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Faculty of Medicine
Institute of Dentistry
Department of Oral Pathology and Oral Radiology
Finnish Doctoral Program in Oral Sciences (FINDOS-Turku)
Department of Biomedicine/Virology
Turku University Hospital
Department of Obstetrics and Gynecology

Supervised by

Professor Stina Syrjänen, DDS, PhD
Department of Oral Pathology and Oral Radiology, Institute of Dentistry,
Faculty of Medicine,
University of Turku and Department of Pathology,
Turku University Hospital, Turku, Finland.

Docent Veijo Hukkanen, MD, PhD
Department of Biomedicine/Virology,
University of Turku, Turku, Finland.

Reviewed by

Docent Eeva Broberg, PhD
European Centre for
Disease Prevention and Control (ECDC),
Stockholm, Sweden.

Docent Eeva Auvinen, MD, PhD
Medicum, Department of Virology,
University of Helsinki, Finland,
HUSLAB, Virology and immunology,
Helsinki University Hospital,

Opponent

Docent Thedi Ziegler, PhD
World Health Organization (WHO),
Global Influenza Program,
Geneva, Switzerland.

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To my family

"Whats the difference between herpes and true love?
- Herpes lasts for ever."

Unknown

ABSTRACT

Johanna Mäki

Herpes simplex virus (HSV) carriage in oral mucosa and HSV serostatus among pregnant Finnish women and their spouses during a six-year follow-up

University of Turku, Faculty of Medicine, Institute of Dentistry, Department of Oral Pathology and Oral radiology, Finnish Doctoral Program in Oral Sciences (FINDOS-Turku), Department of Biomedicine/Virology, Turku University Hospital, Department of Obstetrics and Gynecology

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Epidemiology of herpes simplex virus (HSV) infections is changing in developed countries, including Finland, where primary HSV infection is now currently being acquired close to reproductive age. HSV infections are usually subclinical, but oral HSV shedding is frequent, which can lead to viral transmission. In addition, HSV could be one of the co-factors of persistent human papillomavirus (HPV) infection, which can lead to oral epithelial dysplasia or oral cancer.

This thesis is part of the longitudinal Finnish Family HPV Study of 329 families. The focus is on oral epithelial HSV carriage and its co-carriage with oral HPV, as well as HSV seroprevalence among pregnant women and their male spouses during a six-year follow-up study. We also evaluated the impact of pregnancy, sexual habits and demographic factors on acquisition of HSV infection.

HSV seroprevalence was 59% (168/285) among these healthy young women near delivery and 53% (64/120) among their male spouses. HSV DNA in oral epithelium was common among seropositive couples, 19% (31/168) of the seropositive mothers' and 20% (13/64) of the seropositive fathers' oral specimens being HSV-1 DNA positive. HPV-HSV co-infection in a single oral specimen was rare. Age, genital HPV and the presence of genital warts were associated with HSV seropositivity, while young age was associated with HSV seroconversion in these women. Oral sex and urethral high-risk HPV was associated with HSV-seropositivity among the male spouses.

To conclude, parents at childbearing age do acquire primary HSV infections in Finland, and HSV-1 DNA in oral epithelium is common among HSV seropositive subjects. Sexual habits may also contribute to HSV transmission among young women and men.

Keywords: herpes simplex virus, HSV, human papillomavirus, HPV, oral cavity, oral sex, seroprevalence, seroconversion, shedding, women, men, pregnancy, follow-up

TIIVISTELMÄ

Johanna Mäki

Herpes simplex -viruksen (HSV) esiintyminen suun limakalvoilla ja seerumin HSV-vasta-aineet kuuden vuoden seurannassa suomalaisilla raskaana olevilla naisilla ja heidän puolisoillaan

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Herpes simplex -viruksen (HSV) epidemiologia on muuttunut viimeisimpien vuosikymmenten aikana kehittyneissä maissa, myös Suomessa. Suurin osa HSV-infektioista on oireettomia, vaikka HSV:tä erittyy suuhun, josta HSV voi syljen välityksellä tarttua toiselle henkilölle. Viimeisimmät tutkimukset osoittavat, että HSV-1-infektio voi olla eräs riskitekijä, joka vaikuttaa ihmisen papilloomavirus-infektion eli HPV-infektion kroonistumiseen epiteelissä. Krooninen HPV-infektio voi pahimmillaan johtaa suusyövän kehittymiseen.

Väitöskirjatutkimus on osa seurantatutkimusta, jossa on selvitetty HPV-infektioiden dynamiikkaa ja sen säätelytekijöitä 329 suomalaisessa perheessä. Väitöskirja keskittyy selvittämään HSV- ja HPV-yhteisinfektion yleisyyttä suun limakalvon irtosolunäytteissä sekä seerumin HSV-vasta-aineiden esiintyvyyttä raskaana olevilla naisilla ja heidän puolisoillaan 6 vuoden seurannassa. Lisäksi arvioitiin raskauden, seksikäyttäytymisen ja muiden demografisten tekijöiden vaikutusta HSV-infektion esiintyvyyteen.

Nuorista, loppuraskaudessa olevista äideistä 59 % (168/285) ja heidän puolisoistaan 53 % (64/120) oli HSV-seropositiivisia. Heillä HSV:n esiintyvyys suun limakalvon irtosolunäytteissä oli suuri; 19 % (31/168) seropositiivisilla naisilla ja 20 % (13/64) heidän seropositiivisilla puolisoillaan. Samanaikainen suun HSV- ja HPV-infektio oli sen sijaan harvinainen. Naisilla ikä ja genitaalialueen HPV sekä syylät eli kondyloomat olivat yhteydessä HSV-seropositiivisuuteen. Naisen nuori ikä liittyi HSV-serokonversioihin. Naisten puolisoilla suuseksi ja virtsaputken HPV liittyi HSV-seropositiivisuuteen.

Yhteenvetona, HSV primaari-infektioita esiintyy lisääntymisiässä olevilla henkilöillä Suomessa. HSV:n erittyminen suun pintaepiteeliin on yleistä HSV-vasta-ainepositiivisilla henkilöillä. Lisäksi seksikäyttäytyminen saattaa vaikuttaa HSV:n esiintymiseen.

Avainsanat: herpes simplex virus, HSV, ihmisen papilloomavirus, HPV, suu, suuseksi, seroprevalenssi, serokonversio, erittyminen, nainen, mies, raskaus, seuranta

TABLE OF CONTENTS

ABBREVIATIONS

AIM2	absent in melanoma 2
CMV	cytomegalovirus
cGAS	cyclic GMP-AMP synthase
CNS	central nervous system
DAMP	damage-associated molecular pattern
EBV	Epstein-Barr virus
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
gC / D / E / I …	glycoprotein C / D / E / I …
HHV	human herpesvirus
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HPV	human papillomavirus
hrHPV	high-risk HPV
HVEM	herpesvirus entry mediator
ICP	infected cell protein
IFI16	interferon-gamma-inducible protein 16
IFN	interferon
IgG, IgM, IgA	immunoglobulin G, M and A
LAT	latency associated transcript
LSIL, HSIL	low-grade/high-grade intraepithelial lesion
lrHPV	low-risk HPV
MDA-5	melanoma differentiation-associated protein 5

MHC	Major histocompatibility complex
NF-κB	nuclear factor kappa B
NK	natural killer (cell)
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PRR	pathogen recognition receptor
p53	tumor suppressor protein 53
vDNA, vRNA	viral deoxyribonucleic acid, viral ribonucleic acid
(HN)SCC	(head and neck) squamous cell carcinoma
qPCR	real-time quantitative PCR
RIG-I	retinoic acid-inducible gene I
Rb	retinoblastoma proteins
SD	standard deviation
TG	trigeminal ganglia
TLR	toll-like receptor
TMD	temporomandibular disorders
US	Unique short (segment)
UL	Unique long (segment)
VZV	varicella-zoster virus

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals (I-III), and on additional data presented in this thesis.

I Mäki J, Paavilainen H, Grénman S, Syrjänen S, Hukkanen V. Carriage of herpes simplex virus and human papillomavirus in oral mucosa is rare in young women: A long-term prospective follow-up. 2015. Journal of Clinical Virology. 70, 58–62.

II Mäki J, Paavilainen H, Kero K, Hukkanen V, Syrjänen S. Herpes simplex and human papilloma virus coinfections in oral mucosa of men – a six-year follow-up study. 2018. Journal of Medical Virology. 90, 564-570.

III Mäki J, Vuorinen T, Rautava J, Kero K, Syrjänen S, Hukkanen V. Herpes simplex virus seroprevalence among pregnant women and their spouses – a six-year follow-up study. Manuscript.

1 INTRODUCTION

Herpes simplex virus (HSV) is a large DNA virus which infects humans worldwide. HSV exists as two serotypes: HSV-1 and HSV-2. Characteristic for HSV is its ability to cause vesiculoulcerative infections at various body sites. HSV can become latent in the host for life and can reactivate to cause lytic or subclinical infections. Orofacial infections are mostly caused by HSV-1 and only a fraction of infections are caused by HSV-2. In the oral region HSV infections are usually subclinical and only shedding of HSV to the saliva can be detected, but sometimes can occur as gingivostomatitis or herpes labialis.

Shedding of infectious HSV occurs intraorally at different mucosal sites without any clinical symptoms of disease. HSV DNA can be detected regardless of the detection of HSV antibodies. Approximately 70% of the population shed HSV into saliva at least monthly (or even more frequently). Oral shedding is observed in cross-sectional time-points in 2-9% of total cases. HSV shedding declines with age and is most prevalent among children and immunocompromised subjects. The role of saliva in controlling reactivations and horizontal transmission of HSV-1 *in vivo* is unclear.

In developed countries, HSV seroprevalence has changed during recent decades. Currently, it is known that there is no significant increase in HSV-1 seroconversion after childhood until the second and third decades of life. In developing countries and among lower socioeconomic populations, most of the primary HSV-1 infections are still acquired at early childhood. The most important factors influencing HSV seroprevalence are age, geographic location, socioeconomic status and behavioral factors, e.g. sexual behavior.

Human papillomavirus (HPV) is another DNA virus that infects oral epithelial cells, and persistent infection with HPV has been associated with an increased risk for the development of oral squamous cell carcinoma and its precursors. Several factors, for example tobacco and alcohol, are known exogenous risk factors for HPV persistence. Recent studies implicate that epithelial co-infection with HSV-1 might be an additional risk factor for HPV persistence.

This thesis focuses on the frequency of HSV-1 and -2 shedding in oral mucosa. The seroprevalence and seroconversion of HSV infections and their association with coincident oral HPV among healthy Finnish pregnant women and their male spouses were examined during a six-year follow-up study. In addition, this work aimed to identify the risk determinants of HSV infection according to behavioral data such as smoking, alcohol intake and sexual habits. The possible effects of pregnancy on the incidence of HSV infections were also studied.

2 REVIEW OF LITERATURE

2.1 Herpesviruses

Historically all herpesviruses have been classified into one family, *Herpesviridae*, based on the virion architecture, and into three subfamilies, *Alpha-, Beta-* and *Gammaherpesvirinae*, based on their biological properties (Roizman et al., 1981). However, these morphologically similar viruses differ from each other in terms of their host-cell range, viral life cycle, mechanisms of infection and the diseases they cause. Availability of nucleotide sequence data led to changes in the taxonomy and to the establishment of the order *Herpesvirales* (Pellett et al., 2012), which includes three virus families based on their host. The *Herpesviridae* family include herpesviruses which infect mammals, reptiles and birds. Herpesviruses with human hosts (Human herpesviruses, HHV) are the Herpes simplex viruses (HSV-1 and -2 or HHV-1 and -2), Varicella-zoster virus (VZV or HHV-3), Epstein-Barr virus (EBV or HHV-4), Cytomegalovirus (CMV or HHV-5), Human herpesviruses 6A, 6B and 7 (HHV-6A, HHV-6B, HHV-7) and Kaposi sarcoma associated herpesvirus (HHV-8).

More than 200 herpesviruses have been identified to date. In general, the herpesviruses have a wide range of hosts, but only a minority infect more than one host species (Pellett and Roizman, 2013). Herpesviruses are therefore highly adapted to their hosts, as they have evolved side by side. For example, the migration and evolution of humans from their origins in Africa is in line with the global distribution of herpesviral genetic clades (Black et al., 1974; Hayward, 1999). Herpesviruses are ancient viruses whose evolution is millions of years old and can be traced to times when there was only one continent on earth (Grose, 2012). In addition, the human herpesviruses have been found in isolated Brazilian tribes, which implicates that herpesviruses infect humans worldwide (Black et al., 1974).

Herpesviruses share many biological properties: they specify a wide range of different nucleic acid metabolizing enzymes, their viral DNA synthesis is similar, the gene transcription and nucleocapsid assembly occurs in the host cell nucleus, and most virions acquire the tegument and the envelope from the host cell. Production of progeny herpesviruses takes place in lytic infections, which lead to destruction of the host cell. Herpesviruses also share the ability to establish cellular latency and therefore persist for life (Forghani et al., 1977), or even after life (Ouwendijk et al., 2012).

Herpes simplex viruses (HSV-1 and -2)

Hippocrates was the first to describe symptoms that could have been caused by the Herpes simplex virus (HSV), when he used the word *herpes* to describe lesions that seemed to "crawl along the skin". However, most of the key findings of HSV infection and treatment have been made since the early 20th century and more research is still needed to fully understand and treat HSV infections (Roizman and Whitley, 2001). HSVs were the first human herpesviruses found and may be the most intensively studied of all viruses. Interest in HSV might come from their biological properties, as HSV can cause asymptomatic or vesiculoulcerative infections at various mucocutaneous or neuronal tissues. HSV can become latent in the host for life, and can reactivate to cause lytic or subclinical infections at or near the primary infection site. HSVs are also great tools for the study of lipid membranes, synaptic connections, gene regulation, gene therapy and cancer therapy, where HSVs serve as vectors (Roizman et al., 2013).

Herpes simplex viruses (HSVs) are included in the family *Herpesviridae,* in the subfamily *Alphavirinae* and within the genus *Simplexvirus*. HSV appears as two serotypes: HSV-1 and HSV-2. HSVs are human pathogens which infect mostly oral and genital mucosa as well as the skin and sensory neurons (Whitley and Roizman, 2001). However, HSV can also infect other species when it is inoculated experimentally, e.g. to mice or rabbits (Hill et al., 2012). Previously, HSV-1 has infected mostly the oro-facial area, but during recent decades it has also become more prevalent in the genital area (Lafferty et al., 2000). HSV-2 infects almost solely the genital area, but it can rarely also infect oral mucosa. Both HSV-1 and HSV-2 have a fast replication cycle and a broad host cell range, and are a major cause of morbidity and mortality globally (Looker and Garnett, 2005).

2.1.1 The structure of the herpes simplex virus virion

The simplified structure of the large and structurally complex herpes simplex virus (HSV) virion is shown in Figure 1. The most detailed analysis of the virion structure was made with cryomicroscopy to 7-nm resolution, which defined the average HSV virion diameter of 186 nm, extending to 225 nm with glycoprotein spikes included (Grünewald et al., 2003). The virion consist of a spherical shaped outer lipid bilayer membrane envelope with 600-750 varying length glycoprotein spikes on its surface (Grünewald et al., 2003). The envelope contains approximately 11 different viral glycoproteins, of which four (gD, gH, gL and gB) are essential for entry to the host cell (Campadelli-Fiume et al., 2012). The envelope is received from the host cell and possesses similar lipid content to that found in

the cellular cytoplasmic membrane (van Genderen et al., 1994; Heming et al., 2017). Under the envelope, there is an asymmetric proteinaceous tegument which occupies about two-thirds of the volume within the virion (Grünewald et al., 2003; Heming et al., 2017). The tegument, consisting of short actin-like filaments, contains around 23 different proteins and serves as a delivery compartment for proteins needed early in the infection cycle (Grünewald et al., 2003). Some studies show that the tegument also contains cellular gene transcripts (Sciortino et al., 2001; Loret et al., 2008). The tegument surrounds a symmetric (T=16) icosahedral lattice protein capsid, which is 125 nm in diameter (Brown and Newcomb, 2011; Heming et al., 2017). The capsid consists of four main viral proteins, at least five other structural proteins and 161 capsomers (Wildy et al., 1960; Heming et al., 2017). The viral DNA is located in the electron opaque core inside the capsid and the negative charge of the DNA molecule is balanced by polyamines, spermine and spermidine (Gibson and Roizman, 1971; Pohjanpelto et al., 1988).

HSV genome

The HSV genome comprises an average 152-kbp double-stranded and linear DNA molecule (Kieff et al., 1971), which is wrapped as a toroid (Furlong et al., 1972) or a spool (Zhou et al., 1999) in a liquid crystalline arrangement (Booy et al., 1991). However, when the HSV DNA molecule is transferred to host cell nucleus, it associates with histones and the viral DNA arranges into the shape of a circular episomal DNA molecule (Jackson and DeLuca, 2003; Sandri-Goldin, 2003; Kent et al., 2004). It has been suggested that the viral DNA exists in circular form mainly in latent or quiescent infections (Jackson and DeLuca, 2003; Sandri-Goldin, 2003). The simplified model of the HSV genome is shown in the Figure 1. The HSV genome structure consists of long (L) and short (S) covalently joined segments, and of repetitions R_L (a b) and R_S (c a) and inverted repetitions IR_L (b'a') and IR_S (a'c') (Wadsworth et al., 1975). Short "a" sequences are present as a direct repeat at both termini and as an inverted form at the L/S joint (Quinn et al., 2000). The "a" sequences are highly conserved and control the packaging and cleavage of HSV DNA (Umene et al., 2008). The genomes of HSV-1 and HSV-2 show 83% identity, and differences can be seen in the location of their endonuclease cleavage sites and the sizes of their encoded proteins (Dolan et al., 1998). The genomes of different strains of HSV-1 and -2 show polymorphism and even within i.e. the virus strain 17+, there seems to be a mixture of slightly different genomes (Parsons et al., 2015). HSV has at least 90 transcriptional units, of which 84 encode different polypeptides needed in the viral life-cycle (Whitley and Roizman, 2001). The HSV genes can be classified in three kinetic classes: α (immediate-early), β (early) and ɣ (late) genes.

