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HUMAN PAPILLOMAVIRUS INFECTIONS IN EARLY CHILDHOOD – IMMUNE RESPONSE AND DISEASE OUTCOME IN THE FINNISH FAMILY HPV STUDY COHORT

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To my Family

ABSTRACT

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Human papillomavirus infections in early childhood – Immune response and disease outcome in The Finnish Family HPV Study Cohort
University of Turku, Faculty of Medicine, Institute of Dentistry, Department of Oral Pathology and Radiology and Finnish Doctoral Program in Oral Sciences (FINDOS-Turku)
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Human papillomavirus (HPV) infects epithelial cells on the skin or mucosa. It is an important risk factor for cervical cancer and head and neck cancers. A child may acquire HPV infection during childhood, even before birth. However, our knowledge of HPV prevalence and HPV-specific immunity in children is limited.

In the present study, the prevalence of oral HPV infection among 331 infants was elucidated. Further, HPV 16-specific cell-mediated immune response was studied in 56 children, aged from 10 to 16 years, who either 1) had a mother with a cervical intraepithelial neoplasia or 2) had a HPV-negative mother or 3) had HPV DNA detected in placenta and/or cord blood or 4) had persistent oral HPV or 5) remained constantly HPV-negative during a six-year follow-up period.

HPV was detected in 18% of the 331 infants up to two months of age: HPV 16 was the most prevalent genotype, followed by HPV 6, 11, 18, 33, and 66. The HPV genotypes and the serum antibodies for HPV capsid protein L1 were concordant between mother and newborn. HPV DNA in the placenta was the most powerful predictor (OR=14.0;95%CI,3.7-52.2;P=.0001) of oral HPV in the newborn. A majority of 56 children showed HPV 16-specific proliferative T cell response. The cytokine production of T cells indicated a more predominant Th2 response in children who had had HPV-positive placenta and/or cord blood.

These results support the view that an infected mother transmits HPV to her newborn. The placenta is a substantive route for HPV transmission and might also play a role in the development of HPV-specific immunity. HPV 16-specific proliferative T cell response was common in children, indicating a prior HPV 16 infection.

Keywords: Cell-mediated immunity, child, cord blood, cytokine, Human papillomavirus, HPV antibodies, HPV DNA, mother, newborn, placenta, pregnancy, T cell, vertical transmission

TIIVISTELMÄ

Hanna-Mari Koskimaa
Ihmisen papilloomavirusinfektiot varhaislapsuudessa ja niihin liittyvä immuunivaste – HUPA-kohorttitutkimus
Turun yliopisto, Lääketieteellinen tiedekunta, Hammaslääketieteen laitos, Suupatologian ja -radiologian osasto ja Suun terveystieteiden tohtoriohjelma (FINDOS-Turku)
Annales Universitatis Turkuensis, Medica-Odontologica, Turku, Suomi, 2016

Papilloomavirus (HPV, human papillomavirus) infektoi ihon ja limakalvojen epiteelisoluja. HPV-infektio on tärkein kohdunkaulan syövän ja merkittävä pään- ja kaulan alueiden syöpien riskitekijä. HPV-infektion voi saada jo varhaislapsuudessa tai jopa ennen syntymää. Lasten HPV-infektioista ja niihin liittyvästä immunologiasta tiedetään kuitenkin hyvin vähän.
Tämä väitöskirjatutkimus on osa Finnish Family HPV Study -seurantatutkimusta. Työn tarkoituksena oli selvittää HPV-infektioiden ja veren seerumin HPV L1 -kuoriproteiinille spesifisten vasta-aineiden esiintymistä 331 vastasyntyneellä kahden kuukauden ikään saakka sekä niiden mahdollista yhteyttä äidin HPV-infektioihin. Lisäksi työssä selvitettiin HPV 16 -spesifisen soluvälitteisen immuunivasteen esiintymistä lapsilla 10–16 vuoden iässä. Kuuden vuoden seurannan tulosten perusteella tutkimukseen valittiin 56 lasta seuraavasti: 1) lapset, joiden äideillä oli löydetty syövän esiastemuutos kohdunkaulan limakalvolta, 2) lapset, joiden äidit olivat olleet HPV-negatiivisia, 3) lapset, joiden istukka ja/tai napaverinäyte oli syntymän hetkellä HPV-positiivinen, 4) lapset, joilla oli löydetty persistoiva suun HPV-infektio ja 5) lapset, joiden suunäytteet olivat olleet aina HPV-negatiivisia.
Suun limakalvon HPV-infektio todettiin ensimmäisen kahden elinkuukauden aikana 18 %:lla 331 vastasyntyneestä. Yleisimmin esiintyi HPV 16 yksin tai muiden genotyyppien kanssa. Seuraavaksi yleisimpiä olivat HPV -genotyypit 6, 11, 18, 33 ja 66. Lapselta ja äidiltä osoitetut HPV-genotyypit olivat yhtenevät. Lisäksi lapselta löydettiin yhden kuukauden iässä HPV-spesifisiä vasta-aineita samoille HPV -genotyypeille kuin äidiltä ennen synnytystä. Istukan HPV-positiivisuus oli voimakkain ennustekijä vastasyntyneen suun HPV-positiivisuudelle. HPV 16-spesifinen T-solujen proliferaatiovaste löydettiin suurimmalla osalla tutkituista 56 lapsesta. T-solujen sytokiinieritys ilmensi voimakkaampaa Th2-soluvastetta niillä lapsilla, joiden istukka tai napaveri oli todettu HPV -positiiviseksi.
Tulokset osoittavat HPV-infektion todennäköisesti siirtyvän infektoituneesta äidistä vastasyntyneeseen. Istukalla on tärkeä osuus virustartunnassa ja todennäköisesti myös HPV-spesifisen immuniteetin muodostumisessa. Suurimmalla osalla tutkituista 56 lapsesta todettiin HPV 16 -proteiineja tunnistavia immunologisia muistisoluja, mikä viittaa aikaisempaan HPV 16 -infektioon.

Avainsanat: ihmisen papilloomavirus, HPV DNA, HPV-vasta-aineet, istukka, lapsi, napaveri, raskaus, soluvälitteinen immuniteetti, sytokiini, T-solu, vastasyntynyt, vertikaalinen tartunta, äiti

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ABBREVIATIONS

APC	antigen-presenting cell
CC	cervical cancer
CIN	cervical intraepithelial neoplasia
CPM	counts per minute
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E region	early region of the HPV genome
FCS	Fetal calf serum
GST	glutathione S-transferase
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
iDC	interstitial dendritic cell
IFN	interferon
IMDM	Iscove's Modified Dulbecco's Medium
iTreg	induced regulatory T cell
LC	Langerhans cell
LCR	long control region
LST	short-term lymphocyte stimulation test
MFI	median fluorescence intensity
MRM	memory response mix
nTreg	natural regulatory T cell
OR	odds ratio
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PRR	pathogen recognition receptor
RNA	ribonucleic acid
RRP	recurrent respiratory papillomatosis
SI	stimulation index
STD	sexually transmitted disease
Th cell	helper T cell
TLR	Toll-like receptor
Treg	regulatory T cell
URR	upstream regulatory region

LIST OF ORIGINAL PUBLICATIONS

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

I. KOSKIMAA H-M, Waterboer T, Pawlita M, Grénman S, Syrjänen K, Syrjänen S. Human papillomavirus (HPV)-genotypes in oral mucosa of newborn and their concordance with maternal cervical HPV-genotypes. Journal of Pediatrics 2012; 160: 837-43.

II. KOSKIMAA H-M*, Paaso A*, Welters M, Grénman S, Syrjänen K, van der Burg SH, Syrjänen S. Human papillomavirus 16 E2-, E6- and E7-specific T-cell responses in children and their mothers who developed incident CIN during a 14-year follow-up of the Finnish Family HPV cohort. Journal of Translational Medicine 2014; 12: 44-55.

III. KOSKIMAA H-M, Paaso A, Welters M, Grénman S, Syrjänen K , van der Burg SH, Syrjänen S. Human papillomavirus 16-specific cell-mediated immunity in children born to mothers with incident cervical intraepithelial neoplasia (CIN) and to those constantly HPV negative. Journal of Translational medicine 2015; 13: 370-81.

IV. KOSKIMAA H-M, Paaso A, Welters M, Grénman S, Syrjänen K , van der Burg SH, Syrjänen S. The presence of human papillomavirus (HPV) in placenta and/or cord blood might result in Th2 polarization. Submitted

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1 INTRODUCTION

Human papillomaviruses (HPV) are small DNA viruses that infect epithelial cells of the skin or mucosal sites and cause epithelial lesions varying from common warts, condylomas and papillomas to invasive cancer. HPV is generally accepted as the most important independent risk factor for cervical cancer and a significant risk factor for head and neck cancers, especially oropharyngeal cancer. Currently, over 200 HPV genotypes have been identified and classified into cutaneous and mucosal types according to their infection site, and further into high-risk and low-risk types according to their association with cancer.

HPV infection is common among the adult population and generally regarded as a sexually transmitted disease. Thus, research on HPV has been focused mainly on adults and especially women in the context of cervical lesions and cancer. However, HPV infection is also found in the oral and genital mucosae of infants and children without evidence of sexual abuse. This has raised interest in possible non-sexual HPV transmission routes, especially vertical transmission from mother to child upon delivery or even during the prenatal period. Nevertheless, studies are few in number and provide highly variable estimates of HPV prevalence in early childhood and the significance of early infections to later infections and their outcome. No previous studies exist on the HPV-specific immunity induced by these early infections.

This study aimed first to estimate the prevalence of HPV infections among infants and to assess the incidence of vertical mother-to-child transmission of HPV in the Finnish Family HPV Study cohort, which includes over 300 children and their mothers. The second aim was to estimate the presence of HPV 16–specific memory T cells in these children at the age of 10 to 16 years by testing immunological T cell responses against HPV 16 E2, E6 and E7 proteins. The 56 tested children were selected and grouped according to the data collected during the six-year follow-up period as follows: 1) children whose mothers had developed a cervical intraepithelial neoplasia, 2) children whose mothers had remained HPV DNA-negative, 3) children who had had HPV DNA detected in placenta and/or cord blood, 4) children who had persistent oral HPV and 5) children who remained constantly HPV-negative.

2 REVIEW OF LITERATURE

2.1 HUMAN PAPILLOMAVIRUS

2.1.1 Genomic structure

All human papillomaviruses (HPVs) have circular, double stranded DNA sheltered by an icosahedral viral capsid close to 55 nm in diameter (Figure 1). The genome of most HPVs is approximately 8000 base pairs in size and consists of eight or nine open reading frames (ORFs) encoding the major proteins. These ORFs are organized into two functional regions, and were originally named early (E) and late (L) genes based on their expression and functions during the viral life cycle. The early ORFs E1, E2, E4, E5, E6, E7 and E8 are transcribed and further processed by alternative splicing into proteins, which play their cardinal, regulatory roles at the early stages of the viral infection. They manage the viral gene expression and modulate the cell cycle, signaling, structure, apoptosis control, and the immune response of the host cell. The late proteins L1 and L2 comprise the virus capsid and are expressed prior to the packaging and release of the progeny virions. (de Villiers *et al.* 2004, Doorbar *et al.* 2012, Moody & Laimins 2010, Straub *et al.* 2014). The major roles of viral proteins are presented in Table 1. As HPV has no polymerases or other enzymes necessary for viral replication, it utilizes the replication proteins of the host cell for viral DNA synthesis. The regulation of viral gene expression is controlled by a variety of viral and cellular transcription factors, which have binding sites at the non-coding regulatory region between the L1 and E6 ORFs. This region is called the long control region (LCR) or upstream regulatory region (URR) and is the most variable region among different HPV types. Thus, there are substantial differences between HPV types in the regulation of gene expression and the translation of genes into functionally distinct protein isoforms, despite the highly conserved overall organization of viral genomes (de Villiers *et al.* 2004).

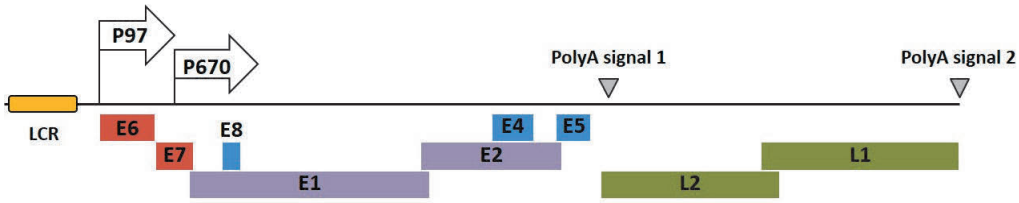


Figure 1. Simplified linearized presentation of the structure of the HPV 16 genome. The transcriptional regulatory elements localize at long control region (LCR). Transcription of the early genes is initiated at the early promoter (P97) and terminated by the early polyadenylation signal (polyA signal 1). The late genes are expressed from the late promoter (P670) to the polyadenylation signal 2.

Table 1. The major functions of HPV proteins.

Protein	Function
E1	ATP-dependent DNA helicase: required for replication of viral DNA, Maintenance of viral DNA as episomal, Mediates cell growth arrest by activation of ATM DNA damage response
E2	Initiation of viral DNA replication: directs E1 to the viral replication origin, Transcriptional activator: regulates viral and cellular gene expression, Induces growth arrest and senescence by down-regulating E6 and E7 expression, Maintenance of viral DNA as episomal, Virion encapsidation
E4	Replication of viral DNA, Cell cycle arrest, Encapsidation of viral genome and remodeling of cell cytokeratin network, Virion release
E5	(Functions studied mainly in high-risk HPVs) Enhances the cell transforming activity of E6 and E7: cell proliferation and productive viral replication and intracellular trafficking, Prevents immune response against infected cell by reducing the levels of MHC I complex at the cell surface
E6	(High-risk HPV) Cell transformation: inhibits cell growth arrest and apoptosis by degradation of p53 and modulation of TNF –reseptor activity, Prevents telomere shortening during cell proliferation by activation of TERT, Induces loss of cell polarity by targeting PDZ proteins
E7	(High-risk HPV) Cell transformation: induces cell hyperproliferation and productive viral life cycle by inhibition of the retinoblastoma (Rb) proteins, Alters cell cycle control and gene expression by interactions with histone deacetylases, cyclins and cyclin-dependent kinase inhibitors
E8	Forms a fusion protein E8^E2C with the C-terminal half of E2, which act as a inhibitor of the replication of viral genome replication
L1	Major capsid protein: encapsidation of viral genome, Cell attachment during infectious entry
L2	Minor capsid protein: encapsidation of viral genome, Virus entry into the host cell, Viral genome transfer into the cell nucleus

2.1.2 Classification

Papillomaviruses are highly host-specific viruses and are named according to their host species. All papillomaviruses belong to the taxonomic family *Papillomaviridae*. Human papillomaviruses are identified and classified by sequence analysis of the viral major capsid protein L1, which is the most conserved gene among papillomaviruses, and divided into five genera (alpha-, beta-, gamma-, mu-, and nu-papillomavirus) with different infection site tropism, life cycle characteristics and disease associations. Within each genus, HPVs are

classified into species or genotypes, subtypes and variants based on sequence homology within the L1 protein. Individual HPV types have at least 10% dissimilarity in their L1 gene sequences, whereas dissimilarity between subtypes and variants is 2-10% and <2%, respectively (de Villiers *et al.* 2004, Doorbar *et al.* 2012). To date, more than 200 HPVs have been classified (de Villiers. 2013).

HPV mainly infects epithelial cells of skin or mucosa. HPV genotypes in the alpha genus are found in mucosal or cutaneous sites, and some are found at both sites. This group includes mucosal HPVs, which are associated with cervical cancer and cancers in the head and neck area (Forman *et al.* 2012, Mehanna *et al.* 2013, Rautava & Syrjänen 2012), and are classified as high-risk types (Table 2). Low-risk HPV types in the alpha genus include both mucosal and cutaneous types, which are associated with benign lesions such as papillomas and skin warts. Most cutaneous HPVs belong to the beta and gamma genera and are not regarded as carcinogenic, although some beta HPV types are associated with skin cancers in immunosuppressed individuals (Doorbar *et al.* 2012).

Table 2. Alpha HPV –genotypes classified by their carcinogenity

Category	**Species**	**Type**
High-risk types	5	51
	6	56
	7	18, 39, 45, 59
	9	16, 31, 33, 35, 52, 58
Potential high-risk types	7	68,73
Potential high-risk types (close phylogenetic similarity to carcinogenic types)	5	26, 69, 82
	6	30, 53, 66
	7	70, 85
	9	67
Low-risk types	1	32
	2	28, 29, 77
	3	61, 62, 72, 81, 83, 84, 86, 87, 89
	4	2, 27, 57
	8	7, 40, 91
	10	6, 11, 13, 44, 74
	14	90, 106

2.2 HPV INFECTION AND PATHOGENESIS

2.2.1 Target cells and viral life cycle

Generally, a virus needs a host cell to manage the viral gene transcription and production of progeny virions. HPVs are considered strictly epitheliotropic, targeting the cells of the squamous cell epithelia of cutaneous or mucosal sites. The squamous epithelium is the outermost constantly renewing layer of the skin or mucosa, consisting of up to 30 layers of either keratinized or nonkeratinized cells called keratinocytes (Figure 2). The cells of the basal layer of the squamous epithelium continuously proliferate and generate new epithelial cells. Subsequently, after cell division one of the daughter cells starts to migrate upwards and differentiates to a fully keratinized corneocyte, which finally sheds off on the surface or superficial layer of the epithelium. HPV targets specifically undifferentiated proliferating basal cells, because progression of the host cell cycle through the early stages of mitosis in particular seems to be mandatory for the initiation of HPV gene transcription (Reinson *et al.* 2015). In addition to proliferation, the differentiation of the host cell also seems to be necessary for a successful viral cycle, as the productive replication and expression of capsid proteins L1 and L2 is known to occur only in terminally differentiated epithelial keratinocytes (Stubenrauch & Laimins 1999). However, placental trophoblasts have been shown to be broadly permissive of the HPV lifecycle despite the absence of epithelial-like differentiation in these cells, as presented later in ***Section 2.3.4 HPV DNA in placenta***, page 24.

The HPV viral lifecycle in general is illustrated in Figure 2. The basal cells of epithelia are exposed to HPV infection upon a minor wound or trauma. The virus gains access to the basement membrane (lamina propria) and binds to the heparan sulfate proteoglycans (HSPGs), which serve as primary attachment receptors (Abban & Meneses 2010, Schiller *et al.* 2010). The following conformational changes in the viral capsid result in virus binding to a yet unidentified secondary receptor, and further exposure of the N-terminus of the minor capsid protein L2 for cleavage by the proprotein convertase furin (Day & Schiller 2009). Binding to both HSPG and the secondary receptor depend on the major capsid protein L1 (Joyce *et al.* 1999, Schiller *et al.* 2010).

After the virus has attached to the host cell, it enters the cell, usually via a clathrin- or caveolae-mediated endocytic pathway, while various HPV types may use different pathways (Horvath *et al.* 2010, Schiller *et al.* 2010). Inside the host cell, the virus is transported and uncoated in endosomes. Then the viral genome forms a complex with L2, escapes the endosome and is transferred into the nucleus (Bergant Marusic *et al.* 2012). In the nucleus, the viral DNA is replicated during the S phase in synchrony with the host cell chromosomes, but the viral replication is also extended into the G2 phase, depending on the stage of the viral lifecycle and host cell differentiation (Reinson *et al.* 2015). In the basal cells, the virus maintains its genome as a low copy number episome, 10-200 copies per cell, under the control of viral E1 and E2 proteins. When infected cells leave the basal layer, they begin to proliferate,

and the viral episomes are divided into daughter cells. The subsequent terminal differentiation is retarded in infected keratinocytes at the same time as the activation of cell cycle progression by increasing the expression of viral early genes E6 and E7 (Banerjee *et al.* 2011). The viral genome amplification for the production of infectious virions occurs in the mid or upper layers of the epithelia, after which the activity of the viral early genes diminishes and cell differentiation is completed. The fully differentiated keratinocytes form new superficial layers, which displace the former superficial layers peeling off. The synthesized genomes are packaged into capsids composed of capsid proteins L1 and L2. Finally the new virions are released when the host keratinocyte comes to the end of its life and is degraded (Doorbar *et al.* 2015, Doorbar *et al.* 2012).

Due to variation in the regulation of viral gene expression and protein products, different HPV types have various lifecycle strategies and preferences for cells of epithelial origin at distinct anatomical sites and tissues. The specialized epithelial sites, such as hair follicles or tonsillar crypts, lack the multiple cell layers shielding the basal target cells beneath and so may serve as more vulnerable target sites for HPV infection as well as the transformation zone, the border between the stratified epithelium and simple columnar epithelium. The well-known infection sites for high-risk HPVs are the transformation zones in the cervix and the anal canal (Egawa *et al.* 2015).

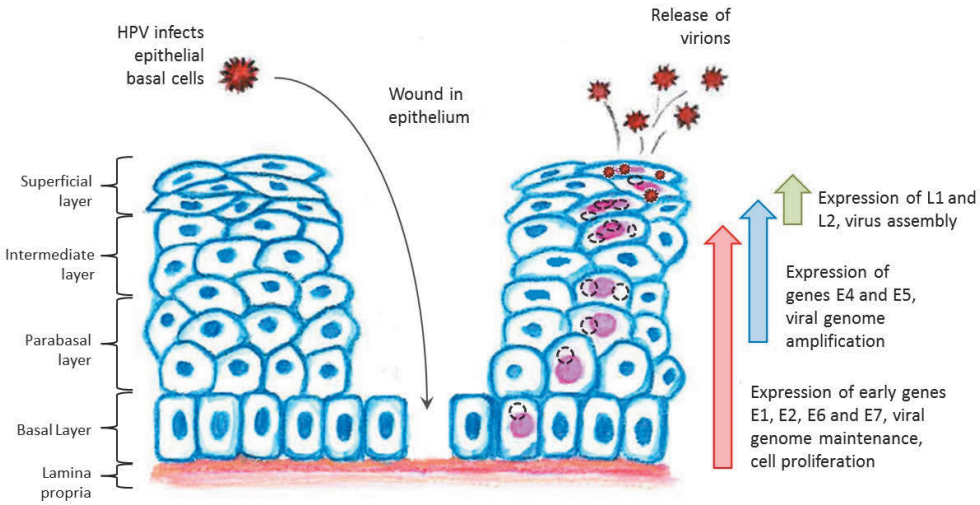


Figure 2. The structure of normal squamous epithelia and schematic view of the productive viral lifecycle. The cells with pink nuclei present infected keratinocytes and black circles indicate replication of the viral genome.

