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HELMI SUOMINEN

Immunological and Serological Aspects of Human Papillomavirus Infection in Finnish Mothers and Their Children

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Joensuu 2024

To my family

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Tampere, February 2024

Helmi Suominen

ABSTRACT

Human papillomavirus (HPV) infects cells of the cutaneous and mucosal epithelia. Most HPV infections are transient and subclinical as they are eliminated by an efficient host immune response. On the other hand, an insufficient immune response may result in persistency and progression of an HPV infection, which may even lead to development of cancer. As HPV infection can be transmitted vertically from a mother to her child, HPV infection may be acquired already at early age.

This thesis is part of the longitudinal Finnish Family HPV Study. The general objective of the present study was to further investigate HPV-related immunology and serology in Finnish mothers and their children. The specific aims of the present study were to characterize peripheral blood T lymphocyte immunophenotypic subsets regarding HPV16 infection status of the mothers and the corresponding effects on their children; to determine maternal antibodies against HPV6 early and late proteins and corresponding HPV seroconversion in children in their early infancy; and finally to evaluate the effect of pregnancy on the seropositivity and antibody levels for HPV types 6, 11, 16, 18, and 45.

Results on T lymphocyte immunophenotypic subsets revealed differences as related to the mother's HPV16 infection status at either genital or oral site. Both genital and oral HPV16 infection were associated with alterations in the peripheral blood T lymphocyte subsets in the mothers. In the children, alterations in the T lymphocyte subsets were observed only in those children whose mother had the persistent oral HPV16 infection. Furthermore, results on HPV6 antibodies against both HPV early and late proteins indicated that there was a clear correlation between maternal and neonatal HPV6 antibodies. After the decay of maternal HPV6 antibodies, seroconversion against HPV6 L1, E2, E4, E6, and E7 proteins did occur in early childhood during the follow-up. Finally, the results of the present study showed that seropositivity for HPV types 6, 11, 16, 18, and 45 was less common in the women who developed second pregnancy than in those who did not.

These results support the view that the mother's HPV infection affects the immune system of her offspring. In addition, these results are in line with that HPV infection may be acquired in early childhood, potentially via vertical or horizontal transmission.

TIIVISTELMÄ

Ihmisen papilloomavirus (HPV) infektoi sekä ihon että limakalvojen epiteelikudosta. Suuri osa HPV-infektioista on ohimeneviä ja ilmenee ilman havaittavia oireita, kun elimistön immuunipuolustus toimii tehokkaasti. Toisaalta riittämättömän immuunivasteen seurauksena HPV-infektio voi kroonistua ja edetä, mikä voi altistaa syövän kehittymiselle. HPV-infektio voi myös välittyä vertikaalisesti äidiltä lapselle, joten HPV-infektion voi saada jo varhaislapsuudessa.

Tämä väitöskirjatyö on osa suomalaista perhekohorttitutkimusta (Finnish Family HPV Study). Tutkimuksen yleisenä tavoitteena oli selvittää HPV-infektioon liittyvää immunologiaa ja luonnollisten HPV-vasta-aineiden muodostusta suomalaisilla äideillä ja heidän lapsillaan. Tässä tutkimuksessa selvitettiin T-lymfosyyttien immunofenotyyppisten alatyyppien ilmenemistä HPV16-infektion saaneilla äideillä ja heidän lapsillaan, varhaisvaiheen ja myöhäisvaiheen HPV6-proteiineja kohtaan muodostettujen vasta-aineiden siirtymistä äidiltä vastasyntyneelle sekä näiden vasta-aineiden ilmaantumista myöhemmin varhaislapsuudessa, ja lopuksi arvioitiin raskauden vaikutusta luonnollisiin vasta-ainetasoihin HPV6, 11, 16, 18 ja 45 osalta.

Tutkimuksen tulokset T-lymfosyyttien alatyypeistä osoittivat muutoksia äidin HPV16-infektiostatukseen nähden. Sekä suun että genitaalialueen HPV16-infektio aiheuttivat vaihtelua perifeerisen veren T-lymfosyyttien alatyyppeihin äideillä. Muutoksia T-lymfosyyttien alatyypeissä havaittiin vain niillä lapsilla, joiden äidillä oli krooninen suun alueen HPV16-infektio. Tutkimustulokset sekä varhaisvaiheen että myöhäisvaiheen HPV6-proteiinien vasta-aineista osoittivat, että sekä äidin että vastasyntyneen HPV6-vasta-aineiden välillä oli selvä yhteys. Äidiltä saatujen vasta-aineiden jo heikennyttyä havaittiin, että HPV6 L1-, E2-, E4-, E6- ja E7-proteiineja kohtaan ilmaantui uusia vasta-aineita jo varhaislapsuudessa tutkimuksen seuranta-aikana. Lisäksi tutkimustulosten perusteella havaittiin, että HPV-tyyppien 6, 11, 16, 18 ja 45 vasta-ainepositiivisuus oli vähemmän yleistä niillä naisilla, jotka tulivat toista kertaa raskaaksi.

Tämän tutkimuksen tulokset tukevat näkemystä siitä, että äidin HPV-infektio vaikuttaa hänen lapsensa immuunijärjestelmään. Lisäksi nämä tutkimustulokset ovat linjassa sen kanssa, että HPV-infektion voi saada jo varhaislapsuudessa, mahdollisesti vertikaalisen tai horisontaalisen välittymisen kautta.

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ABBREVIATIONS

APC	antigen presenting cell
ASCUS	atypical squamous cells of undetermined significance
BCR	B cell receptor
CD	cluster of differentiation
CIN	cervical intraepithelial neoplasia
CMI	cell-mediated immunity
CMV	cytomegalovirus
CSR	class switch recombination
DNA	deoxyribonucleic acid
E	early
EBV	Epstein-Barr virus
FACS	fluorescence-activated cell sorting
FFHPV	Finnish Family HPV Study
GST	glutathione S-transferase
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPV	human papillomavirus
HR	high-risk
HSIL	high-grade squamous intraepithelial lesion
HSV	herpes simplex virus
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ISG	interferon stimulated gene
JO	juvenile-onset
L	late
LC	Langerhans cell
LEEP	loop electrosurgical excision procedure
LR	low-risk

LSIL	low-grade squamous intraepithelial lesion
MFI	median fluorescence intensity
mo	months
n	number
NILM	negative for intraepithelial lesion or malignancy
ORF	open reading frame
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
pRB	retinoblastoma protein
PRR	pathogen recognition receptor
RNA	ribonucleic acid
RRP	recurrent respiratory papillomatosis
SD	standard deviation
STD	sexually transmitted disease
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF-α	tumour necrosis factor alpha
T_{EMRA}	terminally differentiated T cell
T_{reg}	regulatory T cell
VLP	virus-like particle

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by the Roman numerals I–III.

I **Suominen Helmi**, Paaso Anna, Koskimaa Hanna-Mari, Grénman Seija, Syrjänen Kari, Syrjänen Stina, Louvanto Karolina. Peripheral Blood T-lymphocyte Phenotypes in Mother-Child Pairs Stratified by the Maternal HPV Status: Persistent HPV16 vs. HPV-Negative: A Case-Control Study. *Viruses*. 2022;14(12):2633. DOI: 10.3390/v14122633.

II **Suominen Helmi**, Syrjänen Kari, Waterboer Tim, Grénman Seija, Syrjänen Stina, Louvanto Karolina. Serum IgG antibodies to HPV6 L1, E2, E4, E6, and E7 proteins among children prospectively followed-up for three years. Submitted.

III **Suominen Helmi**, Suominen Nelli, Syrjänen Kari, Waterboer Tim, Grénman Seija, Syrjänen Stina, Louvanto Karolina. Effect of a Second Pregnancy on the HPV Serology in Mothers Followed Up in the Finnish Family HPV Study. *Viruses*. 2023;15(10): 2109. DOI: 10.3390/v15102109.

AUTHOR'S CONTRIBUTION

I The author participated in the formal analysis, validation of the data, and conducted a literature search. The author analyzed the results and designed visualization of the results together with the supervisor. The author prepared the original draft of the manuscript and finalized the manuscript for submission.

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1 INTRODUCTION

Human papillomaviruses (HPVs) are small DNA viruses that belong to the *Papillomaviridae* family, and they infect cells of the cutaneous and mucosal epithelia. At present, over 450 HPV genotypes have been isolated and over 200 HPV types have been comprehensively classified. HPV infection can be acquired by both sexual and non-sexual transmission routes, these non-sexual transmission modes including vertical transmission, horizontal transmission, autoinoculation from one site to another, and indirect transmission. Most HPV infections are transient and subclinical as they are eradicated by an efficient host immune response. However, in some cases, HPV infection persists and leads to formation of lesions that may eventually lead to detrimental outcomes and even cancer.

To maintain viral life cycle successfully, HPV must escape from protective immune mechanisms of the host. On the other hand, the host immune system needs to perform effectively in order to avoid being infected with HPV. If infected, both the innate and the adaptive immunity are needed to in order to successfully clear HPV. Innate immune system is responsible of clearing many subclinical HPV infections, whereas regression of an HPV-induced lesion requires an effective adaptive immune response that is activated by innate immunity. More specifically, a cell-mediated immune response that consists primarily of antigen specific cytotoxic T lymphocytes and helper T lymphocytes is needed. In addition to T lymphocytes, adaptive immune system consists of B lymphocytes that are mainly responsible for the antibody-mediated immune response against pathogens that the host has previously encountered.

A failure to develop sufficient immune response can result in the persistence and progression of an HPV infection. In most cases, effective cell-mediated immune response enables recognition of infected cells, T cells infiltrate the HPV induced lesion, and this results in the resolution of the infection. Although antibody response to HPV may contribute to both prevention and elimination of HPV, antibody response resulting from a naturally acquired HPV infection is highly variable. As the antibody response generated by HPV vaccination is stronger than the antibody response resulting from a natural HPV infection, one of the most efficient ways to

primary prevent HPV infection and the development of its clinical manifestations is to vaccinate young individuals before they encounter HPV.

Immunophenotyping of peripheral blood T lymphocyte subsets could cast further light on understanding different outcomes of HPV infection perhaps even without clinical HPV lesions, and knowledge on T lymphocyte immunophenotypic subsets as related to HPV infection status among mothers and their children is practically non-existent. As for HPV antibodies in mothers and their young children and especially from the viewpoint of HPV early protein antibodies, their role is nearly unexplored. In addition, it has not been firmly established whether pregnancy or becoming pregnant for the second time alters HPV antibody levels and HPV seropositivity in general. The prospective Finnish Family HPV Study cohort was used to analyze peripheral blood T lymphocyte immunophenotypic subsets among mothers with persistent HPV16 infection and their children. Furthermore, these mothers and their children were subjected to analyses regarding antibodies to HPV6 L1, E2, E4, E6, and E7 proteins. Lastly, seroprevalence for HPV types 6, 11, 16, 18, and 45 were analyzed in women who developed a second consecutive pregnancy during the follow-up of the study.

2 REVIEW OF THE LITERATURE

2.1 Human papillomavirus

2.1.1 Structure

Human papillomaviruses (HPVs) are small non-enveloped, double-stranded circular DNA viruses that belong to the *Papillomaviridae* family. The HPV genome consists of approximately 7900 base pairs that form eight or nine open reading frames (ORFs) encoding eight protein genes. These genes include six early genes (E1, E2, E4, E5, E6, and E7) and two late genes (L1 and L2) (Doorbar et al., 2015). The E1 and E2 genes are well-conserved core genes involved in viral replication. The L1 and L2 genes are highly conserved, and they function in constructing the viral protein structure. The L1 is the major capsid protein that can spontaneously assemble itself, whereas the L2 is the minor capsid protein that lacks the ability to form virus-like particles (VLPs) (Buck et al., 2013; Wang & Roden, 2013). In addition, high-risk HPVs (HR-HPVs) have oncoproteins E5, E6, and E7 that are involved in cell transformation of the HPV infected cells. Different HPV proteins and their functions are shown in Table 1.

Table 1. Characteristics and major functions of HPV proteins. Modified from McBride (McBride, 2022).

Protein	Characteristics	Function
E1	Origin-binding DNA helicase Site-specific DNA binding protein	Initiates viral DNA replication Recruits cellular replication machinery
E2	Dimeric DNA binding protein	Regulates viral transcription Supports viral DNA replication
E4	Expressed as a fusion protein E1^E4	Promotes viral genome amplification Contributes to viral release
E5	Hydrophobic membrane protein	Reduces immune detection Promotes cell proliferation and productive stages of infection

E6	Degrades p53 (in HR-HPVs)	Prevents growth arrest and apoptosis Modulates cell polarity to promote viral genome amplification and maintenance
E7	Degrades pRb (in HR-HPVs)	Promotes cell proliferation and viral DNA amplification Reduces immune detection and involves in cellular transformation
L1	Major capsid protein	Self-assembles into capsids
L2	Minor capsid protein	Forms capsids Traffics viral genome into nucleus

2.1.2 Classification

At present, over 450 HPV genotypes have been isolated and sequenced and out of these, over 200 HPV types have been comprehensively classified (International HPV Reference Center, 2023) (McBride, 2022). HPV identification is based on sequence analysis of the major capsid protein L1 gene and to be classified as an individual HPV genotype, a dissimilarity of at least 10 % to any other known HPV type is required (de Villiers, 2013). Based on the sequence analysis of the major capsid protein L1, HPVs are further divided and classified in distinct HPV types, species, and genera.

HPVs are divided in five different genera that include alpha-, beta-, gamma-, mu- and nu-papillomavirus according to their phylogenetic origin (McBride, 2022). Phylogenetic tree comprising all HPV types according to their genera is shown in Figure 1. All five genera of HPVs infect cutaneous epithelium, but some alphapapillomaviruses infect also oral and genital mucosal epithelium. Alphapapillomaviruses are clinically most relevant as a subset of their genus are determined as high-risk HPV (HR-HPV) types and it has been approximated that 4.5 % of cancer cases worldwide are caused by a high-risk HPV type (de Martel et al., 2017). Low-risk (LR-HPV) alphapapillomaviruses are considered non-carcinogenic, and they are affiliated with benign lesions such as warts and papillomas. The most important alphapapillomaviruses can be categorized by their carcinogenicity (Table 2). Most cutaneous HPV types from beta- and gammapapillomaviruses are not reported carcinogenic, but some betapapillomaviruses have been associated with UV-mediated skin cancer (Lambert et al., 2020).

Figure 1. Phylogenetic tree of the International HPV Reference Center that presents all official HPV types classified by their genera. Alphapapillomaviruses shown in green, betapapillomaviruses in blue, gammapapillomaviruses in red, mu-papillomaviruses in yellow, and nu-papillomaviruses in black colors. Figure with permission from the International HPV Reference Center (Mühr, 2023).

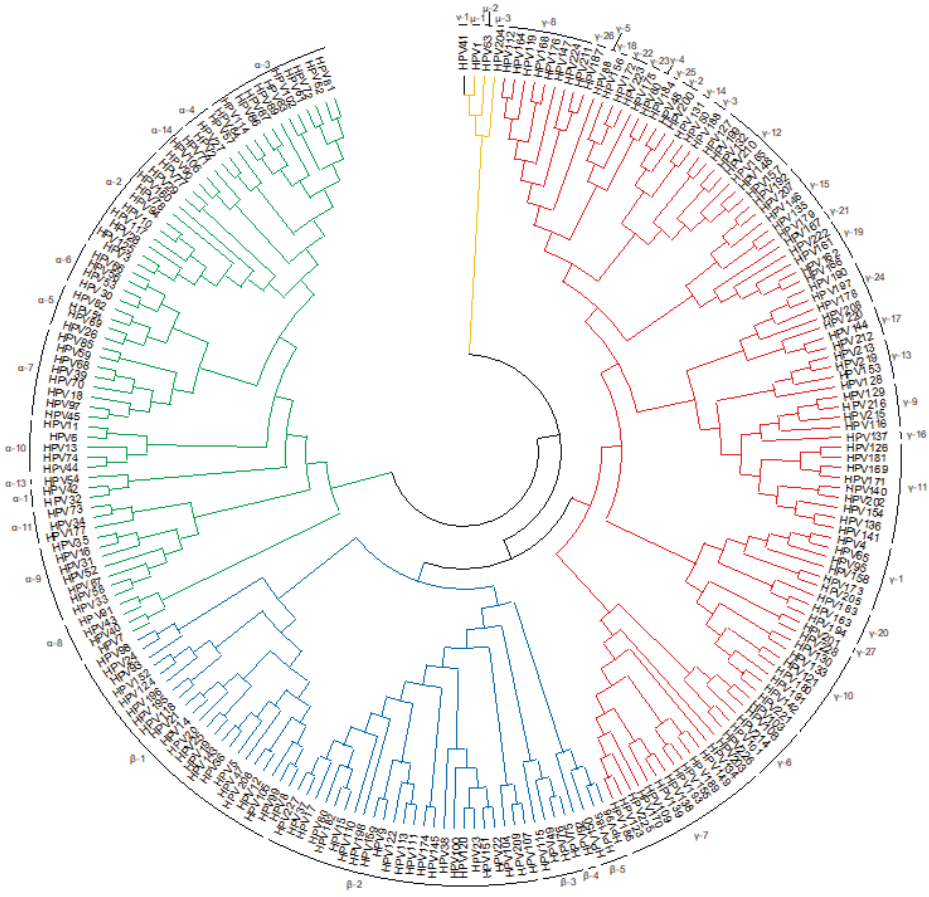


Table 2. Classification of the most important alphapapillomaviruses by their carcinogenicity as defined by the International Agency for Research on Cancer (IARC). (IARC et al., 2012)

Group	Classification	HPV type
1	Carcinogenic	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59[i]
2A	Probably carcinogenic	68 [i]
2B	Possibly carcinogenic	26, 30, 34, 53, 66, 67, 69, 70, 73, 82, 85, 97
3	Not classifiable as carcinogenic	6, 11 [ii]

i) High-risk HPV (HR-HPV) types, ii) Low-risk HPV (LR-HPV) types

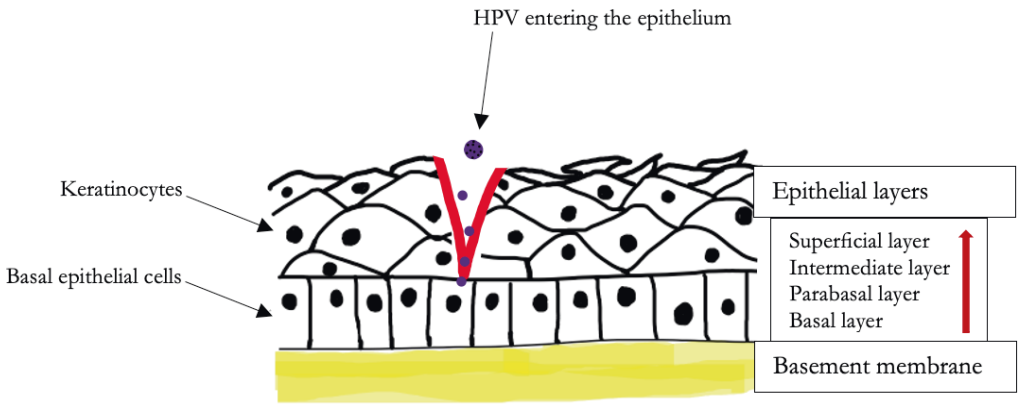
2.1.3 Life cycle

While the ultimate aim of viral life cycle is to produce new virions, viral reproduction requires a host cell in which the virus can replicate itself. As HPVs are regarded as highly epitheliotropic viruses, they infect cells of the cutaneous and mucosal epithelia of those anatomical regions that are characteristic to each specific HPV genus. Stratified squamous epithelium contains a basal cell layer that continuously proliferates and therefore produces new epithelial cells. Some of these daughter cells migrate and by differentiating as keratinocytes, they form differentiated layers of the epithelial tissue. Terminally differentiated keratinocytes (corneocytes) are located in the outermost layer of the epithelium and as they are frequently replaced by new corneocytes, the previous corneocytes shed off from the epithelial tissue.

HPV entries the epithelium via a small fissure or microabrasion, and it targets the undifferentiated cells of the basal cell layer in the epithelium (Figure 2). By infecting these proliferating basal layer cells, the virus promotes efficiency and sometimes this is followed by a long-term HPV infection, also known as a persistent HPV infection. After entering the epithelial cell, the virus may be able to begin its life cycle depending on the anatomical site and the local microenvironment, and if successful, this is initiated by genome amplification (Doorbar et al., 2015).

HPV genome encodes viral proteins that are associated with in different parts of viral reproduction, and viral replication proteins E1 and E2 are the first proteins expressed during infection as they are needed in the initial genome amplification phase. The E5, E6 and E7 proteins regulate the cell cycle progression by manipulating the balance of cellular proliferation and differentiation (Doorbar et al., 2015; McBride, 2022). The E4 protein expression contributes to viral genome amplification, and the E4 protein might be involved in the viral release and transmission as well (Doorbar, 2013). In order to promote late gene expression of the viral L1 and L2 proteins in fully differentiated keratinocytes, the prolongment of the cell cycle in the infected epithelial cell is required (McBride, 2022). These E1, E2, E4, E5, E6 and E7 are also known as HPV early proteins, whereas L1 and L2 are HPV late proteins. The L1 and L2 proteins form the major and minor capsid proteins, correspondingly. After having packaged the replicated HPV genome inside the viral capsid, the newly formed virions are shed from the outermost layer of the epithelium. In addition to the role of basal keratinocytes in viral entry, the infected basal cell layer serves as a reservoir of HPV infection to potentially sustain an HPV-induced lesion (Doorbar, 2018).

Figure 2. A schematic representation of HPV entering the cervical epithelium via a small fissure. The non-keratinized stratified squamous epithelium constructs of distinct layers, and HPV targets undifferentiated epithelial cells of the basal layer.



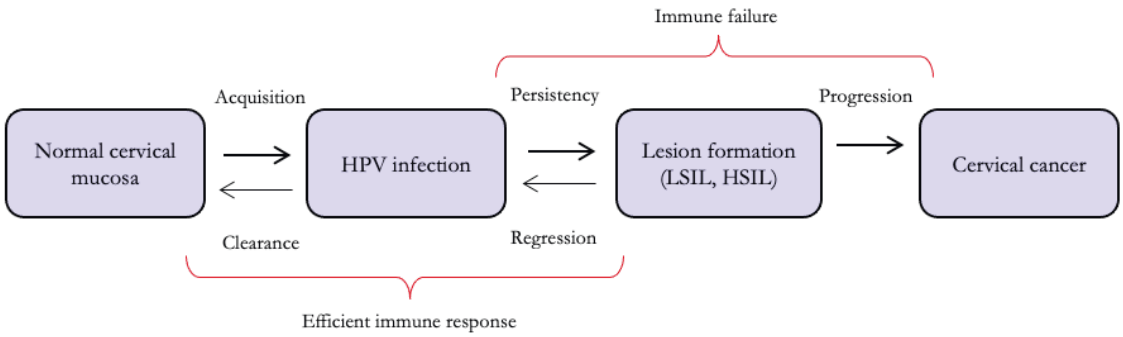
2.1.4 Clinical manifestations

Most HPV infections are transient and subclinical as they are eradicated by an efficient host immune response. However, not all HPV infections are cleared successfully, and in these cases, HPV infection may induce a clinical manifestation that is characteristic to the specific HPV genotype concerned. When the same HPV genotype is found in two consecutive samples, generally the HPV infection is classified as a persistent infection, although the definition of a persistent HPV infection varies. Some define persistency as having two or more HPV positive samples consecutively, and others by having an HPV infection that lasts more than the median duration (de Sanjosé et al., 2018; Marks et al., 2012). Duration of HPV infection varies, but there are some estimations of its clearance rates. Studies have suggested that over 90 % of acquired HPV infections are cleared within 12–24 months (Gravitt, 2011; Woodman et al., 2001).

A persistent HPV infection may lead to a transformation in the epithelial cells resulting in a lesion formation. When considering a HR-HPV induced lesion, the lesion might regress by time or on the other hand, it might progress into a precancerous or cancerous lesion. As for cervical HPV infection, precancerous lesions used to be classified as cervical intraepithelial neoplasia (CIN) on the scale of 1–3, and now in the current Bethesda classification system CIN1 is equivalent to low-grade squamous intraepithelial lesion (LSIL) and CIN2–3 to high-grade squamous intraepithelial lesion (HSIL). CIN1 or LSIL are considered as milder

cervical abnormalities, whereas CIN2–3 or HSIL are cervical abnormalities that show a higher risk of progression to cervical cancer (Gravitt, 2011). A simplified representation of the outcomes of cervical HPV infection is shown in Figure 3.

Figure 3. Outcomes of a cervical HPV infection. Acquisition of HPV infection may lead to persistency and progression as a result of an immune failure. Due to an effective immune response, an HPV infection may lead to clearance or regression of an HPV induced lesion.



The most severe clinical manifestations associated with a HR-HPV infection include several cancers, most strikingly cervical cancer but also certain anal, penile, vulvar, vaginal, and oropharyngeal cancers (Dunne & Park, 2013). The most prominent HR-HPV genotypes inducing carcinogenesis are especially HPV16 and HPV18, but also many other HR-HPV genotypes are detected depending on the specific cancer concerned. HR-HPV genotypes have an ability to express HPV E6 oncoprotein that degrades the tumour suppressor protein p53 and also E7 oncoprotein that binds to the tumour suppressor retinoblastoma protein (pRB) thereby allowing HPV DNA synthesis in the host cell of squamous epithelium (Brianti et al., 2017). Normally, E2 protein is capable to suppress transcription of these E6 and E7 genes and the loss of E2 repression leads to deregulation of viral oncogenes (Brianti et al., 2017; Smith et al. 2014). This in turn is thought to start the malignant transformation of the squamous epithelial host cell and eventually lead to the development of cancer.

Infection with a LR-HPV type can lead to an asymptomatic infection or formation of a benign hyperproliferative lesion (Egawa & Doorbar, 2017). Anogenital warts (condylomata acuminata) in the genital tract are a common

manifestation resulting typically from an infection with HPV6 or HPV11, and these same HPV types are also able to provoke formation of papillomas at oral sites (Doorbar et al., 2015). Common warts of the skin are most frequently associated with HPV types 1, 2, 4, 27, and 57, and other cutaneous HPV infections include manifestations of the skin such as plantar warts, flat warts, filiform warts, and epidermoid cysts (Doorbar et al., 2015). Even though cutaneous HPV infections are most often found to be benign in their nature, some betapapillomaviruses are thought to be involved in the development of cutaneous squamous cell carcinoma (Lambert et al., 2020; Smola, 2020). Recurrent respiratory papillomatosis (RRP) is a disorder in which papillomatous lesions can be located anywhere in the aerodigestive tract, and it is most often caused by HPV6 or HPV11 (Fortes et al., 2017). The juvenile form of RRP can be potentially life-threatening because it shows a tendency of the papillomatous lesions to spread and grow in size which may eventually result in a total respiratory obstruction. A rare disease called focal epithelial hyperplasia affects the oral mucosa, and it is associated with HPV13 and HPV32 infection (Bendtsen et al., 2021). The most frequent HPV types associated with various clinical manifestations are summarized in Table 3.

Table 3. The most common HPV types in different clinical manifestations. Modified from Cubie and Costa-Silva et al. (Costa-Silva et al., 2017; Cubie, 2013).

Clinical manifestation	Most frequently associated HPV types
Common warts	2, 4, 57
Flat warts	3, 10
Plantar warts	1, 2, 4
Anogenital warts	6, 11, 40, 42, 43, 44, 54, 61, 72, 81, 89
Premalignant and malignant anogenital lesions	16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 64, 65, 66, 67, 68, 69, 70, 73, 82
Oral papillomas	2, 6, 7, 11, 16, 18, 32, 57
Laryngeal papillomas	6, 11
Focal epithelial hyperplasia	13, 32
Head and neck carcinomas	16, 18

2.2 HPV epidemiology and transmission

2.2.1 Transmission modes

Transmission of HPV infection occurs primarily through skin-to-skin contact or skin-to-mucosa contact, and success of HPV transmission depends on the intensity of the exposure, susceptibility of the host, duration of infectiousness of the index case, and also other both pathogen and host related factors are involved (Veldhuijzen et al., 2010).

HPV infection is often viewed as sexually transmitted disease (STD), and it is regarded as the most common STD worldwide (Fitzmaurice et al., 2019). Sexual transmission of mucosal HPV infection consists of many possible routes, but the most common transmission route is through penetrative sex. When considering genital HPV infections, vast majority of them are induced by direct genital-to-genital transmission and for example, hand-to-genital transmission is not likely to be an as efficient transmission route (Malagón et al., 2019). Risk factors or cofactors for sexually transmitted HPV infection include early age at the onset of sexual activity, number of lifetime sexual partners, history of STDs, frequency of sex or intimate skin-to-skin contact, and other behavioural and background characteristics of sexual partners (Panatto et al., 2012; Yetimalar et al., 2012).

Despite the commonness HPV acquisition via sexual transmission, HPV infection can also be acquired by multiple non-sexual transmission routes. The significance of these non-sexual HPV transmission modes has been arising interest especially within the last decades as HPV has been detected in individuals prior to their sexual debut, including newborns, children, and virgins. Non-sexual HPV transmission modes include vertical, horizontal, and indirect transmission (Rintala et al., 2005; Liu et al., 2016; Trottier et al., 2016; Niyibizi et al., 2020; 2022; Khayargoli et al., 2023; Suominen et al., 2023).

Vertical transmission of HPV can occur from mother to child, and it may potentially take place periconceptually during fertilization, prenatally during pregnancy, or perinatally during birth or immediately thereafter (Sarkola et al., 2008; Weyn et al., 2011; Park et al., 2012; Trottier et al., 2016; Niyibizi et al., 2020; 2022; Khayargoli et al., 2023). Periconceptual transmission could theoretically occur as HPV DNA has been detected from semen, in both seminal plasma and spermatozoa, and a recent study detected presence of HR-HPV in oocytes as well (Jaworek et al., 2019; Rintala et al., 2004; Syrjänen, 2010). In most cases, vertical transmission occurs

in infants during delivery via direct contact to mothers' HPV infected cells (Trottier et al. 2016). As HPV DNA has been also detected in the amniotic fluid, fetal membranes, and placenta, prenatal intrauterine transmission of HPV may occur due to ascending infection from the birth canal or infection via placenta (Niyibizi et al., 2022; Zouridis et al., 2018). One factor affecting intrauterine vertical transmission rate of HPV infection is the mode of delivery. Caesarean section often shows higher protection from vertical transmission as compared to vaginal delivery, but nonetheless delivery with caesarean section has not shown to prevent vertical transmission of HPV (Chatzistamatiou et al., 2016; Hahn et al., 2013; Park et al. 2012). However, studies have reported widely varying vertical HPV transmission rates, and one systematic review estimated rate for intrauterine vertical transmission with a pooled frequency of 4.9 % (Zouridis et al., 2018). It has been suggested that the mother is the main transmitter of HPV to her newborn (Syrjänen, 2010). In a study assessing the FFHPV Study cohort, a significant concordance between the mother's and their newborn's HPV genotype at any site was observed for HPV types 6, 16, 18, 31, and 56, and this finding could support occurrence of vertical transmission (Suominen et al., 2023). Yet, the concordance between HPV types detected between infants and their mothers varies, which is why HPV infection in infants might be acquired from other sources as well (Hong et al., 2013; Rintala et al., 2005; Suominen et al., 2023).