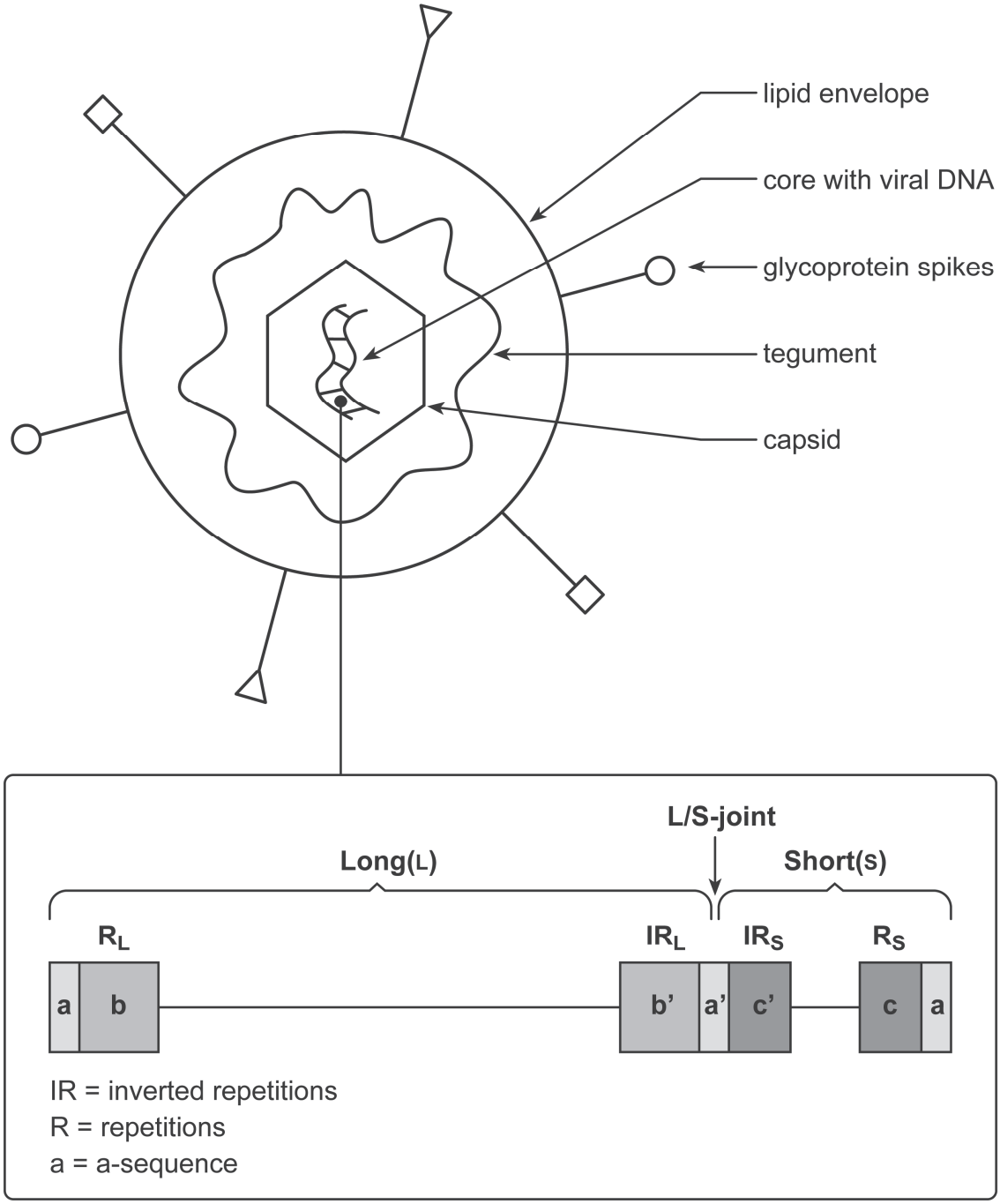

Figure 1. The simplified presentation of the herpes simplex virus (HSV) and HSV genome. The HSV virion constitutes of multiple type surface glycoproteins (n=11), lipid bilayer envelope, proteinaceous tegument, icosahedral capsid and viral DNA. All of the HSV genes consist of long (L) and short (S) segments which comprise DNA repetitions (R) and their inverted DNA repetitions (IR). The repetitions (c, b) and their inverted repetitions (c', b') contain mainly regulatory genes. Short DNA sequences (a, a') are located between the L and the S segments and at both gene termini.

2.2 Life cycle of HSV infection

HSV can be transmitted to susceptible individuals in close contact with a HSV positive individual or mediated by infectious body fluids (saliva, genital fluids, and exudates of active lesions). For transmission, HSV needs to come into contact with a mucosal surface or abraded skin (Arduino and Porter, 2007). At the epithelium of the primary infection site, HSV induces replication of the viruses in the epithelial cells, which causes the epithelial cells to rupture and produce blisters and ulcers on the affected mucosal or cutaneous surface. After the epithelial infection, the sensory nerve endings that innervate the affected area usually become infected as well. HSV uses a retrograde transport (Luxton et al., 2005) to travel via sensory nerves to colonize the trigeminal ganglion (Figure 2) at the orofacial area (Baringer and Swoveland, 1973; Quinn et al., 2000) and the sacral ganglion at the genital area (Johnston and Corey, 2016), where a more restricted HSV replication occurs, culminating in a lifelong latency of the sensory neurons (Decman et al., 2005). Previous infection with other HSV (type 1 or 2) can offer only partial protection against the other HSV type. In addition to sensory nerves, the autonomous nervous system can also harbor HSV persistently, e.g. HSV-1 can reactivate from autonomic ciliary ganglion independently from the sensory trigeminal ganglion (Lee et al., 2015).

The biological cycle of the herpes simplex viruses can be divided into three major steps. First is the initiation of infection, when the virus has managed to bind to the host cell surface of the mucosal or cutaneous epithelium. The second component is lytic replication in the host cell and is generally always accompanied by the third stage of latency within the neuronal cell. Biological selection is made in the infected cell, entering either the lytic replication or the latent pathway. From the latent stage, HSV is able to activate into the lytic stage. However, every biological step is dependent on viable host cell proteins, cellular systems and structures, from which the virus adapts to its own purposes. A schematic representation of the HSVs biological cycle is shown in Figure 3 and the three stages of the HSVs biological cycle are explained in more detail in the next sections.

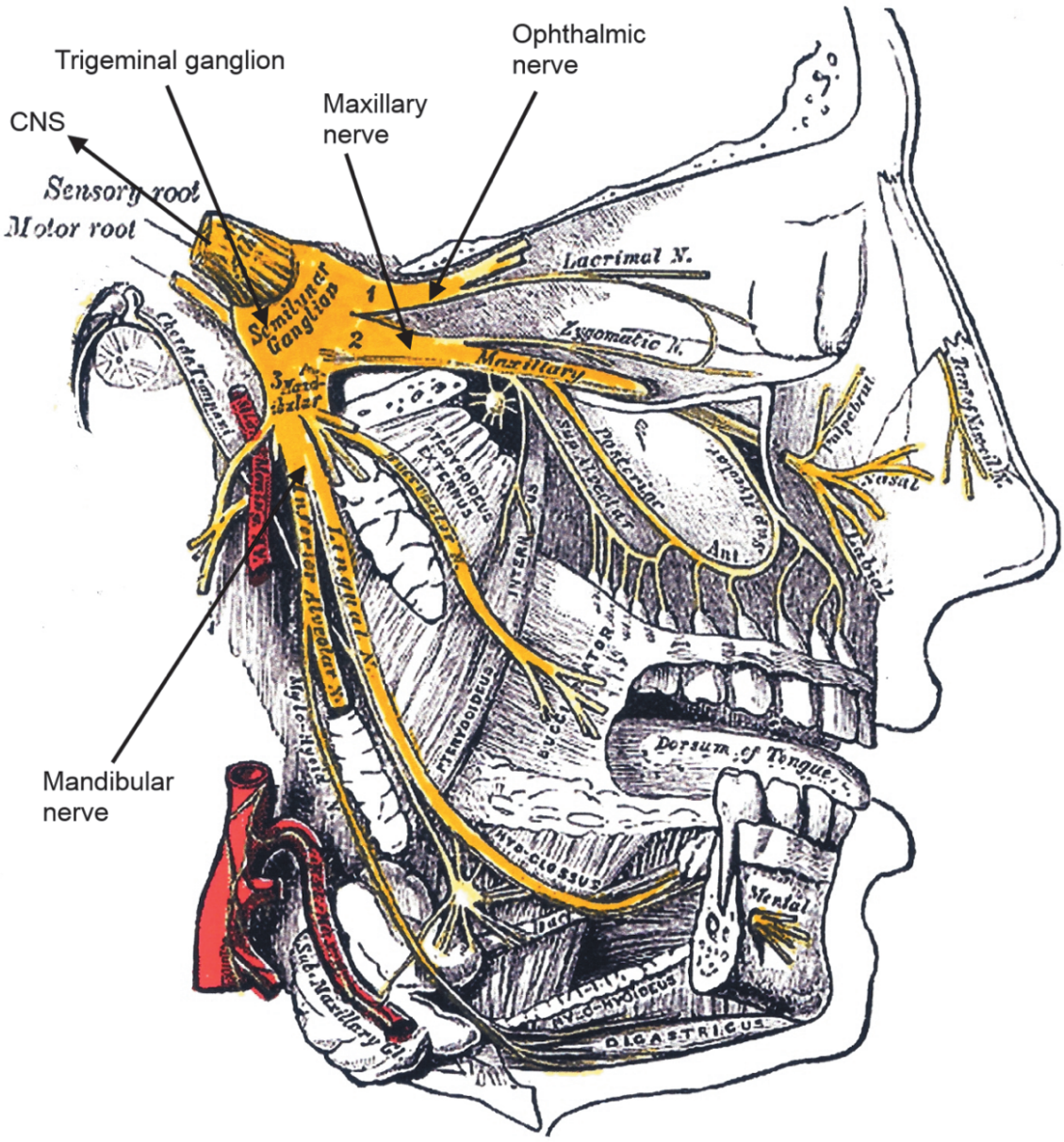

Figure 2. Nervus trigeminus. The trigeminal nerve is divided into three branches: the ophthalmic nerve (eye), the maxillary nerve (upper jaw) and the mandibular nerve (lower jaw). HSV stays latent at the trigeminal ganglion. Modified from a picture from the book *Anatomy of the Human Body*, Philadelphia: Lea & Febiger, 1918; Bartleby.com, 2000, drawn by Henry Gray. The original image modified by the additional arrows.

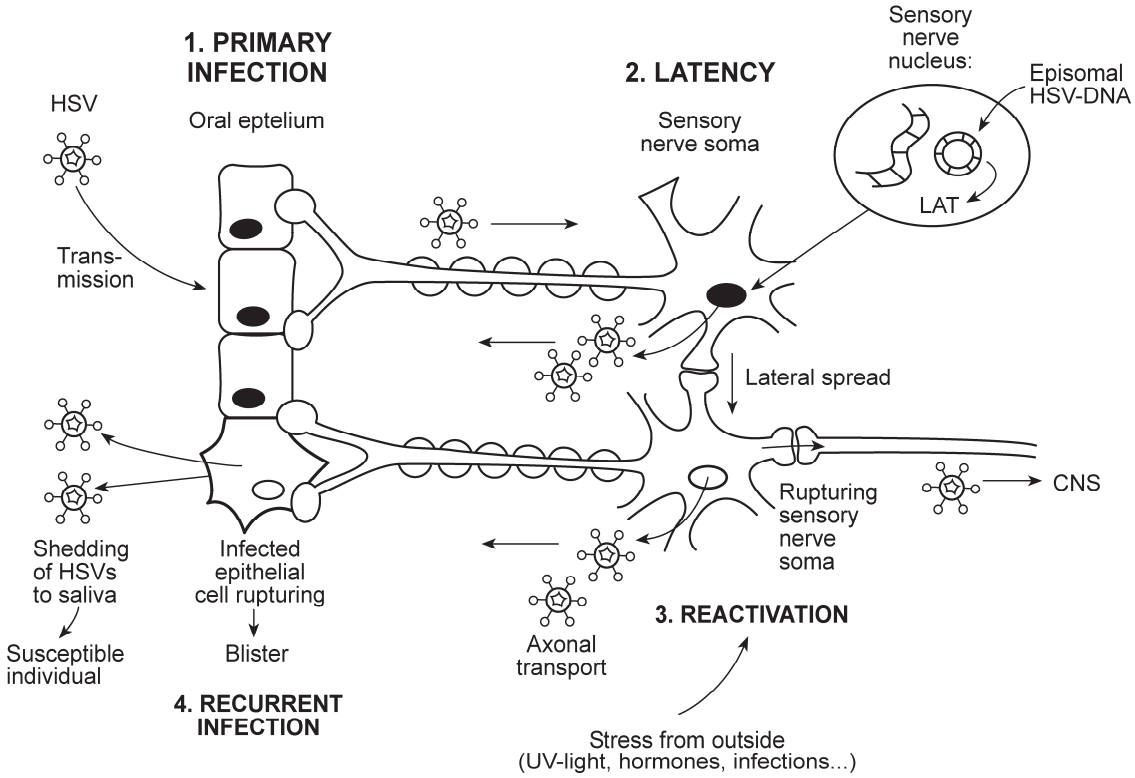

Figure 3. The biological cycle of the HSV infection. Herpes simplex virus causes primary (1) and recurrent (secondary) (4) infections at the orofacial epithelium and skin. During the epithelial infection, the HSVs are shed to the saliva (1 and 4). The shed HSV can be transmitted to susceptible individuals. Between recurrences, HSV stays latent at the nucleus of the sensory nerve, in the form of an episomal DNA molecule (2). Different stimuli can cause reactivation of HSV (3) in the sensory nerve, and HSV uses axonal transport to infect oral epithelial cells, or rarely central nervous system or eye.

2.2.1 Initiation of infection with HSV

Infection initiation begins with receptor binding of HSV to the host cell surface receptors (Nicola and Straus, 2004) and then, according to the host cell type, the HSV envelope either fuses to the cell membrane (Lycke et al., 1988; Wittels and Spear, 1991) or the virus enters the host cell with endocytosis (Nicola et al., 2003). Viral surface glycoproteins have a major and complex role in the entry of HSV into the cell (Heldwein and Krummenacher, 2008; Campadelli-Fiume et al., 2012). HSV-1 entry into cells is mediated by at least two receptors, herpesvirus entry mediator (HVEM) and nectin-1, which both bind to the HSV glycoprotein D (gD) (Petermann et al., 2015; Kurt-Jones et al., 2017). Upon entry to the cell, the tegument-capsid structure is released to the host cell cytoplasm. Then the HSV capsid harboring the viral DNA is transported to the nucleus of the host cell

via microtubules (Kristensson et al., 1986; Sodeik et al., 1997) and the HSV DNA is released to the host cell nucleus (Ojala et al., 2000; Marozin et al., 2004), during which time the linear viral DNA becomes circular (Jackson and DeLuca, 2003; Sandri-Goldin, 2003; Strang and Stow, 2005).

2.2.2 Lytic infection with HSV

Lytic infections occur during primary infection with HSV and also during reactivation from the latent stage in the epithelial and in the neuronal cells (Figure 3). Prior to viral replication, the virus needs RNA polymerase II (Alwine et al., 1974) and other host cell proteins to use those for its own viral replication. During the viral replication, HSV genes are expressed in a cascade fashion. At the beginning of the HSV gene transcription, α genes are expressed and the process is stimulated by a VP16 tegument protein (Batterson and Roizman, 1983). HSV genes are thereafter expressed from α (immediate-early) to β (early) and finally ɣ (late) genes. The α genes regulate viral gene expression and peak rates are detected 2-4 hours after the initiation of HSV infection. The β genes regulate viral DNA replication and are detectable 4-8 hours after the infection onset. Viral DNA synthesis has been initiated, and the ɣ genes start to regulate the assembly of the progeny HSV virions.

HSV capsids are assembled in the host cell nucleus. Four capsid types are formed from the fragile precursors called procapsids (Rixon and McNab, 1999). These different capsid types differ in the minor proteins of the capsid interior and exterior surfaces (Newcomb et al., 2000). The filled HSV capsids continue to maturate by addition of viral and cellular tegument proteins and by final envelopment in the cytosol. There has been debate about how the maturing HSV nucleocapsids reach the cytosol, but the dual envelopment is currently considered the most likely path (Skepper et al., 2001; Mettenleiter et al., 2013). First, the filled capsid buds through the inner lamella of the nuclear membrane driven by two HSV proteins, pUL34 and pUL31 (Sam et al., 2009; Mettenleiter et al., 2013). This process forms the so-called primary envelope, which fuses to the outer nuclear membrane, and the HSV capsid is transmitted to the host cell cytosol (the envelopment-deenvelopment pathway) (Mettenleiter et al., 2013). The HSV virion envelope is then processed by transit through the host cell membranes. The entire viral replication process takes approximately 18-20 hours (Roizman et al., 2013).

When the new infective HSV virions are packaged within the host cell cytoplasm, the virions leave the host cell through membrane fusion to infect neighboring cells by budding and with glycoprotein E/glycoprotein I (gE/gI) complex-mediated lateral spread via cell-to-cell tight junctions (Dingwell et al., 1994),

which ultimately leads to host cell death. Cell death is the cause of HSV infection symptoms, and is also the reason for asymptomatic shedding of the virus to the mucocutaneous surfaces. New HSV virions are transmitted laterally to neighboring cells (Dingwell et al., 1994) or to other susceptible individuals. A HSV plaque is then formed, as seen by light microscopy *in vitro* (Figure 4) and the HSVs can be stained with immunohistochemistry (Figure 5). *In vivo* the HSV plaque can be seen as a mucocutaneous blister.

2.2.3 The histology of the lytic HSV infection

When a mucocutaneous HSV plaque is observed using light microscopy during both primary and secondary HSV infections, the microscopic characteristics represent a combination of virus-induced cell death and associated inflammation (Roizman et al., 2013). Host cells balloon during infection, and their nuclei show condensed chromatin followed by subsequent degradation, mostly at the parabasal and intermediate layers of epithelium. Eventually cells lose their plasma membrane and form multinucleated giant cells, especially in infections with so-called syncytial HSV strains. Vesicular fluid from the cells containing the virus, cell debris and inflammatory cells appear between the epidermis and dermal layer. Intense inflammatory response is seen around the vesicle. Blisters are seen in the skin, but in the mucous membranes the blisters soon rupture and form prominently shallow ulcers. Scarring is uncommon but can be seen in individuals with frequent recurrences. Within the neural cells the same pathologic features are seen, but local analgesia is not caused, as only few individual neurons are damaged at a time, as the other sensory neurons grow to innervate the healed area. In addition, perivascular cuffing and hemorrhagic necrosis can be seen, predominantly in organs other than skin (Roizman et al., 2013).

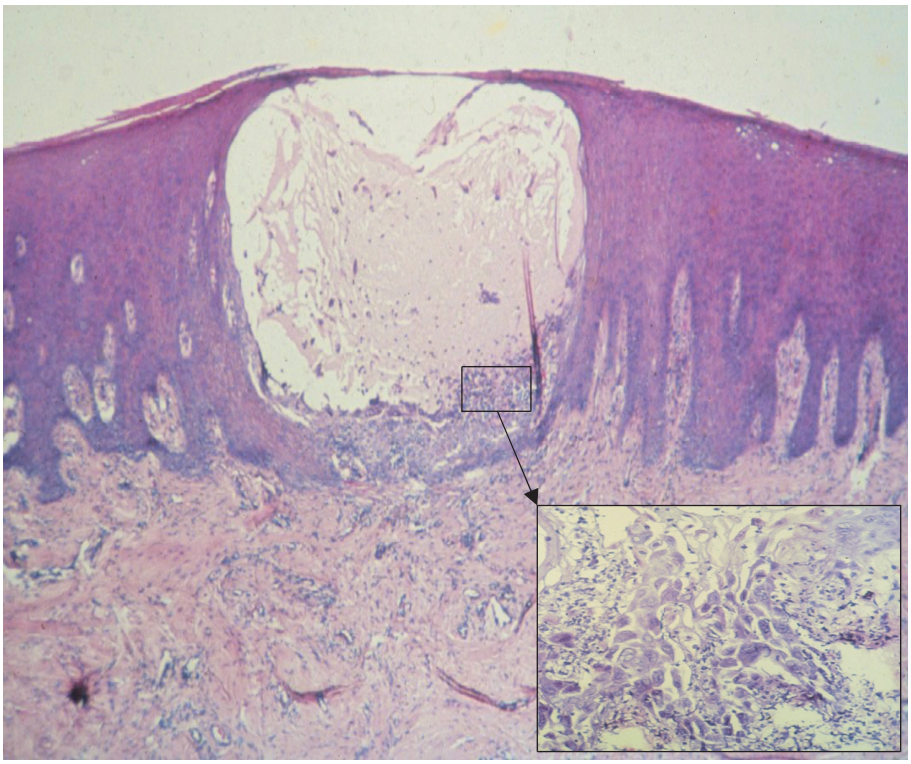

Figure 4. The micrograph of a blister from oral mucosa with the cytopathic features of HSV infection. Courtesy of Stina Syrjänen.