2.2.2 Differences in high-risk and low-risk HPV lifecycle strategies and the development of cancer

The general principles of the viral lifecycle are supposed to be relatively similar in all HPV types, with the main aim being to produce progeny virions by utilizing the replication machinery of the host cell. However, ordered viral protein expression may be disrupted in high-risk HPV-associated lesions. Integration of the viral genome into the host genome can disrupt the E2 gene, which normally suppresses the transcription of E6 and E7, resulting in increased levels of E6 and E7 proteins, which in turn facilitate cell transformation, immortalization and eventually the development of invasive lesion/cancer (Figure 3). Other mechanisms may also drive the deregulation of viral genes, such as hormonal changes and epigenetic modifications (Piccini *et al.* 1997, Vinokurova & Doeberitz 2011).

High-risk and low-risk HPV types show differences in their capability to induce proliferation and maintain a functional cell cycle in the infected host cell. This is due to the different functions of their E6 and E7 proteins (Doorbar *et al.* 2015). A crucial oncogenic function of high-risk E7 is the ability to bind and degrade the proteins of the retinoblastoma (pRb) family (proteins pRb/p105, p107 and p130), which are the central regulators of the normal cell cycle. These tumor-suppressor proteins regulate the activity of a number of important cellular transcription factors, such as the E2F-family, which in turn regulate the expression of genes whose products are important for cell cycle progression. E2F inhibition by the pRb proteins is required for cell cycle exit after terminal differentiation (Chellappan *et al.* 1991, Sidle *et al.* 1996). In the infected cell, the high-risk HPV E7 degrades the pRb proteins, allowing the E2F to also continuously activate the cell cycle progression at the upper epithelial layers, whereas the low-risk E7 has a weaker influence by targeting only one pRb protein (p130) with divergent mechanisms (Barrow-Laing *et al.* 2010, Dyson *et al.* 1989, Münger *et al.* 1989). In addition, the high-risk E7 has various other mechanisms to influence cell cycle deregulation, cell survival, immune evasion, anchorage independence and host genome instability (Pim & Banks 2010).

An important function of HPV E6 is to bind and deactivate the tumor-suppressor protein p53, which is an important factor in the DNA repair and initiation of cell cycle arrest or apoptosis upon aberrant cell cycle progression or DNA damage. Activation of p53 is also enhanced as a result of HPV E7-mediated dysregulation of the cell cycle and DNA synthesis (Doorbar *et al.* 2015, Scheffner *et al.* 1990, Werness *et al.* 1990). All HPVs can abrogate p53 function by inhibiting its acetylation, and low-risk HPVs may also mediate trapping of p53 in the cytoplasm (Pietsch & Murphy 2008, Thomas & Chiang 2005). High-risk E6 is also capable of mediating the degradation of the p53. Another major p53–independent oncogenic function of high-risk HPV E6 is the ability to activate the telomerase complex which maintains the telomeric repetitive ends of the chromosomes (Galloway *et al.* 2005). These chromosomal repeats normally become shorter upon each of the finite number of cell divisions, finally determining cell death. Thus, the E6-mediated activation of the telomerase is suggested to be important for cell immortalization induced by a high-risk HPV (Doorbar *et al.* 2015, Pim & Banks 2010). In

addition, E6 is known to bind a large number of cellular proteins containing a common structural domain PDZ (Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and Zonula occludens-1 protein (zo-1)), involved in the signaling and regulation of cell polarity, cell adhesion and differentiation. This capability to bind PDZ proteins is suggested to be important for E6 contribution to cancer progression (Kranjec & Banks 2011).

2.2.3 Natural course and manifestations of an HPV infection

HPV is the most common sexually transmitted infection worldwide (de Sanjose *et al.* 2007), however most of these infections are eradicated spontaneously as a result of the host's successful immune response (presented later in ***Section* 2.4.4 Regression of HPV infections and HPV-associated lesions due to cell-mediated immune response**, page 31) within 1-2 years (Franco *et al.* 1999, Louvanto *et al.* 2010b). Generally, infections that are not cleared, i.e., the same HPV type is detected in two consecutive examinations, are considered persistent. However, the literature is still lacking a generic definition for persistent HPV infection as well as guidelines for the appropriate interval between examinations. High-risk HPV types have been shown to persist longer than low-risk types (Louvanto *et al.* 2010a, Trottier *et al.* 2008).

Since the establishment of their strong association with cervical cancer, the natural history of genital HPV infections in women is the most studied and described (Figure 4), while the features of HPV infections in other anatomical sites are not that well-known. Clinically, HPV infections are divided according to the presence of viral DNA or clinical manifestations in the epithelial tissue. Asymptomatic HPV infections, which cause no cytological or histological changes, are regarded as latent infections (Syrjänen *et al.* 1990a). In these cases, the viral DNA can only be detected using sensitive molecular methods. In subclinical infections, there are only minor changes in the epithelial histology, which are not consistent with the typical features of clinical HPV lesions. In the case of clinical infection, the productive HPV infection has induced visible cytological and histological alternations, such as abnormal cellular proliferation and koilocytosis (degenerative changes in the cell cytoplasm and nucleus), lesions such as warts and papillomas, or dyplasias from low-grade to cancerous (Muñoz *et al.* 2006, Richart 1990, Syrjänen & Syrjänen 2000).

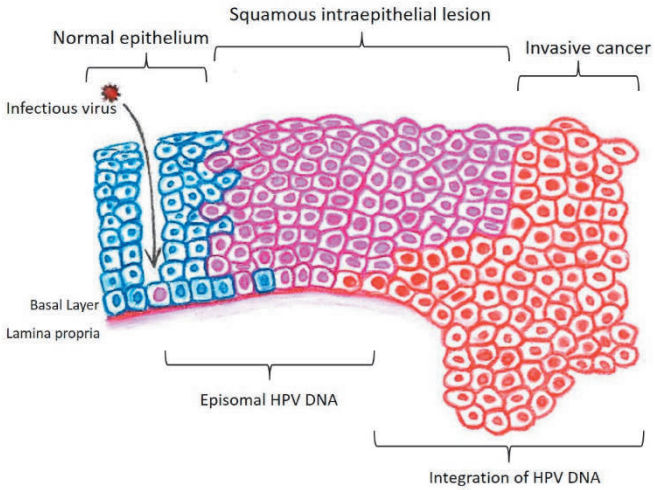


Figure 3. Progression of HPV-induced lesion to invasive cancer. Most HPV infections are eradicated spontaneously as a result of activating an immune response. If this fails, the infection may persist and progress into a high-grade intraepithelial lesion or invasive cancer. This progression is associated with the integration of the human papillomavirus genome into the host genome, loss or disruption of viral E2 gene function and subsequent upregulation of E6 and E7 oncogene expression.

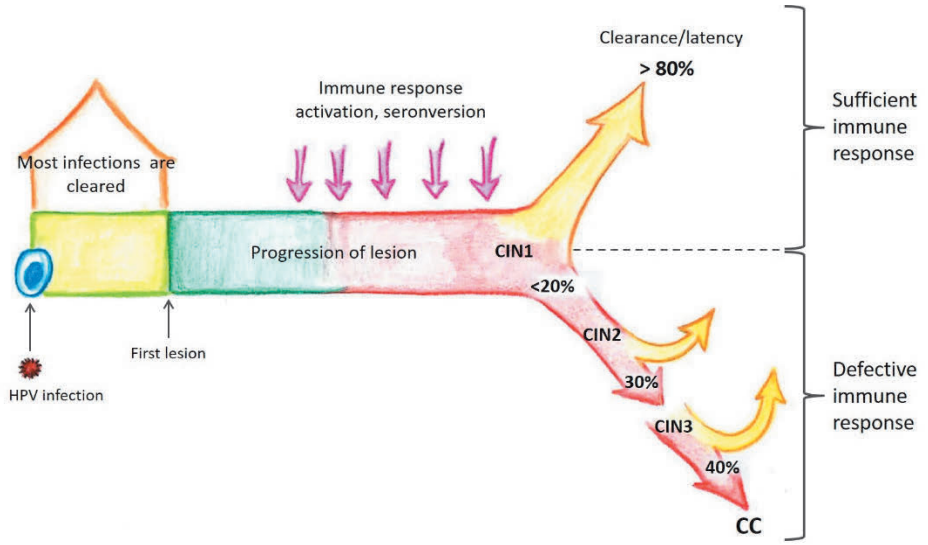


Figure 4. The natural history of cervical high-risk HPV infection. The primary infection is usually followed by months of asymptomatic time during which most of the infections are cleared by an effective immune response. Infection may progress into a low-grade lesion or CIN1 (cervical intraepithelial neoplasia, grade 1). Eventually, in most cases, the host immune response is activated, which in turn results in lesion regression. In the case of a defective or lacking immune response, the lesion will progress into invasive cancer (CC) through a high-grade lesion (CIN2-3).

2.2.4 Detection of HPV DNA and RNA

Papillomaviruses cannot be cultured and the only reliable way to determine the presence of HPV in the tissue is to detect viral nucleic acids by sensitive molecular methods. Southern blot and in situ hybridization (ISH) are nucleic acid hybridization methods and have been widely used in HPV detection. They based on labeled oligonucleotide probes, which have a nucleic acid sequence complementary to that of the target HPV DNA or RNA. Probes recognize and bind their target sequences in the sample after which they can be detected.

The most sensitive methods for HPV DNA detection are based on polymerase chain reaction (PCR). In PCR, HPV DNA present in a sample can be amplified exponentially using consensus oligonucleotide primers, which usually target the highly conserved L1 gene, and the products can be identified on agarose gel electrophoresis. The most commonly used primer pairs for HPV DNA detection are GP05+/GP06+ (Snijders et al. 1990) and MY09/MY11 (Gravitt et al. 2000, Qu et al. 1997). Nested PCR can be used for higher sensitivity and specificity if the sample contains a low copy number of HPV DNA or a limited amount of cells. In nested PCR, the initial PCR products are further amplified using internal primers targeting the sequence within the first PCR product.

Multiplex HPV genotyping is a high throughput hybridization method for the simultaneous detection of multiple HPV genotypes in one sample (Schmitt et al. 2006). The method is based on fluorescent-labeled polystyrene beads coupled to HPV type-specific oligonucleotide probes, which are hybridized with HPV DNA amplified by PCR (GP05/06 primers). Beads in probe-DNA hybrids are identified by their individual fluorescence. Multimetrix® assay is one of the technologies available for multiplex HPV genotyping.

2.3 HPV EPIDEMIOLOGY AND TRANSMISSION

2.3.1 HPV infections in adults

Genital HPV infections are very common among the adult population and the majority of adult individuals will acquire an HPV infection at some time point in their life. Because of the high frequency of subclinical or latent infections, it is difficult to determine the exact lifetime risk of HPV infection (Stanley 2010). According to an epidemiological study conducted among young women in Finland, up to 79% of females acquire an HPV infection during adult life (Syrjänen *et al.* 1990b). Worldwide, 10.2% of women with normal cytology carry cervical HPV, while the prevalence varies between geographical regions (Clifford *et al.* 2005, de Sanjose *et al.* 2007). In men, the prevalence of genital HPV infection is as high or even higher than in women (Dunne *et al.* 2006, Giuliano *et al.* 2010, Hippeläinen *et al.* 1993). The highest prevalence of genital HPV infections is found in young adults, 18-28 years of age (Koutsky 1997), who might be regarded as the most sexually active age group. The association of HPV with a subgroup of head and neck cancers was presented as early as in 1983, but the topic remained controversial

until the late 1990s. The role of asymptomatic HPV infections in the head and neck region has remained less studied than those of genital sites. The overall prevalence of oral HPV infections or HPV DNA among healthy adults varies between 4.5% and 12% (Kreimer *et al.* 2010, Syrjänen *et al.* 2011), with no difference found between genders. In contrast, a cross-sectional study conducted in the United States reported a significantly higher prevalence for any oral HPV infection in men (10.1%) than in women (3.6%) (Gillison *et al.* 2012). HPV 16 has been the most frequently detected HPV genotype in oral and genital sites among both genders (Bruni *et al.* 2010, Gillison *et al.* 2012, Giuliano *et al.* 2010).

The most common diseases caused by low-risk HPVs are benign mucosal lesions such as condylomas and papillomas in anogenital or oral sites (Table 3). A relatively rare HPV-related condition is adult-onset recurrent respiratory papillomatosis (RRP), with the development of multiple papillomas most commonly in the larynx, which can be a life-threatening disease as it obstructs the airways (Derkay & Wiatrak 2008, Kashima *et al.* 1993, San Giorgi *et al.* 2016). The most important HPV-related diseases are cervical cancer in women and a subset of head and neck cancers, especially oropharyngeal cancer, in both genders (Syrjänen *et al.* 2011). In addition, anal cancer is the most common HPV-related anogenital cancer among men (Giuliano *et al.* 2010). HPV infection and HPV-associated lesions and cancers are more prevalent in individuals with HIV (Human immunodeficiency virus) infection, proposing that the natural immunological defense mechanisms against HPV are also failing due to immunodeficiency resulting from HIV infection (Chin-Hong & Palefsky 2005).

Table 3. HPV types in associated diseases.(Modified from Cubie 2013, Forman et al. 2012).

Disease		**Most frequent HPV types**	**Estimated HPV prevalence (%)**
Common warts		HPV 2, 4, 7	
Flat plane warts		HPV 3, 10	
Plantar warts		HPV 1, 2, 4	
Anogenital warts		HPV 6, 11, 40, 42, 43, 44, 54, 61, 72, 81, 89	
Anogenital cancers and precancers	High-risk types	HPV 16, 18, 31, 33, 45, 51, 52	Cervical carcinoma 100.0
	Probable high-risk types	HPV 68	Penile carcinoma 50.0
	Possible high-risk types	HPV 26, 53, 64, 65, 66, 67, 69, 70, 73, 82	Anal carcinoma 88.0
			Vulvar carcinoma 43.0
			Vaginal carcinoma 70.0
Oral lesions	Oral papillomas	HPV 2, 6, 7, 11, 16, 18, 32, 57	
	Laryngeal papillomas	HPV 6, 11	
	Focal hyperplasia	HPV 13, 32	
	Head and neck carcinomas	HPV 16, 18	Head and neck carcinomas 13.0-56.0

2.3.2 HPV infections in children

HPV infections in children have been mostly associated with benign skin and oral warts and oral papillomas (Syrjänen & Puranen 2000, Syrjänen 2010). Skin warts are usually caused by cutaneous beta HPV types, and are most likely acquired during early infancy. However, since warts are rare in young children under 2-5 years of age, it is suggested that these cutaneous HPV types can persist asymptotically over a long time (Hsu *et al.* 2009, Syrjänen 2010).

Common warts (verruca vulgaris) are more prevalent in children than plantar (myrmecias) or flat warts (verruca plana). Estimations of the incidence of skin warts among children vary from 2.8% to 7.1% and have been reported to increase from 5 years to 11-14 years of age and then rapidly decline by the age of 20 years (Syrjänen. 2010). Oral warts and papillomas are considered the most common oral epithelial tumors in children, while systematic studies on their prevalence in children are lacking. Observations of HPV prevalence in these lesions vary from 43% to 100%, including the adult population (Castro & Filho 2006, Syrjänen 2010). Juvenile-onset RRP (JO-RRP) in children is mainly associated with HPV genotypes 6 and 11, and usually emerges before five years of age. JO-RRP has a tendency to appear more severely in younger individuals, often requiring multiple surgical interventions. The estimated incidence of JO-RRP varies from 0.24 to 4.3 per 100,000 children (Campisi *et al.* 2010, Derkay & Wiatrak 2008, Syrjänen 2010).

The presence of anogenital warts in a child usually involves implications about sexual abuse, while in the majority of cases the source of infection remains unknown after the exclusion of the possibility of abuse. The overall prevalence of anogenital warts in children is unknown, while the average age of appearance is 2.8-5.6 years (Sinclair *et al.* 2011). The most common genotypes found in anogenital warts are HPV 6 and 11, followed by cutaneous HPV types and high-risk types HPV 16 and 18 (Syrjänen & Syrjänen 2000). The majority of anogenital warts resolve spontaneously over months to a few years, while longer persistence might indicate defects in the immune system of the child (Culton *et al.* 2009).

In addition to HPV-associated diseases with visible changes in the epithelia, HPV DNA has been detected in samples from mucosal sites of children at various time points starting from birth. The adequate determination of HPV DNA is relatively difficult, because the detection of viral DNA is restricted to the cells in the sample. The varying sampling methods may partially explain the high variation in the reported HPV prevalence rates. The detection rates of oral HPV DNA in children aged 0.3-11.6 years varies from 0% to 47% (Jenison *et al.* 1990, Koch *et al.* 1997, Puranen 1996, Rice *et al.* 1999, Rice *et al.* 2000, Watts *et al.* 1998), while in infants from birth to four days of age the variations were even higher, from 0.9% to 56% (Cason *et al.* 1995, Kaye *et al.* 1994, Pakarian *et al.* 1994, Puranen *et al.* 1997, Smith *et al.* 2010, Smith *et al.* 2004, Smith *et al.* 1995, Tseng *et al.* 1998). Similarly, the same studies report distinct prevalence rates for genital HPV infections varying between 0%–53% in newborns and between 0%-33% in infants (from six weeks to six months old). It seems that the majority of early HPV infections are transient and clear within one year after delivery (Syrjänen 2010)

2.3.3 HPV transmission modes

Alpha-papillomavirus infections are considered to be transmitted primarily through genital-to-genital or genital-to-oral contacts during sexual intercourse. Still, the detection of HPV infection in virgins, infants and children calls for studies on non-sexual transmission: vertical and horizontal transmission and autoinoculation. Horizontal transmission may take place

through breast-feeding and other close contacts such as from siblings, caretakers and friends (Cason 2005, Czeglédy 2001, Syrjänen 2010). However, in some studies no association was established between HPV DNA in breast milk and HPV detection in newborns (Cazzaniga *et al.* 2009, Sarkola *et al.* 2008b). Autoinoculation of HPV denotes the relocation of the virus from one body site to another by scratching (Sonnex *et al.* 1999, Syrjänen & Puranen 2000).

Vertical transmission

Vertical transmission of HPV from mother to child can be assumed to take place during regular nursing and childcare procedures. A recent meta-analysis of twenty published studies of vertical transmission showed that children of HPV-positive mothers had a 33% higher risk of becoming HPV-positive during the first weeks after birth than children of HPV-negative mothers (Merckx *et al.* 2013). Vertical transmission can be divided into three subcategories.

Peri-conceptual transmission is suggested to take place during fertilization or thereupon. Several studies have reported the detection of HPV DNA in different sites of the reproductive tract, in the endometrium and in ovaries in females (Fedrizzi *et al.* 2009, Lai *et al.* 1992, O'Leary *et al.* 1998) and in the urethra, vas deferens, penile brushes and semen in males (Foresta *et al.* 2010a, Foresta *et al.* 2010b, Rintala *et al.* 2004, Rintala *et al.* 2002). *In vitro* studies have shown that spermatozoa are able to transport HPV into oocytes, and also reported the transcription of HPV genes in fecund oocytes (Bodaghi *et al.* 2005, Foresta *et al.* 2011, Giovannelli *et al.* 2007, Griffiths & Mellon 2000, Rintala *et al.* 2002). Further, a study by Lai and co-workers also reported the transcriptional activity of HPV 16 in sperm *in vivo* (Lai *et al.* 1996).

Prenatal transmission of HPV is considered to occur in utero during pregnancy, which could explain the observations of HPV-induced lesions in newborns as early as at birth and the birth of HPV DNA-positive newborns to mothers tested negative for HPV (Syrjänen 2010). Intrauterine transmission can take place through at least two alternative modes (Figure 5). The placenta and chorionic tissue can be infected, presumably through maternal blood, after which HPV may infect amniotic cells that are subsequently ingested by the fetus. However, it is still unclear as to whether viremia and hematogenous transmission of HPV take place. Alternatively, infection could spread via the ascending route from the maternal genital tract and through microtears in fetal membranes or in blood through the placenta. (Freitas *et al.* 2013, Syrjänen 2010). Several studies reporting HPV DNA detection in amniotic fluid, placenta and umbilical cord blood support the view of intrauterine transmission (Armbruster-Moraes *et al.* 1994, Burguete *et al.* 1999, Favre *et al.* 1998, Gajewska *et al.* 2005, Gomez *et al.* 2008, Rombaldi *et al.* 2008, Tseng *et al.* 1992, Uribarren-Berrueta *et al.* 2012, Wang *et al.* 1998, Weyn *et al.* 2010, Worda *et al.* 2005)

Perinatal transmission occurs during vaginal delivery when the fetus is in close contact with the infected cervical and vaginal cells of the maternal birth canal. Merkx and co-workers reviewed several studies that reported the detection of HPV DNA in the cervical mucosa of

the mother prior to the delivery and in nasopharyngeal or genital samples of the newborn (Merckx *et al.* 2013). The HPV transmission rate was shown to be higher in vaginal delivery (18%) than in cesarean section (8%) in the systematic review by Medeiros and co-workers (Medeiros *et al.* 2005). In addition, the observations of Kaye and co-workers showed that mothers who transmitted HPV infection to their newborns had significantly higher viral loads than those who did not (Kaye *et al.* 1994). However, the discrimination of true infections from transient contaminations is possible only by subsequent sampling during follow-up (Syrjänen 2010).