As to other non-sexual HPV transmission routes, horizontal transmission may occur from a person to a person via close mucosal or cutaneous contact (other than sexual). For example, horizontal transmission can take place through hands or saliva in close contact between siblings (Petca et al., 2020; Syrjänen, 2010). Horizontal transmission may also be categorized further into heteroinoculation (transmission via close non-sexual contacts as described earlier) and autoinculation. Autoinoculation is one potential transmission route of HPV infection in which one may relocate the virus from one anatomical site to another, and this may occur by scratching, for instance. In addition, indirect transmission of HPV may take place via fomites such as HPV contaminated medical equipment (Ryndock & Meyers, 2014).

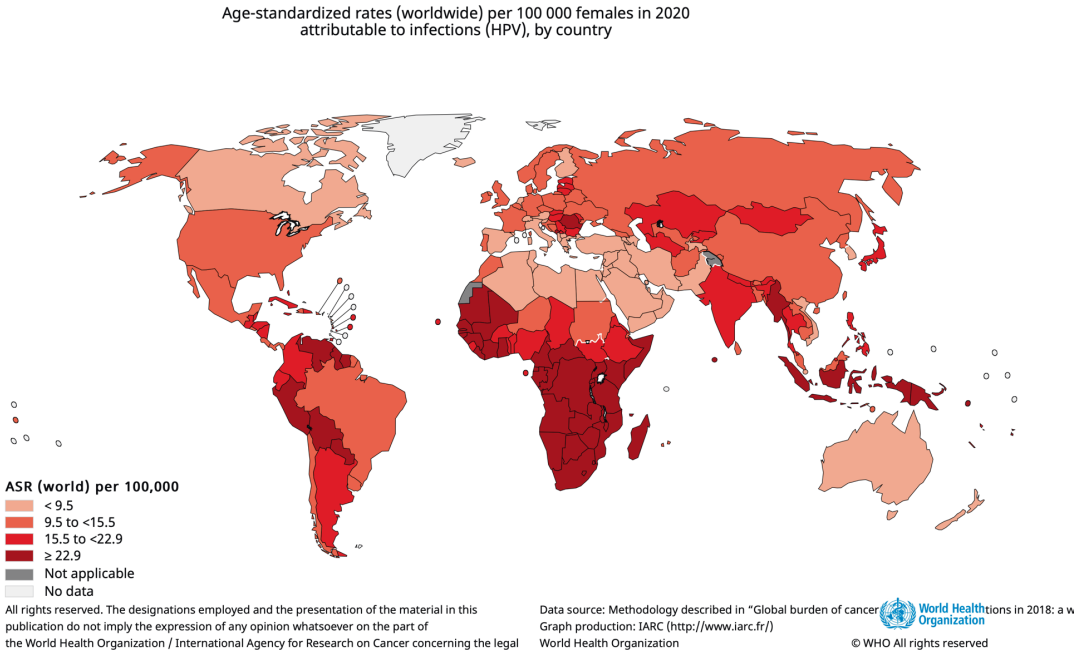
2.2.2 HPV infection in females

In general, the highest prevalence and incidence of HPV infection is recorded usually shortly after debut of sexual activity, and newly detected HPV infections in young

women are often associated with having a new sexual partner (Burchell et al., 2006). If at least one lifetime sexual partner has been recorded, the average lifetime probability of acquiring HPV is 85% in women and 91% in men, (Trottier et al., 2006; Chesson et al., 2014). Almost half of incident HPV infections clear within six months and over 90 % of them are cleared within two years from acquisition (Gravitt, 2011; Winer et al., 2011). Younger individuals have shown higher spontaneous HPV clearance rates (Kang et al., 2014). Worldwide, HPV prevalence is the highest in women under the age of 35, decreasing in women of older age, and a secondary peak of HPV has been detected in women aged 45 or older in Europe, Northern and Southern America, and Africa (de Sanjosé et al., 2007). It has been discussed that this second peak of HPV prevalence could be due to either a new HPV acquisition or reactivation of a previously acquired latent HPV infection, and some studies suggest that HPV latency could be more common cause than the acquirement of a new HPV infection (Fu et al., 2015).

HPV prevalence varies between different countries and continents. The highest prevalence among women without cervical abnormalities is in Sub-Saharan Africa (24.0 %), Eastern Europe (21.4 %), and Latin America (16.1 %) (Formana et al., 2012). Worldwide, the most common HPV types have been estimated to be HPV16 (3.2 %), HPV18 (1.4 %), HPV52 (0.9 %), HPV31 (0.8 %), and HPV58 (0.7 %) (Bruni et al., 2010). A meta-analysis estimated that out of all HPV infections, HPV16 is responsible for 32.3 % in Southern Asia, 28.9 % in Southern Europe, 24.4 % in Western Europe, 24.3 % in Northern America, and 12.0 % in Africa (de Sanjosé et al., 2007). In a study assessing asymptomatic Chinese women, the most prevalent genotypes of all HPV genotypes detected were HPV16 (26.2 %), HPV52 (19.4 %), HPV58 (13.8 %), and HPV53 (13.3 %) (Xue et al., 2015). In another study investigating type-specific distribution of HPV in women aged 25–65 years old who were attending cervical cancer screening in Finland, the most prevalent genotypes were HPV16 (0.9 %), HPV31 (0.7 %), and HPV52 (0.5 %) (Leinonen et al., 2013). As HPV prevalence is known to vary between different countries and continents, its effect may also be observed in the presence of HPV-related diseases. Figure 4 depicts worldwide cancer cases attributable to HPV infection in women (IARC, 2023).

Figure 4. Cancer cases attributable to HPV infection in age-standardized rates per 100 000 females worldwide in the year of 2020 (Figure with permission from the IARC, 2023).



One of the most acknowledged manifestations of HPV infection in women is cervical cancer. Cervical cancer remains as one of the most common malignancies worldwide, although HPV screening and vaccination programs have been implemented in many countries targeting to decrease the morbidity and mortality of cervical cancer. It has been estimated that approximately 604 000 new cases and 342 000 deaths resulting from cervical cancer occurred worldwide in 2020 (Sung et al., 2021). Most cervical neoplasms develop in the transformation zone of the cervical epithelium, which is vulnerable to HPV infection and this specific vulnerability is associated with an epithelial cell type named as cervical reserve cell (Doorbar, 2018). A meta-analysis has estimated that the overall prevalence of HPV in the cervix of women with normal cytology is 10.4 % (de Sanjosé et al., 2007). Development of cervical cancer requires persistent genital infection with a HR-HPV genotype, of which HPV16 and HPV18 are both the most prevalent ones and most often associated with neoplasms (de Sanjosé et al., 2007). Development of a cervical neoplasm requires time, and it has been estimated that precancerous cervical lesions may develop into cervical cancer within about 10 to 20 years (Koliopoulos et al., 2017). A great number of risk factors or cofactors have been identified to affect

disease outcome, including HPV genotype, HPV genetic and epigenetic variation, viral load, tobacco smoking, use of hormonal contraceptives, history of STDs, number of past deliveries, and other behavioural and socioeconomical factors (Bowden et al., 2021; de Sanjosé et al., 2018; Jensen et al., 2013; Yetimalar et al., 2012). It has been stated that cervical cancer could potentially be a fully preventable disease with broad and improved HPV vaccination programmes (Roden & Stern, 2018).

Other HPV-related cancers in women include vaginal, vulvar, anal, and oropharyngeal cancer. The proportion of different HPV-related cancer cases differs remarkably by geographical region and economic state of the country (Giuliano, Nyitray, et al., 2015). When compared to men, women have higher overall prevalence of total HPV-related cancers and incident anal cancers, but lower prevalence of oropharyngeal cancer (Giuliano, Nyitray, et al., 2015). However, in recent years there has been an enormous increase with oropharyngeal cancer incidence especially among young women and males (Day et al., 2020; You et al., 2019).

In most cases, HPV infections do not cause clinical disease and many of them are transient by their nature. Infection with a LR-HPV genotype can cause benign lesions such as warts or papillomas at genital or oral sites. It is estimated that HR-HPV genotypes are nearly as frequent in women as LR-HPV genotypes are, and that HPV prevalence is higher at the genital site than at the oral site (Gavriatopoulou et al., 2020). In women, the highest HPV prevalence anatomically is in the cervix and in the vagina, and HPV prevalence anatomically is lower at the vulvar epithelium (Giuliano, Nyitray, et al., 2015). As for oral HPV infections, their prevalence, incidence, and clearance also vary between geographical regions. Data suggests that oral HPV prevalence is lower in women when compared to men, and in a study conducted at the United States, HPV prevalence rates to any oral HPV infection were 3.6 % in women and 10.1 % in men (Gillison et al., 2012; Wood et al., 2017).

In pregnant women, infection with HPV has been associated with adverse outcomes including increased risk for preterm birth and preterm rupture of membranes, and additionally HPV infection might increase the risk for placental abnormalities, intrauterine growth restriction, low birth weight, and fetal death (Ardekani et al., 2022; Niyibizi et al., 2020; Racicot & Mor, 2017). Estimates on HPV prevalence during pregnancy as compared to non-pregnant women vary, but there is evidence that HPV prevalence increases during pregnancy possibly due to changes in hormonal activity or other pregnancy-related factors (Ardekani et al., 2022; Chilaka et al., 2021). A recent meta-analysis estimated that the overall HPV prevalence rate during pregnancy is 30.4 % in cervico-vaginal samples, 32.1 % in

serum samples, 17.8 % in placenta samples, and 2.3 % in amniotic fluid samples, and the highest prevalence rates were for African countries and the lowest for European and Eastern Mediterranean regions (Ardekani et al., 2023). Pregnancy is considered as an immunosuppressive state, which is why HPV-associated diseases may be more severe in pregnant women. In addition, the risk of vertical transmission of HPV infection from mother to child during pregnancy is to be considered.

2.2.3 HPV infection in children

Most HPV-associated diseases in children are benign conditions in either mucosal or cutaneous sites, such as skin warts, oral warts and oral papillomas. HPV infection occurs in children primarily due to non-sexual transmission routes of HPV, such as vertical transmission. Skin warts are usually caused by HPV genotypes 1, 2, 3, 4, 27, and 57, and common warts (verruca vulgaris) represent around 70 % of all skin warts in children (Syrjänen, 2010). Estimates on the prevalence of common warts vary remarkably, from 4 % to 33 % in school children (Gerlero & Hernández-Martín, 2016). In a study investigating cutaneous warts and HPV presence in a small population of Dutch primary school children, prevalence of skin warts was 44 % and detection rates of HPV were even higher, indicating HPV presence even in a clinically normal skin (De Koning et al., 2015). When observing transmission of HPV in relation to children's skin warts, transmission takes place especially within families and school classes (Van Haalen et al., 2009). Oral papillomas are common lesions typically associated with either HPV6 or HPV11 infection in the oral mucosa, and as for oral warts in young children, the transmission of HPV infection associated is thought to occur vertically from direct contact to the mother's infected cells during delivery (Orenuga et al., 2018). Epidemiological studies concerning specifically oral papillomas are very limited, however. In a study assessing children aged 2–17 years old in the United States, the prevalence of any oral mucosal lesion was 10.3 %, and correspondingly, 28.9 % in another study assessing children aged 0–12 years old in Italy (Majorana et al., 2010; Shulman, 2005).

Juvenile-onset recurrent respiratory papillomatosis (JO-RRP) is difficult to treat especially in young children and it may be potentially life-threatening. JO-RRP is observed clinically as papillomatous lesions usually restricting in the larynx, but in some cases the disease progresses and becomes more aggressive as it involves other parts of the aero-digestive tract as well (Fortes et al., 2017). JO-RRP appears in children before the onset of puberty and most often before the age of 5 years old,

and the disease severity increases in relation to younger age at the onset. JO-RRP in children is associated with a vertically acquired infection most often to HPV6 or HPV11, although HR-HPV types such as 16, 18, 31, 33 and 39 have also been identified (Benedict & Derkay, 2021). The mother's history of genital warts during pregnancy and delivery has been determined to be a risk factor for developing JO-RRP at earlier age and therefore increasing the risk for a more severe disease (Niyibizi et al., 2014). Nonetheless, majority of the children suffering from JO-RRP have a mother with no history of diagnosed genital warts, although the mother's subclinical infection remains a possibility in these cases as well (Syrjänen, 2010).

Presence of anogenital warts can be an indicator of sexual abuse, and therefore the possibility of sexual abuse must be considered when anogenital warts are diagnosed in children. In children with anogenital warts, reports on sexual abuse have ranged widely from 0 % to 80 %, but epidemiological data concerning children with anogenital warts is somewhat scarce (Costa-Silva et al., 2017). If sexual abuse is not considered likely, possible non-sexual transmission routes for HPV in anogenital area include vertical transmission or autoinculation from one anatomical site to another (Costa-Silva et al., 2017, 2018; Syrjänen, 2010). HPV6 and HPV11 are the most commonly found HPV types in anogenital warts, but HPV16, HPV18 and cutaneous HPV genotypes have been identified as well (Costa-Silva et al., 2017).

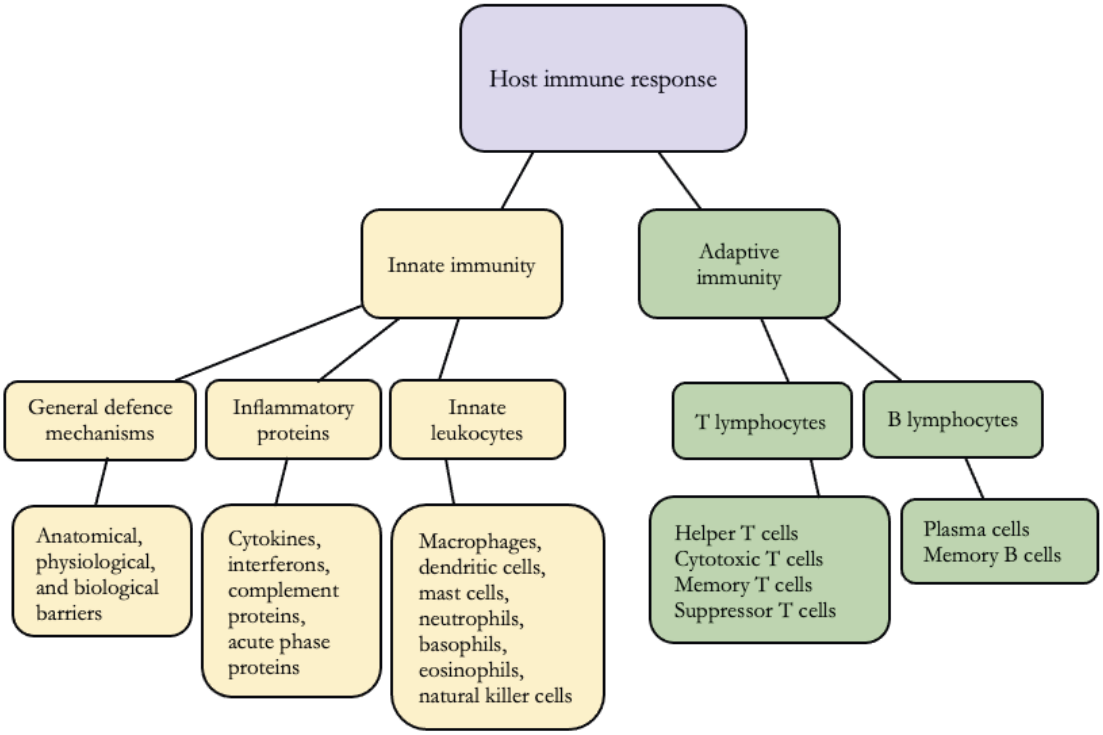
Current knowledge implicates that HPV prevalence is relatively high in infancy and childhood, although no proper global estimates on HPV prevalence in children exist (Ardekani et al., 2022). Detection of HPV in children even without clinical disease supports the hypothesis that many HPV infections in children are asymptomatic and studies investigating the duration of HPV infection have shown that many of these infections are transient. HPV DNA has been detected in neonates in multiple sites, including anogenital area and oropharynx (Chilaka et al., 2021; Elósegui et al., 2022). In neonates, vertical transmission from the mother is the most likely HPV transmission route, and many of these neonatal HPV infections are cleared as HPV detection rates have been observed to decrease with increasing age of the infant (Syrjänen, 2010).

2.3 Host immune response to HPV

2.3.1 Innate and adaptive immunity

To maintain viral life cycle successfully, HPV must escape from protective immune mechanisms of the host and on the other hand, the host immune system needs to perform effectively in order to avert being infected with HPV. Innate immune system is the first line of host immune defence, and it consists of general defence mechanisms, inflammatory proteins, and innate leukocytes. General defence mechanisms include anatomical barriers (such as intact mucosal and cutaneous epithelial structure), physiological barriers (such as low pH and temperature) and biological barriers (such as microbiome of the epithelium). Numerous proteins are involved in the function of the innate immune system, including cytokines, interferons (IFNs), and chemokines, and one of the major components in the innate immunity is the complement system that consists of proteins which enable an inflammatory chain reaction against the pathogen. Leukocytes of the innate immune system include macrophages, dendritic cells, mast cells, neutrophils, basophils, eosinophils, and natural killer cells (Marshall et al., 2018). As the innate immune system is evolutionarily older than the adaptive immune system, its response is rapid and nonspecific, and it is unable to memorize (Marshall et al., 2018; Tomar & De, 2014). A schematic representation of the components of the host immune response is depicted in the Figure 4.

Figure 5. A schematic representation of the components of the host immune response. The host immunity consists of innate and adaptive immunity, and it is important to notice that these two systems have complex interactions between them, which are not shown in this simplified figure.



HPVs infect keratinocytes that express innate immune system receptors, including pathogen recognition receptors (PRRs) and pathogen associated molecular patterns (PAMPs). These PRRs enable keratinocytes that are infected with HPV to recognize the foreign pathogen and they participate in the activation of the innate immune response. PAMPs are common structures that pathogens share, and the innate immune system recognizes microbes' PAMPs through PRRs (Marshall et al., 2018). As for HPV, the viral capsid proteins L1 and L2 may act as PAMPs, for example. Ligand activated PRRs can bind on adaptor proteins and recruit protein kinases and thereby activate an antiviral response against the foreign pathogen inside the cell. These protein kinases initiate signalling cascades and thereby activate transcription factors that stimulate antiviral gene transcription, such as the production of IFNs and inflammatory cytokines. IFNs in turn affect transcription of interferon stimulated genes (ISGs) that stimulate neighbour cells to an antiviral state. (Stanley, 2021)

HR-HPVs tend to actively escape from the innate immune response. The early proteins E6 and E7 of HR-HPV types 16, 18, and 31 can interfere directly with the

signalling cascade of the innate immune response by inhibiting the PRR ligation, ISG transcription, and disturbing the IFN receptor activation (Stanley, 2021). These effects of HPV E6 and E7 oncoproteins result in the modulation of cytokines, the downregulation of IFN pathways, the impairment of antigen presentation, and the reduction of adhesion molecules' expression (Nunes et al., 2018). cGAS/STING pathway is an innate sensing pathway that reacts after detection of double-stranded cytosolic viral DNA, and it has been shown that HPV is able to uniquely evade this pathway by delivering HPV DNA in a vesicle to the nucleus of the cell and in addition, HR-HPV E2 and E7 proteins are able to interact with proteins of this pathway (DiGiuseppe et al., 2016; Uhlorn et al., 2020).

Studies on mouse models suggest that the expression of pro-inflammatory molecules is dependent on MyD88 pathways via signalling through the interleukin-1 (IL-1) receptor and Toll-like receptors (TLRs), and that HPV oncoproteins are able to downregulate a PRR called TLR9 (Hasan, 2014; Pacini et al., 2015; Scagnolari et al., 2020). The downregulation of TLR9 prevents production of immunostimulatory and pro-inflammatory cytokines, such as Type I IFN (Hasan, 2014). A subtype of dendritic cells called the Langerhans cells (LCs) are professional antigen presenting cells (APCs) in squamous epithelium, and they participate in HPV antigen recognition and activation of the HPV-specific cell-mediated immunity (CMI) (Dai et al., 2022). It has been demonstrated that the number of LCs or their maturation levels are reduced after HPV infection, and it has been suggested that the LCs might be associated with HPV clearance (Dai et al., 2022).

Both the innate and adaptive immunity are needed to affect outcome of an HPV infection. Innate immunity is considered being responsible of clearing many subclinical HPV infections, but regression of an HPV-induced lesion needs an effective adaptive immune response, more specifically a cell-mediated cytotoxic T cell response that consists primarily of antigen specific cytotoxic T cells and helper T cells. Adaptive immune system consists of B lymphocytes that are mainly responsible for the antibody-mediated (humoral) immune response and T lymphocytes which are in charge of the CMI against a specific pathogen such as HPV. Adaptive immune response is activated by innate immunity, and it is highly specific to each pathogen that has been previously encountered. An effective adaptive immune response may result in regression of a persistent HPV infection and on the contrary, a failure to develop sufficient CMI can result in the persistence and progression of an HPV infection (Jee et al., 2021; Litwin et al., 2021). Complex interactions between different T cell subsets and other components of adaptive immunity enable clearance of an HPV infection without harming uninfected cells of

the host (Litwin et al., 2021). Function of T lymphocytes and antibody response mediated by B lymphocytes are discussed more detailedly in the following chapters (see chapters 2.3.2 and 2.3.3).

One mechanism that is considered important in the ability of HPV to evade host immune system is the limitation of viral gene expression in the epithelium. This is enabled by the low number of viral proteins needed for basal cell genome maintenance which restricts the ability of antigen presentation to activate adaptive immunity (Doorbar, 2018). As for infection with many LR-HPVs, it has been noted that the viral gene expression is difficult to detect (Doorbar, 2018). In addition, HPVs may also inhibit LC function at the infection site and HPV early proteins may disturb the innate immune system's signalling pathways, which thereby delays activation of adaptive immune system (McBride, 2022).

Together with the mechanisms of innate and adaptive immunity, certain factors of the local mucosal environment, such as hormones and microbiome, may influence the natural course of an HPV infection. Sex hormones are known to modulate immune response, and in women, estrogen receptors regulate cells and pathways of both the innate and the adaptive immune system (Kovats, 2015). As for cervical lesions, both estrogen and progesterone receptors are highly expressed in the cervical transformation zone, and these receptors might also be involved in cervical carcinogenesis (Hong et al., 2017).

2.3.2 T lymphocytes in HPV infection

T lymphocytes (T cells) of the adaptive immune system are important in implementing CMI against a specific pathogen such as HPV. T lymphocytes differentiate from the lymphoid stem cell in bone marrow, and they migrate to thymus for maturation, in which T cell receptors (TCR) are added on their membranes (Tomar & De, 2014). In humans, TCRs recognize only those antigens that are presented by APCs that have human leukocyte antigen (HLA) molecules on their surface and this recognition is crucial in T cell activation (Szeto et al., 2021). Local APCs are necessary for antigen recognition and activation of the adaptive immunity as T cells do not recognize free antigens. Before encountering a specific antigen, naïve T cells circulate in the bloodstream and the lymphatic organs, and after T cell activation they are able to traverse outside the lymphatic tissues.

T lymphocytes are characterized by the expression of cluster of differentiation (CD) cell surface marker CD3, and different T lymphocyte subsets express other

CDs as well. Helper T cells express CD4, and they recognize foreign antigens with APCs that carry HLA2 (Marshall et al., 2018). CD4 T cells have various tasks in adaptive immunity, including antigen recognition, cytokine expression, regulation of antibody production mediated by B lymphocytes, and regulation of CMI. Cytotoxic T cells (killer T cells) express CD8, and they destroy infected cells or cancer cells directly after encountering an APC that carries HLA1 with the corresponding antigen. Memory T cells consist of central memory T cells ($CD45RA^{-}CCR7^{+}$) in lymphatic tissues, stem-cell memory T cells ($CD45RA^{+}CCR7^{+}CD95^{+}CD122^{+}$), and effector memory T cells ($CD45RA^{-}CCR7^{-}$) in periphery that do not require co-stimulated activation by APC, and these memory T cells are part of long-term immunity against the pathogen (Kumar et al., 2018). Other T cell subsets include regulatory T cells (T_{reg}s) that commonly express FoxP3, CD25 and CD4, and terminally differentiated cells (T_{EMRA}s) that express $CD45RA^{+}CCR7^{-}$ (Kumar et al., 2018; Litwin et al., 2021).

When an APC presents a foreign antigen to a naïve T cell, an activated T cell begins to secrete cytokines such as interleukin-2 (IL-2) that induce T cell proliferation and differentiation (Kumar et al., 2018). Clonally expanded T cells may further differentiate as effector T cells or memory T cells, although most effector T cells are short-lived as they undergo apoptosis and only a small fraction survive further as memory T cells (Kumar et al., 2018). Cytotoxic CD8 effector cells are able to express cytotoxic molecules such as perforin, and they can produce proinflammatory cytokines including IFN-γ and tumour necrosis factor alpha (TNF-α), and chemotactic cytokines (chemokines) (Hashimoto et al., 2018). While the majority of effector T cells are short-lived, a population of T_{EMRA} cells can circulate long-termly and stimulate proinflammatory IFN-γ production (Kumar et al., 2018). In infected cells, cytotoxic CD8 T cells are able to activate programmed cell death (apoptosis) by secreting antiviral cytokines.

An activated T cell derived from a naïve T cell may also differentiate as a helper T cell (Th cell), a T cell subtype of CD4 cells that are able to secrete proinflammatory and antiviral cytokines and stimulate other immune cells such as B lymphocytes and cytotoxic CD8 T cells. CD4 helper T cells have been traditionally classified as Th1 cells, Th2 cells, and Th17 cells according to their signature cytokine production and surface marker expression, but various subgroups of T helper cells have been discovered in recent years (Saravia et al., 2019). Th1 cells are essential in antiviral and antibacterial immunity, and they express IFN-γ, IL-2, and TNF-α, whereas Th2 cells express IL-4, IL-5, and IL-13, and they are characteristic in immune response against extracellular pathogens (Saravia et al., 2019). Th17 cells have a task in the clearance

of certain extracellular pathogens, such as bacteria and fungi, and it has been proposed that they might be involved in the development of certain autoimmune diseases as well (Schnell et al., 2023).

In most cases, effective CMI enables recognition of infected cells, T cells infiltrate the HPV induced lesion, and this results in the resolution of the infection (McBride, 2022). Nonetheless, ineffective CMI response appears to influence on the persistency and the progression of an HPV infection. Chronic viral infections are associated with impaired function and decreased proliferative capacity of the CD8 T cells, and this is suggested to be due to overexpression of inhibitory receptors (Hashimoto et al., 2018). T_{reg}s suppress the immune system by affecting other T cells and APCs, and the presence of high number of T_{reg} cells and low number of circulating antigen-specific T cells is associated with persistency of HPV infection (Doorbar, 2018). Abundance of T_{reg} cells may promote carcinogenesis and in cervical cancer, infiltration of the T_{reg} cells increases along with the progression of cervical cancer (Litwin et al., 2021).

Over human lifespan, dynamic changes occur in both T cell development and differentiation, as well as in T cell maintenance (Kumar et al., 2018). In childhood, majority of T cells are naïve T cells and T_{reg} cells. As new pathogens and their antigens are encountered, naïve T cells are needed to construct antigen-specific adaptive immunity and gradually long-term memory T cells accumulate, whereas T_{reg} cells are crucial for developing antigen tolerance (Kumar et al., 2018). In adulthood, fewer new antigens are encountered, and the role of T cells shifts more into maintaining homeostasis and immune regulation (Kumar et al., 2018). In advanced age, T cell function declines and this correlates with an elevated risk for infections, tissue inflammation, and cancer (Goronzy & Weyand, 2017). It has been suggested that naïve T cells seem to retain their functionality over human lifespan, as differences have not been observed for this particular T cell subtype between younger and older individuals while comparing to their distinct thymic function (Thome et al., 2016).

2.3.3 Antibody response to HPV

B lymphocytes are responsible for the antigen-specific humoral immune response, in which antibodies recognize extracellular foreign antigens, and optimally, the produced antibodies participate in the elimination of pathogens. B lymphocytes differentiate from the lymphoid stem cell in bone marrow and migrate into tissues

to wait until their activation. B lymphocytes are then activated after encountering the foreign antigen to which they have an appropriate antigen specific receptor (Marshall et al., 2018). After antigen recognition, B lymphocytes proliferate and their B cell receptors (BCRs) undergo genetic rearrangements such as somatic hypermutations (Tomar & De, 2014). After activation, B lymphocytes differentiate into antibody producing plasma cells or memory B cells. Local helper T cells regulate B lymphocyte proliferation and differentiation by their cytokine expression, and they also direct the type of antibody that B lymphocytes followingly produce (Marshall et al., 2018). Each antibody recognizes a specific part of the foreign antigen. Memory B cells are long-lived cells that remain expressing their antigen-specific receptors, and they enable a quick antibody response if the same antigen is encountered again.

Antibodies (also known as immunoglobulins, Igs) produced by B lymphocytes are glycoproteins that generally consist of two light chains and two heavy chains forming a roughly Y-shaped structure, and they are able to participate in pathogen identification (non-neutralizing antibodies) and neutralization (neutralizing antibodies) (Ma & O'Kennedy, 2015). Antibodies are categorized into five distinct classes according to their different functions in the humoral immune response: IgM, IgG, IgA, IgE, and IgD. IgM antibodies are expressed first as a primary response during an infection, and their main function is to activate the complement system of the innate immunity (Chen et al., 2020). IgG antibodies are expressed in a secondary response during infection, and they participate in the activation of the complement system and the neutralization of toxins and viruses (Marshall et al., 2018). IgG antibodies are transferred from the mother to the fetus via placenta during pregnancy, and some of these antibodies have an important task in protecting the fetus and neonate from infections in the early childhood. IgA antibodies are affiliated with mucosal immunity, whereas IgE antibodies are considered important in allergic inflammation. The role of IgD antibodies is somewhat unclear, but they may affect mucosal homeostasis via both activating and inhibitory immune functions (Chen et al., 2020). B lymphocytes can switch antibody production from one Ig class to another through class switch recombination (CSR) (Tomar & De, 2014).

Although antibody response to HPV may contribute to both prevention and elimination of HPV, antibody response resulting from a naturally acquired HPV infection is highly variable. A type-specific antibody response against HPV L1 capsid occurs in approximately 60–70 % of women who acquire HPV infection (Beachler et al., 2016). Antibodies could ideally protect from subsequent HPV infections, in giving protection especially against reinfections with the same HPV genotype, but the magnitude of this potential protective effect is under debate. As for genital HPV

infection, it has been suggested that the HPV antibodies resulting from a naturally acquired infection offer only modest protection against subsequent genital HPV infection (Beachler et al., 2016). Antibodies generated as a response to a prophylactic HPV vaccination are expressed in high levels, and their protective effect is much greater than with the antibodies produced after a naturally acquired HPV infection. It has been presented that the HPV vaccines provoke a strong response of neutralizing antibodies, but these vaccine-induced antibodies might have other non-neutralizing functions as well (Quang et al., 2022).