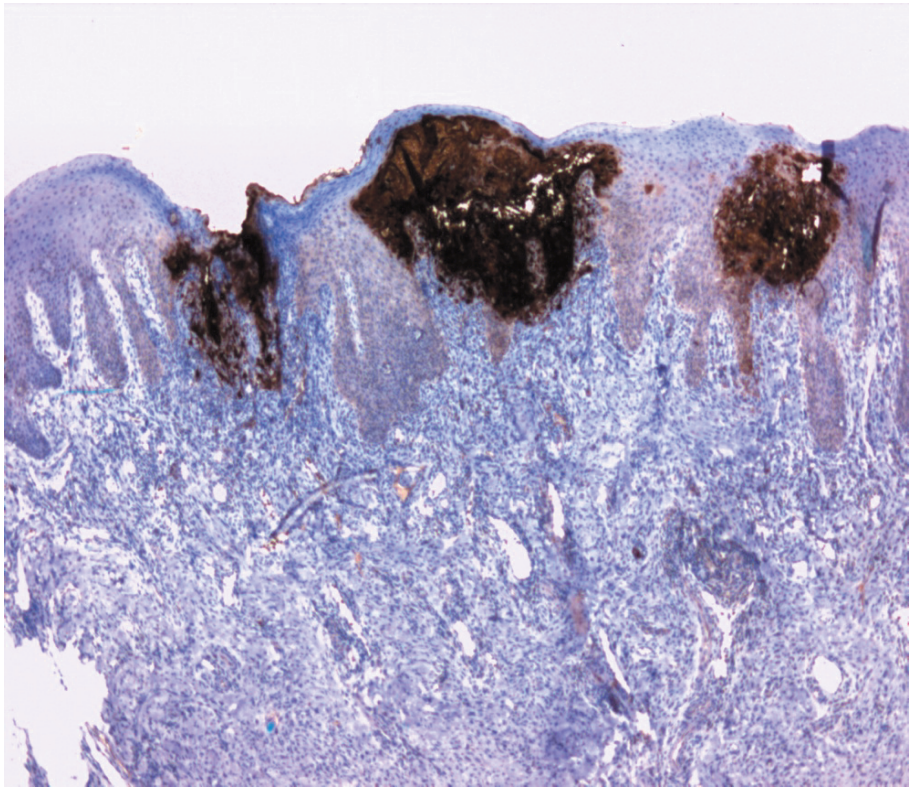

Figure 5. The micrograph of oral mucosa stained with immunohistochemistry to detect the HSV infected cells. The epithelial cells that contain the virus are stained brown. Courtesy of Stina Syrjänen.

2.2.4 HSV latency

Cellular latency is a distinct feature of herpes simplex viruses: HSV hides from its host in the nucleus of the sensory neuron in the trigeminal or in the sacral ganglion. There is no ganglionic site preference for either the HSV-1 or the HSV-2. This implicates that there might be some local host factors that induces HSV-1 to be mostly found in the trigeminal ganglion and HSV-2 in the sacral ganglion (Obara et al., 1997). Recent studies also show that the autonomous nervous system can harbor HSV persistently (Lee et al., 2015). The HSV hides in form of a non-integrated episomal DNA molecule. The HSV genome is repressed and no infective virions are made. No viral genetic encoding is needed for establishment of latency, however, for reactivation HSV needs a full repertoire of viral genes (Ward and Roizman, 1994). However, during latency, a long non-coding RNA called latency associated transcript (LAT) is present in the host cell nucleus (Figure 3) and it has a role in maintaining the latent stage of HSV (Stevens et al., 1987; Cliffe et al., 2009). In addition to LAT, latency-associated viral microRNAs are also expressed, which are thought to down-regulate the genes that are needed for lytic infection (Umbach et al., 2008; Jurak et al., 2010). The activity of the latency-associated HSV gene area and the inactivity of the lytic genes are due to different modifications of histones that bind viral DNA (Kubat et al., 2004; Wang et al., 2005).

The latent HSV genomes retain the ability to reactivate to lytic stage, which is preceded by a non-cascade type of gene expression (Camarena et al., 2010; Mattila et al., 2015). Even during active lytic HSV infection, a part of the sensory neurons harboring HSV genome are found to be at the latent repressed state, while the other neuron populations show lytic HSV genome activity at the same time (Margolis et al., 1992). Previous studies also show that 10-30% of the host neurons in the trigeminal ganglion can be latently infected (Maggioncalda et al., 1996; Sawtell, 1997). A positive correlation is seen between the probability of *in vivo* reactivation of HSV-1 and a higher number of latently infected neurons in the ganglia (Sawtell, 1998). It has been theorized that a fraction of the latency-associated HSV shedding might be due to a low-level of chronic HSV infection in epithelial cells or in the autonomous nervous system, rather than a real latent HSV infection, but the viral persistence in non-neuronal tissues remains controversial (Openshaw et al., 1995; Schiffer and Corey, 2009). For example, in a previous *in vitro* study conducted in keratinocyte culture, expression of the LAT gene was seen by hybridization and HSV DNA by PCR, even though there were no signs of cytopathic effects by light microscopy (Syrjänen et al., 1996). In oral cavity, for example, the gingival sulcus has been implicated as a reservoir for viral replication and latency (Amit et al., 1992).

2.2.5 Host defense against HSV

The host defense mechanisms against HSVs include a partly overlapping inherited intrinsic and innate immunity and an adaptive immune response, which all aim to prevent pathogen entry to the body, to limit the viral replication within the host cells and to eventually eliminate the virus from the body. If the immune system is present and active during the lytic infection phase, the lytic spread of HSV is eventually halted, which is advantageous to the virus, as the host stays alive and it can continue to spread in the future. The symptomatic disease may result and continue for longer period of time if the viral load is too high or if the host is immunocompromised. HSV can manipulate the host's immune system in multiple ways, as described briefly below.

Intrinsic and innate immunity

Host defense mechanisms against HSV include physical barriers such as intact skin, constant tear rinsing of the eyes, protective mucin layer of oral mucosal surfaces, constant cleansing salivary flow in oral cavity and an antiviral neutralizing effect of saliva (Gu et al., 1995; Mikola et al., 1995; Välimaa et al., 2002). These physical barriers play an important role in the evasion of HSV infection, because the virus needs to reach the basal cells of the epithelium in order to replicate. Saliva contains numerous antiviral activities and proteins, i.e. cathelicidin, lactoferrin, lysozyme, mucins, peroxidase, salivary agglutinin, secretory leucocyte protease inhibitor, and α and β defensins which are primarily responsible for innate immunity, while salivary immunoglobulin A and G are involved in acquired immunity (Malamud et al., 2011). Lactoferrin is considered as the most important oral innate defense factor against HSV (Välimaa et al., 2002). However, the role of saliva is controversial in controlling the intraoral HSV and horizontal transmission of HSV *in vivo*, as HSV can be isolated from the saliva of asymptomatic HSV-seropositive individuals (Douglas and Couch, 1970; Spruance, 1984). Salivary immunoglobulin A (IgA) is the most prevalent Ig class in the saliva, but salivary IgG seems to be mostly responsible for the neutralization of HSVs in mouth (Douglas and Couch, 1970; Gyselink et al., 1978). Previous study has also shown that the saliva of asymptomatic HSV-seropositive individuals had higher salivary neutralizing activity than individuals with recurrent symptomatic labial herpes (Välimaa et al., 2002).

The innate defense mechanisms are always present in uninfected cells and include apoptosis, autophagy, antiviral proteins and RNA silencing. The innate immunity comprises signaling receptors and molecules that recognize HSV on plasma membranes, on endosomes and intracellularly. These include pathogen recognition receptors (PRRs), which recognize pathogen-associated molecular

patterns (PAMPs), e.g. viral nucleic acids (vDNA, vRNA) and viral proteins, and damage-associated molecular patterns (DAMPs). Innate PRRs include Toll-like receptors (TLRs), cytosolic RNA sensors (RIG-I and MDA-5) and cytosolic DNA sensors (cGAS, AIM2 and IFI16) (Rasmussen et al., 2009; Thompson et al., 2011; Knipe, 2015; Kurt-Jones et al., 2017). Innate PRRs induce the production of type I interferons (IFN-alpha, -beta and -gamma), cytokines and chemokines. TLR2, -9, and -3 initiate innate responses to HSV-1 infection, leading to type I interferon expression (Peri et al., 2008; Kurt-Jones et al., 2017). The type I IFNs are essential in controlling viral infections, as defects in IFN production or responsiveness result in uncontrolled viral replication, which can lead to severe HSV encephalitis (Schoggins and Rice, 2011; Reinert et al., 2012). TLR2 is the major plasma membrane receptor of HSV detection on the cell surface of antigen-presenting cells. Activation of TLR2 activates the nuclear factor kappa B (NF-κB) pathway, which produces inflammatory cytokines and chemokines in many cells (e.g. in macrophages, monocytes, neutrophils, glial and neuronal cells, epithelial cells and keratinocytes) (Kurt-Jones et al., 2017). The innate immune system is activated within minutes to hours after infection and includes cytokine and chemokine production, activation of complement pathway and recruitment of dendritic cells, macrophages and natural killer (NK) cells. Cytokine and chemokine production leads to recruitment of immune cells, maturation of antigen-presenting cells and development of adaptive immune response.

Adaptive immunity

The adaptive immune system is tailored to pathogens and is based on immunoglobulin (Ig) production by B cells, on T-cell responses and on immunological memory. The immunoglobulins against viruses are produced against the viral antigens, most commonly the viral envelope glycoproteins. Helper $CD4^+$ T-cells and cytotoxic (killer) $CD8^+$ T-cells recognize HSV-antigens, with the aid of antigen class I and II presentation of major histocompatibility complex (MHC). Activated $CD4^+$ T-cells secrete chemokines of which aids activation of $CD8^+$ T-cells and promotes Ig production by plasma cells (activated B-cells). In primary HSV infections, HSV-specific IgA and IgM antibodies become detectable in sera for a month. After that, virus-specific IgG antibody levels rise in the serum during the next one to five months, while the IgA and IgM antibodies decrease (Hashido and Kawana, 1997). IgG and IgM response to HSV infection is found more prominently and long-lasting against an antigen preparation derived from viral envelopes, compared to that from viral capsids (Kalimo et al., 1977). Patients with primary HSV infections show both IgG and IgM responses. During HSV reactivation, usually only IgG antibodies are detected, but if the HSV infection is severe or sometimes in normal HSV reactivations, IgM antibody response can

also be detected (Marttila and Kalimo, 1977; van Loon et al., 1985; Ziegler, 1989). In addition, after the primary HSV infection, tissue-resident HSV specific memory T cells ($CD8^+$) are found at and near the primary infection site, to provide protection for possible successive pathogen entries (Zhu et al., 2007; Gebhardt et al., 2009).

The peripheral mucosal immune system plays an important role in clearing HSV infections, as most of the oral and anogenital mucosal reactivations were shown to last for only for ≤6-12 hours (Mark et al., 2008). Immune responses to HSV gene therapy vectors, with deletion of the HSV neurovirulence gene that inhibits host interferon response, can be different from responses to wild type viruses (Broberg et al., 2002; Broberg and Hukkanen, 2005).

HSV inhibits immune system of the host

HSV prevents host cell signaling in multiple ways. HSV evades the T-cell induced host cell apoptosis (Jerome et al., 1998), which is needed for the maintenance of viral latency. It also inhibits host gene expression, degrades some host cell proteins and inhibits the complement system pathways (Kostavasili et al., 1997). Most importantly, HSV prevents antigen class I presentation of major histocompatibility complex (MHCs) on the host cell surface by ICP47, which prevents the activation of cytotoxic T-cells (Hill et al., 1995; Decman et al., 2005). Thus, the activation of the adaptive immune responses is halted. However, natural killer (NK) cell responses are not evaded that effectively by HSVs, but at least HSV-2 has the ability to disturb NK-cell function (Bellner et al., 2005). In addition, HSV can prevent antibody-mediated immune responses with the aid of viral glycoproteins (Johnson et al., 1988; Hook et al., 2008), for example, viral glycoprotein C blocks complement activation by binding the complement component C3b (Friedman et al., 1986), glycoprotein E together with glycoprotein I binds the Fc fragment of the IgG antibody on the surface of the infected cell, thus preventing the immune recognition of the cell (Johnson et al., 1988), and glycoprotein D inhibits T cell proliferation and induces their death (La et al., 2002).

2.3 Oral HSV shedding

Intraoral shedding of HSV means that an individual excretes the infective virus into the oral cavity and that the HSV DNA can be detected in epithelial cells and in saliva, for example, by the polymerase chain reaction (PCR) method. Due to the fact that HSV-1 is the prevailing infectious agent intraorally and that HSV-2 causes oral infections only rarely (Wald et al., 2004), intraoral HSV shedding is

predominantly caused by HSV-1. In some previous studies, the shedding of intraoral HSV-2 has not been found at all (Tateishi et al., 1994), but oral HSV-2 reactivation has been claimed to be common among immunocompromized, especially among HIV-positive individuals (Kim et al., 2006) and among children on chemotherapy (Aggarwal et al., 2014). Oral HSV shedding occurs during primary and the recurrent productive HSV infections and the shedding is usually clinically asymptomatic. Thus, the individuals do not know that they are transmitting the virus. Mark and coworkers (2008) reported that visible lesions were found only in 1/13 of individuals with oral HSV reactivations and in 6.8% (3/44) of anogenital HSV reactivations during a daily follow-up for 60 days (Mark et al., 2008). This asymptomatic oral shedding of HSV is considered as a major factor in the spread of the virus to susceptible individuals, e.g. from mother to child. Fortunately, during asymptomatic HSV shedding, low amounts of viral DNA are usually detected (Roizman et al., 2013) and the shedding episodes also last for a shorter period of time than in the case of symptomatic productive HSV infections (Mark et al., 2008; Miller and Danaher, 2008). However, Liljeqvist and coworkers (2009) found that among 4 individuals who showed asymptomatic and symptomatic HSV-1 shedding, the PCR copy numbers of HSV-1 DNA were similar during symptomatic and asymptomatic periods (Liljeqvist et al., 2009).

2.3.1 Anatomical sites of shedding

The specific site for intraoral HSV shedding is not known to date, but several cell types have been suggested as origins of viral excretion. For example, oral epithelium and gingival sulcular epithelium (Zakay-Rones et al., 1973; Ehrlich et al., 1983; Amit et al., 1992) (Figure 6). It has been also hypothesized that HSV in saliva could originate from the salivary glands (Kaufman et al., 1967). However, previous study found that 3.6% (22/611) of oral secretions were HSV positive by culture, but all parotid saliva samples were HSV negative (Douglas and Couch, 1970), which suggests that HSV might not be shed to the saliva from the parotid glands. Also, the neutralizing effect of saliva (IgA, IgG and neutralizing proteins) on HSV-1 has been demonstrated (Heineman and Greenberg, 1980; Gu et al., 1995; Mikola et al., 1995; Välimaa et al., 2002), but the decrease seen in viral infectivity (within 2 min and increasing over time) seemed to be minimal in the submandibular saliva (Malamud et al., 1993). Other study suggested that both submandibular/sublingual and parotid saliva possess HSV-neutralization and host cell-protective activities, but submandibular/sublingual saliva seemed to cause a greater reduction of HSV-1 replication than parotid saliva (Bergey et al., 1993).

From all intraoral shedding sites, HSV easily sheds into the saliva, causing high viral detection rates in saliva. In a rabbit model, the shedding of HSV is similar to humans, and a previous study showed that HSV-1 latent rabbits also shed HSV DNA into the saliva (Hill et al., 2012). Due to this, most of the oral HSV prevalence studies have been performed with salivary samples and little is known of the original shedding sites in the oral cavity. One study among twenty-five dental students found no statistical difference between the frequencies of asymptomatic HSV-1 DNA on the buccal mucosa (30%), lower lip (31%) and dorsum of the tongue (33%), sampled with a brush at the same time-points during 30 to 60 days, and analyzed with nested PCR (da Silva et al., 2005). In another study on 8 HSV seropositive individuals with a history of symptomatic recurrences at orofacial area, epithelial brush samples were collected daily for 5 weeks from nares, eyes and 9 oral sites and analyzed with PCR. The oral area was the most frequent site of HSV shedding, as of 271 swabs that detected HSV, 256 (95%) were from oral cavity. Detection rates in oral cavity were as follows: left lower lip (23/199), right lower lip (24/199), left upper lip (20/198), right upper lip (32/199), left palate (22/199), right palate (21/199), pharynx (28/199), tongue (32/198) (Ramchandani et al., 2016). However, in another study the percentages of HSV-1 positive eye and mouth swabs were approximately equivalent: 34% (941/2806) and 38% (1020/2723), respectively (Kaufman et al., 2005). Extraorally, HSV DNA has also been found in genital mucosa (Johnston and Corey, 2016), skin (Brice et al., 1994), neuronal tissue (Baringer and Swoveland, 1973; Ganzenmueller et al., 2012), blood (Brice et al., 1994), ocular tissue (Crouse et al., 1990) and tears (Ramchandani et al., 2016).

