third most common type in Asia, but in Europe its estimated prevalence is below 1% (Bosch et al., 2008). HPV 18 which is the second most common type in Europe was uncommon in our cohort, confirming the observations in a recent study from Estonia, where HPV18 prevalence was also quite low (Uuskűla et al., 2010). In our cohort, HPV 70 was frequent at the second and third visits, 8.7% and 6.6% respectively, closely matching the 5.7% prevalence reported recently (Brandão et al., 2009). Not unexpectedly, species 9 was the most common HPV species, followed by species 7, as also reported before (Trottier et al., 2008).

The proportion of multiple-type HPV infections was high, second only to HPV 16 in overall prevalence. Recently, multiple infections were shown to be more prevalent in a young Danish cohort, 20-29 years of age (Nielsen et al., 2008), representing similar age coverage as in our cohort. This is in sharp contrast to menopausal women (>50 years), among whom single-type infections predominate, frequently associated with high-grade CIN (Syrjänen et al., 2008). It has to be noted that all the women in the present study cohort were pregnant at baseline, which might have affected the genotype distribution**.** HPV 16 was found in 50% to 87.5% of the multiple-type infections during the FU. This dominant role of HPV16 was also confirmed in another recent study, where HPV16 was involved in 61.9% of all multiple-type infections (de Ona et al., 2010).

6.2 HPV INCIDENCE

This is among the first studies to assess type-specific incident HPV infections (Franco et al., 1999; Giuliano et al., 2002; Muñoz et al., 2004; Winer et al., 2003; Chao et al., 2010; Nielsen et al., 2009; Fukuchi et al., 2009; Oh et al., 2008; Goodman et al., 2008), in contrast to earlier studies where acquisition of HPV infection has been analysed collectively for all HPV types, or acquisition of HR and LR types has been compared (Moscichi et al., 2001; Sellors et al., 2003; Syrjänen et al., 2004; Syrjänen et al., 2005). Similarly, in the available literature, it is difficult to find the exact times for the first incidence infections at the genotype-level, most reports being focused on the duration of incident infections (Franco et al., 1999, Giuliano et al., 2002; Goodman et al., 2008).

In our previous analysis of the NIS cohort, the crude incidence times were provided collectively for 13 HR-HPV types detected by HC2 assay (Syrjänen et al., 2004). It took an average of 16.6 months to develop the first incident HR-HPV event. The present study provides these times for individual HPV genotypes (and species) both in actuarial and crude format. To calculate the former, also included here are baseline HPV-negative women who never develop an incident event, while the latter is calculated only for women who develop incident events. Thus, the actuarial times reflect the real-life situation, while indicating how long it takes in a cohort of baseline HPV-negative women to develop the first incident infections. On the other hand, the crude times indicate the time that these first incident infections take to develop among women who all experience such an event. These crude (type-specific) incidence times provide a robust measure to compare different HPV-genotypes in their speed to develop the first incident events. Indeed, individual genotypes showed marked differences in their crude incidence times, varying within the range of 2 to 90 months, with the mean of 20.3 and median of 13.5 months (data not shown). Generally speaking, LR-HPV types had shorter mean incidence times than HR-HPV genotypes. However, despite the significant overall difference ($p=0.0001$) between the individual genotypes, these results are hampered by multiple comparisons and a small number of cases in most of the genotypes. Of interest is the observation that no significant differences were observed between HPV 16, HPV 18 and multiple-type infections, each being represented by sufficient numbers of cases.

The incidence rate (IR) can also be calculated as actuarial and crude, as done in this study. These two IRs are markedly different also because of the significantly different person months at risk. This also makes comparison between the individual studies difficult, particularly when the FU times and cohort size are substantially different. For instance a setting where 1000 women are followed up for 10 months (10.000wmr), and an other where 100 women are followed up for 100 months (10.000wmr) will result in higher IRs in the latter. This is because the median incidence time is around 12-13 months and only a fraction of events are accumulated within a short FU time. In addition, it is essential to know the age profile of the cohort because the IR of HR-HPV genotypes is critically age-dependent (Syrjänen et al., 2005).