2.4 HPV detection

2.4.1 Detection of HPV DNA and RNA

Molecular methods for assessing the presence of HPV depend strongly on viral DNA detection due to the fact that HPV cannot be cultured (Abreu et al., 2012). One method for HPV detection is conducted through polymerase chain reaction (PCR). PCR assays are based on genome amplification, and they may be coupled with microarray analysis. PCR techniques are widely used as they have been shown to be both sensitive and specific, and using real-time PCR techniques enables determination of HPV viral load (Abreu et al., 2012). Another molecular method for HPV detection is nucleic acid-hybridization assay, which is based on target HPV-DNA hybridization using synthetic type-specific RNA probes (Faulkner-Jones et al., 1993). Southern blot and other nucleic acid-hybridization techniques have been traditionally used in HPV detection, and Southern blot is considered as gold standard in HPV genomic analysis, but these techniques have a lower sensitivity, and they are generally more time-consuming (Abreu et al., 2012). There are also molecular methods for assessing HPV messenger RNA in order to detect HPV (Arbyn et al., 2022).

Varying methodological approaches between different studies may lead to differences in the results for HPV incidence, prevalence, and clearance. In addition, there may be some differences with regard to the samples from which HPV is detected, as HPV DNA may be detected from both smear specimens and biopsy specimens. As for detecting HPV DNA in cervical cancer screening settings, using cytological smear samples is preferrable than using solely cervical biopsy samples (Auvinen et al., 1989; Webersinke et al., 2013).

Detection of HPV DNA is an indicator of HPV's presence in the sampled tissue. It has been shown that HPV DNA detectability is transient and decreases over time (Artemchuk et al., 2018; Ramanakumar et al., 2016). Individual who has acquired an HPV infection, may shift from being HPV positive to HPV negative in HPV testing. A loss of HPV detection may be an indicator of a successful clearance of the HPV infection by the host immune system, but having a latent infection also might be the case. In latency, HPV may hide in the basal epithelial cells while expressing only low levels of viral proteins that are below the limit for detection, and this results in being out of sight from the immune system (Doorbar, 2018). In an individual with a latent HPV infection, viral reactivation may appear at some timepoint. Altogether, a detected positivity for HPV may be a sign of a new HPV infection, a sign of a reactivation of previously acquired latent HPV infection, a sign of re-infection with the same genotype or a sign of a new infection due to autoinoculation from another anatomical site (Gravitt & Winer, 2017).

Alongside detecting HPV's presence, HPV genotyping provides important information concerning HPV infection. Detection of either LR-HPV or HR-HPV may lead to different treatment and follow-up approaches in the clinical practice. HPV DNA detection is relevant in for example in the triage of women with cytological findings, in the cervical cancer screening program with or without Pap smear, and in the follow-up of abnormal cervical cancer screening results (Abreu et al., 2012).

2.4.2 Serology

Antibodies produced by the host's immune system can be measured by serological methods from the individual's blood sample. Naturally acquired HPV infection may result in antibody production against HPV viral proteins, and most commonly these antibodies are measured as a type-specific antibody response against the HPV L1 capsid protein. Although antibodies against the L1 capsid protein are most frequently used, antibodies against other viral proteins such as HPV early proteins may also be measured. As for naturally acquired HPV antibodies, a confirmed seroconversion (change from a seronegative state to a seropositive one) against HPV proteins can be used as an indicator for previous exposure to HPV infection. Being seropositive implies that a test positivity for specific antibodies has been measured from the blood sample. Definition of seropositivity depends on the used serological assay and the comparison population (Giuliano, Nyitray, et al., 2015). In some

studies, it has been observed that persistent HPV infections result in seroconversion more commonly than transient HPV infections do (Giuliano, Viscidi, et al., 2015).

Optimally, serology may reflect cumulative lifetime exposure to HPV. Despite its advantages, HPV serology has its limitations. It is well established that all individuals do not seroconvert after a naturally acquired HPV infection (Beachler et al., 2016). Another limitation in HPV serology is that different serological analyses performed on a serum sample vary methodologically and currently there is no gold standard method for assessing HPV antibodies, which limits comparison between different HPV serology studies. Problems regarding distinctive serological assays have been identified, and actions have been initiated in order to improve standardization in HPV serology (Park et al., 2023). Another limitation in HPV serology is that the timing of HPV acquisition or the duration of an HPV infection are difficult to assess, and therefore their potential effect on HPV serology and in its interpretation is not considered. It has been suggested that HPV serology does not predict current HPV infection as there is no concordance between type-specific HPV antibodies and HPV DNA detection in cervical or oral mucosa (Paaso et al., 2011). In addition, there has been some discussion whether the stability of HPV antibodies over time could be variable. A longitudinal study assessing serology of cutaneous HPV types concluded that the seroprevalence to cutaneous HPV types is relatively stable over time (Antonsson et al., 2010). One study reported that serum IgG antibodies against HPV16 are relatively stable, but IgA antibodies show more variation over time (Pirttilä et al., 2022). In clinical practice, serological tests are not commonly used as diagnostic tests for HPV infection as they tend to have low sensitivity (Costa-Silva et al., 2017).

It has been suggested that HPV seroprevalence due to naturally acquired infection is higher in women when compared to men, and this has been observed especially when investigating antibodies against HPV types 6, 16, and 18 (Beachler et al., 2016; Giuliano, Nyitray, et al., 2015). Interestingly, it appears that the immune protection induced by HPV antibodies could be greater in women than men (Giuliano, Nyitray, et al., 2015). In addition to its role in evaluating cumulative exposure to HPV, measurement of HPV antibodies may also have a potential role as a biomarker. Increasing evidence suggests that antibodies against HPV16 E6 may be used as potential biomarkers in oropharyngeal cancer as to identify HPV-driven cancers from other oropharyngeal cancers, and antibodies against E1, E2, and E7 may also be used as biomarkers (Hibbert et al., 2021). In addition, after the treatment of HPV-associated oropharyngeal cancer, measuring high levels of HPV16 L1

antibodies may predict patient outcome with a higher overall survival (Prétet et al., 2023).

2.5 Prevention strategies

2.5.1 HPV screening

As the importance of HPV infection in cervical carcinogenesis was revealed, the battle against this infectious agent has resulted in the development of screening programmes, HPV testing, and vaccinations. HPV screening is part of secondary prevention against HPV-associated diseases, most notably cervical cancer as its screening programmes been proven to be highly influential. Cervical cancer screening programmes have been traditionally based on cytological Pap smear samples (Papanicolaou's test, Pap test), but HPV testing has now replaced the Pap smear as the primary screening test in many countries.

Pap testing is performed on a cytological scraping sample, and it can be used to detect abnormal or malignant cells in the cervix. A cytologist examining a Pap smear sample can distinguish these malignant cells as they have a deformed nuclear morphology that seems to result from the loss of structural proteins (Smith et al., 2018). As most cervical cancers or their precursors develop in the cervical transformation zone that is especially vulnerable to HPV infection, this area is specifically examined when collecting a Pap smear sample. Pap testing has also different methodological variations, as it can be performed as conventional cytology or liquid-based cytology (Koliopoulos et al., 2017). Screening programmes using Pap testing require repeated sampling as cytological tests are less efficient in detecting cervical cancer cases, because they have a lower sensitivity than HPV testing (Bhatla & Singhal, 2020).

Gradually, HPV testing has been introduced in co-testing with the cytological Pap smear sample and furthermore, in some cases, it even has replaced the cytological testing as the primary screening method for cervical cancer. Benefits of screening with an HPV test include a higher sensitivity to premalignant lesions, a higher specificity, and a safe prolongation of screening intervals (Bhatla & Singhal, 2020). There are multiple types of HPV tests available, and the screening algorithms vary between countries. One of the limitations in HPV testing is that it is not able to differentiate between whether a test positive case is associated with a transient or a

persistent HPV infection, as the latter is more clinically relevant when assessing the risk of cancer. Assessment of HPV genome methylation markers has been introduced as a new molecular test method that may help in triaging HPV positive cases, and some methylation tests may even differentiate between a transient and a persistent HPV infection (Kremer et al., 2022; Louvanto et al., 2020). In addition, HPV methylation markers may be promising as independent screening methods for cervical precancerous or cancerous lesions (Hillyar et al., 2022).

HPV screening protocols vary between different screening programmes. In Finland, women aged from 30 to 65 years old are invited to participate in cervical cancer screening every five years. HR-HPV testing is used as the primary test with Pap smear triage for those women that are HR-HPV positive. HR-HPV positive women with a Pap smear sample that has a LSIL or worse as a finding are referred straight to colposcopy, while women that are HR-HPV positive with a Pap smear containing NILM (negative for intraepithelial lesion or malignancy) or ASCUS (atypical squamous cells of undetermined significance) as a finding are re-screened in 12 to 24 months with HR-HPV testing. Persistent HR-HPV positive women at the re-screening are then referred to colposcopy. During the cervical examination by colposcopy, punch biopsies are taken and if precancerous lesions are observed, required treatment with a loop electrosurgical excision procedure (LEEP) can be done. A systematic Cochrane review assessed that about 20 women out of 1000 women screened will have precancerous lesions, and HPV testing correctly identifies 18 women and Pap testing 15 out of these women, whereas these tests result in 99 and 95 cases with false positivity, respectively (Koliopoulos et al., 2017). Therefore, HPV testing slightly increases more unnecessary colposcopy referrals, although more test positive cases (cases with cancer or precancerous lesions) are identified.

The success of a screening programme depends not only on having highly efficient screening tests but also on its participation rate. In Finland, the overall coverage of the cervical cancer screening program in all age groups together is around 70 %, and among younger generation (participants aged 25–40 years) only around 60 % (The Finnish Cancer Registry, 2023). In recent years, a new approach into improving cervical cancer screening availability and participation has emerged. Traditionally, HPV detection has been based on samples that are collected by a healthcare professional, but in HPV self-sampling, cervicovaginal samples can be collected by the patient themselves. HPV self-sampling is recommended as an additional screening method, and it may improve cervical cancer screening participation rates as it may reach better those individuals who do not attend in the traditional screening (Nishimura et al., 2021).

2.5.2 HPV vaccination

One of the most efficient ways to primary prevent HPV infection and the development of its clinical manifestations is to vaccinate young individuals before they encounter HPV. Prophylactic HPV vaccines have been successfully developed in order to battle against HPV-associated diseases. The first bivalent and quadrivalent vaccines became available in 2006, and both of these vaccines have been shown to have more than 90 % efficacy in preventing HPV types 16 and 18 (Cohen et al., 2019). As the bivalent vaccine offers protection only against HR-HPV types 16 and 18, the quadrivalent vaccine covers these two HR-HPV types as well and additionally, it offers protection against HPV types 6 and 11, which are the two most common HPV types known to cause anogenital warts. In addition, a nine valent vaccine preventing HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58 was commercially introduced in 2018. In addition to the prophylactic vaccines, therapeutic HPV vaccines are being attempted to develop.

Optimally, HPV vaccination induces long-term protection against future infections. HPV vaccines consist of VLPs such as inactive L1 capsid proteins that induce B lymphocytes to produce neutralizing antibodies (Handler et al., 2015). HPV VLPs are highly immunogenic and antibodies against these VLPs have been shown to persist for years after vaccination (Prabhu et al., 2022). Memory B lymphocytes and long-lived plasma cells are both needed in maintaining the immune protective effect (Prabhu et al., 2022). Vaccine-induced antibody response is stronger and more efficient than the antibody response resulting from a naturally acquired HPV infection, and it has been estimated that the magnitude of the antibody response following HPV vaccination is about 40-fold stronger than after natural HPV infection (Prabhu et al., 2022).

It is possible that cervical cancer could be completely prevented in the future with efficient HPV vaccination programmes (Roden & Stern, 2018). Currently, vaccination programmes are expected to decrease incidence of new cervical cancer cases and other HPV-associated cancers, but these outcomes will be observed gradually as HPV-related cancers typically take years to develop after initial infection. Therefore, a reduction in the incidence of HPV-related cancers might be measurable in decades after initiation of a vaccine programme. Vaccination programmes were initially targeted for young females, but young males have also been included in these programmes. In Finland, both girls and boys aged 10 to 12 years old are the primary group to be vaccinated against HPV with a bivalent vaccine.

Efficacy of a vaccination programme is dependent on achieving sufficient vaccination coverage to ensure herd immunity and this requires having enough individuals who willingly choose to be vaccinated against HPV. Vaccinating with a three-dose and in some cases with a two-dose schedule, has been proven to be effective, but simpler vaccination schedules are investigated as an attempt to increase vaccine uptake (Bergman et al., 2019). Even with a suboptimal vaccine coverage, it has been shown that the prevalence of HPV infection with genotypes covered by HPV vaccination has decreased remarkably (Oliver et al., 2017). Vaccination programmes have been initiated in many high-income countries, but there is a great need for HPV vaccinations in middle-income and low-income countries as well. This is true especially when taking into consideration that the majority of cervical cancer cases worldwide occur in middle-income and low-income countries, and the related mortality is 18 times higher than in high-income countries (Cohen et al., 2019). Globally, it has been estimated that only 15 % of girls in the target age are sufficiently vaccinated against HPV (Bruni et al., 2021). Therefore, increasing HPV vaccine uptake is crucial in the battle against HPV-related diseases.

3 AIMS OF THE STUDY

The Finnish Family HPV Study was originally designed to evaluate the dynamics of HPV infection and HPV transmission within regular Finnish families. The general objective of this particular study was to further investigate HPV immunology and serology in Finnish mothers and their children. The study hypothesis was that the mother's immunological status as related to the HPV infection would have a crucial role in the outcome of her offspring's immune system against HPV infection.

The specific aims were:

1. To characterize T lymphocyte immunophenotypic subsets regarding different HPV16 infection outcomes of the mothers and the corresponding effects on their children, and to evaluate what immunophenotypic alterations can be observed in T lymphocyte subsets in relation to the mother's persistent HPV infection.

2. To examine vertical transmission of HPV infection by determining maternal antibodies against HPV6 early and late proteins, and HPV seroconversion in children in their early infancy, and to assess whether there is a clear concordance between maternal antibodies and seroconversion of the children.

3. To evaluate seropositivity and antibody levels for HPV types 6, 11, 16, 18, and 45 in women who develop a second pregnancy, in order to elucidate if second pregnancy has an effect on HPV seropositivity or does it affect HPV antibody levels in general.

4 MATERIALS AND METHODS

4.1 Subjects

4.1.1 The Finnish Family HPV Study

The Finnish Family HPV (FFHPV) Study is a longitudinal cohort study that was designed to clarify the dynamics of HPV infection within Finnish families. The study cohort has been conducted jointly by the Department of Obstetrics and Gynaecology, Turku University Hospital and the Institute of Dentistry, Faculty of Medicine, University of Turku.

The participants were originally recruited in the study between the years of 1998–2001. In total, 329 families were enrolled, consisting of 329 mothers who were at their third trimester of pregnancy at the time of enrolment, 131 fathers-to-be, and their 331 newborns (including two sets of twins). The mothers were recruited at a minimum of 36 weeks of pregnancy, and the follow-up time was up to six years after the delivery. The mean age of the mothers at the time of enrolment was 25.5 years (ranging from 18 to 38 years). Recruitment of the children and the mothers for the specific cell-mediated immunity studies began in the year of 2012. None of the participants had received a prophylactic HPV vaccination.

Written informed consent was obtained from each participant. The original study protocol and its amendments were approved by the Research Ethics Committee of Turku University Hospital (#3/1998, #2/2006, 45/180/2010, TO7/008/2014, 45/1801/2018).

4.1.2 Demographic data

The parents filled a detailed questionnaire concerning their demographic and behavioural data at the study onset, at the 36-month follow-up visit, and at the 6-year follow-up visit. These questionnaires included information on their

socioeconomic status, general health, sexual habits, smoking habits, use of alcohol, use of medication, and history of STDs.

4.2 Sample collection

4.2.1 Mucosal scraping samples (I)

Scraping samples from the genital and oral mucosa were taken with a cytobrush (MedScand, Malmö, Sweden) at the baseline of the study and during the follow-up visits. The follow-up visits that included sample collection were at day three after the delivery before leaving the hospital, and at the 1-, 2-, 6-, 12-, 24-, 36-month and the 6-year follow-up visits. Oral scraping samples were taken from the buccal mucosa of both cheeks, and also from the upper and lower vestibular area using a small cytobrush (MedScand), after which the oral brushes were placed in 80 % ethanol. Cervical brushes were placed in 0.05 M phosphate-buffer saline with 100 μg/ml of gentamycin. After collection, the samples were immediately fixed, frozen first at –20 °C and then stored at –70 °C.

4.2.2 Blood samples (I, II, III)

The participants were recalled for the collection of venous blood samples. The mean age of the children was 12.2 years at the time of blood sample collection. The venous blood samples were cryopreserved after collection, and they were later used in the flow cytometric analysis. (Study I)

Blood samples were collected from the mothers at their third trimester of pregnancy, and at the 12-, 24-, and 36-month follow-up visits and from their offspring at the 1-, 2-, 6-, 12-, 24-, and 36-month follow-up visits after birth. The blood samples were centrifuged at 1150 g for 10 minutes (Sorvall GLC-2, DuPont Instrument). The blood serum was divided into three 1 ml aliquots and stored first at –20 °C for no longer than a week and then at –70 °C until analysis at the German Cancer Research Center (DKFZ) in Heidelberg, Germany. (Studies II and III)

4.3 HPV testing

4.3.1 HPV DNA testing (I)

HPV DNA was extracted by using the high salt method protocol (Miller et al., 1988). HPV testing was performed with PCR using GP05+/GP06+ primers for cervical scrapings, and with nested PCR using MY09/MY11 and GP05+/GP05+ primers for oral scrapings (Snijders et al., 1990). The PCR was carried out in a 25 µl reaction mixture using Amplitaq Gold DNA polymerase (Perkin Elmer, NJ, USA). The PCR products were hybridized with digoxigenin-labelled HR-HPV oligoprobe cocktail (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56, 58) to categorize whether the sample was positive or negative for HR-HPV (Anttila et al., 1999).

4.3.2 HPV genotyping (I)

HPV genotyping was performed by using the Multimetrix Kit (Progen Biotechnik GmbH), which detected 24 LR- and HR-HPV genotypes, including HPV types 6, 11, 42, 43, 44, 70, 26, 53, 66, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 (Schmitt et al., 2006). The manufacturer's instructions were otherwise followed, but only half of the volumes were used. A 100 µl blocking buffer was used when reading in a Luminex 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 set in the sample. The cut-off value was defined for each HPV probe as follows: 1.5 x background MFI (negative control) + 5 MFI. Retesting of the HPV16 positive samples was done in order to identify samples that might have become contaminated during previous testing or reamplification.

4.4 Laboratory analyses

4.4.1 Flow cytometric analysis (I)

Peripheral blood mononuclear cells (PBMCs) from previously cryopreserved venous samples were used. PBMCs were stained in 96-well U-bottom plates in the concentration of 200,000–500,000 cells/well. The plate was centrifuged for 5

minutes with 450 g at 4 °C. This was followed by a washing step with fluorescence-activated cell sorting (FACS) buffer (PBS/0.5 % BSA/2mM EDTA), and then the plate was centrifuged again. After the supernatants were removed, the diluted antibody mix (Table 4) was added to the samples (50 µL/well) and incubated for 30 minutes (dark, 4 °C). After washing again with the FACS buffer, the supernatant was removed and resuspended to 5 µL of CellFix (BD Bioscience). The samples were analyzed within 2 hours in a BD 4-laser LSR Fortessa™ cell analyzer (BD Bioscience, New York, NJ, USA), and gating was performed with Flow-Jo Software (BD Bioscience, New York, NJ, USA).

Table 4. Antibodies used for immunological phenotyping of peripheral blood mononuclear cells by flow cytometry. (Suominen et al., 2022), Table 1.

Cell marker	Dilution	Fluorochrome	Antibody clone	Source
CD3	1/15	APC-Cy7	SK7	Biolegend
CD4	1/15	PerCP-Cy5.5	OKT4	Biolegend
CD8	1/15	FITC	SK1	Biolegend
CD25	1/15	Alexa 700	M-A251	BD Bioscience
CD27	1/15	APC	L128	BD Bioscience
CD45RA	1/15	BV50/BV510	HI100	BD Bioscience
CD45RO	1/15	PE	UCHL1	Biolegend
CD57	1/33	PE Dazzle	HNK-1	Biolegend
CD38	1/15	BV605	HB7	BD Bioscience
CD69	1/15	BV421	FN50	BD Bioscience

T lymphocyte cell populations were defined by the presence or absence of different cell surface markers to either early or late activated cells, memory or naïve cells, differentiated or undifferentiated cells, following a previously reported protocol (Rodríguez et al., 2011). T lymphocyte populations were defined as shown in Table 5. CD69 markers in $CD4^+$ and $CD8^+$ T cells were reported as percentages of the total number of lymphocytes, the lymphocyte population being selected on the basis of the forward scatter/side scatter pattern. The CD45RO, CD45RA, CD27 and CD57 markers were reported as percentages of $CD3^+CD4^+$ or $CD3^+CD8^+$ lymphocytes. HLA-DR and CD38 marker populations were estimated as a percentage of the $CD3^+CD4^+$ or $CD3^+CD8^+$ lymphocytes. The CD57 subpopulation was estimated as a percentage of $CD3^+CD4^+$ or $CD3^+CD8^+$ lymphocytes that were $CD27^+CD45RA^+$ or $CD27^+CD45RO^+$.

Table 5. T lymphocyte definitions used in the analyses. T lymphocytes were categorized by their expression of different cluster of differentiation (CD) surface markers. (I)

Category	Cluster of differentiation surface marker

Early activated T cells	$CD4^+CD69^+$
	$CD8^+CD69^+$
Late activated T cells	$CD4^+CD25^+$
	$CD8^+CD25^+$
	$CD4^+HLA\text{-}DR^+$
	$CD8^+HLA\text{-}DR^+$
	$CD4^+CD38^+$
	$CD8^+CD38^+$
Memory T cells	$CD45RO^+CD45RA^-$
Naïve T cells	$CD45RO^-CD45RA^+$
Central memory cells and naïve-like memory T cells	$CD45RO^+CCR7^+$
	$CD45RA^+CCR7^+$
Effector memory cells and naïve-like effector T cells	$CD45RO^+CCR7^-$
	$CD45RA^+CCR7^-$
Resting memory cells and differentiated naïve T cells	$CD27^+CD45RO^+$
	$CD27^+CD45RA^+$
Differentiated memory cells and undifferentiated naïve T cells	$CD57^+CD45RO^+$
	$CD57^+CD45RA^+$

4.4.2 Serological analyses (II, III)

After blood sample collection as mentioned above (see chapter 4.2.2), antibodies to HPV6 proteins E2, E4, E6, E7, and L1 were analyzed by multiplex serology based on glutathione S-transferase (GST) fusion protein capture to fluorescent beads as followed by a previously documented protocol (Schmitt et al., 2006; Waterboer et al., 2005). Multiplex HPV serology combines fluorescent bead array with a method that enables in situ purification of any GST fusion protein that have been developed for conventional ELISA method (Waterboer et al., 2005). Cut-off value for seropositivity for E2, E4, E6, and E7 was median fluorescence intensity (MFI) of ≥ 100, and ≥ 200 MFI for L1. Seroconversion was defined by measuring at least a two-fold increase from the previous MFI value and the new MFI value exceeding the cut-off level. (Study II)

Similarly to Study II, antibodies to the major capsid protein L1 of HPV6, 11, 16, 18, and 45 were analyzed with multiplex HPV serology based on GST fusion protein capture on fluorescent beads. For all HPV types, seropositivity was defined as MFI > 200 or > 400 (stringent cut-off). (Study III)

4.5 Statistical analyses

Study I: The mothers had to have a persistent HPV16 infection either in the genital or oral mucosa to be eligible for the analysis in the case group. Persistent HPV16 infection either at the genital or oral site was defined by testing HPV16 positive at least in two consecutive follow-up visits. As the reference group, the mothers who tested continuously HPV negative during the follow-up time were selected. The children of these mothers were subjected to similar analyses. The group of 42 mothers and their 28 children were divided in subgroups based on the mother's HPV infection status: Group 1 included 10 mothers who developed an incident CIN with persistent genital HPV16 infection during the follow-up and their 10 children. Group 2 consisted of seven mothers with a persistent oral HPV16 infection and their seven children. Group 3 included 20 mothers who tested constantly HPV negative at the genital site during the follow-up and eight children of these women. Group 4 consisted of five mothers who tested repeatedly HPV negative at the oral site and their three children. In the statistical analyses, the HPV16 positive groups (Group 1 and Group 2) were also analyzed as a combined group of persistent HPV16 infection and correspondingly, a combined group that included those women that remained always HPV negative (Group 3 and Group 4) was analyzed as well.

All statistical analyses were performed by using SPSS (IBM SPSS Statistics for Windows, Armonk, NY, USA) version 25.0 statistical software. All tests were performed two-sided, and statistical significance was declared at the p-value < 0.05. Between all the groups mentioned above, differences in the mother's mean age, follow-up time, gender of the children, and oral HPV status of the children were addressed. The proportion of different T lymphocyte subsets were compared between these groups, first between the mothers, secondly between the children, and thirdly between mother-child pairs. Bonferroni post hoc tests were used to control for multiple comparisons. In null hypothesis testing by this post hoc test, the same significance level (alpha) was used as the settings in options.

Study II: All children of the FFHPV cohort that had participated in the blood sample collection and their mothers were eligible for the present analysis. The number of the children's blood samples varied between the follow-up visits: n=232 at the 1-month, n=239 at the 2-month, n=263 at the 6-month, n=272 at the 12-month, n=250 at the 24-month, and n=243 at the 36-month follow-up visits.

All statistical analyses were performed by using SPSS (IBM SPSS Statistics for Windows, Armonk, NY, USA) version 26.0 and STATA (Stata Corp., College

Station, TX, USA) version 14.1 statistical software. All tests were performed two-sided, and statistical significance was declared at the p-value < 0.05. Differences in the means of continuous variables were analyzed using the Mann-Whitney test for two samples and the Kruskal-Wallis test for multiple independent samples. Mean antibody levels to HPV6 L1 were compared to those of HPV6 E2, E4, E6, and E7 by using Pearson's R correlation coefficient. Seroconversion was defined by measuring at least a two-fold increase from the previous MFI value and the new MFI value exceeding the cut-off level. Correlations between the HPV6 E2, E4, E6, E7, and L1 antibody levels in the baseline samples of the mothers and those of the children at the age of one month were analyzed by using the Spearman R correlation coefficient. The same test was used to analyze correlations between the HPV6 L1 and individual E2, E4, E6, and E7 antibody levels of the children at each follow-up visit.

Study III: For the analysis, all of the 89 women who became pregnant for the second time during the follow-up and the remaining 238 women who did not develop a second pregnancy were included from the FFHPV cohort. Two out of the original 329 women were lost to follow-up, so they were excluded from the analysis of the present study.

All statistical analyses were performed by using SPSS (IBM SPSS Statistics for Windows, Armonk, NY, USA) version 26.0.1 and STATA (Stata Corp., College Station, TX, USA) version 16.1 statistical software. All tests were performed two-sided, and statistical significance was declared at the p-value < 0.05. Frequency tables were analyzed by using the Chi-square (χ^2) test or the Fisher's exact test for categorial variables (seropositivity and seronegativity). Differences in the means of continuous variables (MFI titers) were analyzed by using ANOVA (analysis of variance) after controlling their normal distribution with the Kolmogorv-Smirnov test. The two groups were also compared by the distribution of potential HPV-associated covariates that were recorded by a detailed questionnaire at the enrollment of the study.

5 RESULTS

5.1 T lymphocyte immunophenotypic subsets stratified by the maternal HPV16 status (I)

5.1.1 Immunophenotypic subset distribution of the mother-child pairs (I)

Baseline characteristics of the subgroups are shown in Table 6. The mean age of the mothers varied between 37.0 and 40.0 years in the subgroups, whereas the mean age of the children varied between 12.2 and 14.7 years. 17 out of the 28 children included in the present analysis developed an oral HPV infection during the follow-up, and out of these children, three had a persistent oral HPV infection.

Table 6. Baseline characteristics of the subgroups including the mothers (n=42) and their children (n=28). (Suominen et al., 2022), Table 2.

		Genital HPV16 infection		**Oral HPV16 infection**	
		Incident ≥CIN+ with persistent* infection	Always negative	Persistent* infection	Always negative
Mothers	N	10	20	7	5
Children	N	10	8	7	3
Mean age	Mothers	37.0	40.0	38.7	38.7
	Children	12.2	12.3	14.7	14.7
Gender of the children	Girls	3	4	4	2
	Boys	7	4	3	1
Oral HPV status of the children	Always negative	5	3	3	0
	Incident	1	3	4	2
	Persistent	2	0	0	1
	Fluctuation	1	2	0	0
	Clearance	1	0	0	0

*Persistent defined as testing two or more consequent follow-up visits HPV16 positive.
Abbreviations: CIN = cervical intraepithelial neoplasia

The $CD4^+$ immunophenotypic subsets as defined by 23 $CD4^+$ T lymphocyte surface markers in mother-child pairs are shown in Table 7. Correspondingly, $CD8^+$ immunophenotypic subsets as defined by 25 $CD8^+$ T lymphocyte surface markers in mother-child pairs are shown in Table 8. The highest proportion of T lymphocytes ($CD3^+$ T lymphocytes) presented as median percentages of cells among PBMCs was measured in those mothers who had a persistent genital HPV16 infection (incident CIN), while the lowest proportion of T lymphocytes was seen in the mothers who had a persistent oral HPV infection, 73.0 % vs. 47.0 %, respectively. T lymphocyte counts differed significantly between the mothers who had a persistent genital HPV16 infection and their HPV negative controls (p=0.019). In the children, no significant differences were observed in the distribution of $CD3^+$ cells across the comparison groups. In addition, no statistically significant differences were observed in the distribution of $CD4^+$ and $CD8^+$ T lymphocytes between the combined groups of persistent HPV infection and the combined groups of always HPV negative, as seen in Tables 7 and 8.

As for the mothers' $CD4^+$ lymphocyte distribution in the mother-child comparisons, the median percentage for $HLADR^+CD3^+CD4^+$ (a marker of T lymphocyte activation) was significantly lower in those mothers who had a persistent oral HPV16 infection when compared to their HPV negative counterparts, 4.27 vs. 6.28 (p=0.038). The children of these mothers who had a persistent oral HPV16 infection had higher median levels $CD38^+HLADR^+CD3^+CD4^+$, when compared to those children whose mother remained oral HPV negative, 2.70 vs. 1.06 (p=0.038).

In the $CD8^+$ lymphocyte distribution among the mother-child comparisons, the median levels of $HLADR^+CD3^+CD8^+$, $CD38^+HLADR^+CD3^+CD8^+$, and $CD38^-HLADR^+CD3^+CD8^+$ immunophenotypic subsets were significantly higher in the children of those mothers who had a persistent oral HPV16 infection as compared to their HPV negative counterparts, 10.30 vs. 3.80 (p=0.006), 5.63 vs. 2.91 (p=0.008), and 4.38 vs. 1.20 (p=0.018), respectively. Furthermore, the median percentage of $CD45RO^+CD8^+$ subset (a marker of memory T lymphocytes) was significantly higher among those children whose mother had the persistent HPV16 infection as compared to their HPV negative counterparts, 33.20 vs. 23.00 (p=0.033).

Table 7. $CD4^+$ T lymphocyte immunophenotypic subset distribution of the mother-child pairs stratified according to the mother's genital and oral HPV status. Significant median comparisons between the subgroups are shown in bold. (Suominen et al., 2022), Table 3.