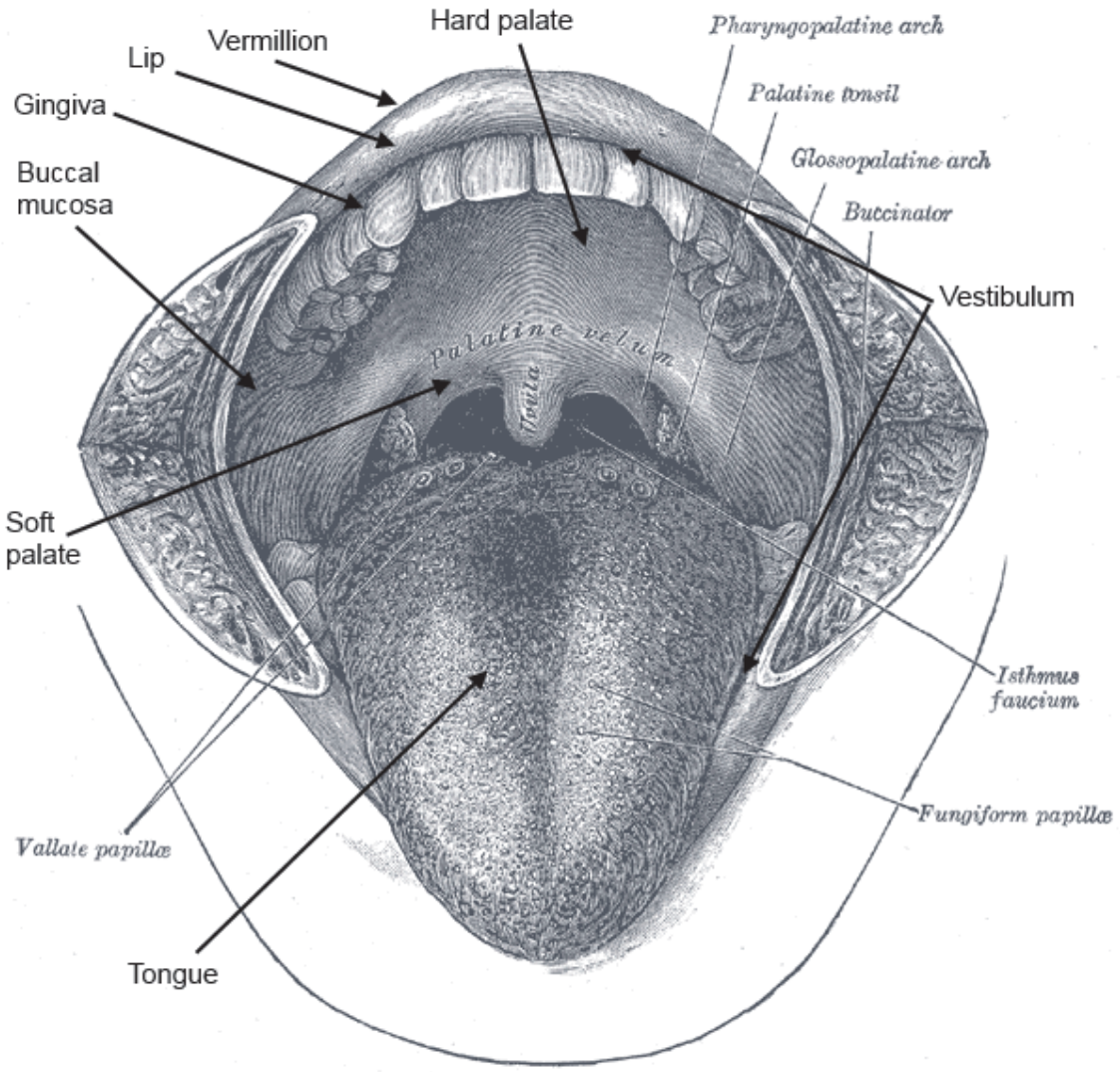

Figure 6. Oral cavity. The oral cavity is limited to the border of hard and soft palate and to the vallate papillae at the dorsum of tongue. The soft palate and the back one-third of the tongue and tonsils belong to the oropharynx, which differs from the oral cavity in the case of epithelium and disease entity. Modified from a picture from the book *Anatomy of the Human Body.* Philadelphia: Lea & Febiger, 1918; Bartleby.com, 2000, drawn by Henry Grey. Original image modified by the additional arrows.

2.3.2 Prevalence of oral HSV shedding

Oral shedding of HSV varies among individuals in different cohorts (Table 1) (Spruance et al., 1977; Miller and Danaher, 2008). In the earliest studies the asymptomatic shedding of HSV-1 into the oral cavity was found 3.6% (22/607) (Douglas and Couch, 1970) and 7.4% (47/637) (Spruance, 1984) of the specimens, based on HSV detection in cell cultures after sample inoculation. As ex-

pected, more sensitive HSV detection methods such as PCR increased the prevalence of HSV-1 shedding, from 2.7% (27/1000) detected with viral culture to 4.7% (47/1000) with PCR in the same oral samples (Tateishi et al., 1994). The detected rate of oral HSV shedding increases also according to the sampling frequency, as HSV recurrences are mostly short in clinical duration (from 4 hours to 12 days, mean 1-3 days) (Mark et al., 2008; Miller and Danaher, 2008), being rapidly cleared by the immune system of immunocompetent adults (Zhu et al., 2007). Among immunocompromised, the duration of asymptomatic oral HSV shedding is increased, to a mean of 5.8 days (Kameyama et al., 1988). To compare, among healthy individuals with primary symptomatic non-treated oral HSV disease, the shedding of HSV lasted for up to 23 days (Dodd et al., 1938; Amir et al., 1997).

The latest meta-analysis on the global prevalence of asymptomatic oral shedding showed that at least 70% of the population shed HSV to the oral cavity at least monthly or even more regularly, regardless of the level of circulating HSV antibodies (Miller and Danaher, 2008). In cross sectional surveys, HSV shedding ranged from 2 to 9% among the healthy adult population (Wheeler, 1988; Wald et al., 2004; Roizman et al., 2013). The HSV shedding is found most frequently among young, immunosuppressed and oral surgery patients, as described in more detail in next chapters. Oral shedding is also found to be lower among Asian versus Western individuals (Okinaga, 2000), and symptomatic labial herpes is seen more frequently in white individuals than in non-white (Embil et al., 1975). Between genders, no significant differences in oral HSV shedding have been noted, but in one study, men (45%, 457/1009) had a higher frequency of shedding in saliva compared to women (33%, 563/1703) (Kaufman et al., 2005).

Sometimes HSV DNA can be detected in oral cavity of HSV seronegative individuals, but obviously HSV seropositive individuals are the shedders of reactivated HSV. For example, among HSV-1 seropositive healthy individuals, 76% (19/25) tested oral HSV-1 DNA positive at least once over a period of 58-167 days with nested PCR, while HSV seronegative individuals tested HSV-1 negative in their oral samples (Knaup et al., 2000). Approximately 20-30% of HSV seropositive persons have recurrent symptomatic HSV infections in the oral cavity 1-4 times annually, especially after stressful events (Embil et al., 1975; Spruance et al., 1988; Miller and Danaher, 2008). Previous study of seropositive people without a history of orofacial blisters showed less HSV-1 shedding than did people who had symptomatic herpes labialis in the past (Knaup et al., 2000). However, other study showed that asymptomatic oral HSV shedding occurs independently of herpes labialis recrudescence, as asymptomatic oral HSV shedding was found equally both in patients with history of symptomatic

herpes labialis and in a control group without a history of oral herpetic lesions (da Silva et al., 2005). Asymptomatic oral HSV shedding is most detected during the prodromal phase (i.e. burning, tingling) of the primary disease and HSV shedding occurs in 60% of individuals who do not develop vesicles after prodromal symptoms (Scott et al., 1997a). During the acute phase of localized symptomatic oral HSV infections, HSV DNA was detected in the saliva and blood (systemic viremia) of (9/10) and (7/10) of the individuals, respectively (Youssef et al., 2002).

Table 1. The prevalence of oral HSV shedding

Study (et al.)	n (cohort)	Seropositive	Follow-up	Sampling	Method	Sampling site	Shedding (oral HSV+ /total samples)
Kameyama, 1988	110 (healthy), 55 (oral surgery), 21 (im.def.)		1-2 months	every day	culture	sputum	<1% (6/5318), 2.5% (64/2490), 6.2% (25/398)
Tateishi, 1994	1000 (oral surgery)		none	1 time (entry to hospital)	PCR, culture	saliva	PCR: HSV-1: 4.7% (47/1000), culture: HSV-1: 2.7% (27/1000)
Knaup, 2000	30 (healthy)	25 (HSV-1)	58-161 days	8-12 times	PCR	oral swab	HSV-1: 14.1% (41/290)
Druce, 2002	477 (frequent symptomatic recurrences, oral)		none	1 time (entry to hospital)	PCR	oral swab	HSV-1: 9.0% (43/477)
Kaufman, 2005	50 (healthy)	37 (HSV-1)	30 days	2 times/day	PCR	saliva	HSV-1: 37.5% (1020/2723)
Kim, 2006	109	50 (HIV)	64 (30-127) days	every day	PCR	oral swab	HSV-1: 6.6% (141/2122), 4.2% (79/1896), HSV-2: 3.5% (103/2954), 1.3% (45/3468)
Miller, 2008, meta-analyzis	>3500 (22 studies)		none to 6 months	Twice daily to 1 time	culture, PCR	saliva, oral swab	HSV: 0-92% of days tested, HSV-1: >70% of population 1 time per month, daily 33.3%
Mark, 2008	43 (healthy)	18 (HSV-1), 25 (HSV-2)	60 days	4 times/day	PCR	oral swab	HSV: 9% (98) days, 7% (254) time-points, HSV-2: once
Ramchandani, 2016	8 (healthy)	8 (HSV)	5 weeks	1 time/day	PCR	oral swab	HSV: 13.7% (256/1874), symptomatic recurrences detected

2.3.3 Association of age with frequency of oral HSV shedding

HSV shedding is most frequent during childhood, after which it declines with age. An early study showed that asymptomatic shedding of oral HSV-1 was most frequent among infants when compared to individuals 15 years old or older (Buddingh et al., 1953). The oral salivary HSV-1 shedding frequency on patient visit days was 20% (11/55) among infants aged 7 months to 2 years, 9.0% (18/199) among children aged 3-14 years and 2.4% (5/185) among adolescents aged 15 years or older (Buddingh et al., 1953). In another study, the shedding of HSV-1 to the saliva was most frequent in the age group of ≤10 years olds 17% (2/12) when compared to those >10 years olds 4.6% (45/988) ($p<0.05$) (Tateishi et al., 1994). In addition, oral HSV-1 shedding among dental patients was detected in younger patients (mean age 28.1 years), with the mean age of patients without HSV-1 shedding at 39.7 years ($p<0.02$) (Miller et al., 2005). The shedding of oral HSV is most common during childhood, but at the same time an increasing proportion of young individuals are HSV seronegative, as primary infection with HSV-1 is being acquired later in life (Bradley et al., 2014) and in the majority of the cases seronegative individuals don't shed HSV intraorally. The higher shedding among younger individuals may be explained by the reduced efficiency of reactivations among older individuals. Those who have most recently experienced primary HSV infection also have the highest shedding frequency rates (Miller and Danaher, 2008). Accordingly, among genital HSV-2 shedders, the shedding frequency decreases on average by 70% during the 10-year period after the primary genital HSV-2 infection (Wald et al., 2000).

2.3.4 The influence of immunosuppression, trauma and inflammation on oral shedding of HSV

The frequency and duration of oral HSV shedding is highest among immunosuppressed individuals due to their weakened immune system. Among 21 immunocompromised adult patients in Japan (a high use of corticosteroids or multiple chemotherapies due to malignancies), asymptomatic HSV-1 shedding was noted in 6.2% (25/398) of salivary specimens compared to 0.1% (6/5318) shedding in healthy individuals during 1-2 months, with daily samplings analyzed with cell cultures, which is less sensitive method than PCR (Kameyama et al., 1988). The duration of oral HSV shedding was increased from 1.2 days among healthy individuals to 3 days among the immunocompromised and to 5.8 days among oral surgery patients (Kameyama et al., 1988). Among HSV-2- and HIV-seropositive men, 48% (24/50) had oral HSV-2 positive epithelial samples collected with a brush during a median of 64 days, compared to 34% (20/59) HSV-2 positive oral

epithelial samples in HIV-negative men (Kim et al., 2006). In another study, human herpesviruses (EBV, HHV-8, CMV, HSV) were more prevalent in the saliva of HIV infected individuals (HSV-1 in 16% of samples) when compared to the healthy controls (HSV-1 in 2% of samples) ($p<0.008$) (Miller et al., 2006). And among HIV-infected individuals, oral HSV shedding episodes were found frequent and short in duration lasting in median 7.5 (range 4-253) hours (Mark et al., 2008). The increased reactivation of latent HSV among immunosuppressed individuals is of great importance because it can cause disseminated or even fatal outcome in these individuals (Faden et al., 1977).

Trauma and inflammation increase oral shedding of HSV. Among oral surgery patients, oral HSV shedding has been shown to be increased due to traumatic stress to the trigeminal nerves innervating the orofacial area (Figure 3) (Kameyama et al., 1989b). The traumatic stress acts as an inducing factor for the HSV recurrence. In cross-sectional study with one sampling, the oral surgery patients shed HSV-1 in the saliva in 4.7% (47/1000) of the cases, analyzed with PCR (Tateishi et al., 1994). Patients with inflammation (8.0%) and patients with oral cysts (7.2%) had HSV-1 in their saliva more often than healthy oral surgery patients (4.7%) (Tateishi et al., 1994). The severity of the oral trauma also affected HSV-1 shedding as 20% (11/55) of oral surgery patients, 17% (14/83) of orofacial fracture patients and 40% (12) of patients with oral malignant tumors shed HSV to the saliva for up to one month, analyzed with cell culture (Kameyama et al., 1988, 1989a, 1989b). One study also showed that oral HSV shedding increased during the common cold (21%) and after oral trauma (17%; $p=0.001$ and 0.04, respectively) (Spruance, 1984). More recent study also showed, that 63.5% (20/32) of patients attending for oral surgery shed HSV asymptotically after treatment. And 53% (21/40) of patients in control group attending for noninvasive dental treatment (migraine or temporomandibular disorder (TMD) -pain patients) shed HSV to saliva after treatment (Hyland et al., 2007). The number of patients positive for oral HSV-1 DNA statistically significantly increased after treatment from 10 to 20 in the oral surgery group, but not in the control group (18 to 21) (Hyland et al., 2007). These findings indicate that the frequency of asymptomatic oral shedding of HSV not only increase by direct surgical trauma, but also appears to be common among TMD and migraine patients, who experience orofacial pain.

After routine dental procedures the HSV shedding into saliva also increases for a week to months among otherwise healthy patients. The oral shedding rate of asymptomatic HSV increased within one week afterwards from 8.9% to 14% (Miller et al., 2004) and within a month afterwards from 2.7% to 13% (Hatherley et al., 1980). Clinicians should take into consideration the increased frequency and duration of HSV shedding when planning dental and orofacial operations,

especially for patients at risk of experiencing a recurrence. And previous studies recommend antiviral therapy to minimize individual morbidity as well as the transmission of the HSV (Perkins and Sklarew, 1996; Miller et al., 2004).

2.3.5 Timing of sampling and duration of oral HSV shedding episodes

The detected incidence of asymptomatic intraoral HSV shedding episodes increase when the sampling frequency is increased (Miller and Danaher, 2008). To compare, in a meta-analysis asymptomatic HSV-1 shedding was detected in 6.3% (117/1861) of healthy patients examined at one visit only, and in 12% (212/1708) of patients in multiple day samplings (Miller and Danaher, 2008). However, only 0.4% (3/744) of the patients at one day samplings and none (0/247) of patients in multiple-day samplings shed HSV-2 to the oral cavity (Miller and Danaher, 2008). Of note, HSV-1 was found in 10% (161/1548) of the patients that were sampled ≤5 times weekly and in 32% (51/160) of the patients that were sampled >5 times weekly ($p<0.001$) (Miller and Danaher, 2008).

There are only few studies that have been done to evaluate the duration of asymptomatic HSV shedding. Previously, four studies have been performed with cell culture assays and all of them reported that asymptomatic oral HSV-1 and HSV-2 shedding lasted on average 1-1.2 days (Spruance, 1984; Kameyama et al., 1988; Okinaga, 2000; Wald et al., 2004). Studies that have been conducted with PCR showed longer duration of asymptomatic oral shedding, lasting between 1 to 3 days (Miller et al., 2004; Kaufman et al., 2005). Previous study conducted with PCR showed that at least one-third of individuals shed HSV-1 intraorally for at least 3 subsequent days and 10% of individuals shed HSV-1 for 3-7 subsequent days during one-month follow-up period (Kaufman et al., 2005). A more recent study of healthy adults (25 were HSV-2 seropositive and 18 were HSV-1 seropositive), followed-up for 60 days with four specimens collected daily from oral and anogenital area and analyzed with PCR, reported that 33 oral HSV episodes detected lasted for median 24 hours and the shedding episode time ranged individually between 4 hours to 12 days (Mark et al., 2008). The median reactivation detection of oral HSV was 1.4 reactivations per person per 30 days and 16.2 reactivations per person annually, and if the sampling is decreased from 4 times daily to 1 time per day the reactivation detection per person decreases to 0.9 per 30 days and 10.8 annually (the reactivation was defined as positive HSV DNA detected in oral mucosa) (Mark et al., 2008). Visual HSV lesions were found only in 7% (3/44) of anogenital reactivations and in 8% (1/13) of oral reactivations (Mark et al., 2008). Among individuals with primary symptomatic non-

treated oral HSV disease, the shedding of HSV lasted for up to 23 days (averagely 7 to 10 days) (Dodd et al., 1938; Amir et al., 1997).

2.3.6 The effect of antiviral therapy to oral HSV shedding

The efficient use of antiviral drugs (valacyclovir, acyclovir) before or during the HSV reactivations decreases the amount and duration of oral HSV shedding (Amir et al., 1997). Antiviral drug use could minimize the risk of HSV transmission to susceptible individuals, as shown in genital mucosa (Corey et al., 2004). HSV shedding among children experiencing gingivostomatitis was significantly shorter in the acyclovir treatment group than in the placebo group (1 versus 5 days) (Amir et al., 1997). Antiviral drugs also relieve and shorten the duration of symptoms of the HSV disease (Välimaa et al., 2013). Among gingivostomatitis children, those who received acyclovir treatment had oral lesions for a shorter time period when compared to the placebo group (4 versus 10 days) (Amir et al., 1997). Especially among orofacial surgery patients who have a history of oral herpetic lesions, it has been shown that the incidence of postoperative herpetic lesions were noted more often in an absence of prophylactic acyclovir (50%, 6/12) than among patients who received prophylactic acyclovir (8.3%, 4/48) (Perkins and Sklarew, 1996). Also, 6.6% of patients (no acyclovir treatment) with no history of previous orofacial herpes lesions developed postoperative HSV recurrences (Perkins and Sklarew, 1996). The authors concluded that all patients going through perioral surgery should be treated with prophylactic acyclovir. However, antiviral drugs cannot prevent HSV reactivations completely (Johnston et al., 2012), and therefore new medications are needed to be developed to fully prevent HSV shedding. Treatment of HSV infection is described in the chapter 2.5.4 and reviewed previously (Vere Hodge and Field, 2013; Birkmann and Zimmermann, 2016).

2.4 HSV epidemiology

HSV infections occur worldwide in both developed and developing countries, without seasonal variation, in all age groups. HSV has also been found in desolated tribes (Black et al., 1974; Whitley and Roizman, 2001). The primary factors that influence the acquisition of HSV infections are geographic location, socioeconomic status and age (Dodd et al., 1938). Seroprevalence for both HSV-1 and HSV-2 has decreased during recent decades, especially in developed countries, as described in more detail below.

2.4.1 HSV-1 seroepidemiology

Globally, seroprevalence of HSV-1 increases gradually from childhood and reaches 70-80% prevalence among adult population (Miller et al., 1998). In earlier decades primary HSV-1 infections were acquired during early childhood, before the age of 5 years. In contrast, during 20th and 21th centuries, primary infections have been delayed in the western world, not occurring until adolescence or young adulthood (Smith and Robinson, 2002; Pebody et al., 2004; Sauerbrei et al., 2011; Bradley et al., 2014; Puhakka et al., 2016). Socioeconomic status (Corey and Spear, 1986), geographic location, ethnic background and age are the main factors affecting HSV-1 seroprevalence worldwide.