In this study cohort, actuarial IR was by far the highest for HPV 16, 15.6/1000wmr. This is consistent with other studies, where HPV 16 had the highest IR, albeit the absolute IRs were lower. A young women's health study-cohort from the USA reported an IR of 5.9/1000wmr for HPV 16 (Giuliano et al., 2002). In the Ludwig-McGill low-income women's cohort, the IR for HPV 16 was 1.4/1000wmr (Franco et al., 1999), which is very similar to another cohort consisting of sexually active women from Hawaii, 1.77 /1000wmr (Goodman et al., 2008). Due to the dominant role of HPV16, the actuarial IR of species 9 was also the highest 17.9/1000wmr, which is three times higher than recently published by Goodman and co-workers. (5.4/1000wmr) (Goodman et al., 2008). As pointed out above, these differences are explained by different cohort sizes and particularly by much shorter FU-times of 10-15 months only (Franco et al., 1999; Giuliano et al.,

2002; Goodman et al., 2008). Another factor is the different age-profile; the mean age of our cohort was 25.5 years which is close to the 24.2 years in the study by Giuliano and co-workers (Guiliano et al., 2002), whereas the mean age in the study by Goodman and co-workers was 35 years (Goodman et al., 2008). The IRs are higher in the present study and in Giuliano's cohort (Guiliano et al., 2002) than in studies on older women (Franco et al., 1999; Goodman et al., 2008), which gives further support to the close age dependence of the IRs (Syrjänen et al., 2004; Syrjänen et al., 2005).

This is one of the first studies on crude IRs, which reflect the differences in the rates at which individual genotypes accumulate incident events among women who have these events. Although firm conclusions on single genotypes are difficult because of the rarity of many individual genotypes, it is interesting to note that the crude IR of the key LR-HPV types (HPV 6, 11), is almost twice as high as that of the two main HR types (HPV 16, 18). This indicates that the former develop incident infections at a much higher rate, which is also shown by their significantly shorter times to incident events. Using rate-ratio (RR) statistics, one cannot detect significant differences between the different HPV-species in their crude IRs. An much larger cohort is needed to establish whether such differences exist between individual HR-HPV-genotypes. If this speculation could be confirmed, however, such data would have important implications, for instance in the selection of the most aggressive genotypes with high IRs as targets of primary prevention.

Predictors of incident HPV infections

In analysis of the risk factors for incident species 7 and 9 infections, seven variables were significant predictors in univariate analysis, and three of these remained significant also in the multivariate model.

Seroconversion to HR-HPV

There was a link with HPV-serology (Syrjänen et al., 2009); mothers who failed to seroconvert to HR-HPV during the FU were at increased risk for incident infections. It is well established that serological response to HPV infections needs several months to become detectable (Ho et al., 2004; Syrjänen et al., 2009). Thus, women who experience an incident event close to the end of the FU have not enough time to become seroconverted during the observation period. There is some evidence that seroconversion may fail with transient and even with some persistent infections (Carter et al., 2000). It is commonly believed that high levels of HPV antibodies detected after seroconversion will protect against a new infection. Thus, seroconversion detected during the early months of the FU could implicate an incident event that took place before the baseline visit, and these high antibody levels, except for being incompatible with new incident events, might also neutralise the virus and make these women baseline HPV DNA-negative.

Initiation of smoking

Initiation of smoking older than 13 years of age was associated with an increased risk of incident HPV-infection, but lost its significance in the multivariate model. Some studies have found no association between smoking and incident infection (Muñoz et al., 2004; Goodman et al., 2008), whereas others implicate current smoking to increase the risk (Sellors et al., 2003; Winer et al., 2003; Oh et al., 2008; Nielsen et al., 2009). Analysis of this same cohort also disclosed that initiation of smoking before 13 years of age increased the risk of type-specific persistence. Because incident infections precede viral persistence, the present data are consistent with this observation, implicating that women who start smoking later have had less time to develop persistent infection, and consequently are at the phase when incident infections accumulate.