	HPV16 infection status of the mother

		Genital		Oral		Combined	
		Persistent* infection	Always HPV negative	Persistent* infection	Always HPV negative	Persistent* infection	Always HPV negative
Marker		**Median (%)**		**Median (%)**		**Median (%)**	
$CD3^+$ lymphocytes	Mothers	**73.00** [a]	**54.65** [a]	47.10	60.10	58.30	54.70
	Children	72.65	67.40	54.50	67.80	67.10	67.80
$CD3^+CD4^+$	Mothers	47.50	51.45	36.60	46.90	44.20	51.10
	Children	40.15	36.90	38.20	41.30	39.50	37.10
$CD69^+CD4^+$	Mothers	1.89	1.54	1.54	0.58	1.72	1.39
	Children	0.39	0.41	0.40	0.50	0.40	0.49
$CD25^+CD4^+$	Mothers	0.13	0.15	0.10	0.10	0.11	0.14
	Children	0.25	0.25	0.27	0.25	0.25	0.25
$CD27^+CD4^+$	Mothers	88.90	90.25	82.30	86.00	88.15	89.90
	Children	92.30	89.00	92.30	95.10	92.30	93.40
$HLADR^+CD3^+CD4^+$	Mothers	4.92	4.69	**4.27** [b]	**6.28** [b]	4.45	4.73
	Children	3.06	3.53	3.92	1.81	3.60	2.92
$CD38^+CD3^+CD4^+$	Mothers	53.00	49.65	49.30	34.50	51.25	48.70
	Children	65.70	67.10	63.00	68.80	65.30	67.20
$CD38^+HLADR^+CD3^+CD4^+$	Mothers	2.59	2.74	2.71	3.63	2.70	2.90
	Children	2.12	2.71	**2.70** [c]	**1.06** [c]	2.37	1.80
$CD38^-HLADR^+CD3^+CD4^+$	Mothers	2.33	2.42	1.91	4.29	2.07	2.45
	Children	1.54	1.26	1.46	0.72	1.47	1.11
$CD45RA^+CD4^+$	Mothers	65.20	58.95	59.90	46.00	63.30	58.00
	Children	71.60	72.25	68.10	70.50	69.20	70.50
$CD45RA^+CD27^+CD4^+$	Mothers	93.60	92.50	88.30	95.60	92.30	92.70
	Children	96.50	94.55	97.20	99.10	96.90	97.60
$CD45RA^+CD27^-CD4^+$	Mothers	6.37	7.35	11.30	4.37	7.66	7.05
	Children	3.47	5.42	2.73	0.88	3.04	2.30
$CD45RA^+CD57^+CD4^+$	Mothers	9.18	5.35	5.28	2.37	8.01	5.00
	Children	1.97	4.76	0.96	0.73	1.79	1.69
$CD45RA^+CD57^-CD4^+$	Mothers	90.80	94.60	94.70	97.60	92.00	94.90
	Children	98.00	95.25	99.00	99.30	98.20	98.30
$CD45RA^+CD57^+CD27^+CD4^+$	Mothers	2.51	0.79	1.35	0.72	1.68	0.78
	Children	0.80	0.86	1.02	0.82	0.91	0.82
$CD45RA^+CD57^-CD27^+CD4^+$	Mothers	86.50	91.25	87.90	94.10	87.00	91.30
	Children	95.85	93.35	96.00	98.30	96.00	96.70
$CD45RO^+CD4^+$ memory	Mothers	41.80	52.15	51.10	63.80	45.25	54.40
	Children	40.10	37.35	36.50	36.80	38.50	37.30
$CD45RO^+CD27^+CD4^+$	Mothers	80.40	83.60	76.30	79.50	77.80	82.50
	Children	79.25	74.75	80.10	89.40	79.50	83.00
$CD45RO^+CD27^-$	Mothers	19.60	16.40	23.70	20.50	22.20	17.50
	Children	20.75	25.25	19.90	10.60	20.50	17.00
$CD45RO^+CD57^+CD4^+$	Mothers	13.70	6.71	11.20	4.59	12.45	6.56
	Children	6.46	12.42	5.70	4.06	6.16	5.32
$CD45RO^+CD57^-CD4^+$	Mothers	86.30	93.30	88.80	95.40	87.55	93.40

	Children	93.55	87.60	94.30	95.90	93.80	94.70
$CD45RO^+CD57^+CD27^+CD4^+$	Mothers	3.01	2.30	2.23	2.39	2.72	2.37
	Children	3.75	2.92	4.29	3.21	3.96	3.21
$CD45RO^+CD57^-CD27^+CD4^+$	Mothers	77.20	79.55	75.00	78.10	76.00	78.10
	Children	77.10	69.50	77.20	87.90	77.20	79.80

* Mothers developed an incident ≥ CIN+ during the follow-up.
p-values= [a] 0.019, [b] 0.038, [c] 0.038

Table 8. $CD8^+$ T lymphocyte immunophenotypic subset distribution of the mother-child pairs stratified according to the mother's genital and oral HPV status. Significant median comparisons between the subgroups are shown in bold. (Suominen et al., 2022), Table 4.

		HPV16 infection status of the mother					
		Genital		Oral		Combined	
		Persistent* infection	Always HPV negative	Persistent* infection	Always HPV negative	Persistent * infection	Always HPV negative
Marker		**Median (%)**		**Median (%)**		**Median (%)**	
$CD3^+$ lymphocytes	Mothers	**73.00** [a]	**54.65** [a]	47.10	60.10	58.30	54.70
	Children	72.65	67.40	54.50	67.80	67.10	67.80
$CD3^+CD8^+$	Mothers	22.10	18.35	24.40	25.70	22.10	19.80
	Children	25.15	25.30	20.50	20.50	22.20	20.50
$CD69^+CD8^+$	Mothers	1.62	1.83	1.02	3.22	1.46	1.87
	Children	0.76	0.77	0.92	0.93	0.83	0.93
$CD25^+CD8^+$	Mothers	0.06	0.15	0.085	0.33	0.08	0.19
	Children	0.08	0.22	0.12	0.13	0.09	0.17
$CD27^+CD8^+$	Mothers	73.30	70.15	49.50	68.60	64.85	70.00
	Children	84.65	74.05	86.90	93.90	86.50	85.30
$HLADR^+CD3^+CD8^+$	Mothers	5.65	8.35	6.41	7.74	6.03	8.10
	Children	6.01	7.44	**10.30** [b]	**3.80** [b]	8.41	5.36
$CD38^+CD3^+CD8^+$	Mothers	37.80	40.65	36.30	37.50	36.80	37.50
	Children	42.65	52.45	50.50	57.90	49.90	53.50
$CD38^+HLADR^+CD3^+CD8^+$	Mothers	2.99	4.42	3.94	5.59	3.27	5.03
	Children	2.92	4.09	**5.63** [c]	**2.91** [c]	5.07	3.46
$CD38^-HLADR^+CD3^+CD8^+$	Mothers	3.40	3.53	3.37	2.80	3.39	3.35
	Children	4.05	3.56	**4.38** [d]	**1.20** [d]	4.38	2.32
$CD45RA^+CD8^+$	Mothers	79.20	77.55	80.40	73.20	79.80	77.50
	Children	85.35	90.95	87.80	91.30	86.80	91.30
$CD45RA^+CCR7^-CD8^+$	Mothers	93.80	92.70	95.90	93.90	93.80	93.40
	Children	94.75	96.70	92.90	92.90	94.20	96.60
$CD45RA^+CD27^+CD8^+$	Mothers	67.70	68.55	42.30	64.20	58.60	68.20

	Children	82.90	73.50	86.90	94.00	86.30	84.00
$CD45RA^+CD27^-CD8^+$	Mothers	31.30	30.50	57.30	34.90	41.10	30.50
	Children	17.10	26.40	13.00	5.89	13.50	16.00
$CD45RA^+CD57^+CD8^+$	Mothers	43.60	36.30	53.20	37.20	46.35	36.90
	Children	20.90	30.45	25.90	11.40	25.10	23.10
$CD45RA^+CD57^+CD27^+CD8^+$	Mothers	14.20	10.01	6.30	7.60	11.75	9.72
	Children	9.38	8.53	8.03	7.18	8.03	7.18
$CD45RA^+CD57^-CD8^+$	Mothers	56.30	63.50	46.70	62.70	53.55	62.70
	Children	72.20	69.50	74.10	88.60	74.10	76.90
$CD45RA^+CD57^-CD27^+CD8^+$	Mothers	46.50	56.55	36.60	55.30	44.95	56.40
	Children	69.15	65.35	71.90	86.50	71.70	71.70
$CD45RO^+CD8^+$	Mothers	50.20	54.50	46.20	49.60	48.15	49.60
	Children	43.30	38.45	**33.20** [e]	**23.00** [e]	35.10	32.00
$CD45RO^+CCR7^+CD8^+$	Mothers	8.69	12.65	9.90	12.00	9.30	12.00
	Children	6.05	7.17	8.72	6.28	7.29	6.54
$CD45RO^+CCR7^-CD8^+$	Mothers	91.30	87.35	90.10	88.00	90.70	88.00
	Children	93.95	92.85	91.30	93.70	92.70	93.50
$CD45RO^+CD27^+CD8^+$	Mothers	63.80	61.75	48.80	65.90	60.30	63.00
	Children	71.80	60.45	77.00	80.40	73.50	71.10
$CD45RO^+CD27^-CD8^+$	Mothers	36.20	38.25	51.20	34.10	39.70	37.00
	Children	28.20	39.55	23.00	19.60	26.50	28.90
$CD45RO^+CD57^+CD8^+$	Mothers	50.40	42.20	50.60	35.00	50.50	41.70
	Children	43.25	60.20	45.80	35.70	44.50	48.30
$CD45RO^+CD57^-CD8^+$	Mothers	49.60	56.00	49.40	65.00	49.50	57.30
	Children	56.75	39.80	54.20	64.30	55.50	51.70
$CD45RO^+CD57^+CD27^+CD8^+$	Mothers	8.21	4.40	4.09	2.32	7.11	4.18
	Children	3.34	2.65	3.13	3.87	3.21	2.96
$CDRO^+CD57^-CD27^-CD8^+$	Mothers	4.97	6.12	9.64	13.20	8.93	7.72
	Children	2.23	3.80	2.60	2.85	2.51	3.25

* Mothers developed an incident ≥ CIN+ during the follow-up.
p-values= [a] 0.019, [b] 0.006, [c] 0.008, [d] 0.018, [e] 0.033

The supplemental statistical analysis was performed to control for multiple comparisons by using the classical Bonferroni post hoc test. When using the Bonferroni test, the previously observed statistical significances in the $CD4^+$ lymphocyte distribution changed in the following manner: (1) $CD3^+$ lymphocytes: the mother's persistent genital HPV16 infection vs. always HPV negative, the original p=0.019 changed to p=0.084; (2) $HLADR^+CD3^+CD8^+$: the mother's persistent oral HPV16 infection vs. always HPV negative, the original p=0.038 changed to p=0.668; (3) $CD38^+HLADR^+CD3^+CD4^+$: the children of the mothers

with persistent oral HPV16 vs. always HPV negative, the original p=0.038, changed to p=1.000. When using the Bonferroni test, the previously observed statistical significances in the $CD8^+$ lymphocyte distribution changed in the following manner: (1) $CD3^+$ lymphocytes: the mother's persistent genital HPV16 infection vs. always HPV negative, the original p=0.019 changed to p=0.084; (2) $HLADR^+CD3^+CD8^+$: the children of the mothers with the persistent oral HPV16 infection vs. always HPV negative, the original p=0.006 changed to p=1.000; (3) $CD38^+HLADR^+CD3^+CD8^+$: the children of the mothers with the persistent oral HPV16 infection vs. always HPV negative, the original p=0.008 changed to p=1.000; (4) $CD38^-HLADR^+CD3^+CD8^+$: the children of the mothers with the persistent oral HPV16 infection vs. always HPV negative, the original p=0.018 changed to p=1.000; and (5) $CD45RO^+CD8^+$: the children of the mothers with the persistent oral HPV16 infection vs. always HPV negative, the original p=0.033 changed to p=1.000.

5.1.2 Immunophenotypic subsets of the mothers

The immunophenotypic $CD4^+$ and $CD8^+$ T lymphocyte subsets were compared between the different groups and the combined groups as the number of mothers defined by their HPV status was limited. Significant results of the mothers are shown in Table 9. Of the immunophenotypic $CD4^+$ subsets in the mothers with the persistent oral HPV16 infection as compared to their HPV negative counterparts, only $HLADR^+CD3^+CD4^+$ subset was significantly lower, the corresponding mean percentage values being 4.07 and 6.60 (p=0.038).

As for the $CD8^+$ subsets, the mean percentage value for $CD45RO^+CCR7^-CD8^+$ (a marker of memory cells) subset was significantly higher among the mothers with the persistent genital HPV16 infection than in those who remained always HPV negative, 90.19 vs. 80.56 (p=0.048), correspondingly. This difference remained significant also when the mothers with either persistent oral or genital HPV16 infection were pooled together as a combined group and compared to the pooled group of those who remained always HPV negative (p=0.033). Among the mothers with the persistent oral HPV16 infection, the mean percentage of $CD38^+HLADR^+CD3^+CD8^+$ (a marker of activated T lymphocytes) subset was significantly lower as compared to their HPV negative counterparts, the mean values being 3.67 vs. 7.70 (p=0.036), respectively. In the pooled group of the persistent HPV16 infection, the mean percentage of $CD45RA^+CCR7^-CD8^+$ (a marker of

terminal effector cells) subset was significantly higher as compared to the pooled group of always HPV negative mothers, 89.44 vs. 82.07 (p=0.033).

Table 9. The proportion of CD4+ and CD8+ T lymphocytes by their immunophenotypic subsets* given as a mean (±SD) percentage among the mothers with persistent genital or oral HPV16 infection and HPV negative mothers. Only lymphocyte subsets with statistically significant differences are given, results shown in bold. (Suominen et al., 2022), Table 5.

	Genital HPV16 infection		**Oral HPV16 infection**		**Combined HPV16 infection**	
	Persistent infection (n=10)	Always HPV negative (n=20)	Persistent infection (n=7)	Always HPV negative (n=5)	Persistent infection (n=17)	Always HPV negative (n=25)
	Mean (± SD)		**Mean (± SD)**		**Mean (± SD)**	
Lymphocytes ($CD3^+$)	69.03 (14.17)	56.76 (11.40)	48.17 (7.35)	60.26 (14.26)	59.91 (15.59)	57.46 (11.78)
$CD4^+$ cell population						
$HLADR^+CD3^+$**	5.67 (3.33)	5.17 (2.47)	**4.07 [a] (1.53)**	**6.60 [a] (2.15)**	4.97 (2.74)	5.46 (2.44)
$CD8^+$ cell population						
$CD45RO^+CCR7^-$	**90.19 [b] (4.97)**	**80.56 [b] (13.40)**	88.47 (7.48)	88.12 (2.62)	**89.44 [c] (6.03)**	**82.07 [c] (12.36)**
$CD38^+HLADR^+CD3$***	3.92 (2.44)	5.40 (4.38)	**3.67 [d] (2.18)**	**7.70 [d] (3.61)**	3.81 (2.26)	5.86 (4.27)
$CD45RA^+CCR7^-$	92.96 (3.18)	90.21 (5.63)	95.37 (2.15)	94.14 (0.55)	**94.01 [e] (2.97)**	**91.00 [e] (5.26)**

p-values= [a] 0.038, [b] 0.048, [c] 0.033, [d] 0.036, [e] 0.044

* Percentages of $CD4^+$ and $CD8^+$ T lymphocyte subpopulations. PBMCs were analyzed by flow cytometry by first gating on the total PBMCs and then on the $CD3^+$ T lymphocytes.

** Markers expressed as percentages of total CD3 positive CD4 lymphocytes.

*** Markers expressed as percentages of total CD3 positive CD8 lymphocytes.

5.1.3 Immunophenotypic subsets of the children

As for the $CD4^+$ and $CD8^+$ T lymphocyte subsets, significant differences were only observed between those children whose mother had the persistent oral HPV16 infection and their HPV negative counterparts. These results are shown in Table 10.

The mean percentage value for $CD38^-HLADR^+CD3^+CD4^+CD8^+$ subset (a marker for both activated $CD4^+$ and $CD8^+$ T lymphocytes) was significantly higher in the children whose mother had the persistent oral HPV16 infection as compared to the children of those mothers who remained always HPV negative, 4.25 vs. 1.52 (p=0.018). Similarly, the mean percentage values for $CD38^+HLADR^+CD3^+CD4^+$

and $CD38^+HLADR^+CD3^+CD8^+$ subsets (markers of activated T lymphocytes) were measured significantly higher in the children of those mothers who had the persistent oral HPV16 infection, the corresponding values being 3.22 vs. 1.24 (p=0.038) and 6.44 vs. 3.06 (p=0.0080). In addition, the mean percentage values for $HLADR^+CD3^+CD8^+$ subset (a marker of T lymphocyte activation) and $CD45RO^+CD8^+$ subset (a marker of memory T lymphocytes) were higher in these children whose mother had the persistent oral HPV16 infection, 9.79 vs. 4.17 (p=0.005) and 34.30 vs. 23.40 (p=0.033), respectively.

Table 10. The proportion of $CD4^+$ and $CD8^+$ T lymphocytes by their immunophenotypic subsets* given as a mean (± SD) percentage in the children whose mothers either had the persistent genital or oral HPV16 infection and in the children of HPV negative mothers. Only lymphocyte subsets with statistically significant differences are given, and those results are shown in bold. (Suominen et al., 2022), Table 6.

	Mother's HPV16 status					
	Genital HPV16 infection		**Oral HPV16 infection**		**Combined HPV16 infection**	
	Persistent infection (n=10)	Always HPV negative (n=8)	Persistent infection (n=7)	Always HPV negative (n=3)	Persistent infection (n=17)	Always HPV negative (n=11)
	Mean (± SD)		**Mean (± SD)**		**Mean (± SD)**	
Lymphocyte subsets in children						
Lymphocytes ($CD3^+$)	67.82 (13.99)	72.49 (13.67)	57.99 (12.77)	65.30 (10.67)	63.77 (14.01)	70.53(12.84)
$CD4^+$ cell population						
$CD38^+HLADR^+CD3^+$**	4.86 (8.11)	2.65 (1.08)	**3.22 [a] (1.32)**	**1.24 [a] (0.36)**	4.18 (6.19)	2.26 (1.13)
$CD8^+$ cell population						
$HLADR^+CD3^+$	9.45 (9.60)	7.99 (5.12)	**9.79 [b] (2.42)**	**4.17 [b] (1.05)**	9.59 (7.35)	6.95 (4.67)
$CD38^+HLADR^+CD3^+$***	6.40 (9.03)	4.46 (2.76)	**6.44 [c] (1.62)**	**3.06 [c] (0.35)**	6.41 (6.85)	4.08 (2.41)
$CD38^-HLADR^+CD3^+CD4^+$	11.92 (25.21)	4.19 (3.00)	**4.25 [d] (1.49)**	**1.52 [d] (0.70)**	8.76 (19.33)	3.46 (2.82)
$CD45RO^+$	40.81 (17.42)	38.59 (8.58)	**34.30 [e] (6.90)**	**23.40 [e] (2.82)**	38.13 (14.12)	34.45 (10.17)

p-values: [a] 0.038, [b] 0.005, [c] 0.0080 [d] 0.018, [e] 0.033

* Percentages of $CD4^+$ and $CD8^+$ T lymphocyte subpopulations. PBMCs were analyzed by flow cytometry by first gating on the total PBMCs and then on the $CD3^+$ T lymphocytes.

** Markers expressed as percentages of total CD3 positive CD4 lymphocytes.

*** Markers expressed as percentages of total CD3 positive CD8 lymphocytes.

5.2 Maternal antibodies to the HPV6 proteins and seroconversion in the children (II)

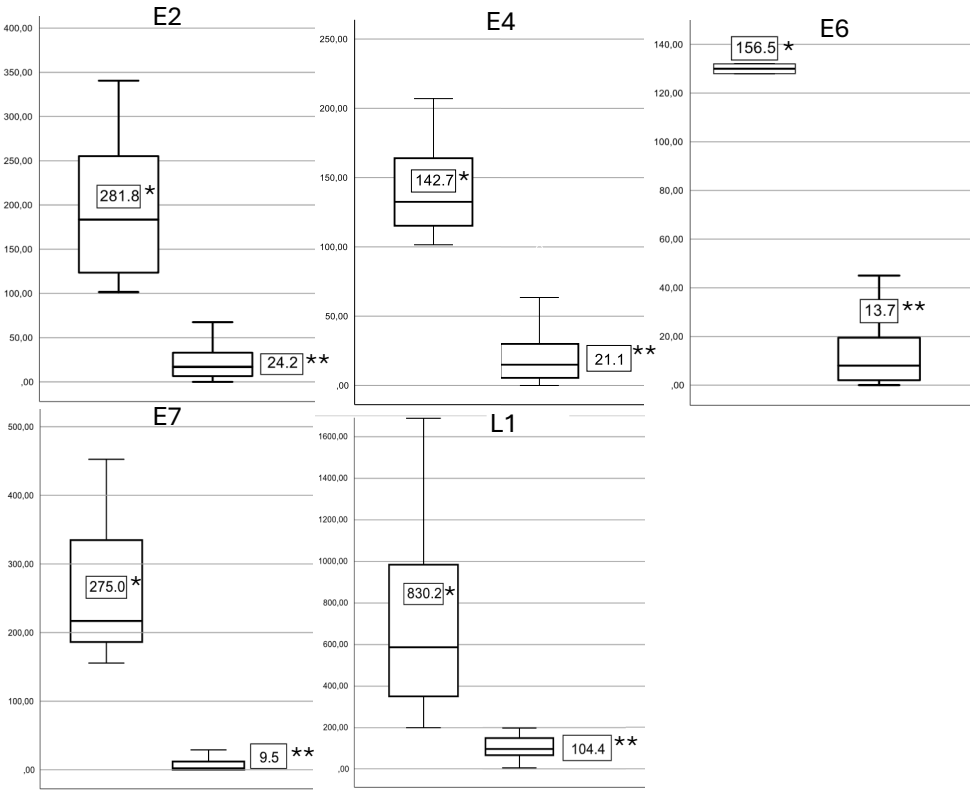
5.2.1 Maternal HPV6 antibodies at their third trimester of pregnancy

Maternal HPV6 antibody levels to HPV6 E2, E4, E6, E7, and L1 proteins were measured at their third trimester of pregnancy. The mean antibody levels as stratified by the maternal serostatus (mother being either seropositive or seronegative) are depicted in Figure 6.

In the seropositive and the seronegative mothers-to-be, the highest mean MFI values were measured for HPV6 L1 antibodies with the mean MFI values being 830.2 and 104.4, respectively. Of the HPV6 E-protein antibodies in the seropositive mothers, the highest mean MFI values were measured for E2 (mean MFI 281.8) and for E7 (mean MFI 275.0). Antibody levels to HPV6 E4 and E6 were the lowest with the corresponding mean MFI values being 142.7 and 156.5 in the seropositive women.

Out of the 231 women included in this analysis, the number of seropositive and seronegative women varied between different HPV6 proteins in the baseline antibody measurements. Whereas 28 women were seropositive for HPV6 E2, 11 women were seropositive for HPV6 E4. As for HPV6 E6 and E7, only 5 and 3 women were seropositive for these E-proteins, respectively. The largest proportion of seropositive women was for HPV6 L1, as 134 women were seropositive for this HPV6-protein.

Figure 6. Antibody levels (MFI mean ± SD) to HVP6 E2, E4, E6, E7, and L1 proteins among pregnant women at their third trimester of pregnancy, as shown separately for the seropositive and the seronegative women. (Modified from publication II)



* Seropositive women; ** Seronegative women
Cut-off value for seropositivity was MFI ≥ 200 for L1 and ≥ 100 MFI for E2, E4, E6, and E7.

5.2.2 Maternal HPV6 antibodies and their correlation in the children at their first month of life

Antibody titers from the mothers' baseline blood samples (taken at their third trimester of pregnancy) were compared to those of the children at the age of one month. The correlation was significant with *p*-value 0.001 for all the tested HPV6 proteins, the Pearson correlation coefficient (r) being 0.854 for L1, 0.640 for E2, 0.679 for E4, 0.546 for E6, and 0.562 for E7, respectively. Therefore, all the tested maternal antibodies to the HPV6 E- and L-proteins were significantly correlated with the children's respective antibody titers suggesting either a strong or a moderate correlation for each HPV6 protein. At the age of 12 months, this observed correlation between the children's antibody levels and the baseline maternal antibody levels was lost.

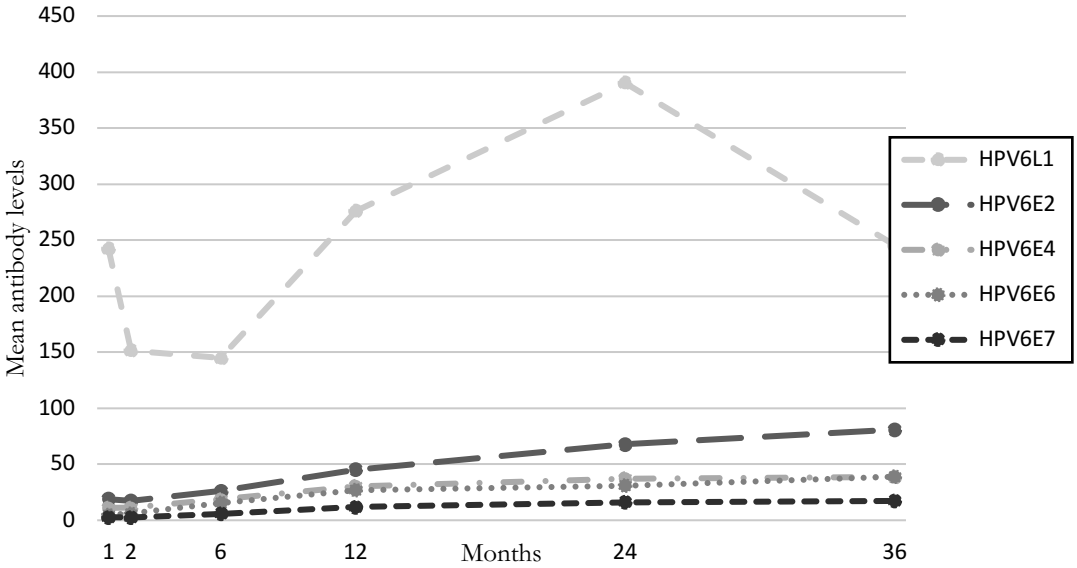
5.2.3 Antibodies to the HPV6 proteins in the children

Antibody levels to the HPV6 E2, E4, E6, E7, and L1 proteins in the children were measured during their first three years of life, and these results are shown in Figure 7.

In the longitudinal setting, the pattern of HPV6 L1 antibodies is different from that of the HPV6 E-protein antibodies as shown in Figure 7. Firstly, the L1 antibody titers are of different magnitude than with the E-proteins, and secondly, a distinct decrease of L1 antibody titer occurs during the children's first six months of life. This L1 antibody titer decline is then followed by an increase in the mean antibody levels, peaking between 12–24 months and then again this is followed by a decline. The mean antibody levels to the HPV6 E-proteins follow a less pronounced pattern, and they remain in a relatively stable state until six months and slowly increase thereafter. Out of the E-protein antibody levels, the increase seems to be the most pronounced for HPV6 E2 antibodies.

IgG antibody levels (mean ± SD) to the HPV6 L1- and E-proteins during the follow-up are also shown in Figure 7. Correlations between the L1 antibodies and E-protein antibodies were measured, and statistically significant differences are shown in bold. According to the Pearson correlation test, antibody levels between the L1 and E2 proteins correlated significantly at the two-month and the 12-month follow-up visits, the corresponding values being r=0.138 (p=0.034) and r=0.145 (p=0.017), suggesting a weak positive correlation. As for the correlations between the L1 and E4 protein antibodies at the six-month and the 12-month follow-up visits, the Pearson correlation values were r=0.176 (p=0.004) and r=0.157 (p=0.01), respectively. At the six-months follow-up visit, also antibody levels between the L1 and E6 proteins correlated significantly, r=0.174 (p=0.005). At the 12-months follow-up visit, antibody levels between the L1 and E7 proteins correlated significantly, r=0.121 (p=0.046).

Figure 7. Antibody levels to HPV E2, E4, E6, E7, and L1 of the children at each follow-up visit. Below are depicted these same antibody levels with mean (± SD) values at each follow-up visit. Numbers shown in bold represent the E-protein antibody levels that are significantly correlated (Pearson correlation) with the protein L1 antibody levels. (Modified from publication II)



	Mean (±SD)					
	1 mo (n=232)	2 mo (n=239*)	6 mo (n=263)	12 mo (n=272)	24 mo (n=250)	36 mo (n=243)
L1	242.5 (422.2)	151.3 (258.8)	144.93 (398.2)	275.8 (604.3)	390.1 (677.4)	245.6 (404.1)
E2	19.0 (50.7)	**17.3 (41.2)**	26.0 (38.8)	**45.2 (51.6)**	67.7 (155.9)	80.9 (139.5)
E4	11.0 (15.1)	11.2 (17.5)	**18.6 (25.9)**	**30.4 (33.8)**	37.2 (43.2)	38.7 (31.9)
E6	4.7 (8.9)	6.0 (12.7)	**15.4 (26.0)**	26.8 (30.5)	30.9 (30.7)	38.9 (114.3)
E7	2.7 (7.0)	2.5 (6.0)	5.9 (13.0)	**11.9 (16.2)**	16.0 (21.0)	17.3 (62.6)

Abbreviations: n = number of children, SD = standard deviation, mo=months
* Only 237 children for E2 and E4.

5.2.4 Seroconversion in the children

The proportion of seropositive children and the median times to seroconversion with range are shown in Table 11. Whereas 159 of the children seroconverted to HPV6 L1, 70 children seroconverted to HPV6 E2. As for the other E-proteins, 32, 19, and 13 children seroconverted to E4, E6, and E7, respectively. Time to seroconversion was the shortest for HPV6 L1 with the median time of 11.8 months (range 0.9–38.1 months). Seroconversion for HPV6 E2, E4, and E6 took slightly longer with the median times being 23.7, 23.2, and 23.4 months, respectively. As for

the few events of seroconversion (n=13) to HPV6 E7, time to seroconversion took longer than with the other E-proteins, the median time being 35.4 months (range 5.8–37.1 months) for E7.

Table 11. Proportion of the children that were seropositive to HPV6 L1, E2, E4, E6, and E7 proteins at each follow-up visit. In addition, the number of children that seroconverted and the median times to seroconversion are shown. (Modified from publication II)

	Proportion (%) of seropositive children						Seroconversion	
HPV6	1 mo (n=232)	2 mo (n=239*)	6 mo (n=263)	12 mo (n=272)	24 mo (n=250)	36 mo (n=243)	n	Median time in months (range)
L1	31.5	18.8	17.8	25.0	37.2	31.3	159	11.8 (0.9 – 38.1)
E2	3.9	2.5	3.4	9.2	12.4	17.3	70	23.7 (1.0 – 42.2)
E4	0.4	0.8	2.3	4.8	8.0	5.3	32	23.2 (5.2 – 42.2)
E6	0.0	0.4	1.5	3.7	6.0	4.1	19	23.4 (5.3 – 36.0)
E7	0.0	0.0	0.4	0.4	1.6	3.3	13	35.4 (5.8 – 37.1)

Abbreviations: mo = months

Seropositivity cut-off was MFI ≥ 200 for L1 and MFI ≥ 100 for E2, E4, E6, E7.