In the USA the average HSV-1 seroprevalence has declined from 58% (6640/11508) to 54% (5983/11100) between 1999-2004 and 2005-2010 ($p<0.01$) (Bradley et al., 2014). The decline was most significant among 14- to 19-year-old adolescents and young adults, from 39% (1814/4650) to 30% (954/3180) ($p<0.01$). The decline was even more significant (43% to 30%) in the same age group during a longer time period, between the years 1976-1980 to 2005-2010 ($p<0.01$) (Bradley et al., 2014). In the USA, HSV-1 infection is the most common among women (33%), among Mexicans (58%) and among non-Hispanic blacks (40%) and national HSV-1 seroprevalence increases with age (Bradley et al., 2014). At a London antenatal clinic, black women born in the Caribbean (92%, 202/220) or in Africa (96%, 102/106) were more likely to be HSV-1 seropositive than Asian women (71%, 835/1177) or women (black 75%, 162/215 or white 80%, 912/1138) born in the British Isles (Ades et al., 1989). It is not known why these ethnic differences in HSV serology have persisted for decades. In Australia HSV-1 seroprevalence was 76% (760/1000) between 1999-2000, and women were more HSV-1 seropositive than men (80% vs 71%, $p<0.001$) (Cunningham et al., 2006).

Across Europe HSV-1 seroprevalence varies highly, as HSV-1 seropositivity was 67% (2608/3892) in Belgium (Pebody et al., 2004), 84% (2688/3200) in Bulgaria (Pebody et al., 2004), 78% (5196/6627) in Germany (Korr et al., 2017), 57% (4085/7166) in Netherlands (Pebody et al., 2004) and 90% (2040/2257) in Poland (Smith et al., 2006). In northern European countries, in Finland, Netherlands, Germany and the United Kingdom (England and Wales) significantly higher age-standardized female HSV-1 seroprevalence compared to male seroprevalence was found. In all studied European countries, including Finland, the probability for HSV infection increased with age. In Finland the age-standardized HSV-1 seroprevalence was 52% (1740/3346) between the years 1997-1998 (Pebody et al., 2004). In Finland, the HSV-1 seroprevalence among pregnant women (aver-

age age 28.5 years) has declined by 25%, from 70% (139/200; in 1992) to 45% (90/200; in 2012) ($p<0.001$) (Puhakka et al., 2016).

Behavioral and lifestyle factors may also influence the acquisition of HSV-1 in selected groups of individuals, but cannot be generalized to population levels. Among 67 men and 41 women from Sweden experiencing first-episode genital herpes, orogenital sex and a history of labial herpes in their partner were significantly associated with HSV-1 infection (Löwhagen et al., 2000). Smoking, lower levels of education and not speaking German were correlated with HSV-1 in women and men living in Germany (Korr et al., 2017). Stock and coworkers reported that use of alcohol might protect from primary HSV-1 infection, while HSV-1 seropositivity and a higher level of mental stress were risk factors for occurrence of oral lesions in 17-41-year-old German and Spanish university students. In addition, students practicing penetrative sex were more likely to be infected with HSV-1 (Stock et al., 2001).

2.4.2 HSV-2 seroepidemiology

The average HSV-2 seroprevalence in the USA was 16% (1743/11100) during 2005-2010 and a decline in HSV-2 seroprevalence from the years 1994-1998 (21%) has been noted (Bradley et al., 2014). Some earlier studies document a decline (Persson et al., 1995) in HSV-2 seroprevalence and in others an increase (Forsgren et al., 1994; Fleming et al., 1997; Armstrong et al., 2001), but during recent years HSV-2 seroprevalence seems to have declined over a long time period; however, this decrease is mostly statistically insignificant. The independent predictors of HSV-2 seropositivity in USA were female gender, black race or Mexican-American ethnic background, older age, lower education, poverty, cocaine use, and a higher number of lifetime sexual partners (Fleming et al., 1997). In the USA, HSV-2 seroprevalence was 20% (741/3650) in women and 11% (372/3506) in men during the years 2007 to 2010. Non-Hispanic black women had the highest rates of HSV-2 (50%, 349/700) (Fanfair et al., 2013). There is no clear explanation for interracial differences in the rate of HSV infections, which have persisted over time. In Australia HSV-2 seroprevalence was 12% (480/4000) between 1999-2000 and women were statistically more HSV-2 seropositive than men (16% vs 8%) (Cunningham et al., 2006). In Haiti among pregnant women, older age and HIV positivity increased the HSV-2 seropositivity (Domercant et al., 2017). Earlier studies also showed that HSV seroprevalence is higher among sexual risk behaviour groups (Nahmias et al., 1990; Smith and Robinson, 2002; Roizman et al., 2013) and the highest HSV-2 seroprevalence

has been noted among female prostitutes (75%) and male homosexuals (83%) (Nahmias et al., 1990).

In Europe, HSV-2 seroprevalence varies, as it was 11% (428/3892) in Belgium (Pebody et al., 2004), 24% (768/3200) in Bulgaria (Pebody et al., 2004), 9.6% (481/5013) in Germany (Korr et al., 2017), 9.3% (210/2263) in Poland (Smith et al., 2006) and 4% (198/4948) in the United Kingdom (England and Wales) (Pebody et al., 2004). Older age, smoking and a history of abortion were associated with HSV-2 among German women (Korr et al., 2017). Among 3533 women at a West-London maternal antenatal clinic during 1980-81, single women (16% 135/821) were more likely to be HSV-2 seropositive than those married or cohabitant (8.6%, 233/2707), and HSV-2 seropositivity was higher among older and black women and lower among white and Asian women (Ades et al., 1989). In all studied European countries, including Finland, women had a higher HSV-2 seroprevalence and earlier age of acquisition than men (Pebody et al., 2004). In Finland, age-standardized HSV-2 seroprevalence was 13% (435/3346) (Pebody et al., 2004). And between the years 1992 and 2012, HSV-2 seroprevalence in pregnant women declined from 18% to 11% in Finland, but the decrease was not statistically significant (Puhakka et al., 2016).

2.4.3 HSV infections among immunocompromised individuals

HSV seroprevalence, and especially HSV shedding, is higher among immunocompromised individuals compared to healthy ones. In a Japanese cohort, 58% (64/110) of healthy and 90% (19/21) of immunocompromised individuals were HSV-1 seropositive (Kameyama et al., 1988). HIV-seropositive individuals (HSV-1: 6.6%, HSV-2: 3.5%) shed HSV to the oral cavity more often than HIV-seronegative individuals (HSV-1: 4.2%, HSV-2: 1.3%) (Kim et al., 2006). Among children with larger burns (and burn-related immunosuppression), *Herpesviridae* infections were statistically significantly more prevalent than among children with smaller burns (53±15% vs. 38±18%; $p<0.001$). HSV-1 was the most prevalent *Herpesviridae* among burnt children (Wurzer et al., 2017).

2.4.4 HSV infections during pregnancy and association with neonatal HSV

Among pregnant women, HSV seroprevalence is well studied. However, the recent decrease in seroprevalence, especially in the HSV-1 seroprevalence among adolescents and young adults, has raised interest in research on HSV seroprevalence during pregnancy. Figure 8 summarizes HSV seropositivity in pregnant

women worldwide. In the USA, 63% (394/626) of pregnant women (mean age 27 years, range 14-49) were seropositive for HSV-1, 22% (138/626) for HSV-2, 13% (81/626) for both and 28% (175/626) were HSV seronegative during the years 1999-2002 (Xu et al., 2007). The HSV-1 seropositivity rate has been 79% (814/1030) in Switzerland (Kucera et al., 2012), 82% (164/200) in Germany (Sauerbrei et al., 2011), 70% (1050/1507) in Netherlands (Gaytant et al., 2002), 77% (25/32) in Serbia (Dordević, 2006), 95% (108/114) in Turkey (Ozdemir et al., 2009) and 100% (n=134) in Vanuatu (island in Pacific Ocean) (Haddow et al., 2006). The HSV-2 seroprevalence rate of pregnant women has ranged from 7.6% (51/673) in Italy (Suligoi et al., 2000), 10% (31/299) in Sweden (Berntsson et al., 2009), 21% (218/1030) in Switzerland (Kucera et al., 2012), 24% (366/1507) in Netherlands (Gaytant et al., 2002), 17% (85/500) in Korea (Kim et al., 2012), 12% (4/32) in Serbia (Dordević, 2006), 8.2% (13/158) in Turkey (Ozdemir et al., 2009), 14% (186/1371) in Australia (Sasadeusz et al., 2008), 28% (214/765) in Papua New Guinea (Vallely et al., 2016) and 59% (229/390) in South Africa (Perti et al., 2014). HSV-1 and -2 seroprevalence in Nigeria was high, 99% (179/180) (Okonko and Cookey, 2015). HSV-2 seroprevalence among pregnant women in Brazil was 31% (39/127) among middle-class socioeconomic individuals and 42% (73/173) among very low-income individuals (Weinberg et al., 1993).

In Finland the HSV-1 seropositivity rate was 70% (698/997) among pregnant women during 1988-89 (Arvaja et al., 1999). Finnish pregnant women giving birth at the Turku University Hospital in the year 2000 were HSV-1 and HSV-2 seropositive in 47% (261/558) and 9.3% (52/558) of the cases, respectively (Alanen et al., 2005). In 2012 the HSV seroprevalence among Finnish pregnant women was 45% (90/200) for HSV-1 and 11% (22/200) for HSV-2. The proportion of either HSV-1 and/or HSV-2 seronegative women increased significantly from 26% (51/200) to 48% (96/200) between the years 1992 and 2012 ($p<0.001$). Thus, these seronegative women are susceptible to primary HSV infection during pregnancy (Puhakka et al., 2016).

HSV-2 has been the causative agent in most genital HSV-infections, which are mostly transmitted via sexual contact after the onset of sexual activity (Johnston and Corey, 2016). However, during recent decades an increasing proportion of genital HSV infections are caused by HSV-1, especially among young women in developed countries, including Finland (Lafferty et al., 2000; Löwhagen et al., 2000; Roberts et al., 2003; Gilbert et al., 2011; Bernstein et al., 2013; Kortekangas-Savolainen et al., 2014; Tuokko et al., 2014). This has been explained by the acquisition of primary HSV-1 infection later in life during fertile years and the first infection with HSV-1n these cases could be genital rather than oral (Bradley et al., 2014), while at the same time the acquisition of genital HSV-

2 infections has slightly decreased in all age groups (Xu et al., 2007). In addition, oro-labial sexual contacts have increased during recent years even in the USA (Copen et al., 2012), which can impact the transmission of oral HSV-1 to the genital mucosa (Lafferty et al., 2000; Stephenson-Famy and Gardella, 2014; Delmonte et al., 2017). In addition, approximately 10% of HSV-2 seronegative pregnant women will have an HSV-2-seropositive partner, which increases the risk of acquiring primary HSV infection during intercourse (Kulhanjian et al., 1992).

An important point of view is that pregnant women are also more susceptible to viral infections due to decreased T-cell-mediated immunity (Mellor and Munn, 2001). A meta-analysis on susceptibility to viral diseases during pregnancy stated that during pregnancy, women are more susceptible to HSV infections (Sappenfield et al., 2013). This may, together with the aspects discussed above, increase the risk for primary HSV-1 genital infections during pregnancy, which has increased risk for neonatal HSV-1 infection, which can be life-threatening for the infant. However, neonatal HSV infections are rare, occurring in less than approximately 1 per 1700-12500 live births globally (Corey and Wald, 2009), but the geographic variation in the incidence of neonatal HSV infection is wide (Brown et al., 1991; Whitley et al., 1991; Tookey and Peckham, 1996; Kropp et al., 2006; Pascual et al., 2011; Stephenson-Famy and Gardella, 2014). The mother receiving a primary HSV-1 or HSV-2 infection at the time of delivery increases the risk of neonatal HSV infection by 57% (Brown et al., 2003; Stephenson-Famy and Gardella, 2014). Among Finnish pregnant women, the odds ratio for high IgM reactivity responses (primary infection) in early pregnancy (mean gestation age 11.1 weeks) to HSV-1 or -2 was 1.94 (95% CI: 0.74, 5.12) (Werler et al., 2016). Clinical symptoms and outcomes of neonatal HSV infections are described in more detail in next chapters.

Figure 7. The HSV seroepidemiology among pregnant women around the world.

2.5 HSV diseases

First exposure to either HSV-1 or HSV-2 causes primary infection of the affected site. Reactivation of HSV from the sensory ganglion leads to recurrent (secondary) infections, of which symptoms are less severe, last shorter and are more localized than with the case of primary infection. Reactivation can be spontaneous, or it can be triggered by stress. An individual with pre-existing antibodies to either HSV-1 or HSV-2 can also be infected with the other type, in which case the infection is known as an initial infection rather than a primary infection. Previous HSV-1 antibodies give only partial protection against HSV-2 infection, and HSV-2 antibodies cannot prevent HSV-1 infection (Pebody et al., 2004). If an individual has not experienced symptoms of primary infection, the first recurrence is identified as a first episode of infection.

2.5.1 Orofacial HSV diseases

HSV-1 causes most orofacial infections, but a fraction of oral infections are also caused by HSV-2 (Looker and Garnett, 2005). The primary HSV infection is mild or subclinical in most cases but approximately 10-12% (Chauvin and Ajar, 2002) to 25-30% (Cesario et al., 1969; Becker et al., 1988; Schmitt et al., 1991) of children who were infected had noticeable symptoms of gingivostomatitis. Primary orofacial infections are experienced mainly during two age periods: the first between 6 months and 5 years of age and the second in the early twenties, of which the latter is more prominent in developed countries (Amir et al., 1997; Arduino and Porter, 2007). The incubation period is 2-20 days depending on the site of infection and on the viral strain (Amir, 2001). Gingivostomatitis is a painful and feverish infection of the oral mucosa, which starts with non-specific malaise and myalgia and after 1-3 days is followed by blistering and ulceration of the tongue, lips, buccal mucosa, gingiva and the soft and hard palate (Huber, 2003). Intraoral blisters are 1-2 mm in size, which rapidly rupture and coalesce to form shallow ulcers covered with pseudomembranes, which are surrounded by erythematous halo (Eisen, 1998). The ulcers heal without scarring in 10-14 days (Amir et al., 1999). Systemic symptoms include pyrexia, lethargy, loss of appetite, fractiousness and hypersalivation and sometimes headaches and bilateral cervical lymphadenopathy (Esmann, 2001; Huber, 2003).

Secondary infection is mostly subclinical, but it can be also symptomatic. Secondary infection can be presented at the mucocutaneous junction, mostly on the lip as “cold sores” in 20-40% of HSV-1-seropositive individuals (Figure 8) (Kameyama et al., 1988; Stock et al., 2001; Shulman, 2004), or as intraoral sores

and ulcers, which can appear on attached gingiva and on the hard palate. Intraoral recurrent HSV-1 lesions have been considered more rare in healthy patients (Holbrook et al., 2001), but iron-related deficiency has been suggested as a predisposing factor (Lamey and Biagioni, 1995). Approximately 25% (2508/10032) of HSV-seropositive 2- to 17-year-old US adolescents had at least one clinically recognized recurrence during a one year period (Shulman, 2004). In another study, involving young adults in 21 countries, conducted through questionnaires, 33% (1965/5918) of men and 28% (1103/3941) of women reported two or more herpes labialis outbreaks during their lifetime, and North American responders mainly from Canada, had the highest prevalence of herpes labial and aphthous ulcers (Embil et al., 1975). Among immunocompromised patients, secondary infection can also be presented as more disseminated gingivostomatitis (Epstein et al., 1990; Woo et al., 1990). Prodromal symptoms at the site of recurrence, such as itching, tingling or burning, can be experienced in 46-60% of recurrences approximately 6 hours prior to the appearance of the clinical signs of HSV infection. An average of 25% of facial recurrences do not progress beyond the prodromal stage and the healing of lesions lasts 1-10 days (Esmann, 2001; Arduino and Porter, 2007). One could also assume that HSV-seropositive pregnant women should have more recurrences due to hormonal changes, but previous study of 3738 pregnant women who had a history of recurrent herpes labialis lesions showed reduced incidence of these lesions during pregnancy (0.11 lesions/subject per month) when compared to outside pregnancy (0.19 lesions/subject per month) ($p<0.0001$), as measured with questionnaires at one time between 10 to 15 weeks of pregnancy (Scott et al., 2003). However, it is not known for sure, if there is some protective effect of pregnancy and how long the protective effect lasts after delivery.

It has also been shown that oral HSV infections are associated with periodontal disease (Slots, 2010), and the bacterial and HSV infections may increase the risk of cardiovascular disease through associated low HDL cholesterol concentration and chronic infection burden within the body (Vilkuna-Rautiainen et al., 2006).

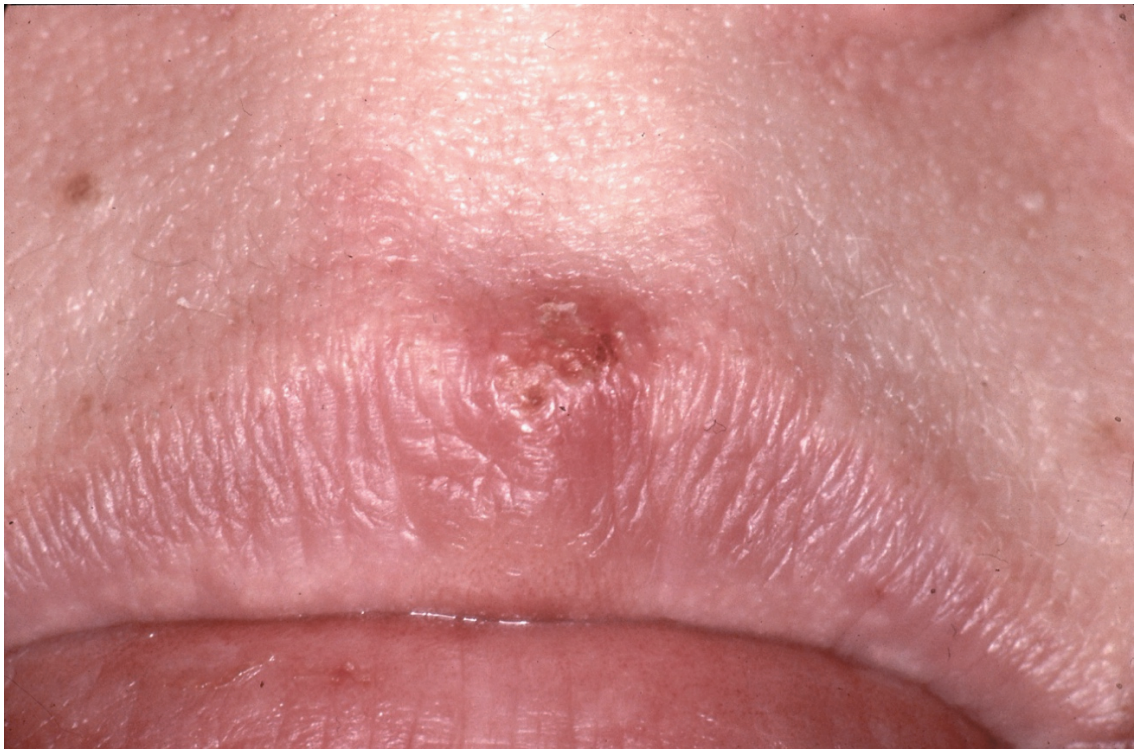

Figure 8. Recurrent HSV infection of the lip. "Cold sore" seen on the vermillion of the lip. Courtesy of Stina Syrjänen.