Sexual behavior
Women who started their sexual activity before 13 years of age were found to have a lower risk for incident infections during the FU. Also this association is in line with the other analyses of these data disclosing early age of sexual debut as a risk factor for HR-HPV persistence. It is likely that women with later onset are at increased risk for incident infections because those with longer sexual experience have already contracted it, and have either cleared or developed a persistent infection by the time of this observation period.

The same analogy applies to the life-time number of sexual partners (another measure of HPV exposure), shown in this analysis to be inversely related to the risk of incident infections i.e. lower partner number was associated with an increased risk. It seems likely that those with more partners had already experienced such an event before being enrolled in the study i.e. are baseline HPV+, and by definition, no longer at risk of incident infection by the same HPV genotype.

Oral contraceptive use
The initiation of OC usage before the age of 20 increased the risk of incident infection and was a significant predictor also in the multivariate model. Previous studies report conflicting results; current use of OCs has been shown to increase (Winer et al., 2003) or decrease (Moscicki et al., 2001) the risk of incident infection. In addition, it has been reported that current users of OCs are not at increased risk while past users of OCs seem to be protected, as shown by a decrease in incident infections with the years of OC use (Goodman et al., 2008). Contradictory to the previous finding, a recent study stated that the risk of incident infection increased with increasing years of OC use (Nielsen et al., 2009). It is evident that further studies are needed to fully elucidate the association of OC and incident infections.

Pregnancy
Another significant protective factor was second pregnancy during the FU. No previous data are available from a similar setting where newly delivered mothers were prospectively followed up. A recent analysis from this same cohort showed that women committed to the second child did not share many of the known life-style behavioural risk factors of HPV infection (Sarkola et al., 2009), and this could be the likely explanation for this significant (IRR=0.32, 95%CI0.17-0.61) protective effect of a new pregnancy against incident species 7 and 9 HPV-infections in the present analysis. Some earlier data suggest that parity was protective especially against LR-HPV-types (Muñoz et al., 2004). In our cohort, increasing parity did not show any such effect. However, in a large screening study (the NIS Cohort), ever being pregnant was an independent predictor of incident HR-HPV infection (Syrjänen et al., 2004).

6.3 HPV CLEARANCE

The present study is the first to provide detailed information on both actuarial and crude clearance times and clearance rates at HPV-genotype- and species-levels. Not unexpectedly, the lowest clearance frequency was recorded for HPV16 and multiple-type infections, of which only 51.6% and 50.5% cleared, respectively. In the Kaplan-Meier analysis, HPV 16 clearance was almost 20% less than that of all other genotypes. In other studies, 80.7%, 69% and 51.9% of HR-HPV infections cleared between 14-19 months of FU (Sellors et al., 2003; Goodman et al., 2008; Rosa et al., 2008). Of the LR-HPV types, 81% were shown to clear within 12 months of FU (Goodman et al., 2008), and the majority of type-specific clearance occured within 2 years (Richardson et al., 2003). When stratified by HPV species, species 10 (LR-types) showed a significantly more rapid clearance as

compared to species 7 and 9. This is in alignment with the results indicating that species 9 has the lowest clearance rate and the longest disease duration (Trottier et al., 2008; Goodman et al., 2008).

In including also women with no clearance, the actuarial times indicate how long it takes among HPV-positive women to clear the infection by a specific genotype. To the best of our knowledge, actuarial clearance of different HPV types has not been previously reported. The crude times for each genotype indicate the time required for clearance in women who experience such an event, providing a robust measure to compare different HPV genotypes. In the present series, the data on HPV 16 is remarkably similar to recently reported, mean clearance times between 17.1-22 months during the 19-month and 48-month FU time, respectively (Insigna et al., 2007; Rosa et al., 2008). For HPV 6 and 11, the crude times were 14.8 months and 12.4 months, respectively, which are also very similar to those reported previously: 9.3 months and 8.4 months (Insigna et al., 2007), respectively, as well as 9.5 months for HPV 6 and 11 (Trottier et al., 2008). These data confirm that the crude clearance times for HR-HPV types are almost twice as long as for LR-HPV (22.1-28.1 months vs. 10.4-14.8 months).