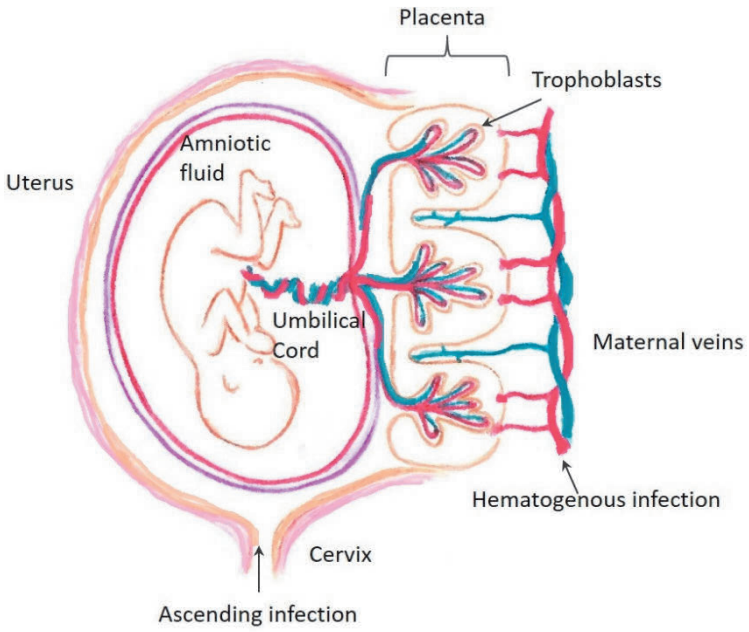


Figure 5. Alternative routes for intrauterine transmission of HPV. HPV can be transmitted to placenta and chorionic cells such as trophoblasts via the hematogenous route through maternal blood or the ascending route from the maternal genital tract. The fetus may then acquire HPV by ingesting infected cells along with amniotic fluid or through umbilical cord blood.

2.3.4 HPV DNA in the placenta

Previous observations in the Finnish Family HPV Study showed that a HPV DNA-positive placenta increases the risk of the newborn carrying oral HPV (Sarkola *et al.* 2008a). This could implicate the important role of placenta in the transmission of HPV, similar to other viruses (Younes *et al.* 2009). However, the reported detection rates of HPV DNA in the placenta show wide variation, from 0% to 47.2% (Eppel *et al.* 2000, Favre *et al.* 1998, Gomez *et al.* 2008, Rombaldi *et al.* 2008, Uribarren-Berrueta *et al.* 2012, Wang *et al.* 1998, Worda *et al.* 2005). Placental trophoblasts have been shown to be broadly permissive for HPV, and HPV types 11, 16, 18, and 31 have been shown to fully replicate in trophoblast cells *in vitro* (You *et al.* 2002, You *et al.* 2008). Reports of the transfer of maternal cells, such as lymphocytes, through the

maternal-fetal barrier suggest the possibility that the virus could pass through as a passenger, as has been observed for hepatitis B virus and herpes virus (Younes *et al.* 2009).

Pregnancy is associated with immunological changes which may increase the risk of infection by certain viruses, such as HIV, cytomegalovirus, Epstein-Barr virus, and HPV (Poole & Claman 2004, Younes *et al.* 2009). In addition, the elevated steroid hormone levels during pregnancy are suggested to increase replication of HPV (Mittal *et al.* 1993). Indeed, a systematic review of HPV prevalence in pregnant and non-pregnant women reported an increased risk of HPV infection in pregnant women (Liu *et al.* 2014). Further, HPV infection is observed to be more prevalent in women whose pregnancy results in an adverse outcome, such as spontaneous abortion or preterm delivery, than in women with normal full-term pregnancy (Ambuhl *et al.* 2016). This could be partly explained by observations that HPV oncogenes affect trophoblast viability and the adherence of endometrial cells (Boulenouar *et al.* 2010, You *et al.* 2002). By contrast, in the Finnish Family HPV Study, pregnancy was associated with both decreased clearance of existing HPV infections and increased protection against new incidents of HPV infections (Louvanto *et al.* 2014, Louvanto *et al.* 2013) However, the reported HPV prevalence rates among pregnant women vary greatly, from 5.5% to 65.0% (Medeiros *et al.* 2005), depending on the type of sample tested and the geographical location of the study population. Thus, studies on HPV infections and their prevalence during pregnancy are controversial and provide no consistent evidence of a difference between pregnant and non-pregnant women.

The concomitant transfer of small doses of virus and maternal neutralizing antibodies from mother to infant through the placenta might establish favorable conditions for the activation of the infant's immune response, while the infection is weakened by the maternal antibodies, as suggested by Zinkernagel (Zinkernagel 2001). Accordingly, the nature of the maternal infection prior to or during pregnancy i.e. immunological response and formation of antibodies, might be the most important determinant for the infection outcome in her infant (Zinkernagel 2001).

2.4 HOST IMMUNE RESPONSE AND HPV IMMUNE EVASION STRATEGIES

2.4.1 Innate and adaptive immunity

The human immune system is made up of two barriers of distinct cells and molecules. Natural or innate immunity is responsible for quickly blocking the entry of or detecting various pathogens invading the body, and finally for activating the cells of the adaptive immunity. The epithelia create the outermost physical and chemical shield against infection, while the cells and proteins of the innate immune system patrol in the tissues and circulation. Neutrophils, monocytes and macrophages are phagocytes which ingest the pathogen and destroy it, while natural killer cells kill the infected and stressed cells. In addition, numerous proteins, such as enzymes and cytokines secreted by different cells, mediate signaling pathways and cellular

reactions (Kupper & Fuhlbrigge 2004). Activation of adaptive immunity is induced by antigen-presenting cells (APCs), which are specialized in capturing foreign antigens at any site in the body and displaying them to the T cells in the lymph nodes. The activated, antigen-specific T cells in turn help the B cells to produce antibodies, which recognize foreign antigens outside the cells and subsequently neutralize and eliminate them. This arm of adaptive immunity is called humoral immunity**.** T cells mediate the defense against intracellular pathogens, extracellular parasites or bacteria and fungi and form the other arm of adaptive immunity, cell-mediated immunity (CMI). Helper T (Th) cells activate and help the functions of other immune cells, for example, phagocytes to destroy pathogens and cytotoxic T cells (CTLs) to kill infected cells (Gasteiger & Rudensky 2014).

2.4.2 Activation of immune response during HPV infection

HPV is not capable of infecting cells of intact epithelia, but it gets access to basal keratinocytes through a minor wound or trauma. Wound healing is associated with infiltration of inflammatory cells such as neutrophils and macrophages, although the role of inflammation in HPV infection is not well-known. Observations are contradictory: infiltration of macrophages expressing the pro-inflammatory cytokine TNF-α correlated with the regression of papillomavirus infection in rabbits (Hagari *et al.* 1995). On the other hand, increased number of macrophages in HPV-induced cervical lesions in humans was shown to be associated with infection persistence or progression (Castle *et al.* 2001, Hammes *et al.* 2007).

Similar to many viruses, HPV also has several mechanisms for evading the immune defense of the host. Because the lifecycle of HPV is strictly intraepithelial and dependent on the differentiation of the target cell, the local immune cells are not alarmed by the ongoing infection. However, the infected keratinocytes can sense pathogens through pathogen recognition receptors (PRRs), which recognize invariant structures in microbes, such as the HPV capsid proteins L1 and L2 and the viral genome. The best studied PRRs are Toll-like receptors (TLRs) expressed on the cell surface or in endosomes. Other PRRs in keratinocytes include protein kinase R (PKR), and RNA helicases RIG-I and MDA5 (Kalali *et al.* 2008). PRR activation by pathogen ligation results in the activation of cellular transcription factors such as NF-κB (nuclear factor kappa-B) and IRF-3 (interferon response factor-3) and the subsequent production of various cytokines and endothelial adhesion molecules (Nestle *et al.* 2009). In resolving cervical HPV infections, the expression of TLRs on keratinocytes is significantly increased, while progressive diseases are associated with the reduced expression of TLRs (Daud *et al.* 2011, Scott *et al.* 2015).

Type I interferons (IFNs) IFN-α and IFN-β are important cytokines produced in response to viral infection and the activation of PRRs. They inhibit cell proliferation and replication of the viral genome. They also have immunostimulatory effects, i.e., they attract and activate surrounding immune cells. Furthermore, IFNs induce the transcription of a multitude of genes, acting as a bridge between innate and adaptive immunity (Kanodia *et al.* 2007, Le Bon & Tough 2002).

However, HPV, as many other viruses, has evolved mechanisms to interfere with IFN signaling. High-risk HPV E6 and E7 proteins inhibit IFN synthesis and alter the host's antiviral response to infection. In addition to IFNs, high-risk HPV is known to alter the production and secretion of other cytokines, chemokines and adhesion molecules evolved to mediate immune cell activation and their migration to the site of infection (Kanodia *et al.* 2007, Karim *et al.* 2011, Stanley & Sterling 2014). Altogether, HPV dampens warning signs from the infected cell to surrounding cells and to the immune system (Figure 6).

Since HPV gene expression is restricted to keratinocytes, the activation of the T cells of the adaptive immune system is dependent on cross-presentation of HPV antigens by local APCs. Dendritic cells (DC) represent professional APCs in distinct tissues. Several different DC types have been identified (Reviewed by Hovav 2014). DCs in the parabasal and lower suprabasal layers of the squamous epithelia are called Langerhans cells (LCs), and DCs in the lamina propria of the oral mucosa or in the dermis of skin are called interstitial DCs (iDCs) (Figure 6). Characterization of oral DCs in humans is still limited and focused mainly on LCs. However, high resemblance is observed between mouse and human oral DCs (Hovav, 2014). According to their function, LCs should take up the HPV antigens and become activated via the induction of signaling cascades, upregulation of co-stimulatory molecules and release of pro-inflammatory cytokines. Subsequently, the activated LCs should migrate to the lymph node and finally present the antigens to naïve T cells (Malissen *et al.* 2014, Stanley & Sterling 2014). However, some observations suggest that LCs are not properly activated *in vitro* by interaction with HPV virus-like particles (VLPs) (Fausch *et al.* 2005, Fausch *et al.* 2002, Fausch *et al.* 2003). This may indicate that LCs are not capable of initiating an epitope-specific immune response against HPV-derived antigens, which is in agreement with the poor immunostimulatory functions of HPV.

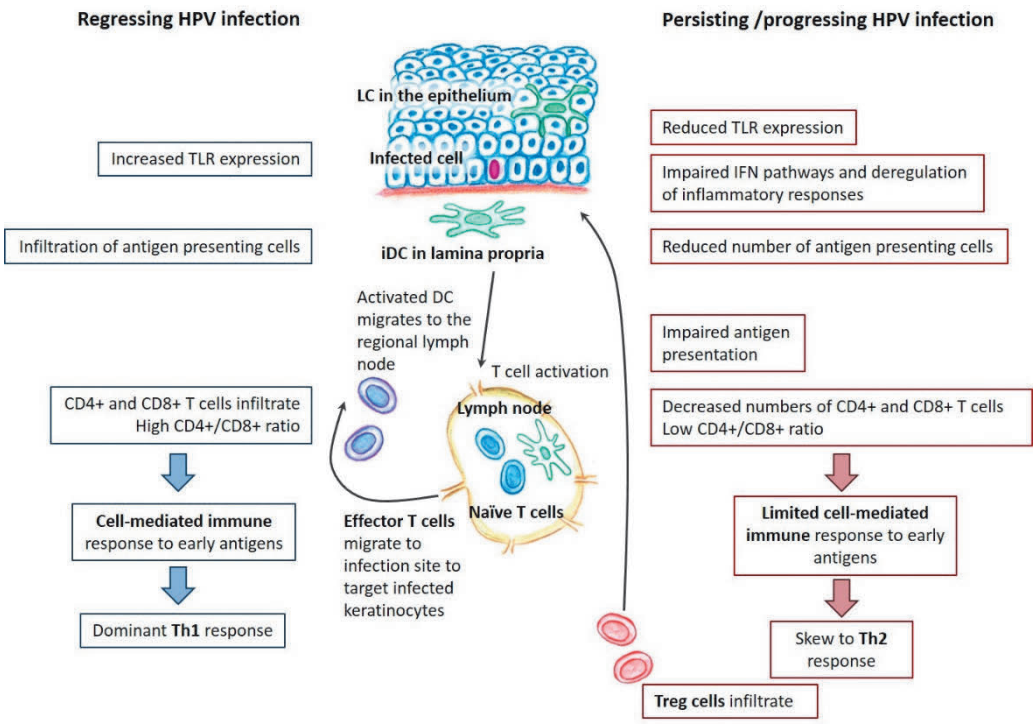


Figure 6. The activation of the host immune response upon HPV infection (in the oral mucosa). The key characteristics of the immune responses in regressing and persisting or progressing HPV infection are presented.

In addition, high-risk HPV infections have been associated with decreased frequency of LCs in the female genital mucosa (Jimenez-Flores *et al.* 2006, Leong *et al.* 2010). Expression of E-cadherin, which is essential for the retention of LCs in epithelia, is decreased in HPV-infected keratinocytes with consequent decreased inadhesion between the keratinocyte and LC (D'Costa *et al.* 2012). Further, in HPV16-infected cells, the expression of chemokine MIP3α is reduced, inhibiting the attraction and migration of LC precursors in the infection site (Guess & McCance 2005). By contrast, the iDCs in the dermis are activated by HPV VLPs and are also able to stimulate T cells (Fausch *et al.* 2003). Indeed, in mice, the migration of the LCs of the oral buccal mucosa to the lymph nodes is much slower than the migration of the buccal iDCs or LCs of the skin. Moreover, buccal LCs express low levels of co-stimulatory molecules (Hovav 2014). Thus, the immune induction mechanisms of mucosal DCs are significantly different from those of skin or other body sites.

2.4.3 T cell differentiation and function

The naïve T cells of adaptive immunity develop in the bone marrow, mature in the thymus and then travel between the blood circulation and lymph nodes. T cells express a wide variety of T cell receptors (TCRs, CD3), which each recognize a specific antigen presented by an APC. If the APC (DC or LC) becomes activated upon viral infection, it subsequently migrates from the tissue to the nearest lymph node, where it encounters a naïve T cell and activates it by presenting the viral antigen (Reviewed by Guermonprez *et al.* 2002) (Figures 6 and 7). The activated T cell starts to secrete autocrine cytokines that induce T cell proliferation. These clonally expanded T cells differentiate into either effector T cells, which have many functions in cell-mediated immunity, or into long-lived pre-activated T cells that are generally defined as memory T cells (Sallusto *et al.* 2004).

T cells are divided into two classes: CD8+ and CD4+ T cells. Activated T cells expressing the CD8 molecule are defined as cytotoxic T cells (CTLs), since they induce programmed cell death in infected cells by secreting antiviral cytokines INF-γ and TNF-α and cytotoxins. T cells expressing CD4 molecule are defined as helper (Th) cells, and they act as important regulators of the immune response by secreting various cytokines, proteins or peptides that stimulate or interact with other cells. Activation of T cell in the lymph node is mediated by a major histocompatibility complex (MHC) on the surface of APC, which is recognized by the TCR of the naïve T cell. There are two classes of MHC proteins, which differ in function. Class I MHC proteins are expressed in all eukaryotic cells, and they present cytosolic peptides that are mostly synthesized in the cell. In the infected cells, Class I MHC presents viral proteins, which are then recognized by the CD8+ cytotoxic T cells. Class II MHC proteins are expressed mainly in specialized APC cells (DCs, macrophages and B cells), where they bind and present extracellular proteins ingested by the APC. The antigen presented in Class II MHC is recognized by CD4+ T cells. Thus, DCs or LCs are the only cells capable of activating naïve CD4+ T cells.

CD4+ T cells can differentiate into distinct functional phenotypes depending on the cytokines secreted by the APC upon T cell activation (Figure 7). Type 1 helper cells (Th1) secrete pro-inflammatory and antiviral cytokines INFγ and TNF-α, stimulating macrophages and promoting inflammation. Th1 cells also stimulate CD8+ CTLs by secreting IL-2. Type 2 helper cells (Th2) produce IL-4 and IL-5 and stimulate the antibody production of B cells and thus the response against extracellular pathogens. (Mosmann *et al.* 1986, Romagnani 2000, Wilson & Barker 2013). Th17 cells are named after the cytokine IL-17 that they secrete upon activation, and are known to play a protective role in the host's defense against pathogens at epithelial and mucosal sites. In addition, Th17 cells have been linked to several autoimmune diseases and seem to have both anti-tumorigenic and pro-tumorigenic activities (Zou & Restifo 2010). Regulatory T cells (Tregs) suppress other T cells or APCs by secreting suppressive cytokines IL-10 and TGFβ or by consuming cytokines needed by other immune cells. Their function is necessary in the control of the immune response and in immunological tolerance (Mills. 2004). Natural nTregs are developed in the thymus while adaptive or induced iTregs are activated by

APCs in the secondary lymphoid organs (Mills & McGuirk 2004). Transcription factor Foxp3 is used as a specific marker for detecting natural and induced Tregs.

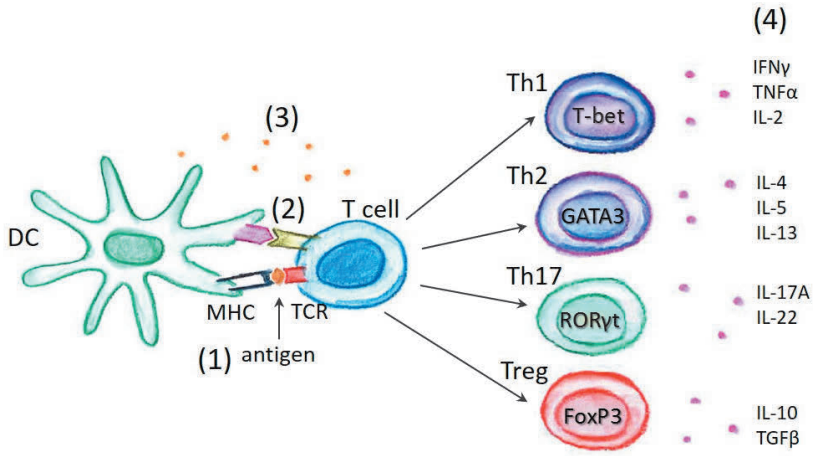


Figure 7. Activation of a naïve T cell in the lymph node and differentiation into effector T cells. LC or DC presents the viral antigen to a T cell via a major histocompatibility complex (MHC), which is recognized by the TCR of the naïve T cell (1). The full activation requires secondary antigenor molecule signals that can be either stimulating or inhibitory (2). In addition, the DC produces a variable panel of cytokines (3), which directs the differentiation of the distinct T cell subsets, which are characterized by the expression of certain transcription factors and cytokines (4).

After the infection has been resolved, the immune system has to return to its steady state. Without the activation signals, the majority of effector T cells die by apoptosis. The pre-activated T cells or memory T cells survive after the elimination of the infection, and are found throughout the body, prepared to respond to new infection. They are functionally silent until they encounter their specific antigen and rapidly start their effector functions. (See review by Sallusto *et al.* 2004).

2.4.4 Regression of HPV infections and HPV-associated lesions due to cell-mediated immune response

Most HPV infections and HPV-induced lesions regress spontaneously as a result of a successful cell-mediated immune response (Stanley & Sterling 2014). Th1 cells are mostly involved in CMI reactions and stimulate the function of CTLs, which in turn play a major role in the control of infection by killing the infected cells. Indeed, studies of HPV 16-specific immunity have shown that both CD8+ CTLs and CD4+ Th cells specific to viral early proteins are present in the circulation in healthy individuals accompanied by a balanced expression of type 1 (Th1) cytokines IFNγ, TNFα and IL-2, and type 2 (Th2) cytokines IL-4 and IL-5 (de Jong *et al.* 2004, Nakagawa *et al.* 1997, van den Hende *et al.* 2008, Welters *et al.* 2003). Similar results have

been reported for HPV 18 (Welters *et al.* 2005). Further, spontaneously regressing HPV-induced genital lesions are found to be infiltrated by CD8+ CTLs and CD4+ cells (Coleman *et al.* 1994, Trimble *et al.* 2010, Woo *et al.* 2008). The ratio between CD4+ and CD8+ T cells also increases in regressing CIN lesions compared to persisting or progressing higher-grade lesions (Monnier-Benoit *et al.* 2006, Väyrynen *et al.* 1985). In contrast, in individuals with progressive HPV-induced disease, the T cells specific to HPV early proteins are lacking or the cytokine environment is skewed toward type 2 cytokines, implicating the predominance of Th2 cells (de Jong *et al.* 2004, Nakagawa *et al.* 1997, Woo *et al.* 2010, Xu *et al.* 2009).

Tregs are suggested to suppress the virus-specific immunity; HPV-specific, adaptive Tregs have been isolated from HPV-induced cervical lesions and cancers and in draining lymph nodes (de Vos van Steenwijk et al. 2008, Jaafar et al. 2009, Scott et al. 2009, van der Burg. 2007). Furthermore, significantly higher Treg frequencies are observed in women with persistent HPV 16 infection than in HPV-negative women (Molling et al. 2007).

2.4.5 Antibody response to HPV infection

The HPV infection-induced cell-mediated immune response should be followed by seroconversion, i.e., B cells being activated and starting to produce antibodies against HPV L1 proteins. The immune function of neutralizing antibodies upon viral infection is the neutralization of viruses by binding the viral capsid and preventing its attachment to a target cell. However, as the infection of new cells by the same HPV type is foiled by the antibodies produced, they cannot neutralize the virus in infected cell. Further, the capability of HPV infection-induced antibodies to provide protection against reinfection by the same type is also currently under debate (Mollers *et al.* 2013). However, the serum antibodies can be used as markers for ongoing or previous infections (Carter & Galloway. 1997, Gissmann 1996).