* Only 237 children for E2 and E4

5.3 Effect of a second pregnancy on HPV serology (III)

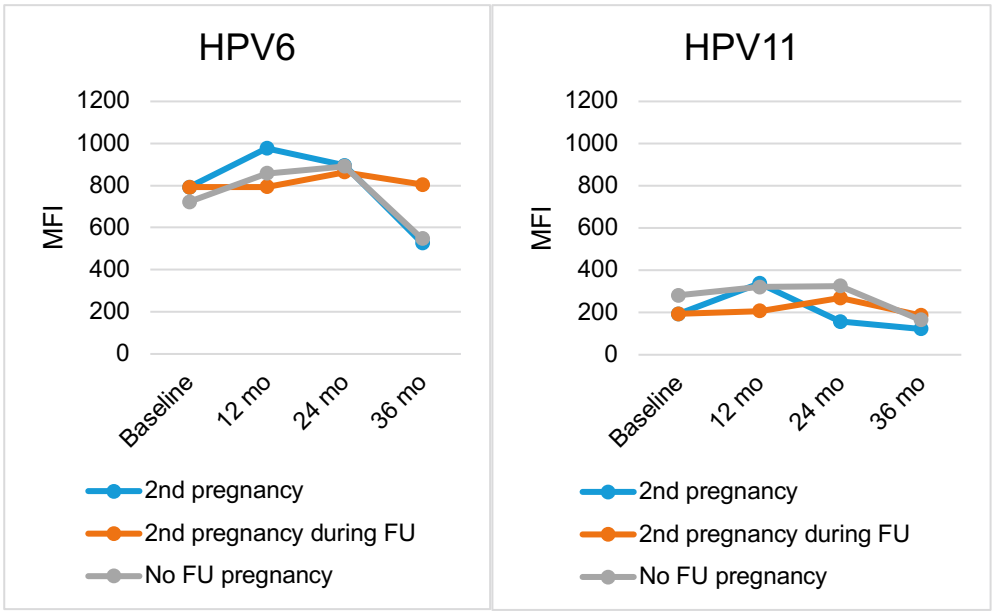
5.3.1 Antibody levels among the seropositive women

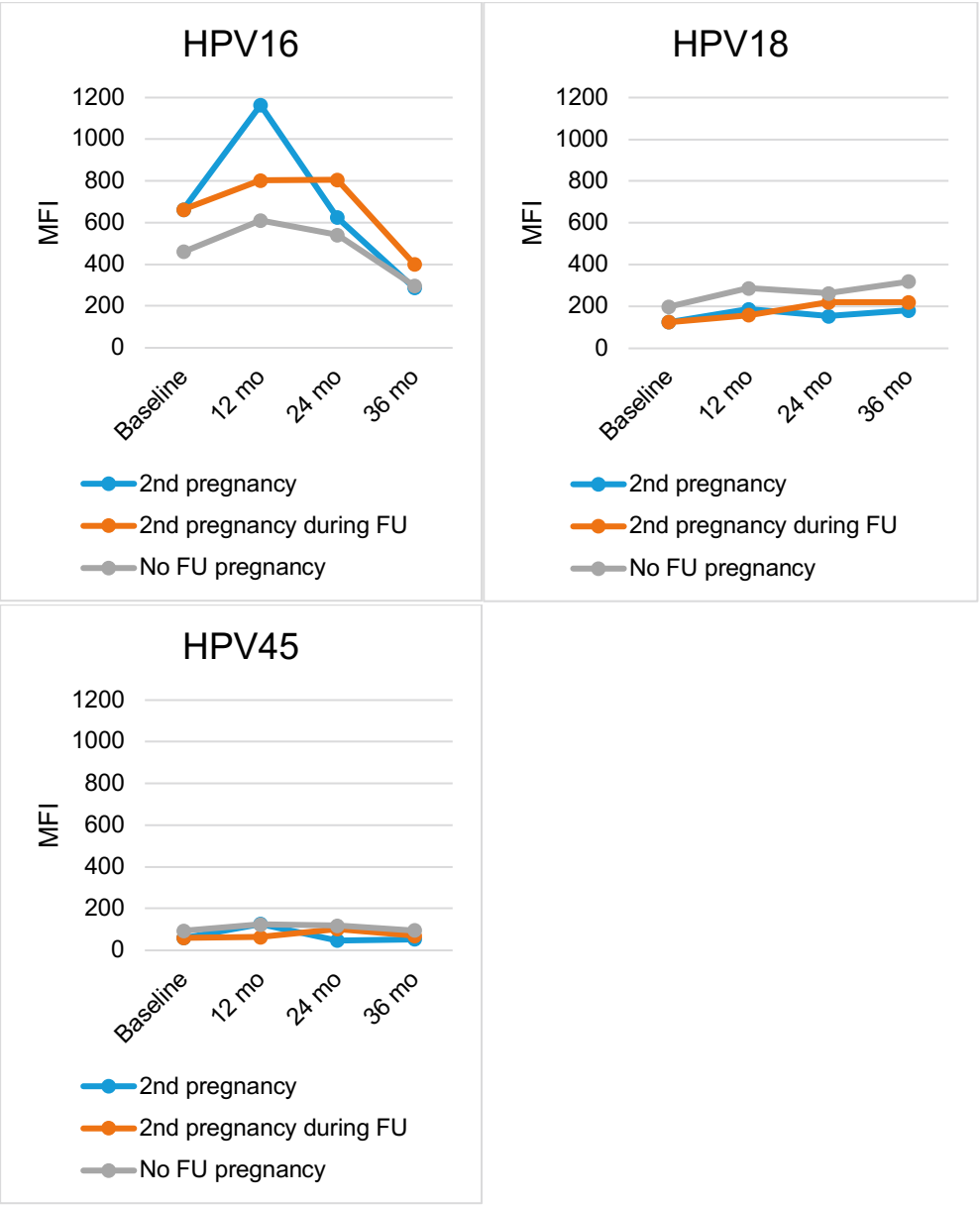
The mean antibody levels to HPV6, HPV11, HPV16, HPV18, and HPV45 in the seropositive women at the baseline and during the follow-up visits as stratified by their status of a second pregnancy are shown in Figure 8. Seropositive women were categorized by either having an ongoing second pregnancy at the time of the follow-up visit, having a second pregnancy at some other timepoint during the follow-up, or not having a second pregnancy at all during the follow-up time (corresponding blue, orange, and gray lines in the figure).

As for the HPV18 antibodies at the baseline of the study, the mean levels (mean MFI ± SD) were 125 (± 117) for those women who developed a second pregnancy, and 199 (± 217) for those who did not have a second pregnancy during the follow-up time (p=0.021). No other statistically significant differences in the HPV18 antibody levels were observed later on between these two groups. As for the HPV16 antibodies in the 12-month follow-up visit, the mean MFI level (1164 ± 2003) was

higher in those women who had an ongoing second pregnancy at that timepoint, whereas the corresponding mean MFI values were 803 (± 971) for those who had a pregnancy at some other timepoint and 611 (± 1217) for those who did not develop second pregnancy during the follow-up (p=0.209).

Figure 8. The mean MFI levels of HPV6, HPV11, HPV16, HPV18, and HPV45 antibodies in the seropositive women at the baseline and during the follow-up visits stratified by the status of their second pregnancy. Blue line depicts mean MFI values of those women who had a second pregnancy at the specific follow-up timepoint, orange line depicts those who had a second pregnancy at some other timepoint, and the gray line depicts those who did not have a second pregnancy at all during the follow-up time. (Suominen et al., 2023), Figure 1.





Abbreviations: MFI = median fluorescence intensity, mo = months, FU = follow-up.

5.3.2 Serostatus of the women

All the 327 mothers included in this study were stratified by their HPV6, HPV11, HPV16, HPV18, and HPV45 serostatus (seropositive or seronegative) as related to

the timing of the possible second pregnancy during the follow-up visits (baseline, 12 months, 24 months, 36 months), as shown in Table 12.

Most statistically significant differences in the serostatuses were observed when the second pregnancy was ongoing at the 24-month timepoint, and this difference between the mothers with second pregnancy and counterparts without a second pregnancy applies to their baseline seropositivity for HPV6 (p=0.008) and HPV11 (p=0.002). As for the HPV11 serostatus among these women, significant differences were measured in the all other timepoints during the follow-up except for the 36-month follow-up visit. At the 36-month follow-up visit, serostatuses for HPV6 and HPV18 differed between those women who had a second pregnancy at the 24-month timepoint and their counterparts with no second pregnancy (p=0.042 and p=0.005, correspondingly).

When the second pregnancy was ongoing at the 36-month follow-up visit, statistically significant differences between these groups of women were observed only in the 24-month serostatus for HPV6 and HPV16 (p=0.021 and p=0.04, respectively). No statistically significant differences were observed when the second pregnancy was ongoing at the 12-month follow-up visit.

The women's HPV6, HPV11, HPV16, HPV18, and HPV45 serostatuses at the baseline and during the follow-up visits were measured with seropositivity cut-offs being MFI > 200 and more stringent MFI > 400. These results are shown in Table 13. At the baseline, only 44.9 % (n=40) of the women included in this study who developed a second pregnancy were HPV6 seropositive (MFI > 200) as compared to 58.4 % (n=139) being seropositive among those who did not develop a second pregnancy, p=0.034. As for HPV11, 6.7 % (n=6) of the women with a second pregnancy were seropositive (stringent MFI > 400) at the baseline, whereas 15.1 % (n=36) of their counterparts with no second pregnancy were seropositive for HPV11, p=0.043. In addition, these two groups differed significantly in their baseline seropositivity (MFI > 200) for HPV18 (p=0.013), and significant differences were also measured at the 12-month and the 36-month follow-up visits.

Table 12. Serostatuses for HPV6, HPV11, HPV16, HPV18, and HPV45 among 327 women during the 36-month follow-up time, as stratified by the timing of the second pregnancy (12 mo, 24 mo, 36 mo). Significant differences in the seroprevalence of the women with a second pregnancy are shown in bold. (Suominen et al., 2023), Table 1.

		Baseline		**12 months**		**24 months**		**36 months**	
		Yes	No	Yes	No	Yes	No	Yes	No
		n (%)		n (%)		n (%)		n (%)	
2nd pregnancy at 12 mo (n= 27)									
HPV6	Seropositive	17 (63.0)	162 (54.0)	18 (66.7)	178 (69.3)	19 (73.1)	159 (67.4)	14 (51.9)	126 (53.8)
	Seronegative	10 (37.0)	138 (46.0)	9 (33.3)	79 (30.7)	7 (26.9)	77 (32.6)	13 (48.1)	108 (46.2)
HPV11	Seropositive	8 (29.6)	62 (20.7)	9 (33.3)	69 (26.8)	7 (26.9)	55 (23.3)	6 (22.2)	33 (14.1)
	Seronegative	19 (70.4)	238 (79.3)	18 (66.7)	188 (73.2)	19 (73.1)	181 (76.7)	21 (77.8)	201 (85.9)
HPV16	Seropositive	10 (37.0)	99 (33.0)	12 (44.4)	104 (40.5)	10 (38.5)	78 (33.1)	8 (29.6)	66 (28.2)
	Seronegative	17 (63.0)	201 (67.0)	15 (55.6)	153 (59.5)	16 (61.5)	158 (66.9)	19 (70.4)	168 (71.8)
HPV18	Seropositive	5 (18.5)	61 (20.3)	5 (18.5)	70 (27.2)	7 (26.9)	52 (22.0)	8 (29.6)	53 (22.6)
	Seronegative	22 (81.5)	239 (79.7)	22 (81.5)	187 (72.8)	19 (73.1)	184 (78.0)	19 (70.4)	181 (77.4)
HPV45	Seropositive	3 (11.1)	28 (9.3)	4 (14.8)	28 (10.9)	5 (19.2)	19 (8.1)	2 (7.4)	18 (7.7)
	Seronegative	24 (88.9)	272 (90.7)	23 (85.2)	229 (89.1)	21 (80.8)	217 (91.9)	25 (92.6)	216 (92.3)
2nd pregnancy at 24 mo (n=43)									
HPV6	Seropositive	**15 (34.9)** [a]	**164 (57.7)** [a]	26 (60.5)	170 (70.5)	23 (54.8)	155 (70.5)	**16 (38.1)** [b]	**124 (56.6)** [b]
	Seronegative	**28 (65.1)** [a]	**120 (42.3)** [a]	17 (39.5)	71 (29.5)	19 (45.2)	65 (29.5)	**26 (61.9)** [b]	**95 (43.4)** [b]

HPV11	Seropositive	**2 (4.7)** [c]	**68 (23.9)** [c]	**6 (14.0)** [d]	**72 (29.9)** [d]	**3 (7.1)** [e]	**59 (26.8)** [e]	3 (7.1)	36 (16.4)
	Seronegative	**41 (95.3)** [c]	**216 (76.1)** [c]	**37 (86.0)** [d]	**169 (70.1)** [d]	**39 (92.9)** [e]	**161 (73.2)** [e]	39 (92.9)	183 (83.6)
HPV16	Seropositive	10 (23.3)	99 (34.9)	14 (32.6)	102 (42.3)	10 (23.8)	78 (35.5)	9 (21.4)	65 (29.7)
	Seronegative	33 (76.7)	185 (65.1)	29 (67.4)	139 (57.7)	32 (76.2)	142 (64.5)	33 (78.6)	154 (70.3)
HPV18	Seropositive	4 (9.3)	62 (21.8)	**4 (9.3)** [f]	**71 (29.5)** [f]	6 (14.3)	53 (24.1)	**3 (7.1)** [g]	**58 (26.5)** [g]
	Seronegative	39 (90.7)	222 (78.2)	**39 (90.7)** [f]	**170 (70.5)** [f]	36 (85.7)	167 (75.9)	**39 (92.9)** [g]	**161 (73.5)** [g]
HPV45	Seropositive	1 (2.3)	30 (10.6)	**1 (2.3)** [h]	**31 (12.9)** [h]	1 (2.4)	23 (10.5)	2 (4.8)	18 (8.2)
	Seronegative	42 (97.7)	254 (89.4)	**42 (97.7)** [h]	**210 (87.1)** [h]	41 (97.6)	197 (89.5)	40 (95.2)	201 (91.8)
2nd pregnancy at 36 mo (n=19)									
HPV6	Seropositive	8 (42.1)	171 (55.5)	12 (63.2)	184 (69.4)	13 (68.4)	165 (67.9)	14 (70.0)	126 (52.3)
	Seronegative	11 (57.9)	137 (44.5)	7 (36.8)	81 (30.6)	6 (31.6)	78 (32.1)	6 (30.0)	115 (47.7)
HPV11	Seropositive	3 (15.8)	67 (21.8)	6 (31.6)	72 (27.2)	**9 (47.4)** [i]	**53 (21.8)** [i]	3 (15.0)	36 (14.9)
	Seronegative	16 (84.2)	241 (78.2)	13 (68.4)	193 (72.8)	**10 (52.6)** [i]	**190 (78.2)** [i]	17 (85.0)	205 (85.1)
HPV16	Seropositive	8 (42.1)	101 (32.8)	11 (57.9)	105 (39.6)	**11 (57.9)** [j]	**77 (31.7)** [j]	8 (40.0)	66 (27.4)
	Seronegative	11 (57.9)	207 (67.2)	8 (42.1)	160 (60.4)	**8 (42.1)** [j]	**166 (68.3)** [j]	12 (60.0)	175 (72.6)
HPV18	Seropositive	1 (5.3)	65 (21.1)	4 (21.1)	71 (26.8)	3 (15.8)	56 (23.0)	3 (15.0)	58 (24.1)
	Seronegative	18 (94.7)	243 (78.9)	15 (78.9)	194 (73.2)	16 (84.2)	187 (77.0)	17 (85.0)	183 (75.9)
HPV45	Seropositive	1 (5.3)	30 (9.7)	3 (15.8)	29 (10.9)	3 (15.8)	21 (8.6)	2 (10.0)	18 (7.5)
	Seronegative	18 (94.7)	278 (90.3)	16 (84.2)	236 (89.1)	16 (84.2)	222 (91.4)	18 (90.0)	223 (92.5)

p-values = [a] 0.008, [b] 0.042, [c] 0.002, [d] 0.028, [e] 0.004, [f] 0.005, [g] 0.005, [h] 0.039, [i] 0.021, [j] 0.04

Abbreviations: MFI = median fluorescence intensity, mo = months

Cut-off value for seropositivity was MFI > 200.

Table 13. Serostatuses (with MFI > 200 and more stringent MFI > 400) for HPV6, HPV11, HPV16, HPV18, and HPV45 during the follow-up time, as stratified by the second pregnancy. Significant differences between the groups are shown in bold. (Suominen et al., 2023), Supplementary Table S1.

			Baseline		12 mo		24 mo		36 mo	
	2nd pregnancy		MFI > 200	MFI > 400	MFI > 200	MFI > 400	MFI > 200	MFI > 400	MFI > 200	MFI > 400
			n (%)		n (%)		n (%)		n (%)	
HPV6	Yes	Seropositive	**40 (44.9)** [a]	29 (32.6)	56 (62.9)	34 (38.2)	55 (63.2)	37 (42.5)	44 (49.4)	37 (41.6)
		Seronegative	**49 (55.1)** [a]	60 (67.4)	33 (37.1)	55 (61.8)	32 (36.8)	50 (57.5)	45 (50.6)	52 (58.4)
	No	Seropositive	**139 (58.4)** [a]	97 (40.8)	140 (71.8)	97 (49.7)	123 (70.3)	79 (45.1)	96 (55.8)	63 (36.6)
		Seronegative	**99 (41.6)** [a]	141 (59.2)	55 (28.2)	98 (50.3)	52 (29.7)	96 (54.9)	76 (44.2)	109 (63.4)
HPV11	Yes	Seropositive	13 (14.6)	**6 (6.7)** [b]	21 (23.6)	9 (10.1)	19 (21.8)	8 (9.2)	12 (13.5)	5 (5.6)
		Seronegative	76 (85.4)	**83 (93.3)** [b]	68 (76.4)	80 (89.9)	68 (78.2)	79 (90.8)	77 (86.5)	84 (94.4)
	No	Seropositive	57 (23.9)	**36 (15.1)** [b]	57 (29.2)	31 (15.9)	43 (24.6)	26 (14.9)	27 (15.7)	12 (7.0)
		Seronegative	181 (76.1)	**202 (84.9)** [b]	138 (70.8)	164 (84.1)	132 (75.4)	149 (85.1)	145 (84.3)	160 (93.0)
HPV16	Yes	Seropositive	28 (31.5)	20 (22.5)	37 (41.6)	25 (28.1)	31 (35.6)	22 (25.3)	25 (28.1)	12 (13.5)
		Seronegative	61 (68.5)	69 (77.5)	52 (58.4)	64 (71.9)	56 (64.4)	65 (74.7)	64 (71.9)	77 (86.5)
	No	Seropositive	81 (34.0)	46 (19.3)	79 (40.5)	48 (24.6)	57 (32.6)	37 (21.1)	49 (28.5)	26 (15.1)
		Seronegative	157 (66.0)	192 (80.7)	116 (59.5)	147 (75.4)	118 (67.4)	138 (78.9)	123 (71.5)	146 (84.9)
HPV18	Yes	Seropositive	**10 (11.2)** [c]	3 (3.4)	**13 (14.6)** [d]	8 (9.0)	16 (18.4)	8 (9.2)	**14 (15.7)** [e]	8 (9.0)
		Seronegative	**79 (88.8)** [c]	86 (96.6)	**76 (85.4)** [d]	81 (91.0)	71 (81.6)	79 (90.8)	**75 (84.3)** [e]	81 (91.0)
	No	Seropositive	**56 (23.5)** [c]	23 (9.7)	**62 (31.8)** [d]	27 (13.8)	43 (24.6)	25 (14.3)	**47 (27.3)** [e]	27 (15.7)
		Seronegative	**182 (76.5)** [c]	215 (90.3)	**133 (68.2)** [d]	168 (86.2)	132 (75.4)	150 (85.7)	**125 (72.7)** [e]	145 (84.3)
HPV45	Yes	Seropositive	5 (5.6)	1 (1.1)	8 (9.0)	4 (4.5)	9 (10.3)	3 (6.3)	6 (6.7)	2 (2.2)

	Seronegative	84 (94.4)	88 (98.9)	81 (91.0)	85 (95.5)	78 (89.7)	84 (96.6)	83 (93.3)	87 (97.8)
No	Seropositive	26 (10.9)	9 (3.8)	24 (12.3)	11 (5.6)	15 (8.6)	11 (6.3)	14 (8.1)	5 (2.9)
	Seronegative	212 (89.1)	229 (96.2)	171 (87.7)	184 (94.4)	160 (91.4)	164 (93.7)	158 (91.9)	167 (97.1)

p-values = [a] 0.034, [b] 0.043, [c] 0.013, [d] 0.002, [e] 0.044

Abbreviations: MFI = mean fluorescence intensity, mo = months

Cut-off value for seropositivity was MFI > 200 or MFI > 400 (stringent).

5.3.3 Demographic and clinical data

Table 14 presents the demographic and clinical data of the women. Those women who developed a second pregnancy and their counterparts without a second pregnancy differed significantly in their marital status, the number of deliveries, the number of lifetime sexual partners, the history of STDs, and the previous contraception method used. Fewer of the women with a second pregnancy were single when compared to those who did not develop a second pregnancy, 2.3 % vs. 8.9 % (p=0.045). Women with a second pregnancy had fewer previous deliveries than the women without a second pregnancy, 11.6 % vs. 30.9 % for two or more previous deliveries, (p=0.002). Accordingly, the use of condom and oral contraceptives in the past was more common among those women who developed a second pregnancy when compared to their counterparts without a second pregnancy, and in this group of women with no second pregnancy (more previous deliveries) having no past method of contraception was more frequent (p=0.032). In addition, those women who developed a second pregnancy had fewer lifetime sexual partners (p=0.038) but having a history of STDs was slightly more common in this group when compared to their nonpregnant counterparts (26.7 % vs. 16.3 %, p=0.041). The mean age with SD was 25.4 ± 3.4 for the women with a second pregnancy and 25.5 ± 3.4 for the women without a second pregnancy (p=0.718).

Table 14. Demographic and clinical data of the women with a second pregnancy compared to those women who did not develop an additional pregnancy during the follow-up time of 36 months in the FFHPV Study cohort. (Suominen et al., 2023), Table 2.

Variable	2nd pregnancy	No 2nd pregnancy	Significance
		n (%)	
Marital status			p = 0.045 *
Single	**2 (2.3)**	**18 (8.9)**	
Other (unmarried couple, married, divorced)	**84 (97.7)**	**184 (91.1)**	
Number of deliveries			p = 0.002 *
0	**1 (1.2)**	**1 (0.5)**	
1	**75 (87.2)**	**138 (68.7)**	
2	**8 (9.3)**	**54 (26.9)**	

3	**2 (2.3)**	**5 (2.5)**	
4	**0 (0.0)**	**3 (1.5)**	
Age at first intercourse			p = 0.179 *
≤ 13	3 (3.5)	4 (2.0)	
14–16	44 (51.2)	117 (57.9)	
17–19	32 (37.2)	75 (37.1)	
≥ 20	7 (8.1)	6 (3.0)	
Number of lifetime sexual partners			p = 0.038
1–2	**28 (32.9)**	**43 (21.3)**	
3–5	**27 (31.8)**	**64 (31.7)**	
6–10	**20 (23.5)**	**45 (22.3)**	
> 10	**10 (11.8)**	**50 (24.8)**	
Number of sexual partners by the age of 20			p = 0.245
0–2	44 (51.2)	80 (39.6)	
3–5	24 (27.9)	74 (36.6)	
6–10	14 (16.3)	32 (15.8)	
> 10	4 (4.7)	16 (7.9)	
Frequency of intercourse, n/month			p = 0.103 *
0–1	0 (0.0)	7 (3.5)	
2–4	27 (31.4)	59 (29.2)	
5–10	53 (61.6)	108 (53.5)	
> 10	6 (7.0)	28 (13.9)	
Oral sex			p = 0.217
Regular	7 (8.1)	28 (13.9)	
Occasionally	64 (74.4)	130 (64.4)	
Never	15 (17.4)	44 (21.8)	
Anal sex			p = 0.179 *
Regular	2 (2.3)	1 (0.5)	
Occasionally	12 (14.0)	40 (19.8)	
Never	72 (83.7)	161 (79.7)	
Age at the onset of oral contraceptive			p = 0.414
Never used	7 (8.1)	17 (8.5)	
≤ 13 years	0 (0.0)	3 (1.5)	
14–16 years	36 (41.9)	81 (40.3)	
17–19 years	31 (36.0)	83 (41.3)	
≥ 20 years	12 (14.0)	17 (8.5)	
Contraception methods used previously			p = 0.032

Condom	**33 (36.7)**	**65 (27.2)**	
Oral contraceptive	**7 (7.8)**	**6 (2.5)**	
Intrauterine device	**17 (18.9)**	**44 (18.4)**	
None	**33 (36.7)**	**124 (51.9)**	
Smoking habits			p = 0.661 *
Not smoker	47 (54.7)	96 (47.8)	
1–10 cigarettes per day	23 (26.7)	61 (30.3)	
11–20 cigarettes per day	14 (16.3)	41 (20.4)	
> 20 cigarettes per day	2 (2.3)	3 (1.5)	
Pack years of smoking			p = 0.685
Lower tertile (< 2.5)	14 (38.9)	31 (33.0)	
Median tertile (< 6.0)	10 (27.8)	34 (36.2)	
Upper tertile (> 6.0)	12 (33.3)	29 (30.9)	
Alcohol use			p = 0.154
Yes	73 (85.9)	185 (91.6)	
No	12 (14.1)	17 (8.4)	
Frequency of alcohol use			p = 0.441
Never	12 (14.1)	17 (8.4)	
Daily	0 (0.0)	2 (1.0)	
2–3 times a week	10 (11.8)	19 (9.4)	
Once a week	26 (30.6)	63 (31.2)	
Once a month	37 (43.5)	101 (50.5)	
History of STDs			p = 0.041
STD history	**24 (26.7)**	**39 (16.3)**	
No STDs	**66 (73.3)**	**200 (83.7)**	
History of genital warts			p = 0.568
Yes	22 (25.6 %)	58 (29.3)	
No	64 (74.4)	140 (70.7)	
Age at the diagnosis of genital warts			p = 0.894
Never	64 (74.4)	142 (71.7)	
< 20 years	9 (10.5)	27 (13.6)	
20–24 years	10 (11.6)	21 (10.6)	
> 25 years	3 (3.5)	8 (4.0)	
Treatment of genital warts			p = 0.840 *
No treatment	12 (40.0)	29 (37.2)	
Topical treatment	6 (20.0)	25 (32.1)	
Electrocautery	1 (3.3)	3 (3.8)	

Cryotherapy	1 (3.3)	2 (2.6)
Laser therapy	4 (13.3)	8 (10.3)
Surgery	0 (0.0)	1 (1.3)
Several treatments	6 (20.0)	10 (12.8)

* Fisher's exact test.

6 DISCUSSION

6.1 T lymphocyte immunophenotypic subsets stratified by the maternal HPV16 status (I)

The analyses regarding T lymphocyte immunophenotypic subsets among the mothers and their children of the FFHPV study cohort revealed differences as related to the mother's HPV16 infection status. In the mothers with the persistent genital HPV16 infection as compared to their always HPV negative counterparts, higher levels of T lymphocytes ($CD3^+$ T lymphocytes) and effector memory $CD8^+$ T lymphocytes ($CD45RO^+CCR7^-CD8^+$) were observed. As to the pooled group of mothers with the persistent HPV16 infection either at the genital or oral site, these mothers had a higher level of terminally differentiated (T_{EMRA}) $CD8^+$ lymphocytes ($CD45RA^+CCR7^-$) as compared to the pooled group of always HPV negative mothers. T_{EMRA}s are the most effective $CD8^+$ lymphocytes that have a function in destroying tumour cells and virus-infected cells (Kumar et al., 2018; Litwin et al., 2021). The mothers with the persistent oral HPV16 infection had markers of activated T lymphocytes ($HLADR^+CD3^+CD4^+$ and $CD38^+HLADR^+CD3^+CD8^+$) in lower levels than their HPV negative counterparts. Both HLA-DR and CD38 molecules are present on immature T and B lymphocytes, and it has been suggested that co-expression of HLA-DR and CD38 is a key marker for $CD8^+$ T lymphocyte immune activation in several viral infections such as influenza and HIV (Jia et al., 2021; Lu et al., 2021).

In previous studies investigating HPV-related T lymphocyte responses, it has been reported that there are differences between naïve ($CD45RA^+$) and memory T lymphocyte ($CD45RO^+$) populations in the women with HPV infection, and this is in line with the present study's results (Fernandes et al., 2021; Williams et al., 2002). One study suggests that the analysis of $CD45RA^+/CD45RO^+$ expression in CIN lesions might be useful as a prognostic biomarker as its increased expression was observed in cases with cervical cancer progression (Fernandes et al., 2021). In a study assessing T lymphocyte surface markers in older women with the persistent HPV infection, expression of $CD45RO^+CD27^-CD8^+$ was associated with an increased

risk of HPV persistence, whereas expression $CD45RO^+CD27^+CD4^+$ was associated with a decreased risk of HPV persistence (Rodríguez et al., 2011). In addition, the same study reported that $HLADR^+CD3^+CD4^+$ was associated with a significant increase in the risk of HPV persistence in genital HPV infection (Rodríguez et al., 2011). As for the results of Rodríguez et al., the present study did not produce similar results, and in the case of $HLADR^+CD3^+CD4^+$, the results of this study were contrary as the mothers with the persistent oral HPV16 infection had lower levels of this T lymphocyte subset as compared to their HPV negative counterparts. According to a study assessing T lymphocytes in HIV infected women, the women who were positive for HR-HPV had higher levels of circulating $CD38^+$ T lymphocytes irrespective of the presence of a CIN (Papasavvas et al., 2016). As for HLA-DR, it potentially participates in down-regulatory signalling and has an effect on T_{reg} cell function (Baecher-Allan et al., 2006; Rosenblum et al., 2016). It has been suggested that the expression of HLA-DR and CD38 can be used in evaluating T lymphocyte activation in multiple viral diseases (Nguyen et al., 2023). In a study assessing an influenza subtype H7N9, it was shown that prolonged expression of $CD38^+HLADR^+$ predicts fatal outcomes of the infection (Wang et al., 2018).

Multiple comparisons might potentially lead to the obtainment of false positive findings. One possible explanation for some of the contradictory results of this study as compared to the study of Rodríguez et al. is the lack of correlation for the multiple comparisons. As for the present study's analysis, the Bonferroni test was used for the correlation of multiple comparisons, which led to previously measured significant comparisons losing their significancy. As several multiple comparison tests are available, the proper use of different post hoc tests for the control of multiple comparisons has been a topic for discussion in the statistical literature, but currently there is no gold standard on which test is the most suitable for each setting (Lee & Lee, 2018). In the present study, the Bonferroni test was used as it is the most used of all post hoc tests, although it is unnecessarily conservative with weak statistical power leading to potential failure of detecting real differences. Nevertheless, the results of this study should be further replicated in a larger study setting to confirm the findings.