The actuarial and crude CRs are markedly different because of the significantly different denominators. Comparison between individual studies is difficult, particularly when the FU time and cohort size are substantially different. In this study cohort, HPV 16 showed a markedly lower crude CR of 45.2/1000wmr as compared to a recent study reporting crude CR of 72.0/1000wmr for HPV16 during 15 month FU (Goodman et al., 2008). When analyzed by species, CRs of 44.9/1000wmr and 59.3/1000wmr were recorded for species 9 and 7, respectively. These are somewhat lower than previously reported for species 9 (143.1/1000wmr and 76.5/1000wmr) and for species 7 (110.7/1000wmr and 94.1/1000wmr) (Goodman et al., 2008; Trottier et al., 2008). As with IRs, these differences in CRs are explained by different study settings, including cohort size and shorter FU times of 15-48 months in those studies (Goodman et al., 2008; Safaeian et al., 2008; Trottier et al., 2008). An additional contributing factor is the different age profile.

Predictors of HPV clearance

Only two significant predictors of species 7 and 9 clearance were found in the univariate model, and one additional predictor emerged as significant in the multivariate Poisson regression model.

Age

Clearance was more common with increasing age, which is consonant with the recent data from several other studies (Franco et al., 1999; Goodman et al., 2008; Safaeian et al., 2008). However, this association was not detected in the study by Trottier and co-workers (Trottier et al., 2008). A previous study evaluating age-specific clearance collectively for 13 HR-HPV types showed that crude clearance rate was relatively constant across the age groups (Syrjänen et al., 2005). These data match the known dynamics of HR-HPV infections. Among young women, incident infections are the dominant pattern, with a distinct duration time, after which clearance is the dominant pattern. Indeed, clearance rate exceeds the incidence rate from the age of 25 years onwards (Syrjänen et al., 2005), explaining why clearance events are more common with increasing age also in the present cohort.

Sexual partners

The number of current sexual partners, recorded at the 36-month midpoint during the FU, was recorded as a significant predictor of clearance. Accordingly, those women who reported no partner during the FU all cleared their infection, as contrasted to those who had two or more partners, of whom none cleared. Previous studies have not found an association between the number of current

sexual partners and clearance (Safaeian et al., 2008; Trottier et al., 2008). Our observation is plausibly explained by the likelihood of higher exposure to HR-HPV types with multiple partnerships.

Oral HR-HPV
Somewhat unexpectedly, the oral HR-HPV status also emerged among the predictors of species 7/9 clearance. HR-HPV DNA-negative oral status at baseline increases the probability of clearance. This observation is completely new and has not even been assessed in any previous studies due to the failure to collect oral samples in these settings. This issue will be explored in detail while analysing the genotype-specific oral HPV data from the Finnish Family HPV study in due course. It is interesting that of the 12 mothers who reported a history of oral warts, none cleared their genital HPV infections, being consonant with the observation that baseline oral HR-HPV DNA-positive status reduces the likelihood of genotype-specific clearance of cervical HPV infections.

6.4 HPV PERSISTENCE

There is no general consensus as to the definition of persistent HPV infection, complicating a direct comparison of different studies. In this study, we defined as type-specific persistence any woman testing positive for the same HPV-genotype in two (or more) consequent samples during the FU. In our cohort, HPV 6 infections persisted 14.1 months, which is longer than reported (6.5 months) in a previous study with only a 24-month FU time (Richardson et al., 2003). In the present series, HPV 35 showed the longest persistence (38.7 months), but there was only one case. Next in order were HPV 58 (32.1 months), HPV 16 (23.9 months) and multiple-type infections (20.9 months). Even if there were only four cases of HPV 58 infections, these findings are in agreement with some recent reports, where HPV 16 and 58 were the two genotypes most the protracted persistence (Kulmala et al., 2007; Ralston Howe et al., 2009; Nielsen et al., 2010). In other studies, HPV 16 alone has shown the most prolonged time of persistence (Schiffman and Castle, 2005; Kulmala et al., 2007; Plummer et al., 2007) ranging from 18.3 months (Richardson et al., 2003), to 16.1 months (Kulmala et al., 2007) and >18 months (Plummer et al., 2007). The persistence of 23.9 months reported for HPV 16 is here even longer, most likely due to the longer FU time than in most of the previous studies. Persistence was longest for species 9 (24.1 months), confirming a recent report (Trottier et al., 2008). This is not surprising since all major HR-HPV types belong to this species.