Antibodies or immunoglobulins are divided into five classes (IgM, IgD, IgG, IgA and IgE) according to their special functions. IgA is the most prevalent antibody class in the body and plays an important role as a first-line barrier against intestinal antigens in the gut mucosa. Further, class IgA controls the intestinal microbiota and pro-inflammatory immune responses. (Pabst 2012) Class IgG is present mainly in the serum in the mg/mL range, and antibodies of this class are also transported across the placenta from mother to immunologically incompetent fetus to provide protection against possibly life-threatening early infections (Zinkernagel 2001). Seroconversion of both IgA and IgG after natural genital HPV infection is considerably slow and takes on average 11-13 months after the detection of HPV types 6, 16 or 18 (Carter *et al.* 2000, Dillner 1999). It is estimated that only 40–60% of women with cervical HPV DNA seroconvert, and in these women, the observed antibody titers are often very low (Mollers *et al.* 2013). Most transient HPV infections do not result in a detectable antibody response in the serum or cervical mucosa (Carter *et al.* 2000, Dillner 1999) implicating that HPV antibodies cannot play an important role in the protection or clearance of infections (Dillner *et al.* 2007).

2.4.6 Immunological tolerance

Immunological tolerance is generally regarded as unresponsiveness to self-antigens or other substances having potential for the activation of the immune response. Central tolerance is established by the negative selection of developing T and B cells in the thymus and bone marrow, respectively, resulting in the deletion of autoreactive cells that recognize the self-antigens of the host. Negative selection of these faulty lymphocytes is most active during fetal development, and dysfunction in this mechanism might result in autoimmunity, where the immune system attacks its own cells or tissues (Goodnow 2007, Palmer 2003). Peripheral tolerance is mediated mainly by Tregs, but other cells, such as B cells and DCs, also have regulatory functions (Ganguly *et al.* 2013). Natural Tregs are specific for self-antigens and their major function is to regulate the self-reactive T cells escaping negative selection, while the induced Tregs recognize allergens, tumor antigens, microbes (commensal bacteria) and allo-antigens. Tregs regulate their target cell through cell-to-cell contact and by either secreting suppressive cytokines (TGF-β and IL-10) or consuming cytokines essential for target cells. Thus, Tregs play an important role in preventing autoimmune diseases and maintaining the immune homeostasis. However, they also seem to be crucial factors in the repression of antitumor or antiviral immune responses resulting in the progression of tumors or infections (Kyewski & Klein 2006, Sakaguchi *et al.* 2010).

2.5 HPV VACCINES

Three prophylactic HPV vaccines are licensed against the most common HPV types: bivalent Cervarix® (GlaxoSmithKline, Belgium) against high-risk HPV types 16 and 18, and quadrivalent Gardasil® (Merck&Co., Inc., USA) against low-risk HPV 6 and 11, and high-risk HPV 16 and 18. Most recently the nona(9)-valent Gardasil9 (Merck&Co) was approved, which targets HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58. These HPV vaccines comprise virus-like particles (VLPs), consisting of L1 proteins assembled into a capsid-like structure. The L1 VLPs are highly immunogenic, generating longer persisting and significantly higher antibody levels than natural HPV infections. A recent meta-analysis showed that the efficacy of the bi- or quadrivalent vaccine against CIN2 or more severe lesions associated with HPV 16 or 18 is over 90% among women who were naïve to the vaccine HPV types upon the first vaccination (Lu *et al.* 2011). Further, in countries with female vaccination coverage of at least 50%, HPV infections and anogenital warts decreased significantly among vaccinated adolescents and adults younger than 20 years (Drolet *et al.* 2015). For example, in Australia, the widespread vaccine coverage has led to a reduction in the incidence of genital warts in young women and, due to herd immunity, also in unvaccinated men (Chow *et al.* 2015). The seven high-risk HPV types covered by nona-valent vaccine cause approximately 90% of all cervical cancers, indicating that even higher efficacy in protection can be expected (Durham *et al.* 2016, Lowy 2016). However, the measurable reduction in cervical cancer and other HPV-related cancers might not be seen before 2030, because of the long progression time from infection to invasive cancer (Lowy 2016).

The better immunogenity of vaccines as compared to natural infection is suggested to result from the different route of the immunization. While natural infection is restricted to the epithelia, where is it difficult to detect by the cells of the immune system, the vaccines are delivered by intra-muscular injection and are thus immediately accessed by the immune cells, which in turn are strongly stimulated by the inflammatory milieu generated by the vaccination (Mollers *et al.* 2013). In addition to the effector cells, vaccination also generates memory B cells, which respond to later encounters with HPV by producing higher levels of antibodies than the initial vaccination (Olsson *et al.* 2009). However, prophylactic HPV vaccination of women who are infected with HPV at the time of vaccination, does not reduce disease progression or influence the viral clearance in HPV DNA-positive individuals (Hildesheim *et al.* 2007).

3 AIMS OF THE STUDY

The Finnish Family HPV Study was originally designed to evaluate the HPV infection prevalence and transmission within regular Finnish families. The current study combines the data collected during the original six years of follow-up and new data from immunological studies to further investigate HPV infections and HPV-specific immunology in children. The study hypothesis was that a child acquires their first HPV infection from the mother as early as at delivery or even during the prenatal period. Further, this early exposure to HPV could affect the developing immune system of the child, and the acquisition and natural history of new HPV infections later in life.

The specific aims were:

i) to elucidate the HPV genotypes present in the oral mucosa of newborns and to measure the serum antibodies to the major HPV capsid protein L1 during the first two months after birth, and to estimate their possible concordance with the cervical HPV genotypes and serum antibodies of the mother before delivery.

ii) to evaluate the HPV16-specific cell-mediated immune reactivity among children born to mothers with an incident of CIN during the follow-up versus children born to mothers who remained constantly HPV-negative.

iii) to evaluate the HPV 16-specific cell-mediated immune reactivity among children who either 1) had a mother with a cervical intraepithelial neoplasia, or 2) had an HPV DNA-negative mother or 3) had HPV DNA detected in placenta and/or cord blood, or 4) had persistent oral HPV DNA detected, or 5) remained constantly HPV DNA-negative during a six-year follow-up period.

4 STUDY SUBJECTS, MATERIALS AND METHODS

4.1 THE FINNISH FAMILY HPV STUDY

The Finnish Family HPV Study is a longitudinal cohort study conducted at the Department of Oral Pathology, Institute of Dentistry, University of Turku and the Department of Obstetrics and Gynecology, Turku University Central Hospital. The study was originally designed to evaluate the dynamics of HPV infections in children and their parents and between family members. The recruitment of families was conducted in 1998-2002. In total, 329 pregnant women in their 3rd trimester of pregnancy, all their newborns (n=331; includes two sets of twins) and 131 men (fathers-to–be) were enrolled in this study at the Maternity unit of the Turku University Central Hospital. The HPV status of the parents was not tested prior to enrollment.

The newborns were followed up for 6 years (Mean 54.9 mo, Median 62.4 mo). Recruitment of children and their mothers for cell-mediated immunity studies started in 2012. In total, 56 children were selected based on the HPV status of the child or their mother during the follow-up period as follows: 1) children whose mothers had a cervical intraepithelial neoplasia, 2) children whose mothers had stayed HPV DNA-negative, 3) children who had had HPV DNA detected in placenta and/or cord blood, 4) children who had persistent oral HPV and 5) children who stayed constantly HPV-negative. In addition, 10 mothers who had had a cervical intraepithelial neoplasia (CIN) were included (Table 4). The mean age of all 56 children was 13.6 years with a range of 10.2-16.2 years, and 30 of them were girls and 26 boys. 10 girls had received three doses and 1 girl one dose of prophylactic bivalent HPV vaccination (Cervarix) before the sample collection, whereas none of the boys had been vaccinated.

The study plan was approved by the Research Ethics Committee of Turku University Hospital (#3/1998, #2/2006, 45/180/2010 and TO7/008/2014).

4.2 DEMOGRAPHIC DATA

At the first study visit after delivery (2.6 months), all the mothers filled in a questionnaire on their demographic characteristics, including age, social status, sexual behavior, gynecological and obstetric history and as other risk factors for HPV infection.

Table 4. Study design: subjects, selection criteria and samples

Original study	Number of subjects	Mean age (range)	Selection criteria	Evaluated follow-up period	Samples taken during 6 years follow-up	Samples at 14-years time point
I	**329 mothers** **and their** **331 newborns**	25.5 years (18-46 years) (birth-2 months)	Pregnant at the 3rd trimester No HPV testing prior to enrollment	Mothers at the 3rd trimester Newborns from birth to 2 months age	Mothers: Routine Pap smear Genital scrapings (HPV DNA) Blood sample (serum HPV antibodies) Placental sample at delivery (HPV DNA)	
II	**10 mothers** **and their** **10 children**	37.0 years (33.2-40.4 years) 12.2 years (11.2-13.2 years)	Mother developed CIN during follow-up	Mothers from the 3rd trimester until 6 years	Breast milk sample at 3 days after delivery (HPV DNA)	Blood sample (cell-mediated immunity)
III	**31 children**	12.3 years (10.3-14.6 years)	Mother developed CIN during follow-up (n=10) Mother HPV-negative during follow-up (n=21)	Children from birth to 6 years age (active follow-up)	Children: Oral scrapings (HPV DNA) Blood sample (serum HPV antibodies) Umbilical cord blood sample at birth (HPV DNA)	Blood sample (cell-mediated immunity)
IV	**33 children**	14.7 years (11.7-16.2 years)	HPV-positive placenta/umbilical cord blood sample and/or repeatedly HPV-positive oral sample (n=16) Always HPV-negative oral sample (n=17)			Blood sample (cell-mediated immunity) Oral scrapings (HPV DNA)

4.3 DETECTION OF HPV DNA AND HPV ANTIBODIES

4.3.1 Samples

The summary of analyzed samples at each follow-up visit is presented in Table 5. A routine Pap smear was taken from all women at baseline using the conventional three-sample technique (vagina, exocervix, and endocervix) using two wooden spatulas and a cytobrush (MedScand, Malmö, Sweden).

Scrapings for HPV DNA testing were taken from the cervical mucosa of the mother and the oral buccal mucosa of the offspring, from both cheeks and the superior and interior vestibule using a small cytobrush (MedScand). The cervical brushes were placed in a tube containing 0.05 M PBS (phosphate-buffered saline) with 100 µg/mL of gentamycin and the oral brushes in a tube containing 80% ethanol and stored at -70˚C.

Blood samples for antibody detection were collected from the mothers and children. The samples were centrifuged (2,400 rpm, 10 minutes), and the serum was separated and divided into three 1 mL tubes, frozen at -20˚C and then stored at -70˚C until serological analysis was performed. Umbilical venous cord blood samples were collected into vacuum EDTA (Ethylenediaminetetra-acetic acid) tubes after delivery, before the placenta was removed. The tubes were frozen at -70˚C.

Two placental samples from the central part of the maternal side of the placenta were taken in the delivery room immediately after delivery and stored at -70˚C until HPV testing. The samples were approximately 1 cm in diameter and penetrated the whole thickness of the placenta. Breast milk samples were collected 3 days after delivery at the hospital. This procedure was done separately from feeding the infant and after careful washing of hands with disinfectant. The milk was collected into 3 ml plastic containers, sealed and immediately frozen and stored at -70˚C.

Table 5. Samples taken during the follow-up. The Roman numerals I-IV indicate the original study in which the samples were used.

Sample	Follow-up visit										
	At 3rd trimester	At delivery	At 3 days after delivery	months after delivery							14 years
				1	2	6	12	24	36	72	
Genital scraping	I, II				II, III	II, III	II, III	II, III	II, III		
Pap smear	II						II	II	II		
Blood sample	I, II						II	II	II		II
Placental sample		I, IV									
Breast milk sample			I								
Oral scraping		I-IV	I-IV	I-IV	I-IV	II-IV	II-IV	II-IV	II-IV	II-IV	II-IV
Blood sample				I-IV	I-IV	II-IV	II-IV	II-IV	II-IV		II-IV
Umbilical cord blood sample		I, IV									

4.3.2 DNA isolation

DNA from the umbilical cord blood and milk samples was extracted using a high pure PCR template preparation kit (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions. The high salt method was used to extract DNA from cervical and oral scrapings, and placental samples (Miller *et al.* 1988) as follows: The samples were lysed in lysis buffer (10mM Tris, 400mM NaCl, 100 mM EDTA, 1% SDS) and digested with proteinase K (10 µg/mL) at 37°C overnight. After digestion, proteins were precipitated with saturated NaCl and ethanol. Purfied DNA was dissolved in 50 µl water, mixed for up to 30 minutes and stored at -20°C.

4.3.3 Detection and genotyping of HPV DNA

HPV DNA was detected from extracted DNA by nested PCR using GP05+/GP06+ and MY09/MY11 (Qu *et al.* 1997) primers for all other samples except the cervical samples, for which single PCR with GP05+/GP06+ primers was used (Snijders *et al.* 1990). Evaluation of possible contamination during the DNA extraction was performed by simultaneous extraction of DNA from cattle lung tissue or HPV-negative cell line, HMK, which is a spontaneously immortalized gingival keratinocyte cell line that does not contain any HPV DNA (Mäkelä *et al.* 1998). HPV-positive CaSki and SiHa cell lines, and non-template samples were used as positive and negative controls, respectively. The PCR was conducted in a 25 µL reaction mixture using Amplitaq Gold DNA polymerase (Perkin Elmer, NJ, USA). The PCR products were run in 2.0% agarose gel.

HPV genotyping was undertaken using Multimetrix® assay (Multimetrix, Regensburg, Germany), which is based on the hybridization of biotinylated PCR products with fluorescent-labeled polystyrene beads. The assay detects low-risk HPV types: 6, 11, 42, 43, 44, 70 and high-risk HPV types: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82 (Schmitt *et al.* 2006). The assay was performed according to the manufacturer's instructions expect that the earlier nested PCR product was re-amplified for biotinylation with GP05+ and bio-GP06+ -primers and only half of the recommended volume was used. In the final step, 100µl of the blocking buffer was used for measuring the hybridized beads with a Luminex LX-100 analyzer (Bio-Plex 200 system, Bio-Rad Laboratories, Hercules, USA). The median fluorescence intensity (MFI) of at least 100 beads was computed for each bead population in the sample. The cut-off value for each HPV probe was defined individually: 1.5 x negative control MFI (background) + 5 MFI. The possible contamination of tested samples was controlled by the simultaneous analysis of HPV-negative control samples.

4.3.4 Detection of HPV antibodies

Blood samples were sent for analysis to the DKFZ (Deutsche Krebsforschungszentrum, German Cancer Research Center, Heidelberg Germany). Serum antibodies for the major capsid protein L1 of HPV types 6, 11, 16, 18 and 45 were analyzed using multiplex HPV serology, based on glutathione S-transferase (GST) fusion-protein capture on fluorescent beads (Waterboer *et al.* 2005, Waterboer *et al.* 2006). Viral L1 antigens of HPV 6, 11, 16, 18 and 45 were expressed as GST-fusion proteins in Escherichia coli bacteria. GST-L1-fusion proteins were affinity-purified by incubating the bacterial lysate with glutathione-displaying beads. L1 antigens of each HPV type were loaded onto spectrally distinct bead sets, which were subsequently washed and mixed. The tested serum was first incubated at a 1:50 dilution in a serum preincubation buffer for 1h at room temperature on a shaker. The incubation buffer (0.1% casein in PBS) contained lysate from bacteria expressing GST alone (2mg /mL) to block antibodies directed against residual bacterial proteins and 0.5% polyvinyl alcohol, 0.8% polyvinylpyrrolidone, and 2.5% Super ChemiBlock (CBS-K, Millipore, Billerica, MA, USA) to block nonspecific binding of antibodies.

The pretreated and diluted serum (50µL) and mixed beads (3000 beads per set) were combined and incubated on a shaker for 1h in the dark at room temperature. Antibodies bound to GST-L1-beads were detected with biotinylated goat-antihuman IgG secondary antibody (Dianova) and fluorescent detection conjugate (streptavidin-R-phycoerythrin). Reporter fluorescence was quantified with the Luminex analyzer and expressed as the MFI of at least 100 beads per bead set per sample. Antigen-specific reactivity was calculated by subtracting the MFI value of the GST tag only (background) from the total MFI value.

Sera were scored positive if the L1 antigen-specific MFI values were greater than the cut-off of 200 or 400 MFI for the individual HPV genotypes (Michael *et al.* 2008). Seroconversion was established if: 1) the MFI value increased at least twofold from the previous serum MFI value and 2) the MFI value exceeded the cut-off levels. The antibody decay was defined if: 1) the MFI value decreased at least twofold from the previous serum MFI value and 2) a positive MFI value declined below the cut-off levels.

4.4 STUDIES ON CELL-MEDIATED IMMUNITY

4.4.1 Isolation of peripheral blood mononuclear cells

Venous blood samples of 72 mL and 54 mL from mothers and children were collected at the final time point after 14 years of follow-up (Table 5), respectively. Blood samples were drained into sodium-heparin collection tubes and processed within 4 hours. In addition, 9 mL of blood was collected into a clotting tube for separation of the serum.

The peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over a Ficoll-Hypaque gradient (GE Healthcare Life Sciences, Uppsala, Sweden) after diluting the blood to 1:1 with PBS. Approximately 13.5 x 10^6 PBMCs were used to determine the proliferative capacity of HPV 16–specific T cells using a short-term lymphocyte stimulation test (LST). The remaining PBMCs were frozen in 80% Fetal Bovine Serum (FBS, Biowest, EU quality) and 20% DMSO, and stored in liquid nitrogen. To separate the serum, nine mL of blood was collected into a clotting tube and centrifuged for 7 minutes at 1800 *g*. Autologous serum was used for short-term T cell proliferation assay (de Jong *et al.* 2004, Welters *et al.* 2008).

4.4.2 HPV 16 peptides

Eight peptide pools of HPV 16 peptides were used to stimulate the isolated PBMCs to determine the proliferative capacity of HPV 16-specific T cells, for cytokine polarization analysis and measuring the HPV 16-specific Foxp3+ regulatory T cells. Panels of overlapping 30-35 mer HPV 16 E2, E6, or E7 peptides were synthesized using a solid phase peptide synthesis (SPPS) method with >95% purity (ChinaPeptides Co. Shanghai, China), with a 14 (for 30-mer) or 15 (for 35-mer) amino acid (aa) overlap. Two pools of E2 peptides (E2.1 and E2.2) consisted of 12 or 11 30-mer peptides, four pools of E6 peptides (E6.1-E6.4) consisted of two

32-mer peptides, and two pools of E7 peptides (E7.1 and E7.2) consisted of two 35-mer peptides (Welters *et al.* 2003). The peptide pools and amino acid sequences of each peptide are shown in Table 6.

Memory response mix (MRM) stock solution (50x), consisting of 0.75 fL/mL tetanus toxoid (Statens Serum Institut, Copenhagen, Denmark), 5 µg/mL Tuberculin PPD (Statens Serum Institut), and 0.015% Candida albicans (Greer Laboratories, Lenoir, USA) was used as a positive control for the proliferation assays and cytokine production capacity of the PBMCs (de Jong *et al.* 2002).

4.4.3 Determination of the proliferative capacity of HPV 16–specific T cells

The proliferation capacity of HPV 16–specific T cells was determined using a short-term lymphocyte stimulation test (LST) (de Jong *et al.* 2002, Welters *et al.* 2003). Briefly, 150,000 freshly isolated PBMCs in 125 µL medium per well were seeded into a U-bottomed 96-well microtiter plate (Nunc, Roskilde, Denmark) in eight replicates for each peptide pool. The PBMCs were cultured in IMDM (Iscove's Modified Dulbecco's Medium, Gibco, Life Technologies, Belgium) containing 10% autologous serum and the indicated peptide pool at a final concentration of 5 µg/mL of each peptide. PBMCs cultured in the presence of MRM were used as a positive control (10 µL/well of 4xMRM) and medium without antigen (medium-only) was used as a background control. After 6 days of culturing, 50 µL of supernatant was collected from all replicative wells and pooled for cytokine analysis. Cells were provided by 50 µL of fresh IMDM supplied with 0.5µCi [3H]-Thymidine (PerkinElmer, Turku, Finland). After 18 hours of incubation, the cells were harvested into Unifilter plates (PerkinElmer) using the FilterMateTM Cell Harvester (PerkinElmer). Subsequently, the filter plates were dried and counted on the 1450 MicroBeta+ counter (PerkinElmer). The cut-off counts per minute (CPM) values were determined by the mean plus 3×SD of the medium-only control wells. The stimulation index (SI) was calculated as the average of tested wells divided by the average of the medium-only control wells. The proliferative response was defined as positive if the CPM values of at least six of the eight wells were above the cut-off value and the SI was ≥3.

4.4.4 Cytokine polarization analysis

The supernatants collected from the eight replicative wells of short-term lymphocyte stimulation test (LST) at day 6 of culturing were pooled and analyzed using a Cytometric Bead Array (CBA) human enhanced-sensitivity flex set system (BD Biosciences, Temse, Belgium) according to the manufacturer's instructions. In this array, the levels of IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-10, and IL-17A were determined. The detection limits for the cytokines were based on standard curves complying with the limit of 274 fg/mL described by the manufacturer. Antigen-induced cytokine production was established if cytokine concentration was more than twice the concentration of the medium-only control (de Jong *et al.* 2002).

4.4.5 Identification of HPV 16–specific CD4+CD25+Foxp3+ regulatory T cells

The frozen PBMCs were thawed and seeded into a 24-well plate (1.0×10^6 cells/well). The cells were cultured in IMDM containing 10% Human AB serum (Sigma-Aldrich, San Louis, USA) with the peptide pools at a final concentration of 5 µg/mL per peptide. PBMCs cultured in the presence of MRM were used as a positive control (80 µL/well of 4xMRM) and medium-only was used as a background control. After 7 days in the culture, the cells were harvested, washed with 0.5% BSA in PBS and stained first for surface marker expression with anti-CD25 FITC (1:25, clone M-A251, BD Pharmingen, San Diego, CA), anti-CD4-APC (1:100, clone RPA-T4, BD Pharmingen), and anti-CD8 PerCP-Cy5.5 (1:30, clone SK1, BD Pharmingen) in a volume of 50µL for 30 minutes. Subsequently, the cells were washed, fixed and permeabilized using an intra-nuclear staining buffer set (FOXP3 Fix/Perm buffer set, Biolegend, San Diego, CA) according to the manufacturer's instructions. After blocking the samples for 15 minutes with 25µL of 2% FBS, intracellular staining for Foxp3 (1:4, PE anti-human FOXP3, Biolegend) or isotype control (1:1, PE Mouse IgG1, κ Isotype Ctrl, Biolegend) in volume of 20µL for 30 minutes were performed. All staining procedures were done on ice and all incubations were performed in the dark. After staining, the cells were washed, resuspended in PBS and measured in a flow cytometer BD FACSCalibur and BD LSRFortessa™ cell analyzer (BD Bioscience). Analysis was performed using Flowing Software, version 2.5.1 (Cell Imaging Core, Turku Center for Biotechnology, Turku, Finland). The fluorescence intensity of the MRM-stimulated and medium-only control cells was used to set the gates for the other samples. Antigen-induced alteration in the population percentage was established if a minimum twofold change was observed as compared to the medium-only control (Heusinkveld *et al.* 2011, Welters *et al.* 2008).