2.5.2 Genital HSV diseases

HSV-2 has usually been the primary cause for genital HSV infection, but during recent decades an increasing proportion of genital HSV infections are caused by HSV-1, especially among young women (Bernstein et al., 2013). Most of the primary and secondary genital HSV infections are subclinical, accompanied by asymptomatic HSV shedding to the genital mucosa (Wald et al., 2000). HSV may also cause painful self-limited genital ulcers and local genital symptoms, e.g. dysuria, cervicitis, lymphadenopathy, and systemic symptoms such as fever, headache and myalgia (Johnston and Corey, 2016). Studies in the past decades have highlighted the importance of genital HSV transmission during subclinical HSV recurrences, as the transmission seemed to happen in 70% of individuals during asymptomatic genital HSV shedding (Mertz et al., 1992). Primary and secondary genital HSV infections recur without antiviral therapy within 3 weeks and 3-5 days, respectively, and the recurrent disease is usually less severe than the primary infection (Johnston and Corey, 2016). However, in most studies only 10-25% of HSV-2-seropositive individuals report a history of genital lesions (Fleming et al., 1997; Mark et al., 2008).

During recent years, several aspects of genital HSV infection have been noted: 1) genital HSV-1 infection is less symptomatic and recurrences occur more rarely than with genital HSV-2 infection, 2) recurrences decrease over time in both HSV-1 and -2 infections being most active near the acquisition of primary infection (Benedetti et al., 1999), 3) pre-existing HSV-1 antibodies reduces the risk for acquisition of genital HSV-2 infection (Mertz et al., 1992; Pebody et al., 2004) and decrease symptoms and shedding of genital HSV-2 infection (Langenberg et al., 1999), and finally 4) HSV-2 infection also enhances HIV-1 acquisition and transmission (Freeman et al., 2006).

2.5.3 Other HSV diseases

Different types of HSV infection have been noted and named according to the anatomical distribution of symptoms or according to the group of people who usually acquire the disease. These HSV diseases include HSV keratoconjunctivitis of the eye, which is the most common viral cause of blindness (Roizman et al., 2013). HSV keratoconjunctivitis can result rarely from direct inoculation of infective HSV to the eye through constant tear rinsing or, more often, from reactivation of latent trigeminal HSV, which is transported to "wrong" direction along the ophthalmic nerve (to the eye instead of the lip), as trigeminal ganglion innervates both the eye and the skin of the face (Labetoulle et al., 2000, 2003; Kuo et al., 2014). Cutaneous herpetic whitlow is an HSV infection near the finger or toe nail, which has been documented among dental personnel and among nurses before the regular use of protective gloves during the treatment of patients (Figure 9) (Stern et al., 1959; Lewis, 2004). HSV can infect the skin of health care personnel through small ulcers at the finger cuticles or in hands. Among athletes, especially among professional wrestlers or rugby players, who are exposed to trauma of the skin, serious facial cutaneous herpes infections have been noted, since the virus can penetrate to the basal layer of the skin through micro-ulcers. These cutaneous infections have been called by many different names during history: herpes gladiatorum (Selling and Kibrick, 1964), herpes luctatorum (Laur et al., 1979) and herpes of wrestlers (Nomikos et al., 2015).

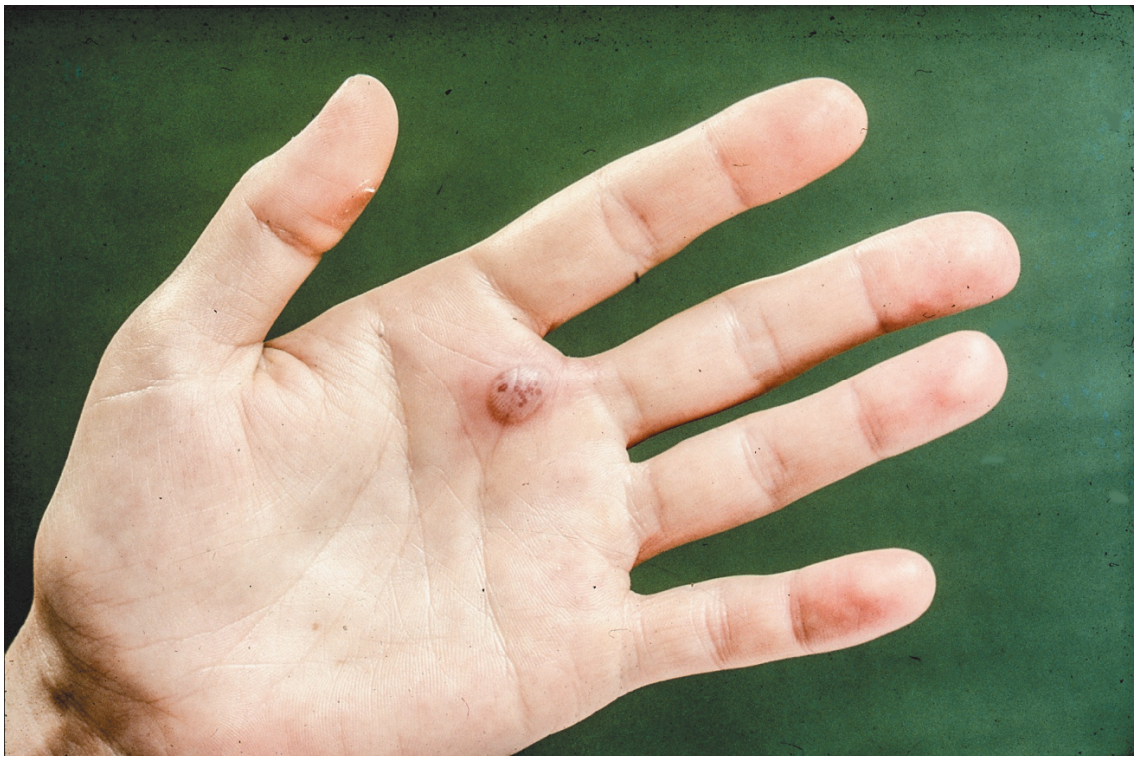

Figure 9. Recurrent HSV infection in the palm of a dentist who acquired the primary infection via an occupational incident with a sharp instrument. Courtesy of Stina Syrjänen.

HSV can also cause systemic diseases; primary infections with HSV may lead to viral spread beyond the dorsal root ganglia, which leads to systemic HSV infection in which the virus can be detected in the blood (Huang et al., 2004; Johnston et al., 2008). The most hazardous but rare infections with HSVs are HSV meningitis and encephalitis (Schlitt et al., 1986; Steiner, 2011; Välimaa et al., 2013; Bradshaw and Venkatesan, 2016) and also neonatal HSV disease, which has high mortality of 85% and a poor neurologic outcome if untreated (Corey and Wald, 2009; Stephenson-Famy and Gardella, 2014). Rare (1:1700-12500 live births) neonatal HSV infections can occur when the mother has an ongoing genital HSV infection during delivery, especially during primary HSV infection (Brown et al., 2003; Kimberlin, 2004). Neonatal HSV diseases can lead to localized skin or eye disease, disseminated multi-organ disease or encephalitis. HSV-1 causes most of the systemic neonatal diseases (Kropp et al., 2006; Välimaa et al., 2013). An increased proportion of primary genital infections caused by HSV-1 (Kortekangas-Savolainen and Vuorinen, 2007; Kortekangas-Savolainen et al., 2014; Tuokko et al., 2014), especially among young women, has raised concern about severe neonatal infections. However, severe neonatal infections are still very rare and recently it has been published that long acyclovir treatment of the newborn with neonatal HSV infection has significantly improved disease prognosis (Kimberlin et al., 2011). In addition, neonatal HSV infection can more rarely also be caused

by the mothers' recurrent HSV disease, but these infections are usually less severe due to protective maternal HSV antibodies (Kulhanjian et al., 1992). Precaution actions against possible neonatal infections in Finland include prophylactic antiviral medical treatment for 3-4 weeks before delivery and it is usually continued 2 weeks after delivery, if a pregnant woman is known to have genital HSV recurrences. Usually antiviral therapy is enough to prevent HSV recurrences, but cesarean delivery might be performed if the pregnant women is suspected to have primary genital HSV infection at the time of delivery (Välimaa et al., 2013; Stephenson-Famy and Gardella, 2014).

2.5.4 Treatment of HSV diseases

Currently no complete cure is available for HSV infection. However, viral medications can diminish the symptoms, decrease the duration of HSV infection and prevent HSV reactivations partially or totally at high doses of prophylactic medication (Välimaa et al., 2013). Traditional treatment and prophylaxis of HSV infections includes topical or systemic administration of acyclovir or its variants, which efficiently halts HSV replication (Amir et al., 1997). Any of the medications needs to be administrated at most 24 hours after the onset of prodromal symptoms for the treatment to be effective; however, its prophylactic use does not entirely prevent HSV reactivations (Johnston et al., 2012). However, prophylactic daily use of valacyclovir significantly reduced the transmission of genital HSV-2 among couples (Corey et al., 2004). Other therapy focuses usually on soothing the symptoms and on avoidance of triggering factors for recurrence; for example, the use of sunscreen can prevent UV-light induced reactivations (Rooney et al., 1991). All prophylactic HSV vaccine approaches have failed so far, including a Phase 3 study with a HSV-2 glycoprotein D (gD2) containing vaccine 'Herpevac' (GSK) that enrolled over 8000 HSV-1 and HSV-2 seronegative women, and lacked efficacy to protect from genital HSV-2 (Belshe et al., 2012). New medications and vaccines against HSV infection are needed because of rarely occurring severe forms of HSV infections and because of partial drug-resistant HSV clinical strains (Biswas et al., 2007; Sukla et al., 2010), especially among immunocompromised patients (Stránská et al., 2005; Frobert et al., 2014). An example of a recent new approach against HSV infection is RNA interference (Nygardas et al., 2009; DeVincenzo et al., 2010; Ozcan et al., 2015), which has shown success in treatment of experimental HSV infections (Romanovskaya et al., 2012; Paavilainen et al., 2017).

2.6 Human Papillomavirus (HPV), HPV and head and neck squamous cell carcinoma and oral co-infections with HPV and HSV

2.6.1 Human papillomavirus (HPV)

Human papillomaviruses (HPV) are small DNA viruses known to infect epithelial cells. The HPV genome is circular and double stranded with approximately 8000 base pairs organized into early and late open reading frames and a long control region. The molecular biology of HPV is summarized in a recent review (Harden and Munger, 2017). Currently over 200 HPV genotypes have been identified and categorized into five genera with the Alpha-, Beta- and Gamma-papillomaviruses representing the largest groups (de Villiers, 2013). The Alpha genus is of main clinical importance as it contains most of the mucosal HPVs. The classification of HPVs is based on the nucleotide sequence of open reading frame coding for major capsid protein L1. Within the alpha genus there are 14 different viral species which share a 60-70% homology of L1 sequence. The most important high-risk HPVs belong to either species 9 (HPV genotypes 16, 31, 33, 35, 52, 55) or species 7 (HPV 18, 39, 45, 59, 68). HPV 16 is the most powerful oncogenic human virus known today. Species 5 contains the most prevalent low risk HPV genotypes 6 and 11.

Benign clinical manifestations of HPV include skin warts and condylomas or papillomas, which can also be seen on oral mucosa in addition to the genital mucosa (usually caused by low-risk HPV types 6 and 11). The HPV life cycle depends on epithelial differentiation and stratification in a partly unidentified way, thus HPV cannot be cultured. HPV needs access to the basal cells of mucosal or cutaneous epithelium to induce infection through an ulcer or microtrauma. Infective matured viral particles are assembled only in the superficial epithelial layers where the HPV virions are released from dying cells when they have reached full differentiation (Stubenrauch and Laimins, 1999; Reinson et al., 2015). In differentiated epithelial cells the HPV oncoproteins E6 and E7 induce a S-phase like environment which supports cell cycle progression through degradation of cell cycle gatekeepers, tumor suppressor protein 53 (p53) and retinoblastoma proteins (Rb) (Moody and Laimins, 2010; Mittal and Banks, 2017). In most cases the HPV infection clears spontaneously, but occasionally HPV infections may persist. HPV can interfere with the differentiation process and immortalize epithelial cells, which can then result in epithelial dysplasia (i.e. low-grade intraepithelial lesion, LSIL or high-grade intraepithelial lesion, HSIL) and even progress to squamous cell carcinoma (SCC), depending on additional exogenous risk factors and the host defense system (McLaughlin-Drubin and Munger, 2008; zur

Hausen, 2009; Rautava and Syrjänen, 2012; Mehanna et al., 2013; Kero, 2014). Transformation by high-risk HPV types (usually HPV types 16 and 18) requires persistent infection, the expression of HPV E6 and E7 genes, and accumulation of cellular mutations over time (Auvinen et al., 1992; Moody and Laimins, 2010). However, less than 1% of women with genital high-risk HPV infection will eventually develop cervical cancer. This indicates that additional biological and environmental cofactors, i.e. hormonal changes, immune status, parity, dietary habits, tobacco and alcohol usage, and co-infection with other pathogens are implicated in the development of HPV-associated cancers (Castellsagué et al., 2002). Indeed, a history of prior infection with herpes simplex virus, human immunodeficiency virus (HIV), *Neisseria gonorrhea*, *Chlamydia trachomatis*, and *Trichomonas vaginalis* have been linked to increased risk of acquisition of HPV infection or to a reduced ability to clear the chronic HPV infection, studied mostly in genital mucosa (Grce et al., 2004; Guidry and Scott, 2017).

2.6.2 HPV induced head and neck squamous cell carcinoma (HNSCC)

Globally, oral and lip cancer is the 15th most common malignant tumor, and the incidence is estimated to be 300373 new cases and 145328 associated deaths annually (IARC., 2012). Head and neck squamous cell carcinoma (HNSCC) is usually divided into HPV-positive and HPV-negative cancers. HPV-association is most evident for oropharyngeal SCC and these patients are usually younger and their survival is better than that for the HPV-negative HNSCC patients (Syrjänen et al., 2017). The prevalence of HPV in HNSCCs is geographically highly variable (Ndiaye et al., 2014). Smoking and excessive use of alcohol are the main risk factors of HPV-negative and also HPV-positive HNSCCs (Shigeishi and Sugiyama, 2016).

The rate of HPV-associated oropharyngeal cancer is dramatically increasing in developed countries, e.g. the incidence in Finland has almost trippled in 30 years (Syrjänen and Rautava, 2015). The prevalence of HPV in oropharyngeal SCC shows varied association, ranging between 45% and 87% globally (Gillison et al., 2015). The most likely sites for HPV-related oropharyngeal cancer are the palatine tonsils and the base of the tongue, followed by the oral cavity, larynx, and sinonasal mucosa (Figure 6) (Rautava and Syrjänen, 2012). The most susceptible site for HPV-associated oropharyngeal SCC is the squamocolumnar epithelial junction of oropharynx. HPV prevalence in laryngeal SCC is 20-30% (Gama et al., 2016), in tonsillar SCC over 50% (HPV 16 being the most prevalent type, 84%) (Syrjänen, 2004) and particularly in the USA, the HPV prevalence in oropharyngeal SCC is up to 80% (Singhi and Westra, 2010). In Finland, 61%

(37/61) of HNSCC biopsies were positive for HPV, HPV 16 being the most frequent type (84%) (Koskinen et al., 2003). A recent study reported that 48% (51/118) of consecutive patients diagnosed with primary oropharyngeal SCC between 2012 and 2014 at the Helsinki University Hospital were HPV positive (Carpén et al., 2017). In non-malignant tonsils high-risk HPV was found in only 1% (5/477) of the samples, and all HPV 16 infected tonsils harbored mutations relevant in development of HNSCC (Ilmarinen et al., 2017). HPV vaccination during childhood has shown success in reducing female anal and genital dysplasias, and vaccination might also give protection for HPV-associated HNSCCs in the future (Syrjänen and Rautava, 2017).

2.6.3 HPV and co-infection with HSV

Mucosal herpes simplex virus (HSV) infection has been suggested as a predisposing factor for subsequent HPV infection. Lytic HSV infection can either allow HPV better access to the basal cell layer of epithelium trough an ulcer to establish infection, or alternatively HSV replication in the same mucosal area may influence persistence, clearance and oncogenic activities of HPV. In vitro, HSV co-infection has been shown to alter HPV life cycle. For example, in HPV 18 positive HeLa cells and HPV 18 transfected A431 cells, the presence of HSV-1 or HSV-2 were shown to enhance HPV replication and to increase HPV genome integration in host cells (Hara et al., 1997). In another study, HPV positive cervical epithelial cells in organotypic raft culture were infected with either HSV-1 or HSV-2. The co-infected cells exhibited cytopathic effects associated with HSV replication, but maintained tissue integrity and architecture. These raft cultures had reduced E6 and E7 and undetectable E2 messenger RNA levels, but HPV replication and genomic copy-number values were maintained (Meyers et al., 2003). These studies indicate, that HSV may replicate in HPV-infected cells in opportunistic manner, of which may disturb regulation of HPV oncogene expression and replication, or HSV may affect HPV integration. These events may in some cases lead to HPV persistence in epithelial cells and to carcinogenesis (Guidry and Scott, 2017).

Prevalence and outcome of co-infection

HSV and HPV co-infection has been studied mostly in association to genital and oropharyngeal carcinogenesis and little is known of the prevalence of co-infection in healthy individuals. In genital mucosa, HSV-2 seropositive women have been shown to have 2- to 9-fold increased risk of developing squamous cell carcinoma (SCC) and adenocarcinoma (Castellsagué et al., 2002; Smith et al., 2002). In addition, the frequency of HSV-2 specific antigens and DNA has been

detected to be higher in exfoliated cells of dysplastic or cancerous cervical (genital) tissues compared to that in normal tissues (Pacsa et al., 1976; Prakash et al., 1985). Also, HSV-2 and HPV 16 or 18 co-infection has been found in 25-30% of cervical intraepithelial dysplasias (CIN), in 13-25% of invasive cervical squamous cell carcinomas and adenomas, and in 0-4% of normal cervical tissues (Zhao et al., 2012). Some studies have shown that HSV-1 could also be implicated in cervical cancer (Finan et al., 2006), while others have shown a lack of association with HSV-1 or HSV-2 and cervical carcinogenesis (Lehtinen et al., 2002).

In head and neck cancers, similar inconsistent association between HSV and HPV has been found. However, recent observations implicate that 1) the patients with HPV16- and HSV-1-positive head and neck squamous cell carcinoma (HNSCC) had the worst disease outcome after radiotherapy, less than one year since the primary diagnosis (Kim et al., 2007; Rautava et al., 2012a; Ndiaye et al., 2014) and 2) HSV-1 infection may modulate the radiation resistance of HPV16 positive HNSCC cells *in vitro* by increasing cell survival after irradiation treatment. The effect might be related to the inhibition of host cell apoptosis (Turunen et al., 2014, 2016). Since the oral mucosa is also in constant exposure to carcinogens, such as tobacco, alcohol and X-rays due to dental radiography, premalignant cells are likely to be present in healthy oral mucosa. Even though the HSV has not been implicated in direct carcinogenesis in oral cavity, HSV-1 infection, carcinogen burden and irradiation treatment for oral cancer combined could be clinically relevant in the pathogenesis or recurrence of HNSCC. Thus, oropharyngeal co-infection could be a prognostic indicator for cancer therapy outcomes or a serological marker for increased risk of HPV-induced HNSCC (Rautava et al., 2012a).