Predictors of HPV persistence
Species 7 and 9 genotype-specific persistence was used as an endpoint in the GEE-model, because these are the clinically most important and globally most frequent HPV types. We disclosed three significant predictors in both the univariate- and multivariate GEE-models.

Sexual behavior
In the univariate GEE-model, there was a significantly increased risk of Species 7 and 9 infections to persist among those who had started their sexual activity younger than 13 years of age. In fact, seven mothers (2.4%) had their first sexual intercourse before the age of 13, and of those 42.9% (n=3) had a persistent type-specific infection by species 7 and 9 genotypes. This corroborates recent data implicating that those HR-HPV-positive girls younger than 15 years of age have an increased risk to develop a persistent infection (Nielsen et al., 2010). In our multivariate analysis, the early onset of sexual activity lost its significance as an independent predictor, however, being in line with another recent study (Herrero et al., 2005). We suspect that an early onset of sexual activity could be a proxy for “high-risk” sexual behavior (oral sex, smoking), and it is the latter that are the true

predictors of HR-HPV persistence as seen in multivariate GEE. Indeed, this was shown to be the case, when the onset of sexual activity (using 16-year cut-off) was controlled for smoking and further stratified by oral sex in Mantel-Haenszel test, and a significant common odds (OR=3.10, 95%CI 1.89-5.07, p=0.0001) was obtained, indicating that these three variables are closely interrelated.

Oral contraceptives

Early initiation of OC use was another factor associated with an increased risk of HPV persistence, which is in line with many other studies (International Collaboration of Epidemiological Studies of Cervical Cancer, 2007; Nielsen et al., 2010). In most studies, OC use has been reported as years of use, and longer than 5 years of use has been associated with an increased risk of CC (Moreno et al., 2002). In our cohort, early initiation of OC use also closely correlated with the total time of OC usage, the latter variable being omitted from the multivariate GEE-model due to collinearity. It is not clear how OCs increase the risk of HPV persistence. In a transgenic mouse model, however, continuous (unopposed) estrogen exposure together with HPV16 E6/E7 expression induced the development of CC in all animals within 7-8 months (Arbeit et al., 1996)

Smoking

Smoking is another well-established risk factor of CIN and CC (Castellsague et al., 2002). There are not many studies, however, assessing the role of smoking and type-specific HPV persistence. A cohort study from Canada concluded that women who smoked one or two packs of cigarettes per day at least for one year were only half as likely to clear their HPV infection as non-smokers (Richardson et al., 2005). In our study, early initiation of smoking (<13 years of age) was closely correlated with the total time (years) of being a smoker, implicating a dose/exposure relationship (data not shown), and was a significant predictor of type-specific persistence even in the multivariate model. Similar data were previously reported in our NIS Cohort, where being a current smoker was the only predictor of persistent HR-HPV infection, analyzed collectively for 13 HR-HPV-types with HC2 assay (Syrjänen et al., 2005). It has been proposed that smoking adversely affects the host immunological surveillance system against viral infections (Poppe et al., 1995). Thus, women smokers <30 years of age have been shown to be less likely to either seroconvert or maintain HPV16/18 antibodies when compared to non-smokers (Simen-Kapeu et al., 2008). Initiation of smoking at a young age may interfere with the immunological system under development, thus contributing to its failure to eradicate viral infections. Also the mucosa, especially the transformation zone, might be more vulnerable at an early age than later.