4.5 STATISTICAL ANALYSIS

Original study I Statistical analyses were run using the SPSS® (SPSS, Inc., Chicago, IL, USA) and STATA (Stata Corp., College Station, TX, USA) software packages (PASW Statistics for Windows, version 18.0.1 and STAT/SE 11.1). Frequency tables were analyzed using the Chi-square test or Fisher's exact test (when appropriate) with Pearson's R or likelihood ratio (LR) statistics to assess the significance of the correlation between categorical variables. Odds ratios (OR) with 95% confidence intervals (95%CI) were calculated where appropriate. Differences in the means of continuous variables between the study subjects were analyzed using ANOVA (analysis of variance, when appropriate) or non-parametric tests (Mann-Whitney, Kruskal-Wallis). For all analyses, p-values less than 0.05 were regarded as statistically significant.

Original study II Statistical analyses were run using the IBM SPSS® (IBM, Inc., New York, USA) software package (IBM SPSS Statistics for Windows, version 22.0.0.1). Frequency tables were analyzed using the Chi-square test, with the likelihood ratio 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 or multiple independent

samples, respectively. A paired-samples test (Wilcoxon) was used to analyze the response levels in mother-child pairs. All statistical tests were two-sided and declared significant at p-value ≤0.05.

Original studies III and IV All statistical analyses were run using the IBM SPSS® (IBM, Inc., New York, USA) software package (IBM SPSS Statistics for Windows, version 22.0.0.1). The means of secreted cytokine concentrations and proliferative responses of all groups were analyzed using a one-way ANOVA. The Bonferroni correction was used to analyze differences between groups. All statistical tests were two-sided and declared significant at p-value ≤0.05.

Table 6. HPV 16 E2, E6 and E7 peptides and amino acid sequences (Welters et al. 2003)

Pool	Peptide	Amino acid sequence	Amino acid position
E2.1	E2-1	METLCQRLNV CQDKILTHYE NDSTDLRDHI	1–30
	E2-2	LTHYE NDSTDLRDHI DYWKHMRLEC AIYYK	16–45
	E2-3	DYWKHMRLEC AIYYKAREMG FKHINHQVVP	31–60
	E2-4	AREMG FKHINHQVVP TLAVSKNKAL QAIEL	46–75
	E2-5	TLAVSKNKAL QAIELQLTLE TIYNSQYSNE	61–90
	E2-6	QLTLE TIYNSQYSNE KWTLQDVSLE VYLTA	76–105
	E2-7	KWTLQDVSLE VYLTAPTGCI KKHGYTVEVQ	91–120
	E2-8	PTGCI KKHGYTVEVQFDGDICNTMH YTNWT	106–135
	E2-9	FDGDICNTMH YTNWTHIYIC EEASVTVVEG	121–150
	E2-10	HIYIC EEASVTVVEG QVDYYGLYYV HEGIR	136–165
	E2-11	QVDYYGLYYV HEGIRTYFVQ FKDDAEKYSK	151–180
	E2-12	TYFVQ FKDDAEKYSKNKVWEVHAGG QVILC	166–195
E2.2	E2-13	NKVWEVHAGG QVILCPTSVF SSNEVSSPEI	181–210
	E2-14	PTSVF SSNEVSSPEI IRQHLANHPA ATHTK	196–225
	E2-15	IRQHLANHPA ATHTKAVALG TEETQTTIQR	211–240
	E2-16	AVALG TEETQTTIQRPRSEPDTGNP CHTTK	226–255
	E2-17	PRSEPDTGNP CHTTKLLHRD SVDSAPILTA	241–270
	E2-18	LLHRD SVDSAPILTA FNSSHKGRIN CNSNT	256–285
	E2-19	FNSSHKGRIN CNSNTTPIVH LKGDANTLKC	271–300
	E2-20	TPIVH LKGDANTLKCLRYRFKKHCT LYTAV	286–315
	E2-21	LRYRFKKHCT LYTAVSSTWH WTGHNVKHKS	301–330
	E2-22	SSTWH WTGHNVKHKS AIVTLTYDSE WQRDQ	316–345
	E2-23	AIVTLTYDSE WQRDQFLSQV KIPKTITVST GFMSI	331–365
E6.1	E6-1	MHQKRTAMFQ DPQERPRKLP QLCTELQTTI HD	1–32
	E6-2	LP QLCTELQTTI HDIILECVYC KQQLLRREVY	19–50
E6.2	E6-3	CVYC KQQLLRREVY DFAFRDLCIV YRDGNPYA	37–68
	E6-4	RDLCIV YRDGNPYAVC DKCLKFYSKI SEYRHY	55–86
E6.3	E6-5	CLKFYSKI SEYRHYCYSL YGTTLEQQYN KPLC	73–104
	E6-6	YGTTLEQQYN KPLCDLLIRC INCQKPLCPE EK	91–122
E6.4	E6-7	RC INCQKPLCPE EKQRHLDKKQ RFHNIRGRWT	109–140
	E6-8	DKKQ RFHNIRGRWT GRCMSCCRSS RTRRETQL	127–158
E7.1	E7-1	MHGDTPTLHE YMLDLQPETT DLYCYEQLND SSEEE	1–35
	E7-2	LYCYEQLND SSEEEDEIDG PAGQAEPDRA HYNIVT	22–56
E7.2	E7-3	GQAEPDRA HYNIVTFCCK CDSTLRLCVQ STHVDIR	43–77
	E7-4	TLRLCVQ STHVDIRTLE DLLMGTLGIV CPICSQKP	64–98

5 RESULTS

5.1 HPV GENOTYPES IN CHILDREN AND MOTHERS

5.1.1 Prevalence of HPV-genotypes in oral mucosa of the infant

We analyzed the HPV prevalence and genotype distribution in the oral mucosal samples of infants (n= 331) taken immediately when born, at day 3, 1 month and 2 months. The point prevalences of HPV genotypes found are presented in Figure 8. The prevalence of any HPV type at the four different sampling points was 22.5%, 13.0%, 18.7%, and 16.9%, respectively. HPV 16 was the most prevalent individual genotype at any visit, being found in 32.2%, 75.0%, 51.8%, and 36.0% of HPV-positive samples. HPV 18 was more frequent at months 1 and 2 (16.1% and 22.0%) than at delivery and at the age of 3 days (2.8% and 5.5%). The HPV 6 genotype was detected at every visit within 15.4%, 13.9%, 5.4%, and 14.0% of the HPV-positive samples.

Co-carriage of multiple HPV genotypes was detected most frequently (16.4%) at delivery, whereas in newborns at day 3, month 1, and month 2, multiple infections were much less frequent (2.8%, 3.6%, and 8.0%, respectively). Among multiple infections, no genotype combination was more common than others. However, HPV 16 was the most frequent type in all combinations, found in 25-100% of multiple-infected newborns at the different time points. When all HPV genotypes also found within multiple infections were taken into account, the HPV genotype distribution changed slightly. HPV 16 was still the most prevalent genotype and was detected in 39.7%, 77.8%, 54.5%, and 38.0% of infants at delivery, day 3, month 1, and month 2, respectively. The prevalence of HPV 18 was now more prevalent in newborns and at month 2 (8.2% and 24.0%, respectively), as was HPV 6 at delivery, day 3, and month 2 (16.4%, 16.7%, and 18.0%, respectively).

5.1.2 Prevalence of HPV-genotypes in genital mucosa of the mother before delivery

The genital scraping samples were taken from the mother (n = 329) in the 3rd trimester of her pregnancy for HPV detection and genotyping. All in all, 16.4% of the samples were HPV -positive. Multiple HPV infections were detected more frequently than any individual HPV genotype in HPV-positive mothers (45.3%). HPV 16 was the most prevalent individual HPV type (20.8%), followed by HPV 6 and HPV 45 (both 5.7%). HPV 16 was also the most frequent type in multiple infections, and was detected in 83.3% of multiple infections in mothers. HPV 18 as an individual infection was found in only 1.9% of mothers. When all HPV genotypes found also within multiple infections were taken into account, HPV 16 was still the most prevalent type, detected in 58.5% of mothers. The prevalence of HPV 18 increased to 15.1%

and that of HPV 6 to 13.2%. In addition, HPV 11 and HPV 33 were detected within multiple infections in mothers at delivery (7.6% and 3.8%), although they were not found in mothers as individual HPV types.

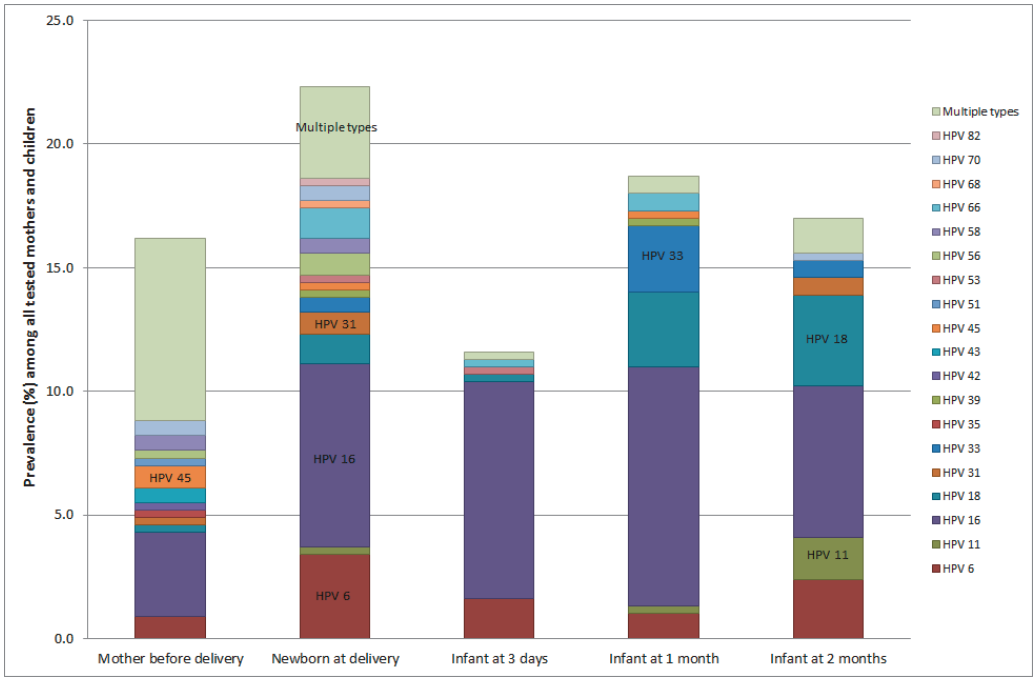


Figure 8. The point prevalences of HPV genotypes detected in the cervical mucosa of the mothers before delivery and in the oral mucosa of the newborns at delivery and at the ages of 3 days, and 1 and 2 months.

5.1.3 Prevalence of HPV genotypes in children and mothers during follow-up

HPV DNA was tested also later during the six-year follow-up among ten mothers and 56 children who were selected for the studies of HPV 16–specific cell-mediated immunity. In children, the oral scrapings for the detection of HPV were obtained at the ages of 6, 12, 24, 36 and 72 months and 14 years, and in mothers at 12, 24 and 36 months and 14 years after delivery (Table 5). During the follow-up from birth to 14 years, 58.9% (33/56) of the children were found positive for oral HPV DNA at some timepoint, and 37.5% (21/56) of the children were positive for HPV 16. HPV DNA for any HPV type was detected in the cervical samples of all 10 mothers, and 9 of them were positive for HPV 16. The detected HPV DNA types during follow-up are presented individually for 10 mothers and 56 children in Tables 7-10.

5.2 HPV GENOTYPE-SPECIFIC CONCORDANCE BETWEEN MOTHER AND INFANT

Figure 9 summarizes the HPV genotype distribution in the infant's mouth during the 2-month follow-up visit and their mother's genital tract at baseline (single and co-infections were pooled). The HPV genotype-specific concordance between the newborns at delivery and the mothers was almost perfect (weighted kappa = 0.988; 95% CI, 0.951-0.997). When the mother and her newborn were compared as pairs, the HPV genotypes of the mother were not significantly different from those of the newborn (Wilcoxon signed-rank test, P = 0.753). However, comparison of mother-infant pairs at just 3 days after delivery revealed a significant difference (P = 0.001), implicating that the HPV genotypes of the infant had started to differ from those of the mother. This difference remained significant at month 1 (P = 0 .02) and at month 2 (P = 0.001). The maternal demographic factors were analyzed to predict the oral HPV status of the infant. The history of genital condylomas (P = 0.021) and the age of the mother (P =0.049) predicted the carriage of oral HPV of the newborn (Table 7). Detection of oral HPV DNA was most frequent in infants of mothers aged 20-34 years (34.3%) than in infants of younger or older mothers (25.0% for both). Oral and cervical HPV DNA status, Pap smear cytology and genital condylomas examined before delivery, and age of sexual debut, age of starting of oral contraception, number of sexual partners, smoking, use of alcohol, history of skin warts, oral warts or papillomas, and history of other sexually transmitted infections showed no significant association with the newborn's carriage of oral HPV.

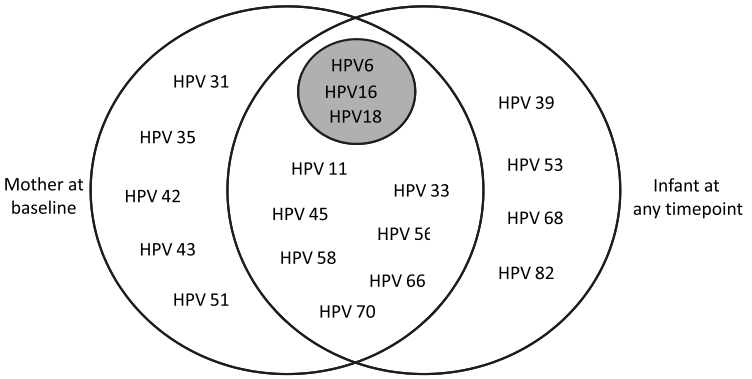


Figure 9. HPV genotypes in infants and their mothers at all time points when single and co-infections are pooled. The HPV types in the middle section were found in both mothers and infants. The grey circle illustrates the most prevalent types (Figure 2, Original publication I).

5.3 HPV DNA IN PLACENTA, UMBILICAL CORD BLOOD AND BREAST MILK

The results of HPV DNA detection in placenta, umbilical cord blood and breast milk have been presented partly by Sarkola and co-workers (Sarkola *et al.* 2008b, Sarkola *et al.* 2008a). HPV DNA was detected in 4.2% (13/306) of placentas. HPV 16 was detected in seven and HPV 6 in five placentas, and one placenta contained HPV 83. All HPV DNA-positive placentas were macroscopically normal. Cesarean section was performed in two cases and 11 other HPV DNA-positive placentas were obtained from vaginal deliveries. The presence of HPV in the oral mucosa of the newborn at delivery was most significantly associated with the presence of HPV in the placenta (OR = 14.0; 95% CI, 3.7-52.2, P = 0.0001), and this association between placental HPV DNA and oral HPV carriage by the infant remained significant during the 2-month follow-up period (OR = 5.1; 95% CI, 1.6-16.4 and OR = 8.1; 95% CI, 2.4-26.7).

HPV DNA was detected in 3.5% (11/311) of cord blood samples. HPV 16 was found in five, HPV 6 in five and HPV 39 in one cord blood sample. In two cases, both the placenta and the cord blood sample were found positive for HPV 16 and in three cases for HPV 6. The presence of HPV in the oral mucosa of the newborn at delivery was significantly associated with the presence of HPV in the umbilical cord blood (OR = 4.7; 95% CI, 1.4-15.9, P = 0.015), and this association disappeared at 1 month (Table 7). On the whole, the risk for oral HPV carriage for the newborn was 6.8–fold (OR = 6.8; 95% CI, 2.6-18.0; P = 0.0001) if HPV DNA was detected in the placenta and/or cord blood. HPV was detected in 4.1% (9/223) of breast milk samples, but the presence of HPV DNA in the breast milk was not associated with the oral HPV status of the newborn (OR = 0.9; 95% CI, 0.2-4.6; P = 1.000).

5.4 HPV ANTIBODIES DETECTED

5.4.1 HPV antibodies in infants and their mothers

Serum antibodies for low-risk HPV types 6 and 11, and for high-risk HPV types 16, 18 and 45 were analyzed from blood samples from 231 infants at the age of one and two months. Altogether, at one month, 34.0% (78/229) of the infants were seropositive for low-risk HPV types and 24.4% (56/229) for high-risk HPV types, and at two months, the frequencies were 21.2% (49/231) and 16.9% (39/231), respectively. The mothers of these 231 infants were tested before delivery at the 3rd trimester and 58.9% (136/231) of them were seropositive for low-risk HPV types and 38.5% (89/231) for high-risk HPV types. The seropositivity of the mother before delivery was statistically significantly associated with that of the infant at the age of one month (P = 0.0001), indicating that both, high-risk and low-risk HPV antibodies in the infants are of maternal origin. The multiple comparisons revealed two correlations between the serum antibodies and the oral HPV DNA status of the infants: first, the detection of oral HPV in newborns at delivery was statistically significantly associated with the presence of high-risk HPV antibodies in the infants at month 1 (*P* = 0.045, *P* =0.035, *P* =0.02). Second, at

the age of two months, the detection of oral HPV in infants was significantly associated with the presence of low-risk HPV antibodies in their serum (*P* = 0.048) (Table 7).

Table 7. Statistically significant predictors of oral HPV carriage and HPV serology of infant (Modified from Tables 1 and 2, Original publication I).

Variable 1	Variable 2	P	OR	95% CI
HPV status of the cord blood	Oral HPV status* of the infant at delivery	0.015	4.7	1.4-15.9
HPV status of the placenta	Oral HPV status of the infant at delivery	0.0001	14.0	3.7-52.4
	Oral HPV status of infant at 1 month age	0.01	5.1	1.6-16.4
	Oral HPV status of the infant at 2 months age	0.001	8.1	2.4-26.7
HPV status of the cord blood and/or placenta	Oral HPV status of the infant at delivery	0.0001	6.8	2.6-18.0
Antibodies to low-risk HPV, mother before delivery	Antibodies to low-risk HPV, infant at 1 month age	0.0001	0.4	0.4-0.5
Antibodies to high-risk HPV, mother before delivery	Antibodies to high-risk HPV, infant at 1 month age	0.0001	68.2	20.1-230.9
Antibodies to high-risk HPV, infant at 1 month age	Oral HPV status of the infant at delivery	0.045	2.0	1.0-4.0
	Oral HPV types of infant at delivery	0.035		
	Oral HPV species** of the infant at delivery	0.02		
Antibodies to low-risk HPV, infant at 2 month age	Oral HPV status of the infant at 2-months	0.048	2.2	1.0-5.0
Genital condylomas of the mother	Oral HPV status of the infant at delivery	0.021	0.3	0.1-0.8
Age of the mother	Oral HPV status of the infant at delivery	0.049		

* HPV status = HPV DNA+ or HPV DNA-

** Tested HPV types were grouped according to their taxonomical classification:
species 7 (HPV 18, 39, 45, 59, 68, 70, 85)
species 9 (HPV 16, 31, 33, 35, 52, 58, 67)
species 10 (HPV 6, 11, 13, 44, 55, 74

5.4.2 HPV antibodies during the follow-up

Serum antibodies against HPV were also measured later during the six-year follow-up among 10 mothers and 56 children who were selected for the studies of HPV 16–specific cell-mediated immunity. In children, the blood sampling for the detection of antibodies was performed at the age of 6, 12, 24 and 36 months, and in mothers at 12, 24 and 36 months after delivery (Table 5). During the follow-up from birth to 36 months, 78.6% (44/56) of the children were found seropositive for one or several of the measured HPV types (HPV 6, 11, 16, 18 and 45) at some timepoint, and 26.8% (15/56) of the children displayed seropositivity for HPV 16. Seropositivity for any HPV type was detected in 8 of 10 mothers, and 7 of them displayed antibodies against HPV 16. The individual results of the serum antibodies of the 10 mothers and 56 children collected during follow-up are presented in Tables 7-10.

5.5 HPV 16-SPECIFIC CELL-MEDIATED IMMUNITY IN WOMEN AND CHILDREN

HPV 16 E2, E6, and E7-specific cell-mediated immune responses were measured among 10 mothers and 56 children. All the mothers had developed an incident of CIN during the follow-up; five mothers had CIN3, three had CIN2, and two had CIN1. All incidents of CIN2 and CIN3 lesions were preceded by long-term persistence of HPV 16. The children were tested and analyzed in four groups:

i) Children whose mothers had developed an incident of CIN (n=10)
ii) Children whose mothers had remained constantly negative for genital HPV (n=21)
iii) Children who had HPV DNA detected in the placenta or/and cord blood or/and had persistent HPV in oral mucosa during the 6-year FU or had oral HPV 16 at the final time point (n=19). Oral HPV was defined as persistent if the same HPV genotype was detected at at least two timepoints within a period of 24 months.
iv) Children who had stayed constantly negative for oral HPV during the 6-year FU (n=14).