Immunological recognition of HPV16 does occur in early childhood, which is why the children of the study were also subjected to similar analyses as their mothers (Koskimaa et al., 2015). In the present study, the median levels of $CD38^+HLADR^+CD3^+CD4^+$, $HLADR^+CD3^+CD8^+$, $CD38^+HLADR^+CD3^+CD8^+$, and $CD38^-HLADR^+CD3^+CD8^+$ immunophenotypic subsets were significantly higher in the children of those mothers who had the persistent oral HPV16 infection

as compared to their HPV negative counterparts. Interestingly, this was an entirely opposite change as compared to their mother's median levels of the same T lymphocyte subsets. Expression of these T lymphocyte subsets may be a sign of an activated immune response in the children whose mother had the persistent oral HPV16 infection as compared to the children of those mothers who remained always HPV negative at the oral site. Previous data on peripheral blood T lymphocyte subsets in mothers with persistent HPV infection and their children is lacking. The results from another study investigating the FFHPV cohort have suggested that the mother seems to be the main HPV transmitter to her offspring via mouth (Syrjänen et al., 2021). Therefore, the present observations could indicate a continuous exposure to maternal oral HPV in the offspring, which could lead to T lymphocyte activation. The observed higher levels of effector memory T lymphocytes ($CD45RO^+CD8^+$) in the children whose mother had the persistent oral HPV16 infection could support this finding. In addition, it has been presented that persistent HPV infection could be associated with an ineffective cell-mediated immune response consisting of undifferentiated memory $CD4^+$ cells and other T lymphocyte subsets that are not capable to eliminate HPV (De Jong et al., 2004; Hewavisenti et al., 2023; Rodríguez et al., 2011).

6.2 Maternal antibodies against the HPV6 early and late proteins and seroconversion in the children (II)

The analyses in the children of the FFHPV study cohort indicated that the maternal IgG antibodies against HPV6 E2, E4, E6, E7, and L1 proteins were transferred to the neonates as the concordance between maternal and neonatal antibody levels was highly significant. These maternal antibodies were observed to vanish by time, and later on, a new seroconversion was observed to occur in some of the children, which could suggest acquisition of an HPV6 infection by vertical or horizontal transmission.

A previous study from the same FFHPV cohort investigated naturally acquired maternal HPV antibodies to L1 of HPV6, HPV11, HPV16, HPV18, and HPV45 in children, and its results suggest that the L1 antibodies are vertically transferred from the mother to her newborn and that these maternal antibodies vanish during the first six months of life, which is the known period of time in the decay of natural immunoglobulins (Syrjänen et al., 2022). Another study assessing the FFHPV cohort

investigated HPV DNA in neonates' oral samples and reported a high concordance between maternal and neonatal HPV genotypes (Koskimaa et al., 2012). There are other previous studies assessing naturally acquired HPV in mothers and children that are in line with these results (Heim et al., 2007; Smith et al., 2010; Zahreddine et al., 2020). These studies refer that there is a close concordance between maternal and neonatal antibodies, which depends neither on the blood sampling of the newborn nor HPV antibody testing (Heim et al., 2007; Kawana et al., 2003; Syrjänen et al., 2022; Zahreddine et al., 2020). However, it is not comprehensively understood to what extent maternal HPV antibodies protect the children against primary HPV infections.

Previous data on HPV serology as related to HPV E-proteins is very limited, as most studies have focused on HPV L1 protein antibodies. The present study aimed to investigate naturally acquired antibodies to HPV6 E-proteins, and HPV6 was chosen in particular as this HPV type is clinically relevant especially in the childhood as it causes majority of juvenile-onset recurrent respiratory papillomatosis, which may be difficult to treat in young children (Benedict & Derkay, 2021; Fortes et al., 2017). In addition, most previous studies on HPV serology in children have focused on HR-HPV types such as HPV16 and HPV18 rather than on LR-HPV types such as HPV6. In this study, the neonates' antibody levels to HPV6 E2, E4, E6, E7, and L1 proteins at the age of one month correlated well with the maternal antibody levels. The maternal samples were taken only a few weeks before delivery. In addition, the MFI levels of the E-protein antibodies were lower than the levels of IgG L1 antibodies, although the levels of E-protein antibodies increased steadily with time during the follow-up of three years. In the present study, the HPV6 L1 antibodies correlated with the HPV6 E4 and E6 antibodies at the six-month follow-up visit and similarly, the L1 protein antibodies correlated with the E2-, E4-, and E7-protein antibodies at the 12-month follow-up visit.

There are a few earlier studies that have assessed antibodies to HPV E-proteins in children. One study reported that HPV antibodies to the E4 protein were observed even in up to 30 % of girls aged 1–10 years (Jochmus-kudielka et al., 1989), whereas another study investigated girls within similar age group and did not record E4 protein antibodies (Köchel et al., 1991). One possible explanation for the varying results between these two studies could result from differences in the use of E4 protein antigen, whether used as truncated or a complete antigen. Another study evaluated HPV16 E4 and E7 antibodies in individuals aged between 1–95 years, and their results suggest that the prevalence of HPV16 E7 protein antibodies increased with age, while HPV16 E4 antibodies were prevalent especially in children (Muller

et al., 1995). The results of the present study concerning the HPV6 E4 protein antibodies in children are in line with the study of Muller et al.

In the present study, the HPV6 E-protein antibodies increased during the follow-up, and significant correlations between the L1- and E-protein antibodies were observed at the six-month and 12-month follow-up visits. At these timepoints, the maternal antibodies had already vanished, which could implicate that the children had developed their own immune response against HPV6. In the children of this study, the antibody levels for HPV6 L1, E2, E4, E6, and E7 were measured relatively low during the early months of life, which could result from low viral loads (no clinical HPV6 lesions) and transient expression of HPV proteins that is not sufficient enough to induce antibody production reaching the MFI cut-off that was used for seropositivity. Generation of sufficient amounts of HPV antibodies that reach the cut-off limit used may require a continuous expression of HPV early gene products during the primary HPV infection. In the present study, the correlations observed between the HPV6 L1, E2, E4, and E7 proteins were recorded significant at the 12-month follow-up visit. This simultaneous appearance of both L1- and E-protein antibodies may result from the first active production of mature HPV particles by the children, which is then followed by an active seroconversion for both L1- and E-proteins a few months later. The measured differences in the median times to seroconversion varied only by months between the HPV6 E-protein antibodies and the L1-protein antibodies, and the median time range for seroconversion was quite similar between both the L1 and E-protein antibodies, although the number of children seroconverting for HPV6 L1 was higher than the number of children seroconverting for the E-proteins. The production of HPV6 L1- and E-protein antibodies may arise from a newly acquired primary HPV infection or from the reactivation of a latent HPV infection that may have been acquired vertically from the mother.

During a child's first years of life, a new confirmed seroconversion to HPV proteins can be an indicator of an early exposure to HPV infection. In the present study, seroconversion occurred slightly earlier to the HPV6 L1 protein than to the E-proteins in the children. Maternal IgG antibodies were transferred from the mother to the neonate, but these maternal antibodies vanished during the following months. This was then later on followed by an active seroconversion against the HPV6 L1, E2, E4, E6, and E7 proteins in the early childhood as a sign of an acquired HPV infection.

6.3 Second pregnancy and HPV antibody levels (III)

The analyses on the second consecutive pregnancy's effect on HPV antibody levels indicated only slight differences in the HPV6, HPV11, HPV16, HPV18, and HPV45 mean antibody levels between the women who developed a second pregnancy during the follow-up and those without a second pregnancy. To current knowledge, this is the first study evaluating the effect of a second consecutive pregnancy on HPV antibody levels in a longitudinal setting.

Another study investigating HPV antibodies in the women of the FFHPV cohort study showed that HPV seroprevalence was the lowest at the baseline of the study when all the women were pregnant at their third trimester, and HPV seropositivity to both HR-HPV and LR-HPV types was associated with the age at onset of sexual activity, the number of sexual partners by the age of 20 years, the lifetime sexual partners, and the history of genital warts (Syrjänen et al., 2009). In the present study, differences between the women who developed a second consecutive pregnancy and those without a second pregnancy were observed as for their marital status, the number of deliveries, the number of lifetime sexual partners, the history of STDs, and the contraception method used previously. These factors are associated as risk factors or cofactors for HPV infection and therefore they represent potential confounding factors (Bowden et al., 2021; Chelimo et al., 2013; Y. Chen et al., 2021; de Sanjosé et al., 2018). Women with the second pregnancy reported less lifetime sexual partners and a smaller number of previous deliveries than those who did not develop a second pregnancy during the follow-up. In addition, women with the second pregnancy reported more contraception methods used previously than their counterparts without a second pregnancy. Due to varying exposure on these HPV-related cofactors, differences in the background factors might predict lower HPV antibody levels and a higher proportion of seronegative outcomes among the women with the second pregnancy.

In the present study, the mean antibody levels to HPV6, HPV11, HPV18, and HPV45 were observed to some extent lower in the women who developed a second pregnancy, although this was not the case for HPV16 in which the mean antibody levels were measured higher among the women with a second pregnancy than among their counterparts without a second pregnancy. Additionally, individual values for HPV6 at the baseline and for HPV45 at the 12-month follow-up visit were higher among those women who developed a second pregnancy. As for the results in the HPV6 antibody levels between these two groups of women at the baseline of the study, the differences might result from some baseline differences between these two

groups of women and therefore they are not related to the second pregnancy itself that takes place later on. On the contrary to the observed changes in the mean antibody levels for HPV16, the general trend in the mean HPV antibody levels was a slight decrease when comparing the women with the second pregnancy to those without a second pregnancy, although the observed differences were relatively slight. However, it is known that the antibody response that results from a naturally acquired HPV infection varies between individuals, which is one possible factor that might explain these results. Also, there has been some discussion on the stability of naturally acquired HPV antibodies, and some suggest that the stability of HPV antibodies is variable, although HPV IgG antibodies are believed to be relatively stable over time (Antonsson et al., 2010; Beachler et al., 2016).

As for the IgG antibody levels during pregnancy, the total IgG levels been suggested to be lower in general (Abu-Raya et al., 2020). It has been observed that the activation of B lymphocytes seems to continue from becoming pregnant to the postpartum period, which has an effect on the antibody secretion of different immunoglobulin classes (Lima et al., 2019). There are studies that have assessed several IgG subclasses, but their results have been somewhat contradictory as some studies suggest that the IgG1 subclass is stable during pregnancy, while others have observed that its levels increase during pregnancy (Abu-Raya et al., 2020). In addition, there is some data referring that the levels of IgG3 subclass increase during pregnancy, while the levels of IgG2 and IgG4 subclasses remain stable (Abu-Raya et al., 2020; Ziegler et al., 2018). One potential explanation for the lower total IgG antibody levels in pregnant women is the physiological hemodilution that occurs during pregnancy. Other possible explanations that may contribute to the lower IgG antibody levels during pregnancy include the suppression of the CMI, the loss of protein in the urine, the placental transfer of IgG antibodies from mother to fetus, and the hormonal changes of pregnancy (Abu-Raya et al., 2020; Zgura et al., 2015).

Results from an earlier study in the FFHPV cohort showed that the IgG antibody levels were lower at the baseline, and an increase was observed in the IgG levels after pregnancy (Syrjänen et al., 2009). In line with this, one study assessing serological responses to HPV16 E4, E6, and E7 proteins in pregnant women suggested that the antibody response against HPV infection could be reduced during pregnancy (Sethi et al., 1998). In another previous study from the same FFHPV cohort, IgG antibodies to HPV16 L1 were recorded lower during pregnancy, whereas IgA antibodies showed a different pattern (Pirttilä et al., 2022). While interpreting these results between different studies concerning HPV antibody levels in pregnant women, potential differences in the HPV prevalence and the possibility of

methodologically varying serology assays should be taken into consideration. According to one meta-analysis, the overall HPV prevalence in pregnant women varies by the study region, the woman's age, and the HPV genotype, and its results suggest that pregnant women are more susceptible to HPV infection than their nonpregnant counterparts (Liu et al., 2014).

Results from the present study suggest that a second pregnancy does not increase HPV seropositivity. The observed changes in the mean HPV antibody levels may result from differences in the women's background factors or individual variations of the immune system's responsiveness to induce HPV antibody production, and therefore the observed changes are not likely to occur due to the second pregnancy itself. As for other DNA viruses' antibodies and their significance in pregnancy, nearly all human herpesviruses have been shown to infect cells at the fetal-maternal interface without crossing the placental barriers (Linthorst et al., 2023). One study investigated IgG antibody titers to Epstein-Barr virus (EBV) infection in pregnant women, and its results demonstrated that the total IgG antibody levels against EBV declined during late pregnancy (Christian et al., 2012). In addition, its results revealed that latent viral reactivation occurred due to the potential stress-induced immune dysregulative state, and this took place especially in the racial disparities (Christian et al., 2012). As for the herpes simplex viruses (HSVs), the overall seroprevalence for both HSV-1 and HSV-2 is thought to be relatively high in pregnant women, and the presence of HSV IgG antibodies in relation to the timing of viral reactivation is associated with pregnancy and neonatal complications (Andrievskaya et al., 2022). Lastly, as for cytomegalovirus (CMV) infections, pre-existing maternal CMV antibodies may offer protection against congenital CMV infection, but viral reactivation or new maternal infection with another virus strain may lead to fetal infection (Davis et al., 2017; Puhakka et al., 2017).

6.4 Strengths, limitations, and future aspects

In order to assess the present study's results, one must take into consideration its strengths and limitations. One of this study's major advantages is its longitudinal setting, that allowed evaluating T lymphocyte immunophenotypic subsets in relation to the mother's genital and oral HPV16 infection status over time. The participants of this study were carefully followed up for six years and their HPV infection statuses were carefully monitored during that time. Previous studies on peripheral blood T lymphocytes in relation to HPV infection in a longitudinal setting in mothers and

children are lacking, and to current knowledge, this was the first study evaluating peripheral blood lymphocyte immunophenotypic subsets among mothers with persistent HPV16 infection and their children. However, the venous blood samples of the participants were collected only once during the follow-up time, therefore possible changes in the T lymphocyte immunophenotypic subsets on different timepoints cannot be analyzed from this data. Moreover, the temporal relationship between the collection of the PBMCs and the acquisition of an HPV infection is not known, and there is no reliable way to clarify the timing of first exposure to HPV. One of this T lymphocyte immunophenotyping study's major limitations is the restricted study size of only 42 mothers and 28 of their children, the latter being even less than the first as some of the children were lost to follow-up. When these mothers and their children were classified into smaller subgroups, the number of participants in each group limits reaching statistical power. Furthermore, multiple comparisons may produce false-positive findings, and the need to control for multiple comparisons should be taken into consideration. Lastly, it is not known whether a persistent HPV infection causes activation of the different T lymphocyte subsets or whether the changes in the activated T lymphocyte subsets cause persistent nature of an HPV infection.

The longitudinal design of the present study also allowed to assess HPV seroconversion to HPV6 early and late proteins in the children in their early infancy and to assess the effects of a second pregnancy on HPV6, HPV11, HPV16, HPV18, and HPV45 serology. The longitudinal perspective is useful in order to gather new information as many previous studies on HPV serology have had a cross-sectional design. However, as for the women's HPV serostatuses and antibody levels to HPV types 6, 11, 16, 18, and 45, these data were measured at four different timepoints (at the baseline and at the 12-, 24-, and 36-month timepoints), and it should be noted that the possible impact of the actual gestational length of the second pregnancy at each timepoint was not taken into consideration, which should be noticed as one potential factor affecting the results.

As generally accepted, all HPV serology studies suffer from a few basic handicaps. Firstly, not all natural HPV infections induce seroconversion, not even in the case of persistent HPV infections (Beachler et al., 2016). Secondly, there is no gold standard method for analyzing HPV antibodies, and varying HPV serology assays between different studies limit their comparison. Thirdly, there has been some discussion on the stability of naturally acquired HPV antibodies, and some suggest that the stability of HPV antibodies could be variable, although HPV IgG antibodies are believed to be relatively stable over time (Antonsson et al., 2010; Beachler et al.,

2016). Also, it should be noticed that the production of HPV antibodies is of different magnitude when it results from a naturally acquired HPV infection as compared to the HPV antibody production induced by an HPV vaccination (Prabhu et al., 2022). In the present study, the multiplex serology assay was used, which is useful for assessing cumulative HPV infection, although it is not equally reliable in assessing immune protection as it does not differentiate between neutralizing and non-neutralizing antibodies (Robbins et al., 2014). Therefore, the role of the HPV antibodies investigated in this study in protecting against future HPV infection is uncertain.

In the future, the results concerning the T lymphocyte immunophenotypic subsets in the mothers and their children should be analyzed within a larger study size, and preferably with using similar grouping in order to be able to compare the results between these studies. If the impact of the mother's persistent HPV infection on her offspring's T lymphocyte immunophenotypic subsets is confirmed, more data is needed on what is the significance of this phenomenon. One may be speculative that if that is the case, it should be further evaluated whether such an exposure to maternal HPV should be taken into consideration in the HPV vaccination programmes of young children. As for the results concerning HPV serology in mothers and their children, more research is needed to determine what is the immunoprotective effect of the naturally acquired HPV antibodies. In addition, as for these young children, further long-term follow-up studies are needed to assess what is the impact of an early exposure to HPV in the long run, especially regarding an infection with the HR-HPV types, and what preventive efforts should be further taken into consideration in the future.

7 CONCLUSIONS

The main conclusions of this study are the following:

1. Both genital and oral persistent HPV16 infections are associated with alterations in the distribution of peripheral blood T lymphocyte immunophenotypic subsets in mothers.

2. Alterations in T lymphocyte immunophenotypic subsets were observed only in those children whose mother had the persistent oral HPV16 infection, which could indicate a continuous exposure to maternal oral HPV resulting in T lymphocyte activation in the children.

3. Maternal and neonatal HPV6 antibodies correlated well indicating maternal antibodies against HPV6 early and late proteins are transferred to her offspring.

4. After maternal antibodies have decayed, seroconversion against HPV6 L1, E2, E4, E6, and E7 does occur in early childhood, which could be a sign of acquired HPV6 infection by vertical or horizontal transmission.

5. Second pregnancy does not seem to have a major impact on HPV antibody levels. Seropositivity for HPV6, HPV11, HPV16, HPV18, and HPV45 was generally less common in those women who had a second pregnancy as compared to those who did not develop a second pregnancy.

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9 PUBLICATIONS

PUBLICATION I

Peripheral Blood T-lymphocyte Phenotypes in Mother-Child Pairs Stratified by the Maternal HPV Status: Persistent HPV16 vs. HPV-negative: A Case-Control Study

Helmi Suominen, Anna Paaso, Hanna-Mari Koskimaa, Seija Grénman, Kari Syrjänen, Stina Syrjänen, Karolina Louvanto

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Article

Peripheral Blood T-lymphocyte Phenotypes in Mother-Child Pairs Stratified by the Maternal HPV Status: Persistent HPV16 vs. HPV-Negative: A Case-Control Study

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Abstract: Only few studies exist on the phenotype distribution of peripheral blood lymphocytes concerning persistent oral HPV infection. T-lymphocyte subsets were phenotyped in women who had persistent genital or oral HPV16 infection, using HPV-negative women as a reference group. A subset of 42 mothers and their children (n = 28), were stratified into two groups according to the mothers' HPV status. PBMCs from previously cryopreserved venous samples were immunophenotyped by flow cytometry. Proportions of the $CD4^+$ or $CD8^+$ lymphocytes by their immunophenotype subsets were compared between HPV-positive and -negative mothers and their children. The mean rank distribution of $CD8^+$ memory cells was significantly higher among mothers with persistent genital HPV16 infection. The median levels of both the antigen-presenting $CD4^+$ cells and activated $CD8^+$ cells were significantly lower in mothers with persistent oral HPV16 infection. When oral and genital HPV16-persistors were analyzed as a group, a marker of terminal effector cells was significantly increased as compared to HPV-negative women. Significantly higher levels of activated $CD4^+$, $CD8^+$ and circulating $CD8^+$ memory cells were found among children whose mothers had persistent oral HPV16 infection. Persistent HPV16 infections are associated with changes in peripheral blood T-lymphocyte subsets. The mother's persistent oral HPV16 infection possibly results in immune alterations in her offspring.

Keywords: human papillomavirus; HPV; T-lymphocyte; immunophenotyping; mother; child; oral infection; genital infection

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1. Introduction

Human papillomavirus (HPV) can be acquired by nonsexual or sexual transmission [1–6], resulting in acute, latent, or chronic infection of the epithelial cells. Persistent HPV infections are known to be the main risk factor for the malignant transformation of the epithelial cells resulting in cervical lesions. Typically, most of these HPV-induced lesions regress spontaneously, by mechanisms that are still incompletely elucidated. Persistent HPV infections in the female genital tract can cause genital warts and squamous intraepithelial lesions (SILs) [7]. Certain HPVs are defined as high-risk (HR) types because of their high propensity to cause persistent infections, high-grade SIL (HSIL) and carcinoma [8]. In addition to HR-HPV, interactions of viral and host-related factors are needed in this carcinogenetic process [9].

The host's immune system is presumed to be a fundamental determinant of the outcome of HPV infection [10]. The adaptive immune system plays an important part in the immune defense against HPV infection along with the innate immune system. The former consists of B-lymphocytes which are mainly responsible for the antibody-mediated immune response and T-lymphocytes which are responsible for the cell-mediated immune response (CMI). When effective, an adaptive immune system contributes to the regression of a persistent HPV infection, whereas a failure to develop sufficient CMI can result in the persistence and progression of an HPV infection [11–15].

Fortunately, most HPV-infected individuals can clear their infection by an efficient CMI response, in which $CD4^+$ T cells are of key importance. Previous studies have shown $CD4^+$ T-cell-mediated immune response decreases in subjects who develop cancer precursor lesions or cancer due to a persistent HPV infection [11,16,17]. $CD4^+$ T cells participate in initiating and maintaining the immune response, whereas $CD8^+$ T-cell function as cytotoxic effectors. However, the role of $CD8^+$ lymphocytes in HPV infection is less clear than that of $CD4^+$ lymphocytes [18]. Regulatory T-lymphocytes (T_{regs}) express $CD4^+$ and $CD25^+$ and because T_{regs} function in suppressing the immune system, these cells are believed to be involved in the progression of persistent HPV infections [19,20]. Compromised $CD8^+$ T-cell function has been associated with chronic viral infections, due to the expression of several inhibitory receptors [21,22].

According to current understanding, the CMI system appears to play an important role in the progression or regression of an HPV infection. Immunophenotyping of peripheral blood T cells could cast further light on understanding the outcomes (persistence, clearance) of HPV infections even without any clinical HPV lesions. The prospective Finnish Family HPV Study (FFHPV) cohort was used to make comprehensive immunophenotyping of peripheral blood T-lymphocyte subsets in mothers with persistent HPV16 infection, accompanied by similar analyses of their children.

2. Materials and Methods

2.1. Study Cohort

The Finnish Family HPV (FFHPV) study is a longitudinal cohort study that was designed to clarify the dynamics of HPV infections within families. Since its onset in 1998, the study has been conducted jointly by the Department of Obstetrics and Gynecology, Turku University Hospital and the Institute of Dentistry, Faculty of Medicine, University of Turku. The original study protocol and its amendments were approved by the Research Ethics Committee of Turku University Hospital (#3/1998, #2/2006 and 45/180/2010).

Altogether, 329 pregnant women in their third trimester and their newborns were enrolled in the study between 1998 and 2001, based on written informed consent of all participants. The study design and the key characteristics of the participants have been detailed in a series of previous reports [19,23–25]. In the present analysis, a subset of mother–child pairs (42 mothers and 28 children) was used, selected from the families specially followed-up for HPV16-specific (CMI) responses, as previously described [23,26].

2.2. Samples

Genital and oral scraping samples for HPV genotyping were taken with a cytobrush at the baseline and during the follow-up visits: at day 3 (before leaving the hospital) and at 1-, 2-, 6-, 12-, 24-, 36-month- and 6-year follow-up visits. The samples were fixed and immediately frozen and stored at −70 °C. HPV DNA was extracted by using the high salt method, as previously described [1]. HPV genotyping was performed by using the Multimetrix Kit (Progen Biotechnik GmbH), which detected 24 low-risk (LR-HPV) and high-risk (HR-HPV) HPV genotypes (HPV6, 11, 42, 43, 44, 70, 26, 53, 66, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82 [27]).

2.3. Flow Cytometric Analysis

Peripheral blood mononuclear cells (PBMCs) from previously cryopreserved venous samples of the mothers and their children were used. The participants were recalled for blood sampling, the mean age of the children being 12.2 years [19]. A detailed description of blood sample collection and processing has been published previously [19,23–26,28]. PBMCs were stained in 96-well U-bottom plates in the concentration of 200,000–500,000 cells/well. The plate was centrifuged for 5 min with 450 g at 4 °C followed by a washing step with FACS buffer (PBS/0.5% BSA/2 mM EDTA) and centrifuged again. After the supernatants were removed, the diluted antibody mix (Table 1) was added to samples (50 µL/well) and incubated for 30 min (dark, 4 °C). After washing again with FACS buffer, the supernatant was removed and resuspended to 5 µL of CellFix (BD Bioscience). Samples were analyzed within 2 h in a BD 4-lasers LSR Fortessa™ cell analyzer (BD Bioscience, NJ, USA) and gating was performed with Flow-Jo Software (BD Bioscience, New York, NJ, USA).

Table 1. Antibodies used for immunological phenotyping of peripheral blood mononuclear cells by flow cytometry.

Cell Marker	Dilution	Fluorochrome	Antibody Clone	Source
CD3	1/15	APC-Cy7	SK7	Biolegend
CD4	1/15	PerCP-Cy5.5	OKT4	Biolegend
CD8	1/15	FITC	SK1	Biolegend
CD25	1/15	Alexa 700	M-A251	BD Bioscience
CD27	1/15	APC	L128	BD Bioscience
CD45RA	1/15	BV50/BV510	HI100	BD Bioscience
CD45RO	1/15	PE	UCHL1	Biolegend
CD57	1/33	PE Dazzle	HNK-1	Biolegend
CD38	1/15	BV605	HB7	BD Bioscience
CD69	1/15	BV421	FN50	BD Bioscience

T-cell subpopulations were defined by the presence or absence of different cell surface markers to (i) either early or late activated cells, (ii) memory or naïve cells, as well as (iii) differentiated or undifferentiated cells, closely following the protocol reported by Rodriguez et al. [16]. The T-cell subpopulations in this study were defined as follows: (1) Early-activated T cells: $CD4^+CD69^+$ and $CD8^+CD69^+$; (2) Late-activated T cells: $CD4^+CD25^+$, $CD8^+CD25^+$, $CD4^+HLA\text{-}DR^+$, $CD8^+HLA\text{-}DR^+$, $CD4^+CD38^+$ and $CD8^+CD38^+$; (3) Memory T-cells: $CD45RO^+CD45RA^-$; (4) Naïve T cells: $CD45RO^-CD45RA^+$; (5) Central memory ($CD45RO^+CCR7^+$) and -naïve cells ($CD45RA^+CCR7^+$); (6) Effector-memory ($CD45RO^+CCR7^-$) and-naïve cells ($CD45RA^+CCR7^-$); (7) Resting memory cells and differentiated naïve cells: $CD27^+CD45RO^+$ and $CD27^+CD45RA^+$; (8) Differentiated memory cells and undifferentiated naïve cells: $CD57^+CD45RO^+$ and $CD57^+CD45RA^+$.

CD69 markers in $CD4^+$ and $CD8^+$ T cells are reported as percentages of the total number of lymphocytes, the lymphocyte population being selected based on the forward scatter/side scatter pattern. The CD45RO, CD45RA, CD27 and CD57 markers are reported as percentages of $CD3^+CD4^+$ or $CD3^+CD8^+$ lymphocytes. HLA-DR and CD38 marker populations were estimated as a percentage of the $CD3^+CD4^+$ or $CD3^+CD8^+$lymphocytes. The CD57 subpopulation was estimated as a percentage of $CD3^+CD4^+$ or $CD3^+CD8^+$ lymphocytes that were $CD27^+CD45RA^+$ or $CD27^+CD45RO^+$.

2.4. Statistical Analyses

To be eligible for the present analysis, the mother had persistent HPV16 infection either in the genital or oral mucosa. As a reference group, the mothers who tested constantly HPV-

negative during the follow-up time were selected as described earlier [23,26]. In addition to the mothers in these two groups, the children were subjected to similar analyses. Altogether, a group of 42 mothers and their children (n = 28) was divided into four subgroups based on the mothers' HPV infection status: Group (1) the first group included 10 mothers, who developed an incident CIN with persistent genital HPV16 infection during the follow-up and 10 children of these mothers; Group (2) the second group consisted of 7 mothers with persistent oral HPV16 infection and their 7 children. Persistent HPV16 infection (genital or oral) was defined by testing HPV16 positive at least in two (or more) consecutive follow-up visits. As to the reference groups, Group (3), consisted of 20 mothers who tested genital-HPV-negative during the follow-up and 8 children of these mothers; Group (4) consisted of 5 mothers who tested repeatedly oral-HPV-negative and their 3 children. To be eligible in these reference groups, the mother had to be always HPV-negative, with no HPV-positive genital or oral sample during the follow-up time.

In statistical analysis, the two HPV16-positive groups (oral and genital) were combined as the group of HPV16 carriers, to be compared with the combined group of always HPV-negatives. Between all the groups above, differences in the mothers' mean age, follow-up time, gender of the children as well as oral HPV status of the children were assessed. The proportion of the $CD4^+$ or $CD8^+$ lymphocytes by their immunophenotype subsets were compared between the groups described above; first between the mothers, secondly between the children, and across the mother–child pairs. Bonferroni post hoc tests were used to control for multiple comparisons. Bonferroni uses t-tests to perform pairwise comparisons between group means but controls the overall error rate by setting the error rate for each test to the experiment-wise error rate divided by the total number of tests. Hence, the observed significance level is adjusted for the fact that multiple comparisons are being made. In null hypothesis testing by this post hoc test we used the same significance level (alpha) as the settings in options. All statistical analyses were performed by using SPSS statistical software (25.0). All tests were performed two-sided, and statistical significance was declared at the p-value < 0.05.

3. Results

The baseline characteristics of the subgroups of the 42 mothers and their children (n = 28) included in the present analysis are shown in Table 2. The mothers' mean age ranged between 37 and 40 years and their children's mean age ranged between 12.2 and 14.7 years. From the 28 children included in this study, a total of 17 children had an oral HPV infection recorded during the follow-up, of which three had a persistent oral HPV infection.

Table 2. Baseline characteristics of the subgroups of mothers (n = 42) and their children (n = 28) from the Finnish Family HPV cohort.

		Genital HPV16 Infection		**Oral HPV16 Infection**	
		Incident ≥ CIN+ with Persistent * Infection	**Always Negative**	**Persistent * Infection**	**Always Negative**
Mothers	*N*	10	20	7	5
Children	*N*	10	8	7	3
Mean age	Mothers	37.0	40.0	38.7	38.7
	Children	12.2	12.3	14.7	14.7
Gender of the children	Girls	3	4	4	2
	Boys	7	4	3	1
Oral HPV status of the children	Always negative	5	3	3	0
	Incident	1	3	4	2
	Persistent	2	0	0	1
	Fluctuation	1	2	0	0
	Clearance	1	0	0	0
	Incident				
	Persistent				

* Persistent is defined as two or more consequent follow-up visits HPV16 positive. Abbreviations: CIN: cervical intraepithelial neoplasia.