Oral sex

Oral sex was another independent predictive factor for type-specific persistence. Not unexpectedly, non-practice of oral sex had a protective effect against type-specific persistence. It can be reasoned that regular exposure of the genital tract to HPV from the oral mucosa might promote viral persistence. In our previous study, however, we failed to correlate baseline HR-HPV DNA status in the oral mucosa with the practices of oral sex (Rintala et al., 2006). Based on analysis in a longitudinal setting in the present study, oral sex could represent one of the potential mechanisms associated with spouse-to-spouse transmission of HPV, but this needs further assessment by more complex models (e.g. multilevel mixed-effects models).

6.5 HPV PROGRESSION

Persistent HPV infection is a necessary factor for the development of cytological abnormalities or CIN lesions. In this cohort of young mothers persistent HR-HPV infection was detected in 35% (n=115/329). In the present analysis, persistent HR-HPV infection, cytologic abnormality, as well as a new partner was independent predictors of incident CIN/SIL lesions, developed among 4.5% of these mothers during the study period.

Actuarial and crude incidence times
In this cohort, the actuarial time for progression was 55.3 months, whereas the crude time was longer for incident CIN than incident CIN/SIL, 74.5 months and 66.3 months, respectively. Trottier and co-workers recently reported that actuarial times from the first incident HR-HPV infection to the detection of SIL and CIN were 43 months and 50 months and the crude times were 29 months and 34 months, respectively (Trottier and Burchell, 2009). Woodman and co-workers reported the median time to CIN from the study entry to be 36.1 months (Woodman et al., 2001). In the present series, the crude times from incident HR-HPV infection to incident CIN could be calculated for four baseline HR-HPV- women and was 70.3 months (95%CI 50.9-89.7). The corresponding crude time to CIN/SIL in seven baseline HR-HPV- women was 47.8 months (95%CI 17.7-77.9). Thus, both crude and actuarial times in our series are longer than in previous studies. However, an even longer actuarial time has been reported for progression from ASCUS to HSIL: 82.7 months (Schlecht et al., 2003).

These differences are likely to be explained by different study designs and population. When calculated from the study entry, the actuarial and crude times to incident CIN and CIN/SIL were clearly longer than in previous studies. The actuarial time is dependent on the length of the FU time, which was longer in the present series, as compared to 29 months and 45.3 months in the other studies (Woodman et al., 2001; Trottier and Burchell, 2009). Noteworthy is the small number of events in the present series, 10 CIN and 14 CIN/SIL only, which results in wide variation of the 95%CI. The possible impact of pregnancy has to be considered while comparing the present results with the published data because all the women in our cohort were pregnant at study entry, giving this cohort a unique profile. However, second pregnancy (n=78) during the FU did not appear among the risk factors for incident CIN or CIN/SIL in this study. This is in agreement with a previous study reporting that pregnancy was not associated with incident CIN3 (Castle et al., 2005).

Schlecht and co-workers reported a long actuarial time from ASCUS to develop HSIL and also showed that this time was 21 months shorter in women infected with an oncogenic HPV as compared with HPV-negative women (Schlecht et al., 2003). This was confirmed also in our cohort, where survival analysis disclosed a significantly higher cumulative incidence of both CIN and CIN/SIL among HR-HPV+ than HR-HPV- women, with the actual time difference of 15 and 6 months, respectively (see Figure 1 and 2 in original publication IV).

Until now, scant data on IRs are available. The crude IR recorded in our study was 13.4/1000wmr for CIN and 15.1/1000wmr for CIN/SIL. These figures are practically identical with almoust to those in a recent study by Trottier and coworkers, where IRs were recorded for biopsy- and cytology-confirmed outcomes among HR-HPV+ women; 10.3 to 13.0/1000wmr for HPV 16 or HPV 18 (Trottier and Burchell, 2009).

Predictors of incident CIN

In the present study, the Poisson regression for panel data was used to analyse the predictors of incident CIN. In the univariate analysis, there were four significant predictors that remained significant also in the multivariate model.