3 AIMS OF THE STUDY

The Finnish Family HPV Study was originally designed to assess the dynamics of HPV infection in the families with special reference to early infections, natural history of oral and genital HPV infection as well as HPV immunity. The focus in this thesis was to evaluate the frequency of HSV infection in oral mucosa in addition to the HSV seroprevalence and seroconversion among the young pregnant women and their male spouses of this cohort, prospectively followed-up before and after delivery for six years. As the natural history of HPV infections during the follow-up was available from previous studies, it was also possible to relate the HSV data to the known HPV status to assess their potential synergistic actions.

The specific study aims were:

1. To determine the frequency of HSV-1 and HSV-2 infections in oral mucosal samples of the women in the Finnish Family HPV Study during a six-year follow-up and to evaluate the frequency of simultaneous HSV DNA and HPV DNA detection in oral mucosa. The possible effect of pregnancy was also evaluated.

2. To determine the frequency of HSV-1 and HSV-2 infections in oral mucosal samples of the male spouses during the six-year follow-up and to compare the presence of HSV DNA in male oral mucosa to that of his spouse and to determine the predictors of HSV infection.

3. To determine the frequency of HSV seropositivity and seroconversion and their concordance with HSV-1 DNA, as well as their predictors among young women and their male spouses in Finland.

4 SUBJECTS, MATERIALS AND METHODS

4.1 Subjects

The HSV testing was performed retrospectively on the cohort material (The Finnish Family HPV Study) collected earlier for HPV analysis purposes. The Finnish Family HPV Study is a longitudinal cohort study conducted at the Department of Oral Pathology, Institute of Dentistry, University of Turku and the Department of Obstetrics and Gynecology, Turku University Central Hospital. The study had been originally designed to assess the dynamics of HPV transmission within healthy Finnish families in a longitudinal setting, and HPV data has been previously published (Rintala et al., 2005; Syrjänen et al., 2009; Louvanto et al., 2010; Rautava et al., 2012b). In total, 329 mothers-to-be in their third trimester of pregnancy, 131 of their male spouses and 331 newborns (includes two sets of twins) were enrolled between 1998 and 2002 at the Maternity Unit of the Turku University Central Hospital. The inclusion criteria at enrollment were the baseline sampling at a minimum of 36 weeks of their index pregnancy (full-time pregnancy) and an informed consent to participate in the study. The HPV or HSV status of the participants was not tested prior to enrollment. The studies were conducted with the permission of the Research Ethics Committee of Turku University Hospital (#3/1998, #2/2006, 45/180/2010 and TO7/008/2014).

The study subjects included in my thesis comprise the women and their spouses from this cohort. The number of subjects and the samples used in the three original papers (I-III) are given in Table 2.

Table 2. Study subjects, sampling site and the number of specimens at different time points according to separate communications (I-III).

Paper	Number of subjects	Mean age (range)	Sample	Baseline (before delivery)	2 month	6 month	12 month	24 month	36 month	72 month	Mean follow-up time (range)
I	325* women	25.5 (18-46) years	Oral brush samples	304	297	291	285	264	261	177	57.3 (6.7-94.5) months
II	139 male spouses	28.6 (19-46) years	Oral brush samples	117	111	115	116	104	101	58	47.8 (5.0-91.5) months
III**	285 women	25.5 (18-38) years	Sera	285			4	9	44	75	57.3 (6.7-94.5) months
	120 male spouses	28.7 (19-46) years	Sera	120			4	5	30	25	47.8 (5.0-91.5) months

*Previously published n=304 mothers (I), which is the number of mothers at the baseline (before delivery). In total, however, there were 325 women and 21 of them were included in the study later, at the 2-month follow-up visit, because there was not enough base line visit's specimen available due to previous analyses on HPV infections.

**Determination of serum HSV antibodies was done at study entry (baseline). The individuals with seronegative or borderline HSV serology at baseline were tested for HSV using their last follow-up sample available. The number of the follow-up samples according to the last visit is given, as individuals had their last sampling at different follow-up visits.

4.2 Demographic data and specimen collections

4.2.1 Demographic data (I-III)

Demographic data were collected with questionnaires at the first follow-up visit (baseline before delivery). Questionnaires included for example information on marital status, education, general health, tobacco and alcohol use, sexual habits and history of previous sexually transmitted diseases e.g. history of genital warts. The Finnish Family Study was originally planned to study HPV infections; thus, the questionnaires were made for the study of HPV-infections and the existing demographic data were also used in this study. The basic demographic data related to HPV have been published before (M. Rintala et al., 2005; Syrjänen et al., 2009; Louvanto et al., 2010; Rautava et al., 2012b).

4.2.2 Specimens for DNA analyzes (I-II)

Sampling was done for earlier HPV study and the specimens were re-analysed for HSV. Oral scrapings were collected at the baseline (before the delivery) and at 2, 6, 12, 24, 36 and 72 months after delivery (Table 2) with a small brush (Cytobrush®, MedScan, Malmö, Sweden) (Rintala et al., 2005; Louvanto et al., 2010; Rautava et al., 2012b). Oral epithelial brush specimens were collected from the upper and lower vestibular area and from the buccal mucosa of both cheeks. The brush was placed in 80% ethanol, frozen and then stored at -70°C before any analysis (Rintala et al., 2005). In total, 1873 and 722 DNA profiles were available for HSV DNA testing, extracted from oral brush specimens taken from 325 women and 139 men, respectively. The numbers of oral specimens collected at each follow-up visit are given in Table 2. The genital specimens were obtained as described before (Louvanto et al., 2010).

4.2.3 Peripheral blood specimens (III)

Blood specimens were collected at the baseline and at 2, 6, 12, 24, 36 and 72 months after delivery (Syrjänen et al., 2009, 2015). In this study, we analyzed all baseline specimens of the women and men available. To determine the HSV seroconversion rate, only the HSV-seronegative or borderline individuals at baseline were tested using their last serum specimen available during the follow-up. In total, baseline serum specimens were available from 285 women and 120 men. The last follow-up specimen was tested for 132 women and 64 men. The follow-

up visit for the last serum specimen tested varied among the individuals, as given in the Table 2.

4.3 Detection of oral epithelial HSV DNA (I,II)

4.3.1 DNA isolation

DNA was extracted from the oral epithelial brush specimens with the high-salt method, as previously described (Miller et al., 1988; Louvanto et al., 2010). Briefly, the oral brush specimens were lysed in buffer (10mM TRIS, 400mM NaCl, 100mM EDTA, 1% SDS). The lysed specimens were digested with proteinase K (10µg/ml) at 37°C overnight. Saturated NaCl was added to precipitate proteins. DNA was precipitated in the supernatant and kept in 50µl water, and then stored at -70°C until analysis.

4.3.2 Quantitative PCR amplification (I,II)

To evaluate oral HSV shedding, oral epithelial brush specimens were analyzed for HSV-1 and HSV-2 DNA with PCR. HSV-1 and -2 DNA detection was performed with quantitative real-time PCR (qPCR) using Rotor-Gene Q Series Software version 1.7 (Qiagen, Hilden, Germany), as described (Nygårdas et al., 2011). Primer and probe sequences for HSV-1 and -2 were from the glycoprotein D (gD) gene (Hukkanen et al., 2000). The sequences of the primers and probes are given in Paper I.

HSV-1 and HSV-2 qPCR analyzes were done separately with HSV-1 and -2 primers (10pmol/µl in water), respectively. HSV copy-number estimations were done with a HSV-1 17+ DNA standard (10^8 copies/ 2µl) dilution series (10^8-10^1 in water). The qPCR mixture contained HSV-1 or -2 primers, water and Sybr®Green buffer with polymerase (ThermoFisher, Waltham, MA USA). The qPCR started with an initial denaturation step of the DNA at 95°C for 15 min. The qPCR contained 45 cycles; 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 45 seconds. The melting temperature analysis was carried out from 72°C to 95°C at 1°C intervals.

4.3.3 Confirmation of the qPCR results (I,II)

The PCR products were judged positive or negative based on the melting temperature (Tm) analysis of the PCR products. The analysis is based on the different melting temperatures of varying length PCR products of HSV-1 and HSV-2. Possible equivocal results were confirmed by Southern Blot hybridization with HSV type-specific digoxi-genin-labelled oligonucleotide probes as described before (Hukkanen et al., 2000). Briefly, the PCR products were separated with gel electrophoresis (2% agarose gel and 0.5xTBE) and then blotted to nylon-membranes (Zeta-probe®GT Genomic) overnight with a buffer (0.4M NaOH, 0.6M NaCl in water) and neutralized with a neutralizing buffer (1.5M NaCl, 0.5M TRIS, pH 7.5). Dried membranes were then hybridized with HSV-1- or -2-specific oligonucleotide probes and examined by imaging with film.

Detection of HPV DNA from the oral epithelial specimens

As the oral HPV DNA detection and genotyping have been made earlier in the same cohort (Louvanto et al., 2010; Rautava et al., 2012b), the possible HSV and HPV co-infection in the same oral epithelial specimens were studied, in order to evaluate the prevalence of oral co-infection among healthy Finnish adults. Briefly, HPV testing was done previously with nested PCR using external primers MY09 and MY11 and internal primers GP05+/ GP06+ as described (van den Brule et al., 1989; Resnick et al., 1990). HPV genotyping was performed with a Multimetrix kit® (Multimetrix, Progen Biotechnik GmbH, Heidelberg, Germany). The kit detects 24 lr- and hrHPV-genotypes as follows: lrHPV 6, 11, 42, 43, 44, and 70; and hrHPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82.

4.4 Detection of HSV antibodies in serum (III)

4.4.1 Serum preparation

Serum was separated by centrifuging at 2400 rpm for 10 min (Sorvall GLC-2; DuPont Instrument). The serum was taken into multiple storage vessels, and was stored first at -20°C for no longer than 1 week and then at -70°C until analysis, as described earlier (Syrjänen et al., 2009, 2015). For this study, serum specimens were thawed for the first time.

4.4.2 Indirect enzyme immunoassay (III)

To evaluate current HSV seroprevalence and seroconversion among young adults near delivery of their child in Finland, IgG antibodies against HSV-1 and HSV-2 were analyzed with indirect enzyme immunoassay (EIA), as previously described (Ziegler et al., 1989; Alanen et al., 2005). Briefly, the antigens were purified from HSV-1 (strain F) and HSV-2 (strain G) infected Vero cell cultures (American Type Culture Collection) (Alanen et al., 2005). Polystyrene strips (Thermo) were coated with 1.5mg/ml HSV-1 and 1.4mg/ml HSV-2 envelope antigens in phosphate buffered saline (PBS). Serum samples, diluted 1:100 in PBS, were tested as duplicates and incubated for 2 hours at 37°C, after which horseradish peroxidase (HRP)-conjugated anti-human-IgG (at dilution 1:16,000, DAKO, Germany) was added and then incubated again for 1 hour at 37°C. After incubations, the polystyrene strips were washed with 0.1% Tween 20 in PBS. After that, tetramethylbenzidine (TMB) was added and incubated in darkness for 15min at 20°C. The reaction was stopped with 0.2N sulfuric acid and the optical absorbance was measured at wavelength 450nm by a BEP III analyzer (Siemens, Germany).

EIA-units were determined as ratios by comparing the specimens average absorbance to the negative controls (<1 EIA-units) and positive controls (100 EIA-units) average absorbance values. Sera were scored positive when the EIA-unit was ≥10, borderline with 5-9 and negative with ≤4 EIA-units. Seroconversion was defined by appearance of HSV-specific IgG antibodies (≥10 EIA-units) in a previously seronegative individual.

4.5 Statistical analyses (I,II,III)

Statistical analyses were performed with SPSS® (SPSS for Windows, version 22.0.0.1, SPSS Inc., Chicago, IL, USA) and STATA (STATA/SE 13.1, StataCorp, College Station, TX, USA). The $\chi 2$ test was used to analyze frequency tables. Differences in the means of continuous variables were analyzed using the Mann–Whitney test or the Kruskal–Wallis test for two and multiple independent samples, respectively. ANOVA was used to analyze the differences between groups. Generalized estimating equation (GEE) modeling was used to analyze the presence of HSV-1 and HPV in epithelial scrapings during the FU as described (Rintala et al., 2005; Louvanto et al., 2010; Rautava et al., 2012b).

5 RESULTS

5.1 HSV DNA in oral epithelial cells (I,II)

These studies determined the presence of HSV DNA with PCR in the oral mucosa of Finnish adults before and after delivery, in order to evaluate current prevalence of asymptomatic oral HSV shedding among young adults near reproductive age in Finland. Women were HSV-1 DNA positive at least once in their oral epithelial brush specimens in 11% (36/325) of the total number of individuals (I) (Table 3) and the men in 13% (18/139) (II) (Table 4) during the 6-year follow-up. In total, only 2.2% (42/1873) of the women's oral follow-up specimens and 2.8% (20/722) of the men's specimens were HSV-1 DNA positive and none of them tested HSV-2 DNA positive. HSV-1 DNA positivity in women's oral specimens varied between 1.4% at 2-month follow-up visit and 4.0% at 72-month follow-up visit. During pregnancy, the HSV-1 DNA positivity was 2.3% (Figure 10). Correspondingly, among the men HSV-1 DNA positivity in oral specimens varied from 0% at 72-month follow-up visit to 7.2% at 2-month follow-up visit (Figure 10). Only six women (1.8%, 6/325) and two men (1.4%, 2/139) tested HSV-1 DNA positive twice ($p>0.05$, *non-significant*) and none of the participants tested HSV-1 DNA positive more than two times during follow-up (Tables 3 and 4). Five of the seven women who tested HSV-1 DNA positive during the last trimester also remained HSV-1 DNA positive after the delivery for at least one follow-up visit (only one of them at 2-month follow-up visit) (Table 3).

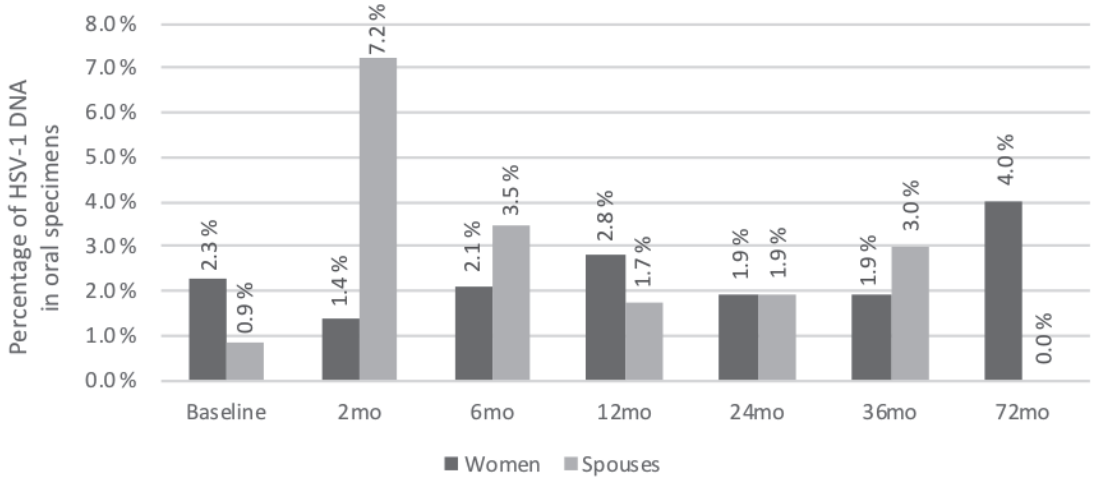

Figure 10. Oral HSV-1 DNA among women and their spouses during follow-up. HSV-1 DNA prevalence was calculated separately for women and their spouses according to follow-up visits. Results previously published (Papers I and II).

Table 3. Concordance of HSV-1 and HPV DNA positive specimens in women with HSV-1 DNA-positive oral specimens during 6-year follow-up*

	Follow-up visit						
ID	BASE	2MO	6MO	12MO	24MO	36MO	72MO
1	1	0 (16)	0 (18)	0	0	0	1 (16)
2	1	1	0 (35,59)	0	0	0	0
3	1	0	0	0	0	0 (16)	0
4	0 (HPV6)	0 (16)	0	1	0 (16)	0	
5	0	0	0	0	0	1	0
6	0	0	1	0	0	0	
7	1	0	0	1	0	0	0
8	0	0	1	1	0	0	0
9	1	0	1 (16)	0	0 (11,16)	0 (16)	0
10	0	0	0	0 (58)	0	1	0
11	0 (6)	0 (59,66)	0	0	0	0	1
12	0	0	0	1	0	0	0
13	1	0	0 (70)	0	0	0	0
14	0	0	0 (16)	0	1	0	0
15	0 (11)	0	1	0	0 (16)	0 (16)	0 (16)
16	0 (16)	0 (16,66)	1 (31)	0 (58)	0	0	0
17	0 (16)	0	0 (16)	1	0	0	0 (16)
18	0	1	0	0	0	0	0
19	0 (6)	1	0	0	0	0	
20	0	0	0	0	1	0	0
21	0	0	0	0	0	0	1
22	0	0	1 (11)	0	0 (16)	0	0
23	0	0 (16)	0				1
24	0		0				1
25	0	0	0	0	1	0	0 (16)
26	0	0	0	0	1	0	
27	0	0	0	0	0	0	1
28	0 (16)		0	0	0	1	0
29	0	1	0	0	0	0	0
30	0	0	0	0	1	0 (6)	
31	0	0	0	1			
32	0	0	0	0			1
33	0	0 (16)	0 (16)	1	0 (16,18)	0	0
34	0	0	0	0	0	1	
35	1	0	0 (16)	0	0	1	0 (16)
36	0	0 (16)	0 (16)	1	0 (16)	0 (16)	0

**1=HSV-1 DNA positive oral specimen, 0=HSV-1 DNA negative oral specimen and ID=patient code. The HPV type is indicated in parentheses, ID numbers 16,18,26,35=Individual's spouse is seen in Table 4 with the same ID number.*

Table 4. Concordance of HSV-1 and HPV DNA positive specimens in male spouses with HSV-1 DNA-positive oral specimens during 6-year follow-up *

	Follow-up visit						
ID	BASE	2MO	6MO	12MO	24MO	36MO	72MO
16	0	0	0	1	0	0	0
18	0	0	0	0 (18)	0	1	0
26	0	1 (16)	0	0	1 (16)	0	0
35	0	1	0	0	0 (70)	0	0
37	1 (HPV16)	0 (16,58)	0	0	0 (16)	0	0
38	0	1	0	0	0	0	0
39	0	1	0	0	0	0	0
40	0	1	1	0 (6)	0	0	0
41	0	1 (16)	0 (16)	0	0	0	0
42	0 (82)	1	0	0	0	0	0
43	0	1	0 (16)	0	0	0	0
44	0 (11)	0	1 (16)	0 (16)	0 (16)	0 (16)	0
45	0	0	1	0	0	0	0
46	0	0 (16)	1 (18)	0 (16)	0	0	0 (16)
47	0	0	0	1	0	0	0
48	0 (16)	0 (16)	0 (33)	0	1	0	0
49	0	0	0	0	0	1	0
50	0 (16)	0	0 (16)	0	0	1 (16)	0 (16)

**1=HSV-1 DNA positive oral specimen, 0=HSV-1 DNA negative oral specimen and ID=patient code. The HPV type is indicated in parentheses, ID numbers 16,18,26,35=Individual's spouse is seen in Table 3 with the same ID number.*

5.1.1 Oral HSV-1 DNA among the couples (II)

The asymptomatic oral shedding of HSV was analyzed in women and their male spouses, in order to evaluate possible risk of transmission and to compare oral HSV shedding prevalence between genders. The oral HSV-1 DNA positivity in epithelial specimens was similar among the men (2.8%, 20/722) and their corresponding female spouses (3.0%, 25/834), ($p<0.05$; *non-significant difference*). Concordant HSV-1 DNA-positive oral epithelial specimens were found in 2.9% (4/139) of the couples (couple=woman and corresponding male spouse) during the follow-up ($p<0.05$; *non-significant*). However, only one of these couples was HSV-1 DNA positive at the same 2-month follow-up visit (Tables 3 and 4). The man of this couple was oral HSV-1 DNA positive twice during the study, at 2-month and 24-month follow-up visits, and both the woman and man in this couple seroconverted at 36-month follow-up visit. The follow-up chart of these four couples with concordant HSV-1 DNA positive oral samples is seen in the Paper II.