Tables 3 and 4 summarize the results of the $CD4^+$ and $CD8^+$ immunophenotypic subgroups stratified by the mother–child dyads, using 23 and 25 $CD4^+$ and $CD8^+$ T-cell surface markers, respectively. The highest proportion of T-lymphocytes ($CD3^+$ T cells; presented as median percentages of cells among PBMCs) was seen in the mothers having persistent genital HPV16 infection (73.0%), while the lowest median proportion (47.0%) was detected in the mothers with persistent oral HPV infection (Table 3). T-lymphocyte counts were significantly different only between the mothers with persistent genital HPV16 infection and their HPV-negative controls (p = 0.019). In children, the distribution of $CD3^+$ T cells was similar across the three comparison groups. No statistically significant differences were found in the distribution of the $CD4^+$ (helper cells) and $CD8^+$ (cytotoxic) T cells within the $CD3^+$ T cells between HPV^+ or HPV^- mothers or the respective groups of children (Tables 3 and 4).

Table 3. CD4+ T-cell immunophenotypic subset distribution of the mother–child pairs stratified according to mother's genital and oral HPV status. Significant median comparisons between the subgroups are bolded.

		HPV16 Infection Status of the Mother					
		Genital HPV16 Infection		**Oral HPV16 Infection**		**Combined HPV16 Infection**	
		Persistent * Infection	**Always HPV Negative**	**Persistent * Infection**	**Always HPV Negative**	**Persistent * Infection**	**Always HPV Negative**
Marker		Median (%)		Median (%)		Median (%)	
$CD3^+$ lymphocytes	Mothers	**73.00** [a]	**54.65** [a]	47.10	60.10	58.30	54.70
	Children	72.65	67.40	54.50	67.80	67.10	67.80
$CD3^+CD4^+$	Mothers	47.50	51.45	36.60	46.90	44.20	51.10
	Children	40.15	36.90	38.20	41.30	39.50	37.10
$CD69^+CD4^+$	Mothers	1.89	1.54	1.54	0.58	1.72	1.39
	Children	0.39	0.41	0.40	0.50	0.40	0.49
$CD25^+CD4^+$	Mothers	0.13	0.15	0.10	0.10	0.11	0.14
	Children	0.25	0.25	0.27	0.25	0.25	0.25
$CD27^+CD4^+$	Mothers	88.90	90.25	82.30	86.00	88.15	89.90
	Children	92.30	89.00	92.30	95.10	92.30	93.40
$HLADR^+CD3^+CD4^+$	Mothers	4.92	4.69	**4.27** [b]	**6.28** [b]	4.45	4.73
	Children	3.06	3.53	3.92	1.81	3.60	2.92
$CD38^+CD3^+CD4^+$	Mothers	53.00	49.65	49.30	34.50	51.25	48.70
	Children	65.70	67.10	63.00	68.80	65.30	67.20
$CD38^+HLADR^+CD3^+CD4^+$	Mothers	2.59	2.74	2.71	3.63	2.70	2.90
	Children	2.12	2.71	**2.70** [c]	**1.06** [c]	2.37	1.80
$CD38^-HLADR^+CD3^+CD4^+$	Mothers	2.33	2.42	1.91	4.29	2.07	2.45
	Children	1.54	1.26	1.46	0.72	1.47	1.11
$CD45RA^+CD4^+$	Mothers	65.20	58.95	59.90	46.00	63.30	58.00
	Children	71.60	72.25	68.10	70.50	69.20	70.50
$CD45RA^+CD27^+CD4^+$	Mothers	93.60	92.50	88.30	95.60	92.30	92.70
	Children	96.50	94.55	97.20	99.10	96.90	97.60

Table 3. *Cont.*

		HPV16 Infection Status of the Mother					
		Genital HPV16 Infection		Oral HPV16 Infection		Combined HPV16 Infection	
		Persistent * Infection	Always HPV Negative	Persistent * Infection	Always HPV Negative	Persistent * Infection	Always HPV Negative
Marker		Median (%)		Median (%)		Median (%)	
$CD45RA^+CD27^-CD4^+$	Mothers	6.37	7.35	11.30	4.37	7.66	7.05
	Children	3.47	5.42	2.73	0.88	3.04	2.30
$CD45RA^+CD57^+CD4^+$	Mothers	9.18	5.35	5.28	2.37	8.01	5.00
	Children	1.97	4.76	0.96	0.73	1.79	1.69
$CD45RA^+CD57^-CD4^+$	Mothers	90.80	94.60	94.70	97.60	92.00	94.90
	Children	98.00	95.25	99.00	99.30	98.20	98.30
$CD45RA^+CD57^+CD27^+CD4^{+3}$	Mothers	2.51	0.79	1.35	0.72	1.68	0.78
	Children	0.80	0.86	1.02	0.82	0.91	0.82
$CD45RA^+CD57^-CD27^+CD4^{+3}$	Mothers	86.50	91.25	87.90	94.10	87.00	91.30
	Children	95.85	93.35	96.00	98.30	96.00	96.70
$CD45RO^+CD4^+$ memory	Mothers	41.80	52.15	51.10	63.80	45.25	54.40
	Children	40.10	37.35	36.50	36.80	38.50	37.30
$CD45RO^+CD27^+CD4^+$	Mothers	80.40	83.60	76.30	79.50	77.80	82.50
	Children	79.25	74.75	80.10	89.40	79.50	83.00
$CD45RO^+CD27^-$	Mothers	19.60	16.40	23.70	20.50	22.20	17.50
	Children	20.75	25.25	19.90	10.60	20.50	17.00
$CD45RO^+CD57^+CD4^+$	Mothers	13.70	6.71	11.20	4.59	12.45	6.56
	Children	6.46	12.42	5.70	4.06	6.16	5.32
$CD45RO^+CD57^-CD4^+$	Mothers	86.30	93.30	88.80	95.40	87.55	93.40
	Children	93.55	87.60	94.30	95.90	93.80	94.70
$CD45RO^+CD57^+CD27^+CD4^+$	Mothers	3.01	2.30	2.23	2.39	2.72	2.37
	Children	3.75	2.92	4.29	3.21	3.96	3.21
$CD45RO^+CD57^-CD27^+CD4^+$	Mothers	77.20	79.55	75.00	78.10	76.00	78.10
	Children	77.10	69.50	77.20	87.90	77.20	79.80

* Mothers developed Incident ≥ CIN+ during the follow-up. *p*-values = [a] 0.019, [b] 0.038, [c] 0.038.

Among mothers, only one significant difference between HPV16 carriers and HPV-negative women was seen in the frequency of immunophenotypic subsets of $CD3^+CD4^+$ T cells; the level of $HLADR^+CD3^+CD4^+$ (a marker for T-cell activation) was lower in the mothers with persistent oral HPV16 (p = 0.038). The same was true with the children: children of the mothers with persistent oral HPV16 infection had higher levels of $CD38^+HLADR^+CD3^+CD4^+$ cells; a marker of T-cell activation (p = 0.038).

When the immunophenotypic subsets of $CD3^+CD8^+$ cells were compared (Table 4), the levels of $HLADR^+CD3^+CD8^+$, $CD38^+HLADR^+CD3^+CD8^+$ and $CD38^-HLADR^+CD3^+CD8^+$ subsets were significantly higher in children of the mothers having persistent oral HPV16 infection as compared with the HPV-negative counterparts; p = 0.006, p = 0.008 and p = 0.018, respectively. In addition, the $CD45RO^+CD8^+$ subset (a marker of memory T cells), was significantly higher among the children of the mothers with persistent oral HPV16 infection as compared with the HPV-negative counterparts.

Table 4. $CD8^+$ T-cell immunophenotypic subset distribution of the mother–child pairs stratified according to mother's genital and oral HPV status. Significant median comparisons between the subgroups are bolded.

		HPV16 Infection Status of the Mother					
		Genital HPV16 Infection		**Oral HPV16 Infection**		**Combined HPV16 Infection**	
		Persistent Infection *	**Always Negative**	**Persistent infection ***	**Always Negative**	**Persistent Infection ***	**Always Negative**
Marker		**Median (%)**		**Median (%)**		**Median (%)**	
$CD3^+$ lymphocytes	Mothers	**73.00** [a]	**54.65** [a]	47.10	60.10	58.30	54.70
	Children	72.65	67.40	54.50	67.80	67.10	67.80
$CD3^+CD8^+$	Mothers	22.10	18.35	24.40	25.70	22.10	19.80
	Children	25.15	25.30	20.50	20.50	22.20	20.50
$CD69^+CD8^+$	Mothers	1.62	1.83	1.02	3.22	1.46	1.87
	Children	0.76	0.77	0.92	0.93	0.83	0.93
$CD25^+CD8^+$	Mothers	0.06	0.15	0.085	0.33	0.08	0.19
	Children	0.08	0.22	0.12	0.13	0.09	0.17
$CD27^+CD8^+$	Mothers	73.30	70.15	49.50	68.60	64.85	70.00
	Children	84.65	74.05	86.90	93.90	86.50	85.30
$HLADR^+CD3^+CD8^+$	Mothers	5.65	8.35	6.41	7.74	6.03	8.10
	Children	6.01	7.44	**10.30** [b]	**3.80** [b]	8.41	5.36
$CD38^+CD3^+CD8^+$	Mothers	37.80	40.65	36.30	37.50	36.80	37.50
	Children	42.65	52.45	50.50	57.90	49.90	53.50
$CD38^+HLADR^+CD3^+CD8^+$	Mothers	2.99	4.42	3.94	5.59	3.27	5.03
	Children	2.92	4.09	**5.63** [c]	**2.91** [c]	5.07	3.46
$CD38^-HLADR^+CD3^+CD8^+$	Mothers	3.40	3.53	3.37	2.80	3.39	3.35
	Children	4.05	3.56	**4.38** [d]	**1.20** [d]	4.38	2.32
$CD45RA^+CD8^+$	Mothers	79.20	77.55	80.40	73.20	79.80	77.50
	Children	85.35	90.95	87.80	91.30	86.80	91.30
$CD45RA^+CCR7^-CD8^+$	Mothers	93.80	92.70	95.90	93.90	93.80	93.40
	Children	94.75	96.70	92.90	92.90	94.20	96.60
$CD45RA^+CD27^+CD8^+$	Mothers	67.70	68.55	42.30	64.20	58.60	68.20
	Children	82.90	73.50	86.90	94.00	86.30	84.00
$CD45RA^+CD27^-CD8^+$	Mothers	31.30	30.50	57.30	34.90	41.10	30.50
	Children	17.10	26.40	13.00	5.89	13.50	16.00
$CD45RA^+CD57^+CD8^+$	Mothers	43.60	36.30	53.20	37.20	46.35	36.90
	Children	20.90	30.45	25.90	11.40	25.10	23.10
$CD45RA^+CD57^+CD27^+CD8^+$	Mothers	14.20	10.01	6.30	7.60	11.75	9.72
	Children	9.38	8.53	8.03	7.18	8.03	7.18
$CD45RA^+CD57^-CD8^+$	Mothers	56.30	63.50	46.70	62.70	53.55	62.70
	Children	72.20	69.50	74.10	88.60	74.10	76.90

Table 4. *Cont.*

		HPV16 Infection Status of the Mother					
		Genital HPV16 Infection		Oral HPV16 Infection		Combined HPV16 Infection	
		Persistent Infection *	Always Negative	Persistent infection *	Always Negative	Persistent Infection *	Always Negative
Marker		**Median (%)**		**Median (%)**		**Median (%)**	
$CD45RA^+CD57^-CD27^+CD8^+$	Mothers	46.50	56.55	36.60	55.30	44.95	56.40
	Children	69.15	65.35	71.90	86.50	71.70	71.70
$CD45RO^+CD8^+$	Mothers	50.20	54.50	46.20	49.60	48.15	49.60
	Children	43.30	38.45	**33.20** [e]	**23.00** [e]	35.10	32.00
$CD45RO^+CCR7^+CD8^+$	Mothers	8.69	12.65	9.90	12.00	9.30	12.00
	Children	6.05	7.17	8.72	6.28	7.29	6.54
$CD45RO^+CCR7^-CD8^+$	Mothers	91.30	87.35	90.10	88.00	90.70	88.00
	Children	93.95	92.85	91.30	93.70	92.70	93.50
$CD45RO^+CD27^+CD8^+$	Mothers	63.80	61.75	48.80	65.90	60.30	63.00
	Children	71.80	60.45	77.00	80.40	73.50	71.10
$CD45RO^+CD27^-CD8^+$	Mothers	36.20	38.25	51.20	34.10	39.70	37.00
	Children	28.20	39.55	23.00	19.60	26.50	28.90
$CD45RO^+CD57^+CD8^+$	Mothers	50.40	42.20	50.60	35.00	50.50	41.70
	Children	43.25	60.20	45.80	35.70	44.50	48.30
$CD45RO^+CD57^-CD8^+$	Mothers	49.60	56.00	49.40	65.00	49.50	57.30
	Children	56.75	39.80	54.20	64.30	55.50	51.70
$CD45RO^+CD57^+CD27^+CD8^+$	Mothers	8.21	4.40	4.09	2.32	7.11	4.18
	Children	3.34	2.65	3.13	3.87	3.21	2.96
$CDRO^+CD57^-CD27^-CD8^+$	Mothers	4.97	6.12	9.64	13.20	8.93	7.72
	Children	2.23	3.80	2.60	2.85	2.51	3.25

* Mothers developed Incident ≥ CIN+ during the follow-up. *p*-values = [a] 0.019, [b] 0.006, [c] 0.008, [d] 0.018, [e] 0.033.

Supplemented statistical analysis was also further performed for the control of multiple comparisons by using the classical Bonferroni test (BT). When BT was used, the three *p*-values that had been declared significant in Table 3, underwent the following changes: (1) $CD3^+$ lymphocytes: Mothers persistent HPV16 infection vs. always HPV-negative, original $p = 0.019$, changed after BT to $p = 0.084$; (2) $HLADR^+CD3^+CD8^+$: Mothers oral persistent HPV16 infection vs. always HPV-negative, original $p = 0.038$, changed after BT to $p = 0.668$; (3) $CD38^+HLADR^+CD3^+CD4^+$: Children of mothers with oral persistent HPV16 vs. always HPV-negative, original $p = 0.038$, changed after BT to $p = 1.000$. When BT was used in Table 4 five *p*-values that were declared significant underwent the following changes: (1) $CD3^+$ lymphocytes: Mothers persistent genital HPV16 infection vs. always HPV-negative, original $p = 0.019$, changed after BT to $p = 0.084$; (2) $HLADR^+CD3^+CD8^+$: Children of mothers with persistent oral HPV16 infection vs. always HPV-negative, original $p = 0.006$, changed after BT to $p = 1.000$; (3) $CD38^+HLADR^+CD3^+CD8^+$: Children of mothers with persistent oral HPV16 infection vs. always HPV-negative, original $p = 0.008$, changed after BT to $p = 1.000$; (4) $CD38^-HLADR^+CD3^+CD8^+$: Children of mothers with persistent oral HPV16 infection vs. always HPV-negative, original $p = 0.018$, changed after BT to $p = 1.000$; and (5) $CD45RO^+CD8^+$: Children of mothers with persistent oral HPV16 infection vs. always HPV-negative, original $p = 0.033$, changed after BT to $p = 1.000$.

Table 5 depicts the immunophenotypic $CD4^+$ and $CD8^+$ T-cell subsets stratified by the defined groups. This was conducted because the number of mothers defined by their HPV status was limited. Of the immunophenotypic subsets of $CD3^+CD4^+$ T cells, only the $HLADR^+CD3^+CD4^+$ T-cell subset was significantly lower ($p = 0.038$) in mothers with persistent oral HPV16 infection than in HPV-negative mothers.

Table 5. The proportion of $CD4^+$ or $CD8^+$ lymphocytes by their immunophenotypic subsets * given as mean (±SD) percentages among the mothers with persistent genital or oral HPV16 HPV-negative mothers. Only lymphocyte subsets with statistically significant differences are given, results are bolded.

	Genital HPV16 Infection		**Oral HPV16 Infection**		**Combined HPV16 Infection**	
	Persistent Infection (*n* = 10)	**Always Negative (*n* = 20)**	**Persistent Infection (*n* = 7)**	**Always Negative (*n* = 5)**	**Persistent Infection (*n* = 17)**	**Always Negative (*n* = 25)**
	Mean (±SD)		**Mean (±SD)**		**Mean (±SD)**	
Lymphocytes ($CD3^+$)	69.03 (14.17)	56.76 (11.40)	48.17 (7.35)	60.26 (14.26)	59.91 (15.59)	57.46 (11.78)
$CD4^+$ cell population						
$HLADR^+CD3^+$ **	5.67 (3.33)	5.17 (2.47)	**4.07 [a] (1.53)**	**6.60 [a] (2.15)**	4.97 (2.74)	5.46 (2.44)
$CD8^+$ cell population						
$CD45RO^+CCR7^-$	**90.19 [b] (4.97)**	**80.56 [b] (13.40)**	88.47 (7.48)	88.12 (2.62)	**89.44 [c] (6.03)**	**82.07 [c] (12.36)**
$CD38^+HLADR^+CD3$ ***	3.92 (2.44)	5.40 (4.38)	**3.67 [d] (2.18)**	**7.70 [d] (3.61)**	3.81 (2.26)	5.86 (4.27)
$CD45RA^+CCR7^-$	92.96 (3.18)	90.21 (5.63)	95.37 (2.15)	94.14 (0.55)	**94.01 [e] (2.97)**	**91.00 [e] (5.26)**

p-values = [a] 0.038, [b] 0.048, [c] 0.033, [d] 0.036, [e] 0.044. * Percentages of $CD4^+$ and $CD8^+$ T-cell subpopulations. PBMCs were analyzed by flow cytometry by first gating on the total PBMCs and then on the $CD3^+$ T cells. ** Markers expressed as percentages of total CD3 positive CD4 lymphocytes. *** Markers expressed as percentages of total CD3 positive CD8 lymphocytes.

Of the immunophenotypic subsets of $CD3^+CD8^+$ T cells, only one statistically significant difference was found: $CD45RO^+CCR7^-CD8^+$ cell population (a marker of memory cells), was significantly more abundant among mothers with persistent genital HPV16 infection than in HPV-negative women ($p = 0.048$). This difference to HPV-negative mothers remained significant also when the oral- and genital HPV16-positive mothers were pooled together, ($p = 0.033$).

Among the mothers with persistent oral HPV16 infection, $CD38^+HLADR^+CD3^+CD8^+$ cells (a marker of activated T cells) were significantly lower (mean 3.67 ± SD 2.18) than in HPV-negative mothers (mean 7.70 ± SD3.61; $p = 0.036$). When the oral and genital HPV16 persisters were pooled, the $CD45RA^+CCR7^-CD8^+$ cell population (a marker of terminal effector cells), was significantly increased in HPV16-positive mothers as compared with their HPV-negative mothers ($p = 0.033$).

Finally, in children, significant differences in the levels of $CD4^+$ and $CD8^+$ T-cell subsets were recorded only between those in the oral (but not genital) HPV16-persistor group and their control group (i.e., children born to mothers who always tested HPV-negative in their oral samples) as seen in Table 6. $HLADR^+CD3^+CD4^+$ $CD8^+$ cells (a marker of both $CD4^+$ and $CD8^+$ activated T cells; MHC II^+ T cells) were significantly higher in children whose mothers had persistent oral HPV16 infection ($p = 0.018$). Similarly, a significant increase in the number of activated T cells was found both for $CD4^+$ helper cells ($CD38^+HLADR^+CD3^+CD4^+$) ($p = 0.038$) and $CD8^+$ suppressor cells ($CD38^+HLADR^+CD3^+CD8^+$) ($p = 0.008$). Additionally, the levels of $HLADR^+CD3^+CD8^+$ cells (i.e., activated T-suppressor cells), were significantly higher ($p = 0.005$) as were the levels of $CD45RO^+$ $CD8^+$ cells ($p = 0.033$), i.e., a population of circulating memory cells.

Table 6. Proportion of $CD4^+$ or $CD8^+$ lymphocytes by their immunophenotypic subsets * given as mean (±SD) percentages among children whose mothers had persistent genital or oral HPV16 infection or were always HPV-negative. Only the lymphocyte subsets that had a statistically significant difference between the groups are given, results are bolded.

	Mother's HPV16 Status					
	Genital HPV16 Infection		**Oral HPV16 Infection**		**Combined HPV16 Infection**	
	Persistent * Infection (*n* = 10)	**Always HPV-Negative (*n* = 8)**	**Persistent * Infection (*n* = 7)**	**Always HPV-Negative (*n* = 3)**	**Persistent * Infection (*n* = 17)**	**Always HPV-Negative (*n* = 11)**
	Mean (±SD)		**Mean (±SD)**		**Mean (±SD)**	
Lymphocyte Subsets In Children						
Lymphocytes ($CD3^+$)	67.82 (13.99)	72.49 (13.67)	57.99 (12.77)	65.30 (10.67)	63.77 (14.01)	70.53(12.84)
$CD4^+$ cell population						
$CD38^+HLADR^+CD3^+$ **	4.86 (8.11)	2.65 (1.08)	**3.22 [a] (1.32)**	**1.24 [a] (0.36)**	4.18 (6.19)	2.26 (1.13)
$CD8^+$ cell population						
$HLADR^+CD3^+$	9.45 (9.60)	7.99 (5.12)	**9.79 [b] (2.42)**	**4.17 [b] (1.05)**	9.59 (7.35)	6.95 (4.67)
$CD38^+HLADR^+CD3^+$ ***	6.40 (9.03)	4.46 (2.76)	**6.44 [c] (1.62)**	**3.06 [c] (0.35)**	6.41 (6.85)	4.08 (2.41)
$CD38^-HLADR^+CD3^+CD4^+$	11.92 (25.21)	4.19 (3.00)	**4.25 [d] (1.49)**	**1.52 [d] (0.70)**	8.76 (19.33)	3.46 (2.82)
$CD45RO^+$	40.81 (17.42)	38.59 (8.58)	**34.30 [e] (6.90)**	**23.40 [e] (2.82)**	38.13 (14.12)	34.45(10.17)

p-values: [a] 0.038, [b] 0.005, [c] 0.0080 [d] 0.018, [e] 0.033. * Percentages of $CD4^+$ and $CD8^+$ T-cell subpopulations. PBMCs were analyzed by flow cytometry by first gating on the total PBMCs and then on the $CD3^+$ T cells. ** Markers expressed as percentages of total CD3 positive CD4 lymphocytes. *** Markers expressed as percentages of total CD3 positive CD8 lymphocytes.

4. Discussion

In the present study, we analyzed the distribution of T cells and their subpopulations in the peripheral blood of mothers with either persistent oral or genital HPV16 infection using constantly HPV-negative mothers as the reference group. Similar analyses were also performed in the children of these mothers because immunological recognition of HPV16 seems to occur in early childhood [19,26,28,29].

These analyses demonstrate that the mothers with persistent genital HPV16 infection had higher levels of $CD3^+$ lymphocytes and effector memory $CD8^+$ T cells ($CD45RO^+CCR7^-$) in their blood as compared with always HPV-negative mothers. When all HPV16-positive mothers were pooled together, they had also higher levels of terminally differentiated (T_{EMRA}) $CD8^+$ cells ($CD45RA^+CCR7^-$), which are the most effective $CD8^+$ cells in destroying tumor cells and virus-infected cells [30–32]. Mothers with persistent oral HPV16 had significantly lower levels of activated helper ($CD4^+$) and suppressor ($CD8^+$) T cells among the $CD3^+$ lymphocyte population.

As to other viruses, a longitudinal study on EBV-infected patients revealed differences in lytic versus latent epitope-specific composition of the $CD8^+$ T-cell population in the chronic carrier stage of the infection [33,34]. Latent epitopes acquired CD45RA in the persistent phase of EBV infection, as also found here for HPV16-persistors [33,34]. In line with our results, there are earlier studies reporting differences between naïve ($CD45RA^+$) and memory T-cell ($CD45RO^+$) populations in peripheral blood in women with HPV infection [11,35,36]. Contradictory to our results, however, Rodriguez et al. reported that $CD45RO^+CD27^-CD8^+$ T cells in PBMCs were positively associated with HPV persistence whereas $CD45RO^+CD27^+CD4^+$ T cells had a negative association [16]. However, there is recent evidence suggesting that local $CD45RA^+/CD45RO^+$ expression in cervical intraepithelial lesions (CINs) might even be a prognostic biomarker because the expression is increasing in parallel with the increasing grade of CIN [37].

We also found that mothers with persistent oral HPV16 infection had lower levels of peripheral $HLADR^+CD3^+CD4^+$ and $CD38^+HLADR^+CD3^+CD8^+$ T cells as compared

with their HPV-negative counterparts. HLA-DR is a histocompatibility antigen from the MHC II family and both $CD4^+$ helper and $CD8^+$ cytotoxic T cells bear HLA-DR molecules as important surface activation markers. Both HLA-DR and CD38 molecules are present on immature T- and B- lymphocytes and are re-expressed during an immune response. Antigen-presenting cells are essential for HLA-DR on $CD3^+CD4^+$ T cells. Co-expression of HLA-DR and CD38 is a key marker of $CD8^+$ T-cell immune activation during several viral infections, e.g., influenza, Dengue, Ebola, and HIV-1 [38].

Contradictory to our results, Rodrigues and coworkers reported a positive correlation between $HLADR^+CD3^+CD4^+$ T cells and persistent genital HPV infection [16]. Additionally, Papasavvas et al. found that irrespective of the presence of CIN, HR-HPV-positive women had higher levels of circulating $CD38^+$ T cells, yet these women had also HIV infection [39]. Importantly, the presence of the human MHCII isotype, HLA-DR, potentially also identifies a regulatory T-cell population. Regardless of an endogenous expression or a protein acquisition, MHCII on T cells has mainly been associated with the induction of down-regulatory signals in the responding T cell [40–44]. It has also been associated with active rather than resting regulatory T cells (T_{regs}) [45]. However, several studies also demonstrate that the $MHCII^+$ T cells can activate other T cells [38,46]. In line with the multipotential function of HLA-DR expressing T cells, it has been shown that activated $CD8^+$ T cells co-expressing of HLA-DR and CD38 accumulated over a prolonged period in HIV-infected lymphopenia patients. Several studies have also indicated that $CD38^+HLA^-DR^+CD8^+$ T cells could play a recovery role in activating immunity and eliminating the virus [37,47].

Our contradictory results above compared to the study by Rodrigues and coworkers might also be due to their studies' lack of correlation for multiple comparisons [16]. Rodrigues and coworkers also compared two groups as factor variables and T cell populations as dependent variables but no correlation was observed for multiple comparisons which might be a potential issue of obtaining false-positive results [16]. Our setting is fortunate in that we had more than two groups of factor variables (both mothers and children), which enables us to use the conventional methods for compensating for the multiple testing. When the Bonferroni test (BT) was used for the correlation of multiple comparisons, none of the significant comparisons stayed significant, as shown in Tables 3 and 4. However, the proper use of different post hoc tests for the control of multiple comparisons has been extensively discussed in the statistical literature [48]. Several different MCTs (multiple comparison tests) are available, and there is no unanimous agreement on which is the most suitable one for each setting. In this study, we used BT, which is the most often used of all post hoc tests. However, BT also has disadvantages since it is unnecessarily conservative with weak statistical power. The adjusted α (alpha) is often smaller than required, particularly if there are many tests and/or the test statistics are positively correlated. Therefore, this method often fails to detect real differences. More liberal methods exist, for example, Fisher's least significant difference (LSD), which does not control the family-wise error rate. In fact, we also tested LSD as the MCT in our calculations, and indeed, the inflation of alpha level was markedly less, around $p = 0.500$ or less in most instances where BT resulted in $p = 1.000$. Considering all the above, our results will need to be further replicated with larger study settings so that we can firmly confirm the findings of this study and the previous studies on this topic.

Interestingly, the children of the mothers with persistent oral HPV16 infection had entirely opposite changes in the proportions of $CD38^+HLADR^+CD3^+CD4^+$, $HLADR^+CD3^+CD8^+$, $CD38^+HLADR^+CD3^+CD8^+$ and $CD38^-HLADR^+CD3^+CD4^+CD8^+$ T cells in peripheral blood, being significantly elevated as contrasted to their mothers with declined levels of these subsets. To the best of our knowledge, this is the first study immunophenotyping the peripheral blood lymphocyte subpopulations among mothers with persistent HPV16 infection and their children. Significant alterations in the distribution of lymphocyte subsets were found only in the children whose mothers had persistent oral HPV16 infection. Activated $CD3^+$, $CD4^+$ and $CD8^+$ T cells expressing HLA-DR with or without CD38 were significantly more abundant in these children, compared with children of HPV-negative mothers, being a sign of activated

immunity. Importantly, HPV-specific T-cell response was not analyzed in the present study. However, based on our previous data from the Finnish Family HPV Study, we know that the mother seems to be the main HP transmitter to her offspring via mouth [19,20]. In line with this, the present observations could indicate a continuous exposure of the offspring to maternal oral HPV, resulting in T-cell activation. This is supported by the fact that also the effector memory $CD8^+$ cells ($CD45RO^+$) were expanded in these children. It has been suggested that persistent HPV infection could be associated with undifferentiated memory $CD4^+$ T cells and other T-cell subsets that are incompetent in eliminating the viral pathogen [11,16,49,50]. In the present series, $CD4^+$ T cells expressing HLADR and CD3 were increased both in mothers with persistent oral HPV16 infection and their children (Tables 5 and 6).

Despite its unique design, the present study has also limitations. First, the number of study subjects is limited, including only 42 mothers and 28 of their children. When stratified into cases and controls, this small number is the key limiting factor to reaching statistical power. In addition, the correlation for multiple comparisons needs to be considered as this might potentially help in obtaining false-positive results as discussed above. Similarly, the temporal relationship between HPV acquisition and collection of the PBMCs is not known. Therefore, the dynamics of adaptive immunity cannot be thoroughly investigated from these data. Finally, as previously affirmed [16], it is not known whether a persistent HPV infection causes the activation of different T-cell subsets or whether it is the activation of these T-cell subsets that causes the persistence of HPV infection.

In general, a detailed dissection of the T-cell immunophenotypes and their relation to the known outcomes of HPV infections could ideally offer important predictive insights in clinical practice. Thus far, the number of studies assessing HPV immunity in relation to natural history is limited. As there is no reliable way to trace the time of the first HPV exposure, the studies on HPV immunology suffer from this inherent handicap. This study is, to our knowledge, the first to conduct T-cell immunophenotyping in mothers and their children in a longitudinal setting. Taken together, these data suggest that both genital and oral HPV16 infections in mothers are associated with alterations in the relative distribution of peripheral blood T-cell subsets. In children of these women, such alterations in T-cell subsets were only found when the mother had persistent oral HPV16 infection.

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PUBLICATION II

Serum IgG Antibodies to HPV6 L1, E2, E4, E6, and E7 Proteins Among Children Prospectively Followed-up for Three Years

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Submitted.