These children were analyzed in case-control setting as follows: group i) together with group ii) (publications I and II), and group iii) together with group iv) (publication IV). Furthermore, in the cytokine analysis, the children of group iii) were divided into further subgroups according to their HPV status as follows: a) Children who had HPV DNA detected in the placenta or/and cord blood with or without oral HPV, b) Children who had only persistent HPV in oral mucosa during the 6-year follow-up, and c) Children who had stayed negative for oral HPV at all visits during the 72-month follow-up until testing positive for oral HPV 16 at the last visit when blood was taken for CMI. Tables 8-11 show the detailed follow-up data for each mother-child pair. Seven children were analyzed in both case-control settings as follows: ID1B=ID003, ID2B=ID301, ID3B=ID302, ID4B=ID012, ID7B=ID017, ID11B=ID201 and ID13B=ID008.

Table 8. Detected HPV-types, serum HPV antibodies and diagnosed Pap smears and colposcopies during follow-up in mothers who had incident CIN

ID		At 3rd trimester	Months after delivery																		
			2	12	18	24	30	36	42	48	54	60	66	72	78	84	90	96	102	108	120	124
1A	Genital HPV DNA	NEG	NA	**HPV 16, 31**		NA		**HPV 16, 56**														
	Pap/Colp			NSIL	NSIL	NSIL		NSIL								NSIL		LSIL	**CIN3**	**CIN1**	NSIL	
	HPV antibodies	**6, 11, 16, 18**		**6, 11, 16, 18**		**6, 11, 16**		**16**														
2A	Genital HPV DNA	**HPV 16, 31, 42**	**HPV 16, 18**	**HPV 31**		**HPV 58**		**HPV 16, 58**									**HPV 16**					
	Pap/Colp		NSIL	ASC-US	NSIL	NSIL	**NSIL CIN1**	NSIL NCIN	NSIL NCIN	NSIL NCIN							NSIL	NSIL				
	HPV antibodies	**6, 16**		**16, 11, 18**		**6, 16**		**6, 18**														
3A	Genital HPV DNA	**HPV 31**	**HPV 31**	**HPV 16, 70**		**HPV 6, 82**		**HPV 16**														
	Pap/Colp			NSIL		NSIL		NSIL									LSIL	**CIN2**	**CIN2**	NSIL		
	HPV antibodies	**6**		**6, 16, 18**		**6, 18**		NA														
4A	Genital HPV DNA	NEG	NEG	**HPV 58**		**HPV 18, 31**		**HPV 16, 39**													**HPV31**	
	Pap/Colp			NSIL		NSIL		NSIL							NSIL		NSIL			HSIL	**CIN2**	**CIN3**
	HPV antibodies	**16**		**6, 16, 18**		**6, 16**		**6**														
5A	Genital HPV DNA	NEG	NEG	**HPV 16, 59**		**HPV 16**		**HPV 16**									**HPV 16**	**HPV 16**				
	Pap/Colp			NSIL		NSIL		NSIL						ASC-US	ASC-US		**CIN3**	**CIN3**	NCIN	NSIL		
	HPV antibodies	neg		NA		NA		NA														
6A	Genital HPV DNA	**HPV 43**	**HPV 18, 31**	**HPV 18**		NA	**HPV 51**	**HPV 51**							NA					**HPV 58**	**HPV58**	
	Pap/Colp			ASC-US	LSIL NCIN	NSIL NCIN	ASC-US	ASC-US	NSIL NCIN	LSIL NCIN	ASC-US NCIN		ASC-US		NSIL	NSIL		NSIL	HSIL	**CIN3**	**CIN3**	NCIN
	HPV antibodies	**6, 16, 18, 45**		**6, 11, 16, 18, 45**		NA	**6, 16, 18**	**6, 16, 18**														
7A	Genital HPV DNA	**HPV 16**	NEG	**HPV 16**		**HPV 16**		NA						**HPV 16**			**HPV 16**	**HPV 16**				
	Pap/Colp			NSIL		NSIL								ASC-US	ASC-US		**CIN3**	**CIN3**	NSIL	NSIL		
	HPV antibodies	**16**		**16**		**16**		NA														
8A	Genital HPV DNA	**HPV 16**	**HPV 16**	**HPV 16**		**HPV 16**		**HPV 16**					**HPV 16**	**HPV 16**								
	Pap/Colp			NSIL		NSIL		NSIL				**CIN2**	NCIN	NCIN NSIL	NSIL	NSIL			**CIN2**			
	HPV antibodies	neg		NA		NA		NA														
9A	Genital HPV DNA	**HPV 16, 59**	**HPV 16, 59**	NA		NA		NA						**HPV 16**	**HPV 16**							
	Pap/Colp					NA								ASC-US	**CIN2**	**CIN2**	**CIN1**	**CIN2**	NSIL			
	HPV antibodies	**6**		NA		NA		NA														
10A	Genital HPV DNA	NEG	NEG	NA		NA		**HPV 16**							**HPV 16, 56**				**HPV 16, 66**	**HPV 16, 66**		
	Pap/Colp			NSIL		NA	NSIL	NSIL						NSIL	LSIL	LSIL NCIN			**LSIL CIN1**	NCIN		
	HPV antibodies	neg		**16**		NA																

Pap = Pap smear, Colp = colposcopy, NEG = no HPV DNA detected, neg = no HPV antibodies detected, NA = no sample available
ASC-US = Atypical squamous cells of undetermined significance
LSIL = low-grade squamous intraepithelial lesion, HSIL = High-grade squamous intraepithelial lesion, NSIL = no SIL/ normal squamous epithelium
CIN = cervical intraepithelial neoplasia, CIN 1 = mild dysplasia, CIN 2 = moderate dysplasia, CIN 3 = severe dysplasia/ carcinoma in situ, NCIN = no CIN/normal cervical epithelium

Table 9. Detected HPV types and HPV antibodies during follow-up period in children, whose mothers had incident CIN (group i) and children whose mothers remained constantly negative for genital HPV (group ii)

	ID		Mother before delivery (genital HPV and antibodies)	Child								
				At birth	At the age of							
					3 days	1 month	2 months	6 months	12 months	24 months	36 months	72 months
i	1B*	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NA
		HPV antibodies	6, 11, 16, 18			NA	neg	neg	neg	neg	neg	
	2B*	Oral HPV DNA	16, 31, 42	HPV 31	NEG	NEG	NEG	NEG	NEG	HPV 70	HPV 18	HPV 31
		HPV antibodies	6, 16			neg	NA	neg	neg	16, 18	neg	
	3B*	Oral HPV DNA	HPV 31	HPV 31	HPV 6	HPV 33	NEG	NEG	NEG	NEG	NEG	HPV 31
		HPV antibodies	6			NA	6	neg	neg	neg	6	
	4B*	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NA
		HPV antibodies	16			16	neg	neg	neg	neg	6	
	5B	Oral HPV DNA	NEG	HPV 6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
		HPV antibodies	neg			NA	neg	neg	6	6, 18	16	
	6B	Oral HPV DNA	HPV 43	NEG	NEG	HPV 18	NEG	NEG	NEG	NEG	NEG	NEG
		HPV antibodies	6, 16, 18, 45			neg	neg	6	6	6	6	
	7B*	Oral HPV DNA	HPV 16	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NA	NA
		HPV antibodies	16			neg	neg	neg	6	6	NA	
	8B	Oral HPV DNA	HPV 16	HPV 16	6.16	HPV 16	NEG	NEG	NEG	NEG	NEG	NEG
		HPV antibodies	neg			neg	neg	6	6	neg	neg	
	9B	Oral HPV DNA	HPV 16, 59	NEG	NEG	NEG	NA	NEG	NA	NA	NA	NEG
		HPV antibodies	6			6	NA	neg	NA	NA	NA	
	10B	Oral HPV DNA	NEG	NEG	NEG	HPV 16	NA	NEG	NEG	NEG	NEG	NA
		HPV antibodies	neg			neg	neg	neg	6	NA	NA	
ii	11B*	Oral HPV DNA	NEG	NEG	NA	NEG	NA	NEG	NEG	NEG	NEG	NEG
		HPV antibodies	11			11	NA	neg	neg	neg	neg	
	12B	Oral HPV DNA	NEG	HPV 16, 39	HPV 16	NEG	NEG	NEG	NA	NA	NA	HPV 39
		HPV antibodies	neg			neg	NA	NA	NA	NA	NA	
	13B*	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
		HPV antibodies	6, 11, 16, 18			11, 16	11, 16	neg	neg	neg	neg	
	14B	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	HPV 16	NEG
		HPV antibodies	18			NA	6, 16, 18	6, 18	6, 18	NA	NA	
	15B	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NA	NEG	NA	NA	NA
		HPV antibodies	6, 11, 16			6	6	NA	16, 45	NA	NA	
	16B	Oral HPV DNA	NEG	NEG	HPV 16	NEG	HPV 11	NEG	NEG	NEG	NEG	NA
		HPV antibodies	6			neg	6, 11	6, 11	6, 11	16, 18	6	
	17B	Oral HPV DNA	HPV 6	HPV 6	NEG	HPV 6	NEG	NEG	NEG	NEG	NEG	NA
		HPV antibodies	neg			NA	neg	neg	neg	neg	18	
	18B	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
		HPV antibodies	18			18	18	6, 18	6	6	neg	
	19B	Oral HPV DNA	NEG	NEG	NEG	HPV 16	NEG	NEG	HPV 16	NEG	NEG	NEG
		HPV antibodies	6, 11, 16			11, 16	16	neg	neg	6, 11	6	
	20B	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	HPV 18	HPV 70	NEG	NEG	NA
		HPV antibodies	6			neg	neg	neg	neg	6, 11	6	
	21B	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	HPV 6	NA
		HPV antibodies	neg			neg	neg	6, 18	6, 18	NA	NA	
	22B	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	HPV 16	HPV 16	NEG	NEG	NA
		HPV antibodies	neg			neg	neg	neg	NA	NA	NA	
	23B	Oral HPV DNA	NEG	NEG	HPV 16	NEG	NEG	NEG	NEG	NEG	NEG	NA
		HPV antibodies	6			neg	neg	neg	neg	neg	18	
	24B	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NA
		HPV antibodies	6			neg	NA	neg	neg	neg	neg	
	25B	Oral HPV DNA	NEG	HPV 6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NA
		HPV antibodies	16, 18			18	neg	neg	neg	6, 11	6	
	26B	Oral HPV DNA	NEG	HPV 18	NEG	HPV 16	NEG	NEG	NEG	NEG	HPV 16	NEG
		HPV antibodies	neg			neg	neg	neg	neg	neg	neg	
	27B	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NA	NEG
		HPV antibodies	6			neg	neg	neg	neg	18	NA	
	28B	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NA	NA	NA
		HPV antibodies	6			neg	NA	NA	6, 11	NA	NA	
	29B	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
		HPV antibodies	6			6	6	neg	NA	neg	neg	
	30B	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
		HPV antibodies	6			neg	neg	neg	neg	neg	neg	
	31B	Oral HPV DNA	NEG	NEG	HPV 6	NEG	NEG	NEG	NEG	NA	NA	NA
		HPV antibodies	6, 11, 16, 18, 45			16	neg	neg	neg	NA	NA	

NEG = no HPV DNA detected, neg = no HPV antibodies detected, NA = no sample available

* Child is analyzed also in group iii or iv

Table 10. Detected HPV types and serum HPV antibodies during follow-up period in children, who had HPV DNA detected in the placenta or/and cord blood or/and had persistent HPV in oral mucosa during the 7-years FU or had oral HPV 16 at the final time point (group iii)

subgroup	ID	HPV DNA in Cord blood	HPV DNA in Placenta		Mother before delivery (genital HPV and antibodies)	Child									Persistent oral HPV DNA	HPV vaccination
						At birth	At the age of									
							3 days	1 month	2 months	6 months	12 months	24 months	36 months	72months	14 years	
a	103		HPV 16	Oral HPV DNA	NEG	HPV 16	NEG	HPV 16	HPV 16	NEG	NEG	NEG	NEG	HPV 16	NEG	HPV 16	YES
				HPV antibodies	6, 11			6	6	neg	neg	neg	NA			
	205		HPV 16	Oral HPV DNA	HPV 16	NEG	NEG	HPV 16	HPV 16	NEG	NEG	NA	NA	NEG	NEG		YES
				HPV antibodies	16			16	neg	neg	neg	NA	NA			
	104		HPV 83	Oral HPV DNA	NEG	HPV 16	NEG	HPV 16	HPV 16	NEG	NEG	HPV 16	HPV 16	HPV 16	NEG	HPV 16	NO
				HPV antibodies	Neg			neg	neg	neg	neg	neg	neg			
	202	HPV 16	HPV 16	Oral HPV DNA	NEG	HPV 16,33,59	NEG	HPV 16	HPV 18	NEG	NEG	NEG	NEG	HPV 33,59	NEG	HPV 33, 59	NO
				HPV antibodies	Neg			neg	neg	neg	neg	6, 11	6			
	203	HPV 16	HPV 16	Oral HPV DNA	NEG	HPV 16,33	NEG	NEG	HPV 16	NEG	NEG	NA	NA	NA	NEG		YES
				HPV antibodies	6			neg	neg	neg	neg	NA	NA			
	204	HPV 6	HPV 6	Oral HPV DNA	HPV 6, 59	NEG	NEG	NEG	NEG	HPV 6	NEG	NEG	NEG	NEG	NEG		NO
				HPV antibodies	6, 16, 18, 45			NA	neg	neg	6	neg	neg			
	206	HPV 6	HPV 6	Oral HPV DNA	HPV 6	HPV 6	NEG	HPV 6	NEG	NEG	NEG	NEG	NEG	NA	NEG		YES
				HPV antibodies	Neg			NA	neg	neg	neg	neg	neg			
	101	HPV 39		Oral HPV DNA	NEG	HPV 39	NA	NA	NA	NA	NA	NA	NA	HPV 39	NEG	HPV 39	NO
				HPV antibodies	Neg			NA	NA	NA	NA	NA	NA			
	102	HPV 16		Oral HPV DNA	NEG	NEG	NEG	HPV 16	HPV 16	HPV 16	NEG	NEG	HPV 16	NEG	HPV 16	HPV 16	NO
				HPV antibodies	6			neg	neg	neg	neg	neg	neg			
	201*	HPV 16		Oral HPV DNA	NEG	NEG	NA	NA	NA	NEG	NEG	NEG	NEG	NEG	NA		NO
				HPV antibodies	11			11	NA	neg	neg	neg	neg			
b	301*			Oral HPV DNA	HPV 16, 31, 42	HPV 31	NEG	NEG	NEG	NEG	NEG	HPV 70	HPV 18	HPV 31	NA	HPV 31	NO
				HPV antibodies	6, 16			neg	NA	neg	neg	16	neg			
	302*			Oral HPV DNA	HPV 31	HPV 31	HPV 6	HPV 33	NEG	NEG	NEG	NEG	NEG	HPV 31	NA	HPV 31	NO
				HPV antibodies	6			NA	6	neg	neg	neg	6			
	303			Oral HPV DNA	NEG	HPV 33	NEG	NEG	NEG	NEG	NEG	NEG	HPV 16	HPV 33	NEG	HPV 33	NO
				HPV antibodies	16			16	NA	NA	18	NA	neg			
	304			Oral HPV DNA	NEG	HPV 16	NEG	NEG	NEG	NEG	HPV 16	NEG	HPV 16	HPV 16	HPV 11	HPV 16	NO
				HPV antibodies	6, 16			6, 16	neg	neg	neg	neg	neg			
	305			Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	HPV 16, 59	NA	NA	NA	HPV 16	HPV 6, 16	HPV 16	YES
				HPV antibodies	neg			neg	neg	neg	NA	NA	NA			
	306			Oral HPV DNA	NEG	HPV 45	NEG	NEG	NEG	NEG	NEG	HPV 16	HPV 16	HPV 45	NEG	HPV 45	NO
				HPV antibodies	neg			neg	neg	neg	neg	neg	6			
c	011			Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	HPV 16		NO
				HPV antibodies	6			6	6	neg	neg	neg	neg			
	013			Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NA	HPV 16		YES
				HPV antibodies	16			16	6, 16	neg	6, 11	6	neg			
	014			Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NA	HPV 16		YES
				HPV antibodies	neg			16	NA	6	neg	6, 11	6, 11			

NEG = no HPV DNA detected, neg = no HPV antibodies detected, NA = no sample available

* Child is analyzed also in group i or ii

Table 11. Detected HPV types and serum HPV antibodies during follow-up period in children, who remained constantly negative for oral HPV (group iv)

ID		Mother before delivery (genital HPV and antibodies)	Child										HPV vaccination
			Baseline	At the age of									
				3 days	1 months	2 months	6 months	12 months	24 months	36 months	72 months	14 years	
001	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NO
	HPV antibodies	**6**			NA	**6**	neg	neg	**16**	**6, 16**			
002	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NO
	HPV antibodies	neg			NA	neg	neg	neg	**6**	neg			
003*	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NA	NA	NO
	HPV antibodies	**6,11,16,18**			NA	neg	neg	neg	neg	neg			
004	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	YES
	HPV antibodies	**6**			**6, 16**	**6, 16**	**6**	**6**	**6, 16**	**6**			
005	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	**HPV 11**	NO
	HPV antibodies	neg			neg	neg	neg	neg	neg	**6**			
006	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NA	NA	NA	NA	NA	NEG	YES ǂ
	HPV antibodies	**6**			NA	NA	NA	NA	NA	NA			
007	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NA	YES
	HPV antibodies	**6.16**			neg	neg	neg	**6**	**6**	neg			
008*	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NA	NO
	HPV antibodies	**6,16,18**			**11, 16**	**11, 16**	neg	neg	neg	neg			
009	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NO
	HPV antibodies	**6, 16**			**6, 16**	neg	neg	neg	**6, 16**	**6**			
010	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NA	NA	NEG	YES
	HPV antibodies	**6, 11,16,18**			NA	NA	neg	**6**	neg	NA			
012*	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NA	NA	NO
	HPV antibodies	**16**			**16**	neg	neg	neg	neg	**6**			
015	Oral HPV DNA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NO
	HPV antibodies	neg			neg	NA	neg	neg	neg	neg			
016	Oral HPV DNA	**18, 45**	NEG	NEG	NEG	NEG	NEG	NEG	NA	NA	NA	NEG	NO
	HPV antibodies	**6, 11, 16,18**			**6,11, 16**	**6, 11**	neg	NA	NA	NA			
017*	Oral HPV DNA	**16**	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NA	NA	NA	NO
	HPV antibodies	**16**			neg	neg	neg	**6**	**6**	NA			

NEG = no HPV DNA detected, neg = no HPV antibodies detected, NA = no sample available
* Child is analyzed also in group i or ii
ǂ 1 of 3 doses of HPV vaccination received before final time point

Table 12. Summary of detected HPV-types and HPV antibodies in children with no T cell response to any HPV 16 peptides

ID	HPV DNA in oral mucosa during follow-up	Persistent HPV DNA in oral mucosa	HPV DNA in oral mucosa at 14 years	Serum HPV antibodies	HPV DNA in Placenta	HPV DNA in Cord blood
16B	HPV 16, 11		NA	6, 11, 16, 18	NEG	NEG
19B	HPV 16		NA	6, 11, 16	NEG	NEG
204	HPV 6		NEG	6	HPV6	HPV6
101	HPV 39	HPV 39	NEG	NA	HPV39	NEG
305	HPV 6, 16, 59	HPV 16	HPV 16	NEG	NEG	NEG
306	HPV 16	HPV 45	NEG	6	NEG	NEG
011	NEG		HPV 16	6	NEG	NEG
001	NEG		NEG	6, 16	NEG	NEG
010	NEG		NEG	6	NEG	NEG

5.5.1 HPV 16-specific proliferative T cell response

PBMCs isolated from venous blood were stimulated for 6 days with peptides spanning the amino acid sequences of the HPV 16 E2, E6 and E7 proteins. HPV 16-specific immunity was determined by measuring the cell proliferation and the cytokine production rates to canvass the Th-polarization of stimulation-activated T cells. In addition, the percentages of Foxp3+ regulatory T cells were determined. These results were related to known oral and genital HPV DNA status and HPV serology during the 6–year follow-up.

Figure 10 shows the proliferation, secreted cytokines and upregulated Foxp3+ regulatory T cells in the mothers and their children with no sexual exposure. The PBMCs of all tested mothers and children, except one child (ID006) showed a proliferative T cell response after MRM stimulation, indicating that the cells had the capability to recognize the common antigens. HPV 16-specific proliferative T cell response against one or both E2 peptide pools was found in the majority of mothers and children (90.0% and 85.7%, respectively). A response against HPV 16 E6 peptide pools was seen in 20.0% of mothers and in 35.7% in all children. In addition, 7.1% of all children had a response against HPV 16 E7 peptides, but no response was seen in mothers.

As seen in Figure 11, the responsiveness against HPV E2 peptides is relatively similar between each specific group of children, from 73.6% to 100.0%. The responsiveness against HPV 16 E6 peptides was significantly less frequent in children of group iii (10.5%) than in children in other groups (47.3%), P=0.0077.

One mother (ID10A) and 9 children had no proliferative response against HPV 16 peptides. Mother ID10A had a persistent HPV 16 infection detectable for at least 72 months and eventually developed a CIN1 lesion with an HPV 16/66 double infection. She had HPV 16 antibodies detectable only once, one year after study entry, despite persisting HPV 16. Table 12 shows the 9 children who did not show any response to HPV 16 peptides. Four of these children had tested negative for oral HPV 16 at every timepoint (IDs 204, 101, 001, and 010). Child 001 had antibodies against HPV 16, but the three others did not have, suggesting that these three children had not encountered HPV 16. In addition, child ID011 did not test HPV 16-positive until the final timepoint, indicating the possibility of a recent infection not yet recognized by the adaptive immune system. The other four children had oral HPV 16 detected during the FU, and two of them (IDs 16B and 19B) also had antibodies against HPV 16. Children ID305 and ID306 had no HPV 16 antibodies detected.

The LST results for all children were also analyzed according to the presence of HPV antibodies in their sera during FU: 1) HPV antibodies of any type detected during follow-up, 2) HPV antibodies of any type detected before the age of 6 months, 3) HPV 16 antibodies detected during follow-up, and 4) HPV16 antibodies detected before the age of 6 months. No statistically significant differences in the frequency of LST responses were found.