Baseline HR-HPV DNA status

Not unexpectedly, women who were baseline HR-HPV DNA+ had an increased risk of progression to CIN. This finding is in line with the published data. Among 20-29 year old women testing positive with HC2, the risk of CIN3 or CC within ten years was 13.6% (10.9-16.2) (Kjaer et al., 2006). The risk of high-grade CIN is greatest for women who were HPV-positive as compared to women with no HPV infection, the highest risk being associated with HPV 16 (Woodman et al., 2001). In our study, HPV 16 was detected in three out of ten women with incident CIN, the remaining seven being multiple-type infections (n=4) or single infections with HPV 31, HPV 45 and HPV 70. However, HPV 16 was also included in all four multiple-type combinations, increasing the HPV 16 involvement to 70%. This was similar in incident CIN/SIL cases as well (9/14, 64.3%). HR-HPV and especially HPV16 have been reported to play a key role also in studies where cytological endpoints of progression have been evaluated (Syrjänen et al., 2004; Kovacic et al., 2006).

Persistent HR-HPV infection

Another risk factor increasing the probability of progression to CIN was persistent HR-HPV infection. Data on HR-HPV persistence have been provided in several recent studies (Kjaer et al., 2002; Trottier and Burchell, 2009), and the risk for progression seems to be highest for HPV16 infections (Muñoz et al., 2009). A recent meta-analysis based on 40 studies evaluating the association between HPV persistence and CIN2-3/HSIL (or CC) disclosed that the relative risk (RR) varies from 1.3 to 813.0, and in 92% of these studies, RRs were above 3.0 (Koshiol et al., 2008). It has been implicated that if an HPV infection has persisted over 6 months or 12 months, it is likely to remain permanent and increase the risk of progressive disease (Plummer et al., 2007; Koshiol et al., 2008; Syrjänen et al., 2009; Syrjänen, 2010). The importance of this subject has increased recently because these 6 months or 12 months viral endpoints could offer potential new surrogates of progressive disease, to be used instead of histological (CIN2+) endpoints in future clinical trials with non-HPV16/18 vaccines (Plummer et al., 2007; Koshiol et al., 2008; Syrjänen et al., 2009; Syrjänen, 2010).

ASCUS+ Pap smear at any FU-visit

The third significant predictor of progression was the ASCUS+ Pap smear at any FU-visit. This observation is consistent with our recent data in the combined NIS-LAMS cohort evaluating potential new surrogates of progression (Syrjänen et al., 2009). ASCUS+ detected at 6-month FU or later was significantly associated with incident CIN1, CIN2 or SIL (Syrjänen et al., 2009). Similarly, 20% of women with persistent LSIL progressed to HSIL or CC during eight years of FU (Schlecht et al., 2003). With ASC-H, this association seems to be even stronger; 40% of women with ASC-H were reported to develop CIN2+ (You et al., 2010). This advocates the use of ASC-H as a surrogate of progression as done in the present study for the CIN/SIL group (i.e., women who developed ASC-H). This leaves little doubt that persistent HR-HPV together with Pap smear abnormality (with ASCUS cut-off) are indicators of progressive disease.

New sexual partner

The fourth significant predictor was a new sexual partner during the FU, shown to be significantly (IRR>9) associated with the risk of incident CIN. This is likely to be associated with exposure to new HPV infection, as shown by the other analysis of this cohort where a new partner increased the

risk of incident HR-HPV infections. Also contradicting results have been reported both for the risk of CIN3 (Deacon et al., 2000) and for the association between sexual partners and disease progression (Woodman et al., 2001; Girianelli et al., 2009).

Other potential co-factors
Smoking and use of OCs are other co-factors considered to be associated with CIN in several studies (Ylitalo et al., 1999; Moscicki et al., 2001; Simen-Kapeu et al., 2008; Simen-Kapeu et al., 2009). Recent data from the LAMS cohort showed that smoking was an independent predictor of incident HR-HPV but not for CIN2 (Syrjänen et al., 2007). In the present series with fewer cases, only a borderline association to incident CIN was ascribed to early initiation of OC use (P=0.082). Smoking clearly had a high IRR (>3.4), but 95% CIs showed wide variation precluding smoking among the significant predictors of incident CIN (or CIN/SIL) in this series.