PUBLICATION III

Effect of a Second Pregnancy on the HPV Serology in Mothers Followed Up in the Finnish Family HPV Study

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Article

Effect of a Second Pregnancy on the HPV Serology in Mothers Followed Up in the Finnish Family HPV Study

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Abstract: The impact of pregnancy on human papillomavirus (HPV) natural antibody levels is not fully understood. We tested the seroprevalence and levels of HPV 6, 11, 16, 18 and 45 antibodies at different time points among 89 women with a second pregnancy and 238 nonpregnant women during their 36-month followup. All participants were unvaccinated for HPV and pregnant at the enrollment of the study. Serum samples were collected from the mothers at baseline and at the 12-month, 24-month, and 36-month followup visits. No statistically significant differences in mean antibody levels were observed in women who developed a second pregnancy compared to their nonpregnant counterparts. Between these two groups, statistically significant differences in serostatus were observed, particularly if the second pregnancy was ongoing at the 24-month timepoint. Accordingly, women with a second pregnancy were more likely to be seronegative for HPV 6, 11, 18, and 45 as compared to the nonpregnant women, the reverse being true for HPV16. In contrast, the women with an ongoing second pregnancy showed a higher prevalence of HPV16 seropositivity at the 36-month followup. These data suggest that a second pregnancy does not seem to have a major impact on the levels of HPV antibodies, but it might influence the serological outcomes.

Keywords: human papillomavirus; HPV; serology; mother; child

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1. Introduction

Acquisition of human papillomavirus (HPV) infection is common especially in young adults [1]. It has been estimated that nearly all individuals become infected with some HPV type at least once in a lifetime [2]. For most individuals, mucosal HPV infections are transient and never result in viral persistence or clinical progression. As HPV infections are most prevalent among young adults, the possible impact of pregnancy on the dynamics of HPV infection is of substantial interest [3].

There is some evidence that pregnancy-related hormonal changes and immunosuppression could have an impact on the prevalence of HPV infection and viral persistence [4–7]. So far, the effect of pregnancy on HPV antibodies is more unclear, as studies have reported contradictory results. In addition, the antibody response resulting from a naturally acquired HPV infection is known to vary remarkably. Confirmed seropositivity can be regarded as a sign of previous exposure to HPV, although seroconversion does not

occur in all HPV-infected individuals [8]. Optimally, antibodies might offer some protection against subsequent HPV infections. In a study evaluating genital HPV infection, it was suggested that HPV antibodies acquired as a result from a natural HPV infection offer only modest protection against a genital HPV reinfection [9]. As for HPV antibodies in pregnancy, it has not been firmly established whether pregnancy decreases or increases HPV antibody levels and HPV seropositivity in general.

Followup data on previously pregnant women are lacking to assess whether becoming pregnant for the second time influences HPV seropositivity and HPV antibody levels. In our previous report from this same cohort, the second pregnancy had little independent impact on the carriage and persistence of oral and cervical high-risk HPV infections [3], but the data on HPV serology were not available at that time. In the present study, we analyzed the seroprevalence for HPV types 6, 11, 16, 18, and 45 in women who developed a second pregnancy during followup in the prospective Finnish Family HPV Study cohort.

2. Materials and Methods

2.1. Participants

The Finnish Family HPV (FFHPV) Study is a longitudinal cohort study, designed to analyze the dynamics of HPV transmission within regular families (mother, father, and the index offspring), conducted since 1998 jointly by the Department of Obstetrics and Gynecology (Turku University Hospital) and the Institute of Dentistry (Faculty of Medicine, University of Turku). The original study protocol and its amendments were approved by the Research Ethics Committee of Turku University Hospital (#3/1998, #2/2006, 45/180/2010). Originally, 329 pregnant women in their third trimester and their 331 newborns (including two twins) were enrolled in the study between 1998 and 2001, and written informed consent was obtained from each participant. The study design as well as the details of the participants have been given in a series of previous reports [10–12]. None of the participants had received a prophylactic HPV vaccination, and therefore all the antibodies resulted from a naturally acquired HPV infection. The present analysis includes, as the study group, all 89 women who became pregnant for the second time during the followup and, as the reference group, the remaining 238 women who did not have a second pregnancy during the followup. Two women out of the original 329 women were lost to followup; so, they were excluded from the present study.

2.2. Serology

Serum samples were collected from the mothers at the baseline (36 weeks of pregnancy) of the study and at the 12-month, 24-month, and 36-month followup visits. Antibodies to the major capsid protein L1 of HPV6, 11, 16, 18, and 45 were analyzed with multiplex HPV serology based on glutathione S-transferase fusion-protein capture on fluorescent beads as previously described [13]. For all HPV types, seropositivity was defined as the median fluorescence intensity (MFI) > 200 or >400 (stringent cutoff).

2.3. Statistical Analyses

Frequency tables were analyzed using the χ^2 test or the Fisher's exact test for categorical variables (seropositivity +/−). Differences in the means of continuous variables (MFI titers) were analyzed using ANOVA (analysis of variance) after controlling for their normal distribution. The Kolmogorov–Smirnov test was used for normality. The two groups were also compared by the distribution of the potential HPV-associated covariates recorded by a detailed questionnaire at the study enrollment. SPSS 26.0.1 (IBM, Armonk, NY, USA) and STATA/SE 16.1 (Stata Corp., College Station, TX, USA) software packages were used. All statistical tests performed were two-sided and declared significant at the *p*-value level of <0.05.

3. Results

The mean antibody levels of the HPV6, HPV11, HPV16, HPV18, and HPV45 seropositive women at baseline and during the followup visits were stratified by their status of second pregnancy and are shown in Figure 1. Seropositive women were categorized by either having an ongoing second pregnancy at the time of the followup visit, having a second pregnancy at some other timepoint, or not having a second pregnancy at all during the followup. These two separate groups in the figure for the women with second pregnancy were used to illustrate the effect of the ongoing pregnancy at the timepoint compared to another time point in the longitudinal followup. As for HPV18 antibodies at the baseline of the study, the mean levels (MFI mean ± SD) were 125 (±117) for women who developed a second pregnancy, and 199 (±217) for women who did not have a second pregnancy during the followup ($p = 0.021$), but no other significant differences were observed later on between these groups. The mean antibody levels to HPV6 and HPV16 showed some variation over time between these groups, but no statistically significant differences were observed. As for antibodies to HPV16, the mean MFI at 12 months was higher in those women who had an ongoing pregnancy at that 12-month timepoint with a mean MFI 1164 (±2003), while the corresponding mean MFI values were 803 (±971) for those who had a second pregnancy at some other timepoint than at 12 months and 611 (±1217) for those who did not develop a second pregnancy during the followup ($p = 0.209$).

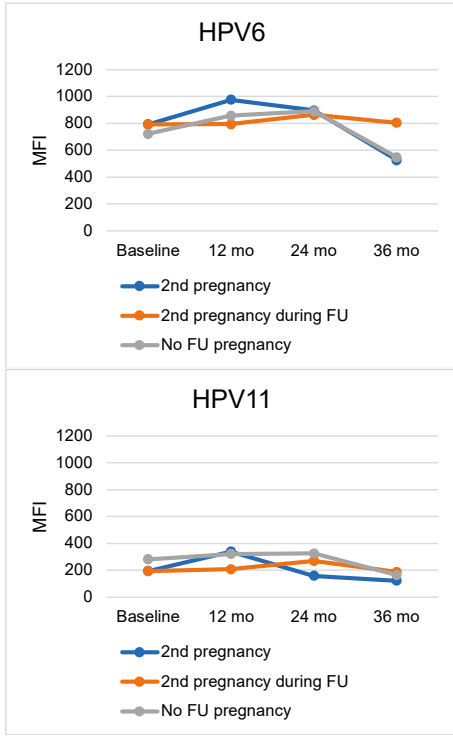

Figure 1. *Cont.*

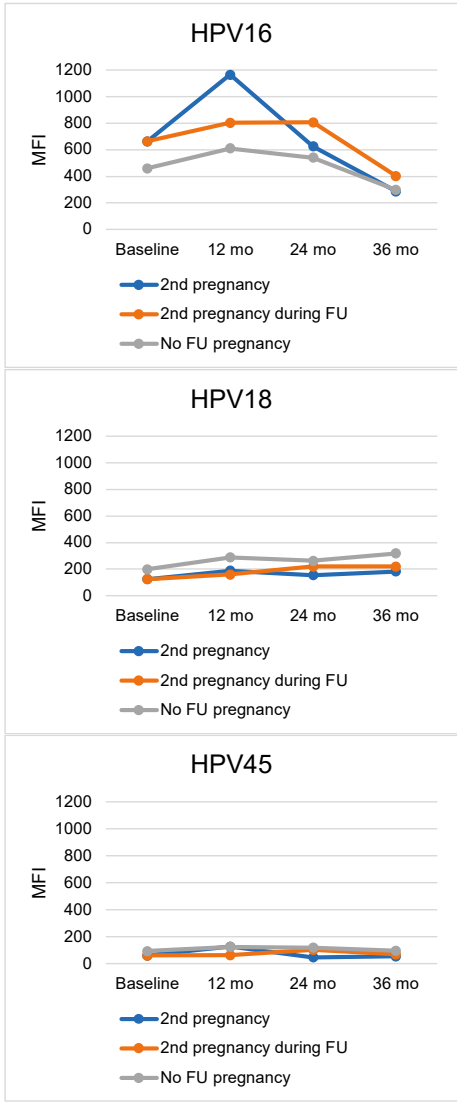

Figure 1. Mean MFI levels of HPV6, HPV11, HPV16, HPV18, and HPV45 antibodies at baseline and at the 12-month, 24-month, and 36-month followup visits among seropositive women stratified by the status of their second pregnancy. The blue line depicts the mean MFI values of those who had a second pregnancy at each specific followup timepoint, the orange line depicts those who had a second pregnancy at some other timepoint during the followup time, and the gray line depicts those who did not have a second pregnancy at all. Abbreviations: MFI = median fluorescence intensity, mo = months, FU = follow-up.

All 327 women included in this study were further stratified by their HPV6, HPV11, HPV16, HPV18, and HPV45 serostatus (seropositive or seronegative), as well as their serostatus during the followup as related to the timing of the second pregnancy (12 months, 24 months, and 36 months) (Table 1). Most of the significant differences in serostatus were observed among the cases when the second pregnancy was ongoing at the 24-month timepoint. This difference between the 24-month pregnant and nonpregnant women applies to their baseline seropositivity for (1) HPV6 ($p = 0.008$) and (2) HPV11 ($p = 0.002$). As to the HPV11 serostatus between the 24-month pregnant and nonpregnant women, significant differences were observed in all timepoints except in the 36-month followup visit. In addition, the HPV6 and HPV18 serostatus at 36 months was different between the 24-month pregnant and nonpregnant women; $p = 0.042$ and $p = 0.005$, respectively. When the second pregnancy was ongoing at the 36-month followup visit, statistically significant differences between pregnant and nonpregnant women were observed only in the 24-month followup visit serostatus: for HPV6 and HPV16, $p = 0.021$ and $p = 0.04$, respectively. No such differences were observed when the second pregnancy was ongoing at the 12-month followup visit.

The serostatus of HPV6, HPV11, HPV16, HPV18, and HPV45 at the baseline and during the 12-, 24- and 36-month followup visits, with both MFI > 200 and stringent MFI > 400 seropositivity cutoffs, are shown in Table S1. At the baseline of the study, only 44.9% (n = 40) of the women who developed a second pregnancy were HPV6 seropositive (MFI > 200) as compared with 58.4% (n = 139) of those who did not develop a second pregnancy ($p = 0.034$). Similarly, 6.7% (n = 6) of the women with their second pregnancy were HPV11 seropositive (with stringent MFI > 400) at baseline, as compared with 15.1% (n = 36) of those who did not develop a second pregnancy ($p = 0.043$). The two groups also differed significantly in their baseline seropositivity (MFI > 200) for HPV18 ($p = 0.013$). In addition, significant differences between women with and women without a second pregnancy were observed in their HPV18 (MFI > 200) serostatus at the 12-month and 36-month followup visits: $p = 0.002$ and $p = 0.044$, respectively.

Table 1. HPV6, HPV11, HPV16, HPV18, and HPV45 serostatus among the 327 women from the Finnish Family HPV study during the 36 months of followup stratified by the timing of the second pregnancy (12 mo, 24 mo, and 36 mo). Significant differences in the seroprevalence of women with a second pregnancy at the given time point and the rest of the cohort are bolded. When the second pregnancy was ongoing at 12 months, no significant differences were observed, but significant differences were observed with a second pregnancy at the 24- or 36-month timepoint.

		Baseline		**12 Months**		**24 Months**		**36 Months**	
		Yes	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**
		n (%)		**n (%)**		**n (%)**		**n (%)**	
2nd Pregnancy at 12 mo (n = 27)									
HPV6	Seropositive	17 (63.0)	162 (54.0)	18 (66.7)	178 (69.3)	19 (73.1)	159 (67.4)	14 (51.9)	126 (53.8)
	Seronegative	10 (37.0)	138 (46.0)	9 (33.3)	79 (30.7)	7 (26.9)	77 (32.6)	13 (48.1)	108 (46.2)
HPV11	Seropositive	8 (29.6)	62 (20.7)	9 (33.3)	69 (26.8)	7 (26.9)	55 (23.3)	6 (22.2)	33 (14.1)
	Seronegative	19 (70.4)	238 (79.3)	18 (66.7)	188 (73.2)	19 (73.1)	181 (76.7)	21 (77.8)	201 (85.9)
HPV16	Seropositive	10 (37.0)	99 (33.0)	12 (44.4)	104 (40.5)	10 (38.5)	78 (33.1)	8 (29.6)	66 (28.2)
	Seronegative	17 (63.0)	201 (67.0)	15 (55.6)	153 (59.5)	16 (61.5)	158 (66.9)	19 (70.4)	168 (71.8)
HPV18	Seropositive	5 (18.5)	61 (20.3)	5 (18.5)	70 (27.2)	7 (26.9)	52 (22.0)	8 (29.6)	53 (22.6)
	Seronegative	22 (81.5)	239 (79.7)	22 (81.5)	187 (72.8)	19 (73.1)	184 (78.0)	19 (70.4)	181 (77.4)
HPV45	Seropositive	3 (11.1)	28 (9.3)	4 (14.8)	28 (10.9)	5 (19.2)	19 (8.1)	2 (7.4)	18 (7.7)
	Seronegative	24 (88.9)	272 (90.7)	23 (85.2)	229 (89.1)	21 (80.8)	217 (91.9)	25 (92.6)	216 (92.3)
2nd pregnancy at 24 mo (n = 43)									
HPV6	Seropositive	**15 (34.9) [a]**	**164 (57.7) [a]**	26 (60.5)	170 (70.5)	23 (54.8)	155 (70.5)	**16 (38.1) [b]**	**124 (56.6) [b]**
	Seronegative	**28 (65.1) [a]**	**120 (42.3)[a]**	17 (39.5)	71 (29.5)	19 (45.2)	65 (29.5)	**26 (61.9) [b]**	**95 (43.4) [b]**
HPV11	Seropositive	**2 (4.7) [c]**	**68 (23.9) [c]**	**6 (14.0) [d]**	**72 (29.9) [d]**	**3 (7.1) [e]**	**59 (26.8) [e]**	3 (7.1)	36 (16.4)
	Seronegative	**41 (95.3) [c]**	**216 (76.1)[c]**	**37 (86.0) [d]**	**169 (70.1) [d]**	**39 (92.9) [e]**	**161 (73.2) [e]**	39 (92.9)	183 (83.6)

Table 1. *Cont.*

		Baseline		12 Months		24 Months		36 Months	
		Yes	No	Yes	No	Yes	No	Yes	No
		n (%)		n (%)		n (%)		n (%)	
HPV16	Seropositive	10 (23.3)	99 (34.9)	14 (32.6)	102 (42.3)	10 (23.8)	78 (35.5)	9 (21.4)	65 (29.7)
	Seronegative	33 (76.7)	185 (65.1)	29 (67.4)	139 (57.7)	32 (76.2)	142 (64.5)	33 (78.6)	154 (70.3)
HPV18	Seropositive	4 (9.3)	62 (21.8)	**4 (9.3)** [f]	**71 (29.5)** [f]	6 (14.3)	53 (24.1)	**3 (7.1)** [g]	**58 (26.5)** [g]
	Seronegative	39 (90.7)	222 (78.2)	**39 (90.7)** [f]	**170 (70.5)** [f]	36 (85.7)	167 (75.9)	**39 (92.9)** [g]	**161 (73.5)** [g]
HPV45	Seropositive	1 (2.3)	30 (10.6)	**1 (2.3)** [h]	**31 (12.9)** [h]	1 (2.4)	23 (10.5)	2 (4.8)	18 (8.2)
	Seronegative	42 (97.7)	254 (89.4)	**42 (97.7)** [h]	**210 (87.1)** [h]	41 (97.6)	197 (89.5)	40 (95.2)	201 (91.8)
2nd pregnancy at 36 mo (n = 19)									
HPV6	Seropositive	8 (42.1)	171 (55.5)	12 (63.2)	184 (69.4)	13 (68.4)	165 (67.9)	14 (70.0)	126 (52.3)
	Seronegative	11 (57.9)	137 (44.5)	7 (36.8)	81 (30.6)	6 (31.6)	78 (32.1)	6 (30.0)	115 (47.7)
HPV11	Seropositive	3 (15.8)	67 (21.8)	6 (31.6)	72 (27.2)	**9 (47.4)** [i]	**53 (21.8)** [i]	3 (15.0)	36 (14.9)
	Seronegative	16 (84.2)	241 (78.2)	13 (68.4)	193 (72.8)	**10 (52.6)** [i]	**190 (78.2)** [i]	17 (85.0)	205 (85.1)
HPV16	Seropositive	8 (42.1)	101 (32.8)	11 (57.9)	105 (39.6)	**11 (57.9)** [j]	**77 (31.7)** [j]	8 (40.0)	66 (27.4)
	Seronegative	11 (57.9)	207 (67.2)	8 (42.1)	160 (60.4)	**8 (42.1)** [j]	**166 (68.3)** [j]	12 (60.0)	175 (72.6)
HPV18	Seropositive	1 (5.3)	65 (21.1)	4 (21.1)	71 (26.8)	3 (15.8)	56 (23.0)	3 (15.0)	58 (24.1)
	Seronegative	18 (94.7)	243 (78.9)	15 (78.9)	194 (73.2)	16 (84.2)	187 (77.0)	17 (85.0)	183 (75.9)
HPV45	Seropositive	1 (5.3)	30 (9.7)	3 (15.8)	29 (10.9)	3 (15.8)	21 (8.6)	2 (10.0)	18 (7.5)
	Seronegative	18 (94.7)	278 (90.3)	16 (84.2)	236 (89.1)	16 (84.2)	222 (91.4)	18 (90.0)	223 (92.5)

p-values = [a] 0.008, [b] 0.042, [c] 0.002, [d] 0.028, [e] 0.004, [f] 0.005, [g] 0.005, [h] 0.039, [i] 0.021, [j] 0.04. Abbreviations: MFI = median fluorescence intensity, mo = months. Cutoff value for seropositivity was MFI > 200.

The demographic and clinical data of the women are shown in Table 2. Women with a second pregnancy and nonpregnant women differed significantly in their marital status, number of deliveries, number of lifetime sexual partners, history of STDs, and previous contraception method used. A smaller number of women with a second pregnancy were single when compared to nonpregnant women: 2.3% vs. 8.9% ($p = 0.045$). Women with a second pregnancy had fewer previous deliveries than the nonpregnant women: 11.6% vs. 30.9% for two or more deliveries ($p = 0.002$). This also explains why more condoms and oral contraceptives were used in the past among women with a second pregnancy compared to the nonpregnant women where no contraception was the most common used method of contraception ($p = 0.032$). Also, women with a second pregnancy had fewer lifetime sexual partners ($p = 0.038$) but having a positive history of STDs was slightly more common in this group ($p = 0.038$) as compared to their nonpregnant counterparts. The mean age with SD was 25.4 ± 3.4 for the women with a second pregnancy and 25.5 ± 3.4 for those who did not develop a second pregnancy ($p = 0.718$).

Table 2. Demographic and clinical data of the women with a second pregnancy compared to the women that did not have an additional pregnancy during the 36-month followup of the Finnish Family HPV Study cohort. Significant comparisons are bolded.

Variable	2nd Pregnancy	No 2nd Pregnancy	Significance
	n (%)		
Marital status			$p = 0.045$ *
Single	**2 (2.3)**	**18 (8.9)**	
Other (unmarried couple, married, divorced)	**84 (97.7)**	**184 (91.1)**	
Number of deliveries			$p = 0.002$ *
0	**1 (1.2)**	**1 (0.5)**	
1	**75 (87.2)**	**138 (68.7)**	
2	**8 (9.3)**	**54 (26.9)**	
3	**2 (2.3)**	**5 (2.5)**	
4	**0 (0.0)**	**3 (1.5)**	

Table 2. *Cont.*

Variable	2nd Pregnancy	No 2nd Pregnancy	Significance
	n (%)		
Age at first intercourse			$p = 0.179$ *
≤13	3 (3.5)	4 (2.0)	
14–16	44 (51.2)	117 (57.9)	
17–19	32 (37.2)	75 (37.1)	
≥20	7 (8.1)	6 (3.0)	
Number of lifetime sexual partners			$p = 0.038$
1–2	**28 (32.9)**	**43 (21.3)**	
3–5	**27 (31.8)**	**64 (31.7)**	
6–10	**20 (23.5)**	**45 (22.3)**	
>10	**10 (11.8)**	**50 (24.8)**	
Number of sexual partners by the age of 20			$p = 0.262$ *
0–2	44 (51.2)	80 (39.6)	
3–5	24 (27.9)	74 (36.6)	
6–10	14 (16.3)	32 (15.8)	
>10	4 (4.7)	16 (7.9)	
Frequency of intercourse, n/month			$p = 0.103$ *
0–1	0 (0.0)	7 (3.5)	
2–4	27 (31.4)	59 (29.2)	
5–10	53 (61.6)	108 (53.5)	
>10	6 (7.0)	28 (13.9)	
Oral sex			$p = 0.217$
Regular	7 (8.1)	28 (13.9)	
Occasionally	64 (74.4)	130 (64.4)	
Never	15 (17.4)	44 (21.8)	
Anal sex			$p = 0.179$ *
Regular	2 (2.3)	1 (0.5)	
Occasionally	12 (14.0)	40 (19.8)	
Never	72 (83.7)	161 (79.7)	
Age at onset of oral contraceptive			$p = 0.544$ *
Never used	7 (8.1)	17 (8.5)	
≤13 years	0 (0.0)	3 (1.5)	
14–16 years	36 (41.9)	81 (40.3)	
17–19 years	31 (36.0)	83 (41.3)	
≥20 years	12 (14.0)	17 (8.5)	
Contraception methods used previously			$p = 0.032$
Condom	**33 (36.7)**	**65 (27.2)**	
Oral contraceptive	**7 (7.8)**	**6 (2.5)**	
Intrauterine device	**17 (18.9)**	**44 (18.4)**	
None	**33 (36.7)**	**124 (51.9)**	
Smoking habits			$p = 0.661$ *
Not smoker	47 (54.7)	96 (47.8)	
1–10 cigarettes per day	23 (26.7)	61 (30.3)	
11–20 cigarettes per day	14 (16.3)	41 (20.4)	
>20 cigarettes per day	2 (2.3)	3 (1.5)	
Pack years of smoking			$p = 0.685$
Lower tertile (<2.5)	14 (38.9)	31 (33.0)	
Median tertile (<6.0)	10 (27.8)	34 (36.2)	
Upper tertile (>6.0)	12 (33.3)	29 (30.9)	
Alcohol use			$p = 0.154$
Yes	73 (85.9)	185 (91.6)	
No	12 (14.1)	17 (8.4)	
History of STDs			$p = 0.038$
STD history	**24 (26.7)**	**39 (16.3)**	
No STDs	**66 (73.3)**	**200 (83.7)**	
History of genital warts			$p = 0.521$
Yes	22 (25.6%)	58 (29.3)	
No	64 (74.4)	140 (70.7)	

Table 2. *Cont.*

Variable	2nd Pregnancy	No 2nd Pregnancy	Significance
	n (%)		
Age at diagnosis of genital warts			$p = 0.903$ *
Never	64 (74.4)	142 (71.7)	
<20 years	9 (10.5)	27 (13.6)	
20–24 years	10 (11.6)	21 (10.6)	
>25 years	3 (3.5)	8 (4.0)	
Treatment of genital warts			$p = 0.840$ *
No treatment	12 (40.0)	29 (37.2)	
Topical treatment	6 (20.0)	25 (32.1)	
Electrocautery	1 (3.3)	3 (3.8)	
Cryotherapy	1 (3.3)	2 (2.6)	
Laser therapy	4 (13.3)	8 (10.3)	
Surgery	0 (0.0)	1 (1.3)	
Several treatments	6 (20.0)	10 (12.8)	

* Fisher's exact test.

4. Discussion

To our knowledge, this is the first study evaluating the effect of a second consecutive pregnancy on naturally acquired HPV antibodies in a longitudinal setting. Our results indicate only slight differences in the mean antibody levels to HPV6, HPV11, HPV16, HPV18, and HPV45 between women who developed a second pregnancy and those women who did not develop a second pregnancy during the followup. Our earlier study on HPV serology in this cohort not stratified by the second pregnancy showed that HPV seroprevalence was lowest at the entry of the study when all women were pregnant at their third trimester. Both low-risk and high-risk HPV seropositivity were significantly associated with the age at onset of sexual activity, the number of sexual partners until 20 years of age, the lifetime number of sexual partners, and the history of genital warts [12].

In the present series, the mean antibody levels to HPV6, HPV11, HPV18, and HPV45 appeared somewhat lower in those women who developed a second pregnancy, but in contradiction, this was not the case for HPV16, in which higher antibody levels were recorded as well as with individual mean values for HPV6 at the baseline and for HPV45 at the 12-month followup visit. As for the differences in HPV6 antibody levels between the two groups of women in the baseline, our results indicate that there could be some baseline differences that affect HPV6 antibody levels between these two groups, and it is important to notice that this difference is not related to the second pregnancy that takes place later on. In contrast, the general trend in the mean antibody levels of our data was a slight decrease when comparing the women with second pregnancy to those without, and this trend was also seen with HPV45 in all timepoints investigated except for one. One possible explanation for this is that antibody response provoked by a naturally acquired HPV infection is known to vary between different individuals; therefore, this could affect our results. In addition, there is some controversy on the stability of HPV antibodies, although HPV IgG antibodies are believed to be relatively stable over time [11].

In this study, we observed that women who developed a second pregnancy during the followup differed from those who did not develop a second pregnancy in terms of their marital status, the number of deliveries, the number of lifetime sexual partners, the history of STDs, and the contraception method used previously (Table 2). These variables are considered as risk factors for HPV infection [5,14,15] and therefore represent potential confounding factors. Women with no second pregnancy reported more lifetime sexual partners and deliveries and no contraception as compared to women who had their second pregnancy during the followup. These background differences might predict lower HPV antibody levels and a higher proportion of seronegative outcomes among the women with second pregnancy, due to less exposure to HPV-related cofactors.

In general, the total IgG level has been suggested to be lower during pregnancy [16]. The activation of B-lymphocytes has been shown to continue from becoming pregnant

to the postpartum period, affecting the antibody secretion of different immunoglobulin classes [17]. Studies on different IgG subclasses have yielded contradictory results; some studies suggest that IgG subclass IgG1 is stable during pregnancy, whereas some suggest its levels are higher during pregnancy, and IgG3 levels are measured to be higher, but IgG2 and IgG4 levels have been thought to remain stable [16,17]. Hemodilution is thought to be one of the causes of lower total IgG levels during pregnancy; however, the suppression of cell-mediated immunity, the loss of protein in urine, the placental transfer of IgGs to the fetus, and hormonal changes, might contribute to this [16,18]. Our earlier study on this same cohort implied that the IgG antibody levels were lower at the baseline, and an increase in the antibody levels was seen after pregnancy [11]. Accordingly, one study assessing serological responses to HPV16 E4, E6, and E7 proteins in pregnant women suggested that the humoral immune response against HPV infections is reduced during pregnancy [19]. In our previous analysis from the FFHPV cohort, IgG antibodies to HPV16 L1 in serum were lower during pregnancy, but the serum IgA antibodies showed a different pattern [20]. However, the possible effect of differing HPV prevalence between different HPV serology studies assessing HPV antibody levels in pregnant women must be taken into consideration. According to one meta-analysis, the overall HPV prevalence in pregnant women varies by study region, age, and HPV type, and its results demonstrated that pregnant women are more susceptible to HPV infection than their nonpregnant counterparts [7].

Our data suggest that a second pregnancy does not increase HPV seropositivity, and the observed changes in mean antibody levels and differences in serostatuses could result from differences in the women's background or immunological factors not the second pregnancy at the followup visit itself. As for other DNA viruses and their significance in pregnancy, nearly all human herpesviruses (HHVs) have been shown to infect cells at the fetal–maternal interface without crossing the placental barriers [21]. In a study investigating IgG antibody titers to Epstein–Barr virus infection in pregnant women, the overall antibody levels declined during late pregnancy, and latent viral reactivation was observed to occur due to the potential stress-induced immune dysregulative state especially in racial disparities [22]. With herpes simplex viruses, the overall seroprevalence for both HSV-1 and HSV-2 is relatively high in pregnant women, and the presence of HSV IgG antibodies in relation to the timing of viral reactivation is associated with pregnancy and neonatal complications [23]. Lastly, with the cytomegalovirus infections, pre-existing maternal antibodies to CMV may act as a protective factor against congenital CMV infection, but viral reactivation or new maternal infection with another virus strain may lead to fetal infection [24].

As many previous studies on HPV serology have had a cross-sectional design, one of the strengths of this study was the use of a longitudinal design that allowed the assessment of the effects of a second consecutive pregnancy on HPV serology with five different HPV genotypes. This study has a unique design on the subject that has not been previously addressed from the viewpoint of a second pregnancy's effect on HPV serology. The women's HPV serostatus and antibody levels were measured at four different timepoints (baseline, 12, 24, and 36 months), but the possible impact of the actual gestational length of the second pregnancy at each time point was not taken in consideration, which is one factor that could also affect our results. Moreover, a known limitation in all serological studies is that not all individuals seroconvert [8], even in the case of a persistent HPV infection, and this limitation must be taken into consideration when interpreting our results. In addition, another limitation in serology studies is that currently there is no golden standard method for assessing HPV antibodies, although efforts have begun in order to standardize HPV serology assays [25]. In this particular study, we used the multiplex serology assay, which is useful in evaluating cumulative HPV infection, although it is not a reliable marker of immune protection, as it does not differentiate between neutralizing and non-neutralizing antibodies [26]. Furthermore, the significance of these measured antibodies in protecting the women of our cohort against future HPV infection is uncertain.

To conclude, HPV antibody levels and HPV serostatus showed only slight variations during the second pregnancy according to our data in the FFHPV cohort. Women with a second pregnancy were less likely to be seropositive for HPV6, HPV11, HPV16, HPV18, and HPV45 as compared to women without a second pregnancy. However, apart from pregnancy, more attention needs to be paid on cofactors that might also impact the serological outcomes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v15102109/s1, Table S1: HPV6, HPV11, HPV16, HPV18 and HPV45 serostatus (MFI > 200 and MFI > 400) during the follow-up, stratified by the 2nd pregnancy.

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