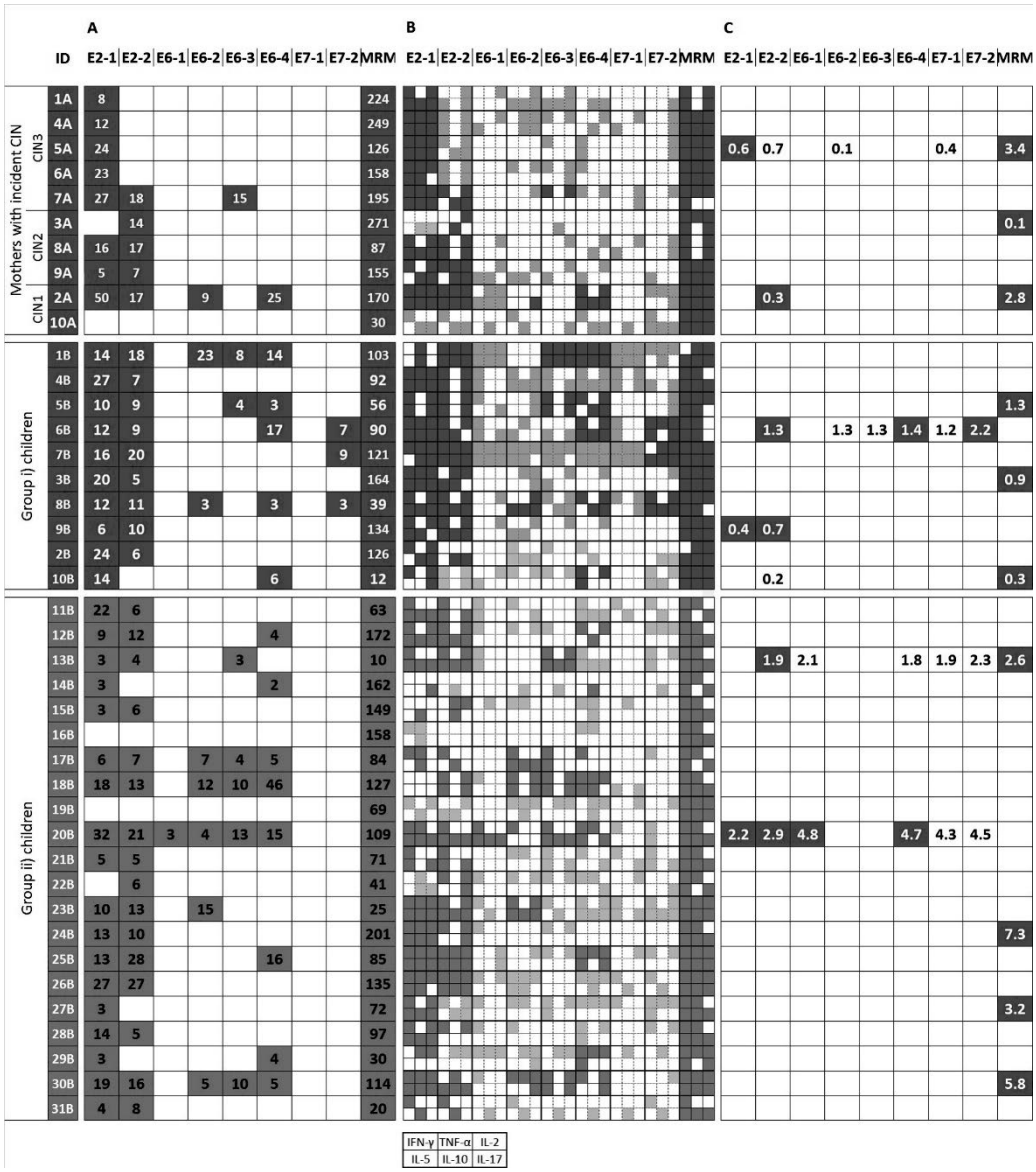
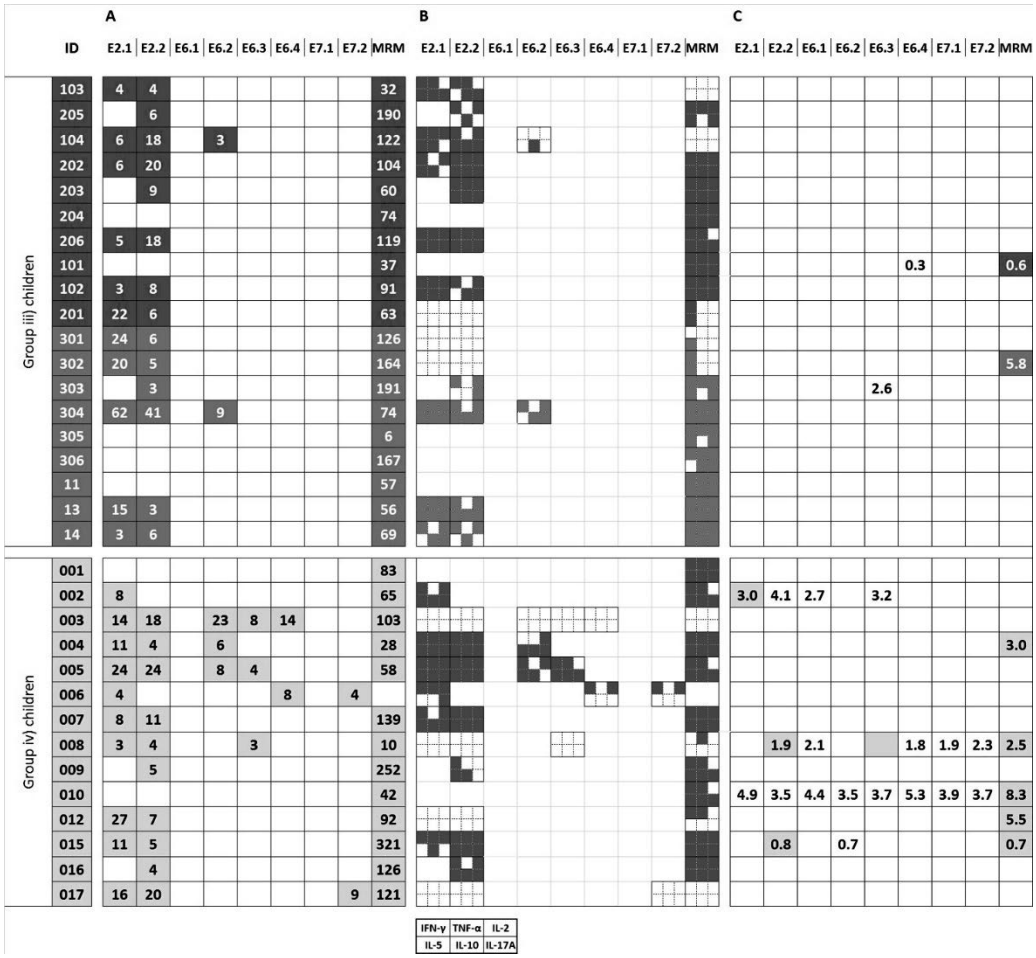


Figure 10. HPV 16 –specific T cell proliferation, cytokine production and Treg frequencies A) Results of lymphocyte stimulation test (LST). Only positive responses are marked with a grey box and stimulation index below each peptide pool. Memory response mix (MRM) was used as a positive control. B) Supernatants from the LST were analyzed for the presence of cytokines IFN-γ, TNF-α, IL-2, IL-5, IL-10, and IL-17. Each small colored square represents the production of one of six cytokines as the layout indicates. The dark grey color indicates cytokine production of proliferating cells (positive response in LST), and light grey indicates cytokine production of cells showing no proliferation. C) The increased frequencies of CD4+CD25+foxp3+ Tregs are shown. A dark colored box indicates a positive response in LST.



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5.5.2 HPV 16-specific cytokine production

Supernatants of the *in vitro* proliferation tests were collected at day 6 and subjected to a Cytometric Bead Array to analyze the cytokines produced. The selected cytokines IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-10, and IL-17A were measured to find out the cytokine profile and to assess the presence of IL-17A producing HPV 16-specific CD4+ T cells.

The mean concentrations and ranges of cytokines secreted by HPV 16 peptide–stimulated PBMCs for the cases and controls are presented in Table 13. Generally, the levels of all measured cytokines were low (in most cases < 100 pg/mL), but the range was wide from 0.27 to 329.0 pg/mL in mothers and to 281.2 pg/mL in children. Cytokine secretion was higher in the cultures displaying cell proliferation after stimulation than in the other cultures. IFN-γ, IL-10 and IL-2 were the most frequently detected cytokines in all children, followed by IL-17A, IL-5 and TNF-α. The concentrations of IFN-γ were also slightly higher than those of other cytokines. IL-4 was rarely detected and therefore not shown. Figures 10B and 11B illustrate the cytokines produced by the PBMC as a response for each peptide pools in the LST. On the whole, the children of mothers who had developed CIN (group i) seemed to the show highest levels of all measured cytokines, but the difference was not statistically significant.

In the case-control setting analysis, the children of mothers who had developed CIN3 showed significantly higher IFN-γ (P = 0.032) and TNF-α (P = 0.008) levels than the children whose mothers had had CIN1 or CIN2 or had stayed negative for genital HPV (groups i and ii). Significantly higher IL-10 levels were detected in children with oral HPV DNA or HPV DNA detected in placenta and/or cord blood (group iii) than in the HPV-negative controls (group iv) (P=0.033). Furthermore, when the children of group iii) were divided into subgroups according to their HPV status, significantly higher HPV 16 E2-induced IL-5 (p=0.043), IL-10 (p=0.013) and IL-17A (p=0.043) levels were detected when HPV DNA had been detected in the placenta or/and cord blood than in children who had only oral HPV. Among cases of HPV-positive placenta and/or cord blood, children without persistent oral HPV showed higher TNF-α (p=0.013), IL-2 (p=0.009) and IL-5 (P=0.006) secretion after E2.1 stimulation than children with persistent HPV (pooled data). However, when the E2-induced cytokine secretion of those children was compared to all other tested children (groups i, ii and iv), no significant difference was found.

Figure 12 shows the mean fractions of each cytokine from the total amount of cytokine secretion and illustrates the distinct cytokine profile of each group. Here, the cytokine profiles of the two control groups, the children of mothers negative for genital HPV and the children negative for oral HPV, are considered to represent the profile of normal, healthy individuals. When comparing the other groups to these controls, the children with HPV DNA detected in the placenta and/or cord blood appear to display a slight shift toward type 2 cytokines (IL-5 and IL-10) and IL-17A, whereas the children with persistent oral HPV show higher IL-2 fractions.

5.5.3 HPV 16-specific CD4+ CD25+ Foxp3+ regulatory T cells

The frequency of CD4+ CD25+ Foxp3+ Tregs after 7-day stimulation of PBMCs was analyzed using flow cytometry. The percentage of HPV 16-specific Tregs was considered to be increased when the measured frequency was twice as high as the background frequency (medium-only control). The increased frequencies of Tregs are shown in Figures 10 and 11.

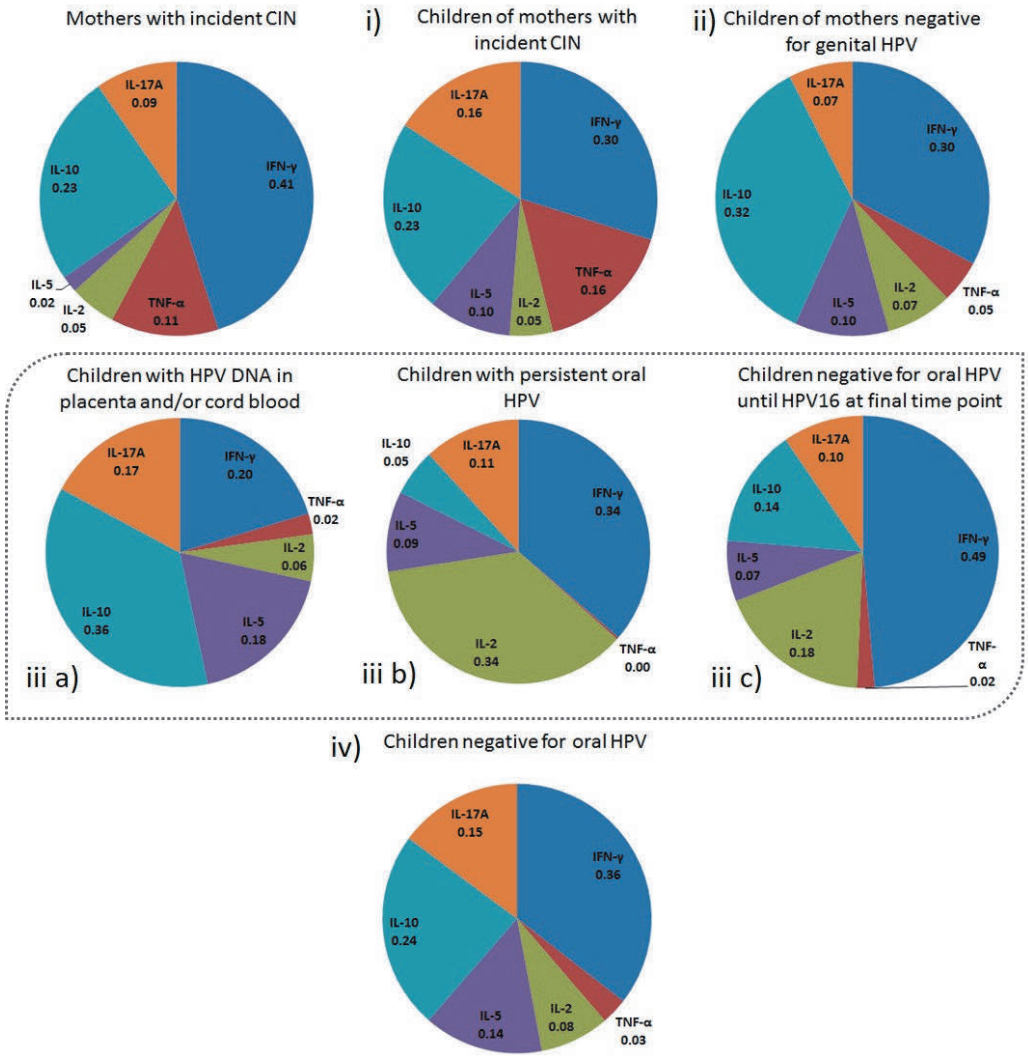

Figure 12. The mean fraction of each cytokine from the total amount of cytokine secretion among each tested group. The children of group iii) are further divided into three subgroups according to their HPV status: a) Children who had HPV DNA detected in the placenta or/and cord blood with or without oral HPV, b) Children who had only persistent HPV in oral mucosa during the 6-year follow-up, and c) Children who had stayed negative for oral HPV at all visits during the 72-month follow-up until testing positive for oral HPV 16 at the last visit when blood was taken for CMI. The fractions of each cytokine produced by each individual against all tested peptide pools were determined, and the mean of those fractions of the whole subgroup were calculated.

Table 13. The mean concentrations (pg/mL) and range of secreted cytokines against HPV 16 peptides.

Groups		IFN-γ						TNF-α						IL-2					
		E2		E6		E7		E2		E6		E7		E2		E6		E7	
		Mean	(Range)	Mean	(Range)	Mean	(Range)	Mean	(Range)	Mean	(Range)	Mean	(Range)	Mean	(Range)	Mean	(Range)	Mean	(Range)
Mothers with CIN	CIN1	7.2	(4.0 - 10.2)					6.0	(1.9 - 10.1)					0.8	(0.5 - 1.1)				
	CIN2	10.8	(1.8 - 20.1)					4.0*						0.9	(0.4 - 1.6)				
	CIN3	86.4	(3.8 - 329.8)	1.64*				14.0	(5.8 - 38.0)					1.9	(0.6 - 4.7)				
	All mothers	48	(1.82 - 329.8)	1.64*				10.3	(1.9 - 38.0)					1.4	(0.4 - 4.7)				
Group i) children	mothers CIN1	46.7*		23.9*				2.6*						2.4	(0.6 - 4.1)	4.3*			
	mothers CIN2	11.7	(3.5 - 22.1)	1.6*		0.7*		5.9	(1.5 - 14.6)					2.5	(0.8 - 3.8)	1.1	(1.0 - 1.2)	2.1*	
	mothers CIN3	46.6 ¶	(1.2 - 141.1)	176	(1.2 - 608.0)	36.7	(0.3 - 73.2)	29.7 ¶¶	(3.7 - 93.1)			75	(0.8 - 149.2)	5.4	(0.5 - 23.9)	2.4	(0.7 - 7.1)	5.6	(2.3 - 9.0)
	all	35	(1.2 - 141.1)	121.5	(1.2 - 608.0)	24.7	(0.3 - 73.2)	19.9	(3.7 - 93.1)			75	(0.8 - 149.2)	4	(0.5 - 23.99	2.3	(0.7 - 7.1)	4.5	(2.1 - 9.0)
Group ii) children		25.9	(1.1 - 281.2)	10.7	(0.6 - 60.3)			4.2	(0.5 - 25.7)	7.3	(0.4-25.0)			4.3	(1.2 - 10.3)	2.4	(0.3 - 7.6)		
Group iii) children	subgroup a	7.2	(0.3 - 45.3)					1.4	(0.3 - 5.6)					1.9	(0.3 - 7.8)				
	subgroup b	129.9	(74.7 - 185.0)					2.0*						4.2	(1.6 - 7.7)	1.0*			
	subgroup c	6.7	(0.6 - 22.7)					1.7*						1.4	(0.4 - 2.1)				
	all	21.5	(0.3 - 185.0)					1.5	(0.3 - 5.6)					2.1‡	(0.3 - 7.8)	1.0*			
Group iv) children		31.1	(0.3 - 230.0)	7.6	(0.6 - 12.2)	0.9*		2.9	(0.3 - 11.2)	0.4*				3.1	(0.5 - 9.8)	1.6	(0.9 - 2.6)	0.8*	

Groups		IL-5						IL-10						IL-17A					
		E2		E6		E7		E2		E6		E7		E2		E6		E7	
		Mean	(Range)	Mean	(Range)	Mean	(Range)	Mean	(Range)	Mean	(Range)	Mean	(Range)	Mean	(Range)	Mean	(Range)	Mean	(Range)
Mothers with CIN	CIN1	0.6	(0.5 - 0.7)					1.4	(0.6 - 2.2)					0.8	(0.8 - 0.9)				
	CIN2	1.1	(1.0 - 1.2)					7.9	(0.5 - 14.0)					7.0	(3.4 - 11.0)	2.8*			
	CIN3	2.5	(0.1 - 9.0)					17.4	(5.3 - 41.6)					17.3	(2.5 - 75.3)				
	All mothers	1.8	(0.1 - 9.0)					11.6	(0.5 - 41.6)					10.6	(0.8 - 75.3)	2.8*			
Group i) children	mothers CIN1			3.2*				2.4	(2.2 - 2.5)					6.7	(2.2 - 9.1)				
	mothers CIN2	9.6	(0.3 - 37.1)	0.3*		0.4*		18.9	(3.1 - 37.9)	16.3	(10.0 - 22.6)	14.3*		8.4	(1.7 - 14.6)	1.5	(1.2 - 1.8)	7.8*	
	mothers CIN3	26.4	(0.6 - 83.4)	7.6	(82.3 - 11.0)	3.7	(1.3 - 6.1)	29.9	(1.9 - 82.0)	15.7	(6.3 - 37.8)	16.7*		9.1	(1.4 - 29.6)	3.1	(1.6 - 5.9)	3.4	(1.7 - 5.1)
	all	19.9	(0.3 - 83.4)	5.6	(0.3 - 11.0)	2.6	(0.4 - 6.1)	22.6	(1.9 - 82.0)	15.9	(6.3 - 37.8)	15.5	(14.3 - 16.7)	8.4	(1.4 - 29.6)	2.6	(1.2 - 5.9)	4.9	(1.7 - 7.8)
Group ii) children		10.5	(0.5 - 38.5)	7.1	(0.8 - 34.5)			23.2	(1.6 - 100.3)	8.3	(3.8 - 18.0)			6.5	(0.3 - 49.5)	2.4	(0.5 - 7.9)		
Group iii) children	subgroup a	10.7*	(0.4 - 37.8)					10.3	(1.4 - 38.4)	0.8*				5.1	(0.5 - 16.7)	0.3*			
	subgroup b	39.6	(34.1 - 45.2)					14.5 #	(0.6 - 22.6)	3.4*				6.7	(0.4 - 16.7)	3.0*			
	subgroup c	2.9	(1.1 - 4.7)					0.8	(0.4 - 1.7)					1.9	(0.4 - 4.8)				
	all	14.0	(0.4 - 45.2)					9.0‡‡	(0.4 - 38.4)	2.1	(0.8 - 3.4)			4.7	(0.4 - 16.7)	1.7	(0.3 - 3.0)		
Group iv) children		18.8	(0.4 - 117.2)	0.9	(0.3 - 2.3)			8.0	(1.1 - 32.4)	3.4	(1.5 - 9.3)			8.7	(0.3 - 48.7)	1.9	(0.5 - 3.2)		

‡ Statistically signicantly lower in group iii than in other groups of children (P=0.027)
‡‡ Statistically signicantly lower in group iii than in other groups of children (P=0.022)
¶ Statistically signicantly higher in children of mothers with CIN3 than in other children (groups i and ii) (P=0.032)
¶¶ Statistically signicantly higher in children of mothers with CIN3 than in other children (groups i and ii) (P=0.008)
# Statistically significantly higher in IV than other children in groups iii and iv p = 0.033
*Only single value detected

6 DISCUSSION

The established conception of HPV infection as a mainly sexually transmitted disease and the well-defined association with cervical cancer has diverted the research mostly to adult women, while knowledge on HPV infections in children under the age of onset of sexual maturity has remained relatively limited. In fact, HPV infections found in children, especially those caused by mucosal HPV types also regarded as genital types, have earlier been associated with sexual abuse (Syrjänen 2010). Today, however, the evidence for additional, non-sexual transmission routes for HPV is relatively consistent. It has been implicated that the mother can transmit HPV to her newborn vertically, but also horizontally, similar to other care takers or siblings, as reviewed by Merckx and co-workers (Merckx *et al.* 2013) and Syrjänen (Syrjänen 2010). Existing data on HPV prevalence in early childhood are limited and highly controversial, and even less is known about HPV-specific immunity invoked by these early infections. However, it now seems that HPV is found in children more often than expected and that the timing and mode of the first HPV infection might have a long-lasting and even crucial impact on later encounters with HPV due to differences in activation and maturation of HPV-specific immunity, as has been observed in other human virus infections during early life (Prendergast *et al.* 2012).