6.6 STUDY STRENGTHS AND LIMITATIONS

As the name implies, the Finnish Family HPV study is a longitudinal cohort study including three members (mother, father, one newborn) of regular families. This study design provides a unique setting to study the transmission dynamics of HPV infections between the mother, father, and their newborn (index) infant followed up until pre-school childhood. In this series of studies, the authors analysed only the cervical HPV infections in the mothers with a focus on assessing the main viral outcomes and their predictive factors.

Strengths
As compared to most previous studies with the FU times reaching only 24 months, the major strength of our study is the long-term follow-up of these young healthy women, reaching up to 6 years and comprising six visits. Secondly, all these women were pregnant at enrolment and 78 women had their second pregnancy during the FU, which offered us an opportunity to investigate the effects of this 2nd pregnancy on the clinical course of HPV infections in a longitudinal setting.

Limitations
The limitation is the relatively small cohort size, consisting of 329 mothers at baseline and 171 completing the 6-year FU visit. The loss of these women may have some effects on our results. The main reasons for losing women to FU were difficulties in attending the visits because of work or family reasons. Because of the rarity of many HPV genotypes, a much larger series is needed to increase the statistical power of the estimates for outcome events at the genotype level. Although pregnancy is considered one of the special strengths of this study, it may also constitute a limitation because the detailed mechanisms of how pregnancy and HPV infections interact are not well understood.

7. CONCLUSIONS

1. Cervical HPV infections among the mothers included in the Finnish Family HPV study were very common. The most prevalent genotype was HPV 16, followed by multiple-type infections.

2. HPV 16 was the most frequent incident genotype followed by multiple-type infections. Among newly delivered mothers, a higher number of life-time sexual partners, initiation of OC use after the age of 20 and becoming pregnant during the FU decreased the risk for incident species 7 and 9 HPV infections. Obtaining genotype-specific data during a long-term follow-up is needed for better understanding of the natural history of HPV-infections as well as for designing tools for their prevention.

3. HPV 16 and multiple-type infections showed the lowest clearance among newly delivered mothers. The significant independent predictors of species 7 and 9 clearance include: 1) age, 2) having >2 current sexual partners and 3) baseline oral HR-HPV DNA status; older age and oral HR-HPV DNA-negative status increases clearance, while multiple current sex partners decreases the probability of clearing species 7 and 9 infections.

4. HPV 16 was the most frequent persisting HPV genotype followed by multiple infections. Early initiation of smoking, practicing oral sex and older age increase the risk for persistence of the species 7 and 9 HPV-genotypes. These data might have important implications e.g. in adolescence and maternity counseling aimed at reducing the risks of persistent HR-HPV-infections.

5. Of the 329 young mothers in the Finnish Family HPV study, ten (3.2%) developed a biopsy-proven CIN within a mean crude time of 74.5 months, and an additional four women showed cytology-confirmed progression. Thus also this special series of young mothers disclosed predictors of progression similar to those detected in large population-based cohorts, namely 1) testing HR-HPV-positive at baseline, 2) type-specific HR-HPV persistence, 3) ASCUS+ Pap smear at any FU visit, and 4) a new sexual partner during the FU. The data indicate that when any of these factors are identified, the increased risk of CIN/SIL lesions needs to be kept in mind, even in women who are young, have delivered relatively recently, and are possibly pregnant. Combined use of Pap smear and HPV testing with prompt referral for colposcopy enables accurate detection of these lesions well before progression to invasive disease.

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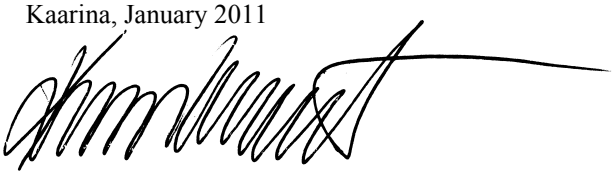

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