In this PhD study, HPV DNA was frequently detected in the oral cavities of over 300 newborns in the Finnish Family HPV Study cohort. The high correspondence between the HPV genotypes and HPV antibodies of mothers and their newborns supports the idea of vertical transmission of the virus and antiviral antibodies. The majority of 56 tested children showed cell-mediated immune responses against HPV 16 early proteins, indicating a past or present exposure to HPV 16 infection. Further, the placenta seems to play an important role in HPV transmission and might also have a role in the development of HPV-specific immunity.

6.1 PREVALENCE AND CONCORDANCE OF HPV DNA AND HPV ANTIBODIES IN NEWBORNS AND MOTHERS

In the present study, 17.9% of oral samples taken during the two first months of infants' lives tested HPV-positive. In general, the literature on the prevalence of oral HPV in infants is relatively controversial. An early cohort study (not included in the present study cohort) conducted by our group reported HPV DNA in 37% of infants' oro-nasopharyngeal aspirates after birth (Puranen *et al.* 1997). Merckx and colleagues recently summarized the observations of 20 studies on the vertical transmission of genital HPV between 1991 and 2010 in a meta-analysis, presenting an overall prevalence of 7.6% for HPV infections in all studied children within the first week after birth (Merckx *et al.* 2013). However, the heterogeneity between the analyzed studies was relatively high, reporting HPV frequencies from 0% to 61%. In a few more recent studies, the prevalence has varied from 3.2 % to 23.6% (Hahn *et al.* 2013, Hong *et al.*

2013, Lee *et al.* 2013, Martinelli *et al.* 2012, Park *et al.* 2012). The wide variation in HPV detection rates might be explained by differences in the study designs, study protocols, sampling techniques and HPV testing methods (D'Souza *et al.* 2005, Kellokoski 1992, Puranen *et al.* 1997).

HPV 16 was the most prevalent genotype in the oral mucosa of infants as well as in the genital mucosa of their mothers. This observation is consistent with most of the earlier studies (Hong *et al.* 2013, Merckx *et al.* 2013, Park *et al.* 2012). The next most prevalent genotypes in infants, HPV 6, 11 and 18, have also been reported in those studies. The variation seen in the reported genotype profiles presumably reflects the variety of HPV genotyping assays covering 2-40 genotypes (Hahn *et al.* 2013, Hong *et al.* 2013, Lee *et al.* 2013, Martinelli *et al.* 2012, Merckx *et al.* 2013, Park *et al.* 2012). The Multimetrix® assay used for HPV genotyping in the present study has one of the broadest range of high-risk and low-risk HPV types. Since the yield of nucleated cells in the oral scrapings of newborns or infants was anticipated to be low, the nested PCR was used to detect even very small amounts of viral DNA. Further re-amplification for biotinylation of the nested PCR products was assumed to increase the HPV detection rate significantly (Remmerbach *et al.* 2004). A meta-analysis implicated that children of HPV-positive mothers have a 33% (ranging from 0% to 70%) higher risk of becoming infected by HPV than children of HPV-negative mothers and that the risk increases up to 45% when analyzing only high-risk HPV types (Merckx *et al.* 2013). In the present study, the HPV genotype spectrum of oral mucosa in newborns was similar, but more restricted than that of the genital mucosa of their mothers. The strong concordance between the HPV genotypes of mother-infant pairs subsequently after delivery suggests vertical transmission from mother to newborn. However, the HPV genotype spectrum of the infant started to differ from that of their mother after the third postnatal day, while the incidence of HPV first declined from delivery to the third postnatal day, and then increased again in months 1 and 2. These changes could be explained by hospital contamination or a transient HPV infection or passage from the mother. The newborn can also acquire HPV horizontally from other family members or caretakers, as has been shown previously (Czeglédy 2001, Syrjänen 2010). Thus, early horizontal transmission cannot be excluded without accurate and repetitive HPV testing of the other family members and care takers (Merckx *et al.* 2013). Comparison of HPV sequences might be required to prove that the mother's HPV genotype has truly infected the newborn and persisted since then.

The evaluation of demographic factors of the mother resulted in only two significant predictors of oral HPV detection in the infant: the age of the mother and her history of genital warts (condylomas). In the present study, detection of oral HPV DNA was most frequent in infants of mothers aged 20-34 years. Previous data shows that the prevalence of genital HPV infections is highest among young women, with the incidence peaking at 20-25 years of age. After this peak, the infection prevalence declines gradually to a plateau in women at middle-age (Bruni *et al.* 2010, Burchell *et al.* 2006). Among young women, HPV infections are usually transient and clear rapidly without persisting long enough to become transmitted to the

newborn. In contrast, among women more than 30 years of age, HPV infections more often remain persistent and might be more prone to being transmitted. This was previously also observed in the mothers of the present study cohort (Louvanto *et al.* 2010a). Further, in older age groups, HPV clearance became more common (Louvanto *et al.* 2010b), even exceeding the incidence rates, explaining the lower probability of maternal-to-newborn transmission among older mothers (Bruni *et al.* 2010). The history of genital warts indicates exposure not only to low-risk HPV types 6 and 11 but also to other genotypes that may remain latent and become activated during pregnancy. Productive genital infection indicates the spread of viral particles in the mucosa of genital tract on a wide scale, favoring virus transmission to the newborn. Genital warts of the mother were earlier shown to predict cord blood HPV positivity (Sarkola *et al.* 2008a). The gender of the infant had no statistically significant association with the acquisition of HPV. In the study by Hong and colleagues, HPV prevalence was higher among female infants than males (17.7 vs. 11.6%, n = 233) (Hong *et al.* 2013), while the ratio of genders among infants is not usually reported in similar studies.

Interestingly, the presence of HPV in the placenta and/or cord blood was found to be a powerful determinant of oral HPV carriage by the newborn. We have earlier shown that HPV DNA 6 and 16-positivity in placenta samples could be localized in syncytiotrophoblastic cells with tyramine amplified by *in situ* hybridization (Sarkola *et al.* 2008a). HPV -types 11, 16, 18 and 31 are able to complete their lifecycle in trophoblastic cells *in vitro* (You *et al.* 2002, You *et al.* 2008), indicating that the crossing of the maternal-fetal barrier is plausible phenomenon for HPV. Thus, these results support the idea of the placenta as an important bridge for the maternal transmission of oral HPV infection to the infant. Recently, several studies have demonstrated microbial presence in the placenta and cord blood (Aagaard *et al.* 2014, Jimenez-Flores *et al.* 2006) and the similarity between oral and placental or amniotic fluid microbiomes (Aagaard *et al.* 2014, Bearfield *et al.* 2002, Fardini *et al.* 2010, Stout *et al.* 2013). The placental microbiome is suggested to be established by the hematogenous spread of maternal oral microbiota (Fardini *et al.* 2010, Rautava *et al.* 2012, Satokari *et al.* 2009). Furthermore, the placenta has been suggested to act as a site where translocated maternal oral microbes are presented to the fetal antigen-presenting cells, which in turn activate fetal Tregs preventing the undesirable reactivity to maternal antigens, thus resulting in the development of prenatal tolerance to the maternal microbiome (Mold *et al.* 2008, Zaura *et al.* 2014). Accordingly, this might implicate that the association between placental and oral HPV infection could have features similar to the development of the oral microbiome. However, because the activities and population dynamics of bacteria are very different from viruses, too straightforward a comparison should be avoided.

HPV infection of the placenta has been associated with pregnancy complications, such as spontaneous abortions and preterm delivery (Ambuhl *et al.* 2016), premature rupture of the membranes (Cho *et al.* 2013) and pre-eclampsia (McDonnold *et al.* 2014, Slatter *et al.* 2015), while *in vitro* studies have demonstrated HPV causing dysfunction in the trophoblast viability and the adherence of endometrial cells (Boulenouar *et al.* 2010, You *et al.* 2002, You *et al.*

2008). However, in this study all HPV-positive placentas were from full-term pregnancies classified as macroscopically normal (Sarkola *et al.* 2008a). The mode of delivery had no predictive value for the detection of HPV DNA in the placenta or umbilical cord blood, as shown in our earlier reports (Puranen *et al.* 1997, Sarkola *et al.* 2008a).

Almost identical HPV L1 antibody profiles were found in mother-infant pairs at 1 month after delivery. This was expected, since infants are known to be entirely dependent on maternal antibodies acquired by active transfer through the placenta (IgG antibodies only) during the first few months of life (Heim *et al.* 2007). These results were concordant with those reported by Smith and co-workers (Smith *et al.* 2010), who found a high concordance (93%) between the antibodies (VLPs for HPV types 16, 18, 31, and 33) of mothers and newborns at delivery, while none of the HPV types detected in the infants matched the infants' HPV antibody types. In the present study, the significant association between the high-risk HPV antibodies in the infants at the age of 1 month and the presence of oral HPV DNA at delivery indicates that the mother transfers both high-risk HPVs and anti-HPV antibodies to her newborn. An association was also found between the presence of low-risk HPV antibodies and the detection of low-risk HPV DNA in infants at 2 months. It has been suggested that the neutralizing antibodies transferred from the mother might not always be completely protective, but they may attenuate the infection, thus generating favorable conditions for the infant's immune system to become activated and generate immunological memory cells against reinfection (Navarini *et al.* 2010, Zinkernagel 2001). This could implicate that detection of HPV DNA concurrently with (maternal) HPV antibodies during the first months of life might predict the development of natural immunization against HPV. However, the viral dose in relation to the antibody levels might be the most crucial factor in the development of virus-specific immunity (Zinkernagel 2001).

6.2 HPV 16 SPECIFIC CELL-MEDIATED IMMUNITY IN MOTHERS AND CHILDREN

The activation of a cell-mediated immune response is essential for the resolution of the HPV infection and diseases induced by HPV (Doorbar *et al.* 2012, Stanley 2009). Thus, the presence of HPV-specific memory T cells in the circulation of the individual might provide information on both infections encountered and possible reactivity to infections to come. A number of studies have described HPV 16–specific cell-mediated immunity in healthy adults and among women with HPV-induced CC or CIN (de Jong *et al.* 2002, de Jong *et al.* 2004, Heusinkveld *et al.* 2011, Jacobelli *et al.* 2012, Welters *et al.* 2003, Welters *et al.* 2008), but no studies of HPV-specific immunity in children have been reported. In fact, children with no sexual exposure to HPV are not expected to present HPV 16-specific immune responses.

Contrary to this hypothesis, the results of the present study revealed HPV 16 E2-specific T cell responses in the majority (85.7%) of the 56 tested children. Since HPV E2 is known to be abundant in HPV-infected cells at the stage of viral amplification, the circulating HPV 16 E2–

specific memory T cells are supposed to originate from prior or present replicative HPV 16 infection (McBride. 2013). Accordingly, these results indicate that the majority of these children must have encountered non-sexual exposure to HPV 16 at some body sites. The possibility of the cross-reactivity of HPV 16 E2, E6 and E7 peptides with T cells specific to other closely related HPV types is a rarely observed event (de Jong *et al.* 2002, de Jong *et al.* 2004, van den Hende *et al.* 2010, Welters *et al.* 2005), and thus cannot explain the frequency of HPV 16-specific cell-mediated immune responses among the children of the present study.

Previous studies among healthy adults have reported responsiveness against both HPV E2 and E6 peptides (de Jong *et al.* 2002, de Jong *et al.* 2004, Jacobelli *et al.* 2012, Welters *et al.* 2003). Similarly, in the present study, over one-third of the children showing a proliferative response against HPV E2 peptides were also responsive against E6 or E7 peptides. However, this responsiveness appeared significantly less frequent in cases of HPV DNA-positive placenta, cord blood or oral mucosa (group iii) than in children negative for oral HPV, indicating that a very early or long-term exposure to HPV can be associated with a lack of E6-specific effector memory T cells. Responsiveness to HPV 16 E2 alone might not be predictive of the outcome of subsequent infection, but both E6 and E2 are needed to successfully resolve the HPV 16 infection in children as well as in adults (Welters *et al.* 2003). However, few studies on HPV-specific immunity in children are available for comparison. An HPV 16 E7-specific response was observed in only 5 children, who were also responsive to E2 peptides. This observation is in line with previous studies among adult women (van der Burg *et al.* 2001, Woo *et al.* 2010), implicating that HPV 16 E7-specific T cell responses are rare in healthy individuals. A possible explanation for the lack of a T cell response against HPV E7 could be the difference in localization of the E proteins of high-risk HPV types. High-risk E7 proteins are expressed in the nucleus of the cell, while E6 and E2 of high-risk HPV are expressed in the nucleus as well as in the cytoplasm (Maitland *et al.* 1998, Guccione *et al.* 2002). The localization of the antigen in the infected cell may be crucial for the accessibility of these antigens for antigen-presenting cells.

Altogether, 5 out of 56 children showed no proliferative responses against HPV 16 peptides even though they tested positive for oral HPV 16 at least once during the follow-up. In the case where HPV had been detected only once, especially at a very early timepoint, the possible explanation for the unresponsiveness could be a transient infection originating, for example, from hospital contamination. Thus, the activation of an adaptive immune response requires a true, productive infection. Also, a very recent infection might not have been recognized by the adaptive immune system prior to blood sampling (Stanley 2009).

Three out of 56 children had HPV 16 DNA in repetitive samples but no serum antibodies to HPV 16 L1 protein after their first months of life. One of these children had received three doses of prophylactic HPV vaccine, while HPV 16 DNA was still detected in her oral samples at the final timepoint. This duly implicates that antibodies elicited by the HPV vaccination cannot neutralize or eliminate persistent HPV 16 infection in children. Thus, these three children,

especially child ID305, carrying persistent oral HPV 16 without any detectable immunological responses, could display conceivable features of HPV tolerance. The exposure of an immunologically immature newborn with no or only low levels of maternal antibodies to a high dose of the virus by vertical exposure could induce immunological tolerance to HPV and result in persistent infection (Syrjänen & Syrjänen 2000, Zinkernagel 2001). Alternatively, the viral lifecycle may remain undetected by the cells of innate immunity. In fact, the infected keratinocyte and the Langerhans cells at the epithelia can act as immunosuppressors and induce tolerance by presenting the HPV-derived peptides to naïve T cells without upregulating the activation markers which are essential co-stimulators in the activation of the T cell response (Bal *et al.* 1990, Fausch *et al.* 2003). In addition, we noticed that in children with persistent HPV during the follow-up, the same HPV type was detected as early as at birth or during the first two months, implying that these very early infections could be more prone to persisting. It has not been established whether repeatedly detected DNA of a given HPV type in the oral mucosa is a sign of persistent HPV infection or whether it is a new encounter with the same HPV type, also applying to the genital samples of the mothers. However, since the recent data on persistent oral HPV 16 infections in adults have shown that HPV 16 is either in an integrated or mixed physical state, while all subject who cleared their oral infection had HPV 16 in an episomal state (Lorenzo *et al.* unpublished), the concept of HPV persistence appears to be more plausible explanation.

In the present study, significantly higher HPV 16 E2-induced levels of type 2 cytokines IL-5 and IL-10 and also Th17-associated IL-17A were observed in children with HPV DNA detected in placenta and/or cord blood than in children who had only oral HPV. Among healthy adults, the HPV 16-specific immune response is characterized by more or less balanced production of T helper type 1 and type 2 cytokines, but this equilibrium is lost in cervical cancer patients, with a concomitant switch toward type 2 cytokines (IL-10 and IL-4) (de Jong *et al.* 2004). These findings raise a question about the significance of the possible prenatal HPV infection for the development of HPV-specific immunity and infection progression.

During normal pregnancy, both the maternal and fetal immune responses are characterized by an adaptation toward tolerance against the antigens of the other party (Prendergast *et al.* 2012). Established by the production of low levels of type I IFNs, IFN-γ and IL-12 (Th1-trophic cytokines) and higher levels of IL-6, IL-23 and IL-1β (Th17-trophic cytokines), the fetal innate immune defense seems to be oriented rather to extracellular microbes than intracellular virus infections. Further, the tolerance of adaptive immunity is associated with predominant Th2 and Treg cell populations suggested to establish a kind of protective homeostasis (Mold *et al.* 2008, Prendergast *et al.* 2012, Prescott *et al.* 1998). This Th2 predominance persists in the newborn's system after birth at least months before the realignment of the Th1/Th2 environment (Abelius *et al.* 2015, Diesner *et al.* 2012). Thus, levels of the key cytokine for Th1 differentiation, IL-12 p70, are shown to be significantly lower in children than in adults at least until the age of 12 years, implicating weaker antiviral responses. In addition, earlier studies in mice have shown that inoculation of newborn mice with high doses of murine retrovirus

induces Th2 cytokine responses, while low viral doses induce Th1 responses. Thus, the lower T cell number in neonates compared to adult mice explains the reduced immune responses to the same antigen. (Forsthuber *et al.* 1996, Ridge *et al.* 1996, Sarzotti *et al.* 1996).

Altogether, the infant seems to remain particularly vulnerable to intracellular pathogens, such as viruses that are transmitted during pregnancy (Prendergast *et al.* 2012). Thus, HPV infection or exposure to viral antigens during the prenatal period, especially via the placenta, might influence the activation and type of immune responses and the outcome of HPV infection. Indeed, the timing of infection seems also to be crucial to the outcomes of other virus infections that are known to be transmitted during pregnancy via the placenta or upon delivery, such as hepatitis B virus (HBV), cytomegalovirus (CMV), herpes simplex virus (HSV), varicella zoster virus (VZV), enterovirus, respiratory syncytial virus (RSV) and influenza virus (Prendergast *et al.* 2012).

On the whole, the cytokine levels measured in this study were relatively low and variable among individuals also within a same study group. Thus, inspection of the secretion profiles, i.e. the mean fractions of each cytokine from the total amount of all measured cytokines, might provide a better view of the polarization of type 1 and type 2 cytokines between these study groups than the comparison of the concentrations of a particular cytokine alone. Importantly, all of the children studied for cell-mediated immune responses were defined as healthy individuals, without any HPV-related disease at the time of sampling, while in earlier studies the alterations in cytokine levels have been observed in adult patients with CIN or cervical cancer (Bais *et al.* 2005, Clerici *et al.* 1997, de Jong *et al.* 2004, Woo *et al.* 2010).

Since HPV 16-induced CIN and cervical cancer lesions are assumed to produce both HPV 16 E6 and E7 proteins (Bosch *et al.* 2002), the memory responses against these proteins should have been expected in the four tested mothers with HPV 16 detected in their CIN lesions. The lack of E6 and E7 responses have been observed earlier in patients with CIN and cervical cancer (de Jong *et al.* 2004, Heusinkveld *et al.* 2011, van der Burg *et al.* 2001, Woo *et al.* 2010). Although the mothers of this study might not be fully comparable to patients with CIN or cervical cancer, since they were all treated several years before obtaining samples for studies on cell-mediated immunity, these results might indicate that the treatments of CIN lesions had no activating effect on the cell-mediated immune responses against the early proteins of HPV 16.

Several studies have shown that the local cytokine milieu in the HPV-induced lesions is shifted toward the Th2 type. This may further change the cytokine secretion pattern of systemic T cells. Accordingly, the removal of the lesion might have a reviving effect on the systemic cytokine polarization, resulting in more or less balanced Th1/Th2 secretion (Bais *et al.* 2005, Giannini *et al.* 1998, Hazelbag *et al.* 2001, Mota *et al.* 1999, Sheu *et al.* 2001). Such a phenomenon might also explain the observations in the mothers of the present study.

Finally, HPV 16 peptide-induced upregulation of Foxp3+ Tregs was found in 7 out of 56 children and 2 out of 10 mothers, but the observed levels were relatively low. While the Tregs

are known to play an important role in normal immune homeostasis, increased frequencies of Tregs are reported in the peripheral blood of individuals with different types of cancers (Whiteside 2012), and also women with persistent HPV infection or progressive CIN and cervical cancer (Molling *et al.* 2007, Visser *et al.* 2007). Because the children of the present study represented more or less healthy individuals, they were not expected to display deviation in their Treg frequencies. Thus, according to these results, Tregs might not be significant predictors of HPV-specific immunity in children. Among mothers, as discussed in the previous paragraph, the possible CIN-induced upregulation of Tregs might have also been suppressed by the treatment/removal of CIN several years earlier. This might implicate that upregulation of Tregs in CIN and cervical cancer patients is rather induced during lesion development than pre-existing in these individuals.

7 CONCLUSIONS

The main conclusions of the present study were:

i) HPV is prevalent in oral samples of newborns. The HPV genotype profile of oral mucosa in newborns is similar, but more restricted than that of the maternal cervical samples at birth and starts to differ from the mother during the follow-up period, suggesting that an infected mother transmits HPV to her newborn.

ii) The serum antibodies for HPV L1 antigens in an infant during the two first months of life were similar to those of the mother before delivery, indicating that the HPV antibodies of the the child are of maternal origin.

iii) The presence of HPV in the placenta was the single most powerful determinant of oral HPV carriage by the newborn at delivery, implying that the placenta might be the most important source of the oral HPV infections of the infants.

iv) HPV 16-specific T cell responses were found in the majority of tested children, indicating that these children must have been exposed to HPV 16 infection at some body sites already before the onset of sexual activity.

v) Dominance of the Th2 cytokine profile in children infected with HPV 16 during early life was established, suggesting that exposure to HPV and/or the specific environment created by the placenta may have a significant impact on the type of HPV-specific immunity developed. However, the HPV-related disease of the mother did not show any statistically significant effect on the HPV-specific immunity of her child.

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