5.1.2 HSV-1 DNA load in the oral specimens (I,II)

Viral load in the oral epithelial specimens were demonstrated with copy number values, of which reflect the amount of amplified HSV DNA in a specimen, analyzed with real-time quantitative PCR. The mean copy numbers of the women followed a pattern where the mean copy numbers were highest during the pregnancy (772 copies), and was followed by a decline after delivery during 2-month (34 copies/specimen) and 6-month (13 copies/specimen) follow-up visits. A rise in the mean copy numbers is seen again at 12-month (124 copies/specimen without an outlier) follow-up visit, followed by stabilization of the mean copy numbers during 24-, 36- and 72-month follow-up visits (Figure 11). During follow-up, women had averagely higher copy numbers than men (p=*non-significant)* ranging individually between 2 and 2537, without and outlier (copy number 134 054). The mean copy numbers of the HSV-1 DNA-positive specimens of the male spouses (II) varied between 4 and 552 viral genome copies/specimen, being highest at 2-month and lowest at 36-month follow-up visits, the 2-month follow-up visit having the widest standard deviation (SD) of ±2560 and the 6-month follow-up visit having the second widest SD of ±220. The widest standard deviation is due to two men, who had higher copy numbers of 4002 and 344 at 2-month follow-up visit, and one man had copy number 378 at 6-month follow-up visit. Other men had copy numbers ranging between 2 and 73 during follow-up. The mean copy numbers during the follow-up are seen in the Figure 11.

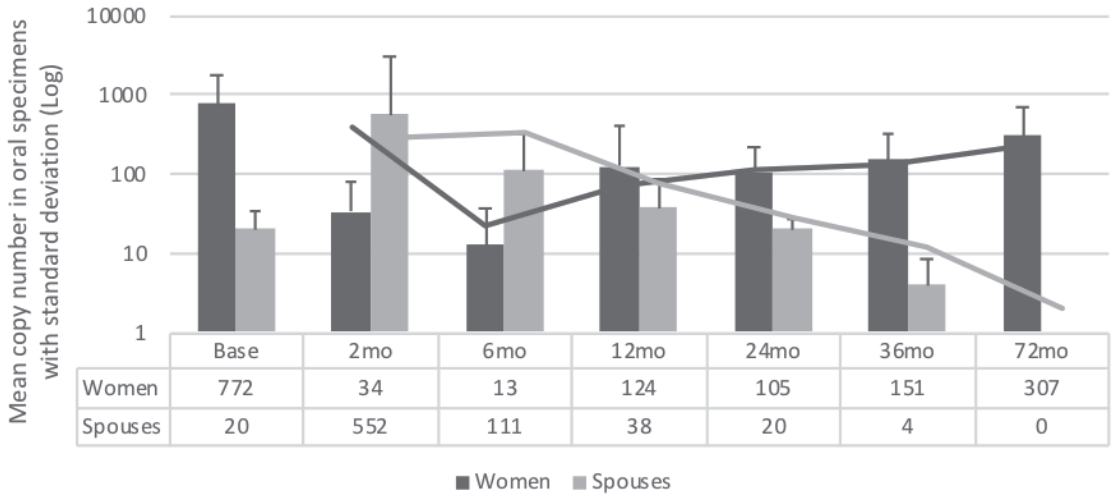

Figure 11. Mean copy numbers of the HSV-1 DNA-positive specimens among women and their spouses (Log, SD +1). An outlier at the 12-month visit (copy number 134 054) has been excluded from this chart. Results previously published (Papers I and II).

5.2 HPV DNA in oral epithelial specimens (I,II)

The oral HPV DNA detection and genotyping in the same oral specimens have been made and published earlier (Louvanto et al., 2010; Rautava et al., 2012b). For this study, the number of specimens was less than previously published in HPV analyzes, because some specimens were exhausted. In this study cohort, altogether 52% (170/325) women and 52% (72/139) men were HPV positive in at least one oral brush specimen during follow-up. Of all the oral brush specimens of women and men, 20% (523/2595) were HPV DNA positive. In total, 20% (367/1873) of the oral brush specimens from the women were HPV DNA positive (Paper I). In male spouses, 22% (156/722) of the oral brush specimens were HPV DNA positive (Paper II). The HPV DNA point prevalence of the women varied between 24% at 6-month follow-up visit to 15% at 36-month follow-up visit, while in male spouses the HPV DNA point prevalence varied between 30% at 2-month follow-up visit and 16% at 36-month follow-up visit.

5.2.1 HSV-1 and HPV co-infection in oral epithelial brush specimens

In this study, previously published results of oral HPV in the same cohort were used, in order to gain further insights into the frequency of coincident oral HSV and HPV infection, in the same oral epithelial samples among young adults in Finland. Altogether, only 0.2% (4/1873) of the women's ($p<0.001$, *significant*) and 1.0% (7/722) of the male spouses' ($p>0.05$; *non-significant*) oral epithelial brush samples contained both HSV-1 DNA and HPV DNA. At an individual level, 1.2% (4/325) of women and 4.3% (6/139) of men had HSV-1 DNA and HPV DNA dual positive samples. All six of these men had a persistent oral HPV infection. Among the four HSV-1 and HPV positive women, two were HSV-1 DNA positive twice during the follow-up and also had a persistent oral HPV 16 infection. In this study, both oral HSV-1 DNA positive and negative participants had persistent high-risk HPV infections in their oral cavity (mostly HPV 16) during the follow-up period. In Tables 3 and 4, all the HSV-1 DNA positive individuals and HPV types for each visit are presented.

The demographic data were collected with questionnaires at the baseline visit, and are described in Paper III. There was no statistically significant association between any of the demographic data (e.g. age, gender, sexual habits or the use of tobacco or alcohol) with the oral carriage of HSV DNA in women or in their spouses ($p>0.05$; *non-significant association*).

5.3 HSV seroprevalence (III)

To demonstrate current HSV seroprevalence and seroconversion in young Finnish adults before and after delivery, their blood specimens were tested for IgG antibodies against HSVs with indirect enzyme immunoassay method (EIA), during a 6-year follow-up. At the baseline visit, the HSV seropositivity was 54% (153/285) among women and 47% (56/120) among their spouses. In total, 12% (23/196) of all the participants who were seronegative or borderline at baseline (129 seronegative women, 3 borderline women and 64 seronegative spouses) seroconverted during the follow-up. According to gender, 11% (15/132) of women and 13% (8/64) of their spouses seroconverted. If all participants (seropositive, seronegative and borderline at baseline visit) are included, the fraction of seroconverted was 5.3% (15/285) for women and 6.7% (8/120) for their male spouses. Total HSV seropositivity (seropositive participants at baseline + seroconverted participants) among all the women and their spouses was 57% (232/405); the total HSV seropositivity among women was 59% (168/285) and 53% (64/120) in their male spouses. In total, 1.7% (7/405) of participants remained borderline during the follow-up. The summary table of the HSV serology among women and their spouses is presented in the Paper III.

5.3.1 Antibody levels of the participants (III)

IgG antibody levels against HSVs are presented as EIA-units of the participants, which demonstrate the concentration of IgG antibodies in blood specimens. EIA-units are ratios of optical absorbance values analyzed with indirect enzyme immunoassay, where small absorbing molecules are hybridized to IgG molecules. EIA-units were determined as ratios by comparing the specimens' average absorbance to the negative controls (<1 EIA-units) and positive controls (100 EIA-units) average absorbance values (chapter 4.4.2). Median EIA-units in HSV serology at baseline were 72 (range 2-119) for the women and 84 (range 2-114) for their spouses. The median EIA-unit was statistically lower in HSV-seropositive women than in their spouses (p=0.0017). The total median EIA-unit for all participants was 74 (range 2-119) at the baseline follow-up visit. Among all the seroconverted participants, the median EIA-unit was 37 (range 10-104) at their last follow-up visit. According to gender, the median EIA-unit was 70 (range 10-104) for the seroconverted women and 16 (range 10-58) for their seroconverted spouses. Among the seroconverted, the EIA-units were significantly higher in women than in their spouses (p=0.0007), which is contradictory to that found at baseline.

5.3.2 HSV seropositivity and seroconversion among the couples (III)

The HSV seropositivity among the couples was assessed, in order to compare prevalence between genders and to evaluate possible risk of transmission between couples. In 39% (47/120) of the couples, both individuals were HSV seropositive. The male spouses of the HSV-seropositive women were HSV seropositive in 60% (47/78) of the cases, while 73% (47/64) of the female spouses of the seropositive men were HSV seropositive (Paper III). HSV seroconversion was detectable at the same follow-up visit in only one couple. Seven of eight of the seroconverted men were the spouses of HSV-seropositive women. On the contrary, only 27% (4/15) of the seroconverted women were spouses of seropositive men.

5.3.3 HSV seroprevalence and demographic factors (III)

Demographic and behavioral factors were assessed with questionnaires at the baseline visit. The questions were previously intended for the study of HPV-infection, but the same questionnaire that were once filled, were also used for this study of HSV-infections in the same cohort. The older age (26.0±3 years; p=0.006) and positive history of genital warts (self-reported in questionnaire) (p=0.006) of the women were associated with HSV seropositivity. A younger age in women (23.7±2 years) was associated with HSV seroconversion (p=0.023). Also, among the men, older age (29.6±5 years) seems to be associated with HSV seropositivity and younger age (26.5±4 years) with HSV seroconversion, however, the difference between these groups were statistically insignificant because of wide standard deviation. Frequent oral sex (self-reported in questionnaire with the scale: frequently, sometimes, never) practiced by the men was associated with their HSV seropositivity (p=0.033). Other demographic factors such as education, age at sexual debut, number of sexual partners or the use of alcohol or tobacco had no statistically significant association to HSV serology. The complete listing of the association of demographic factors with HSV serology is presented in Paper III.

5.4 HSV serology associated with oral HSV-1 DNA (III)

This study analyzed the prevalence of oral HSV-1 shedding among seropositive and seronegative young women and men, to evaluate the prevalence of oral HSV shedding among HSV seropositive individuals in Finland. Altogether 19% (31/168) of the seropositive women and 20% (13/64) of their seropositive spous-

es tested HSV-1 DNA positive in their oral samples and none tested HSV-2 DNA positive. Of the women and their spouses who remained HSV seronegative during the follow-up (borderline results excluded), 2.7% (3/112) and 7.4% (4/54) tested HSV-1 DNA positive in their oral samples, respectively. These four HSV seronegative but HSV-1 DNA positive men had low viral loads in their oral specimens, ranging between 2-4 genome copies per specimen. Also, one of the three seronegative women had low viral load in her oral specimen, but the other two women had viral loads of 576 copies per specimen at 72-month follow-up visit and 1071 561 copies at 12-month follow-up visit (the outlier that has been described before in the chapter 5.1.2).

13% (2/15) of the seroconverted women and none of the seroconverted spouses had oral HSV-1 DNA detectable during the follow-up. One of these two women seroconverted at 36-month follow-up visit and had an EIA-unit of 70, and oral viral load was detected at 222 copies per specimen at 24-month follow-up visit. Interestingly, the spouse of this woman had also HSV-1 DNA positive oral specimen at the same 24-month follow-up visit. He was HSV seronegative at baseline, but had a borderline EIA-unit value 9 at 36-month follow-up visit, which means that he could be HSV seronegative or seropositive, but specimen at 72-month follow-up visit was not collected.

5.5 HSV serology associated with HPV (unpublished data)

To identify the possible association between HSV serology and previously published results of HPV infection (oral and genital), statistical comparison has been performed. At the baseline visit, HSV-seropositive women had statistically more frequent oral HPV infections with low-risk species 10 (HPV 6 and 11) and high-risk 9 (HPV 16 and 58) than with other HPV species (p=0.007) (Table 6). Statistical difference between genital HPV types and HSV seropositivity were not found, however at baseline visit, high-risk species 9 (HPV 16, 31, 35 and 58) and 7 (HPV 18, 45 and 70) were the most prevalent types in genital mucosa. At 12-month (p=0.048) and 72-month (p=0.047) follow-up visit, women's genital HPV positivity was associated with HSV seropositivity. Especially at 12-month follow-up visit, genital HPV positive women were more prone to HSV seroconversions, as 77% (10/13) of the HSV seroconverted women were genital HPV positive (p=0.044) (Table 6). 70% (52/74) of women with a positive history of genital warts were HSV seropositive, compared to 48% (92/190) among women who had no history of genital warts (p=0.006) (Table 6).

Among men, most common HPV species in oral mucosa at baseline visit was high-risk species 9 (HPV 16, 31, 33) (p=*non-significant*). In urethral mucosa

most common HPV species at baseline visit were the high-risk HPV species 9 (HPV 16 and HPV 33) and low-risk HPV species 10 (HPV 6 and HPV 11), but no statistical difference were detected among HSV seropositive and HSV seronegative individuals (Table 7). Urethral high-risk HPV types among men at baseline were associated with HSV seropositivity (p=0.001). In total, among men with high-risk urethral HPV, HSV seropositivity was 80% (16/20), while only 20% (4/20) were HSV seronegative (Table 7).

Table 6. Association of HPV DNA carriage and HSV seroprevalence of 285 women during 6-year follow-up period in Finland*

Variables	Total n* (%)	HSV seropositive n(%)	HSV seronegative n(%)	HSV Seroconverted n(%)	Borderline HSV positive n(%)	p**
Oral HPV at baseline	283	152	112	14	5	0.74
HPV-negative	237 (83.7%)	125 (52.7%)	94 (39.7%)	13 (5.5%)	5 (2.1%)	
HPV-positive	46 (16.3%)	27 (58.7%)	18 (39.1%)	1 (2.2%)	0	
Oral HPV species* at baseline**	43	26	16	1	-	**0.007**
Species 10 (HPV 6, 11)	7 (16.3%)	6 (85.7%)	0 (0%)	1 (14.3%)	-	
Species 9 (HPV 16, 58)	33 (76.7%)	20 (60.6%)	13 (39.4%)	0	-	
Species 7 (HPV 18, 39)	1 (2.3%)	0	1 (100%)	0	-	
Species 6 (HPV 56, 66)	2 (4.7%)	0	2 (100%)	0	-	
Genital HPV at baseline	285	153	112	15	5	0.31
HPV-negative	237 (83.2%)	122 (51.5%)	98 (41.4%)	12 (5.1%)	5 (2.1%)	
HPV-positive	48 (16.8%)	31 (64.6%)	14 (29.2%)	3 (6.3%)	0 (0%)	
Genital HPV species at baseline*						
Species 10 (HPV 6, 11)	2	2	0	0	0	0.63
Species 9 (HPV 16, 31, 35, 58)	15	8	6	1		
Species 8 (HPV 43)	1	1	0	0	0	
Species 7 (HPV 18, 45, 70)	6	4	2	0	0	
Species 6 (HPV 56)	1	0	0	1	0	
Species 5 (HPV 51)	1	0	1	0	0	
Species 1 (HPV 42)	1	1	0	0	0	
Genital HPV at 12-month visit	281	153	111	13	4	**0.048**
HPV-negative	147 (52.3%)	76 (51.7%)	65 (44.2%)	3 (2.0%)	3 (2.0%)	
HPV-positive	134 (47.7%)	77 (57.5%)	46 (34.3%)	10 (7.5%)	1 (0.75%)	
Genital HPV at 12-month visit	281	**not seroconverted (n=268)**		**seroconverted (n=13)**		**0.044**
HPV-negative	147 (52.3%)	144 (98.0%)		3 (2.0%)		
HPV-positive	134 (47.7%)	124 (92.5%)		10 (7.5%)		
Genital HPV at 72-month visit	167	93	62	8	4	**0.047**
HPV-negative	114 (68.3%)	69 (60.5%)	38 (33.3%)	3 (2.6%)	4 (3.5%)	
HPV-positive	53 (31.7%)	24 (45.3%)	24 (45.3%)	5 (9.4%)	0	
History of oral warts	261	141	102	14	4	0.83
No	253 (96.9%)	137 (54.2%)	98 (38.7%)	14 (5.5%)	4 (1.6%)	
Yes	8 (3.1%)	4 (50.0%)	4 (50.0%)	0	0	
History of genital warts	264	144	102	14	4	**0.006**
No	190 (72.0%)	92 (48.4%)	84 (44.2%)	10 (5.3%)	4 (2.1%)	
Yes	74 (28.0%)	52 (70.3%)	18 (24.3%)	4 (5.4%)	0 (0%)	
History of cutaneous warts	158	84	64	7	3	0.84
hands	58 (36.7%)	34 (58.6%)	20 (34.5%)	3 (5.2%)	1 (1.7%)	
feet	62 (39.2%)	30 (48.4%)	28 (45.2%)	2 (3.2%)	2 (3.2%)	
Multiple location	38 (24.1%)	20 (52.6%)	16 (42.1%)	2 (5.3%)	0	

**n=Number of women at each variable group according to HSV serology status. In total, there were 285 women available for HSV serology analysis, but the total n is variable among different variables, as not all participants had answered the individual questions.*

***p-values were calculated separately for each variable. The statistically significant difference between HSV serology groups according to each variable has been calculated.*

****Detected oral/genital HPV types at baseline are given in parenthesis. Multiple HPV infections were detected in 21 oral and in three genital samples, not included in species table.*

Table 7. Association of HPV DNA carriage and HSV seroprevalence of 120 male spouses during 6-year follow up period in Finland*

Variables	Total n* (%)	HSV seroposi-tive n(%)	HSV seroneg-ative n(%)	HSV Serocon-verted n(%)	Borderline n(%)	P**
Oral HPV at baseline	118	55	53	8	2	0.91
HPV-negative	96 (81.4%)	45 (46.9%)	43 (44.8%)	6 (6.3%)	2 (2.1%)	
HPV-positive	22 (18.6%)	10 (45.5%)	10 (45.5%)	2 (9.1%)	0 (0%)	
Oral HPV species at baseline* **	22					0.60
Species 10 (HPV 6, 11)	2	0	2	0	0	
Species 9 (HPV 16, 31, 33)	12	7	4	1	0	
Species 8 (HPV 43)	1	0	1	0	0	
Species 7 (HPV 18, 70)	2	0	2	0	0	
Species 5 (HPV 82)	2	1	0	1	0	
Multiple HPV types	3	2	1	0	0	
Urethra HPV at baseline	115	55	52	6	2	0.70
HPV-negative	89 (77.4%)	42 (47.2%)	40 (44.9%)	5 (5.6%)	2 (2.2%)	
HPV-positive	26 (22.6%)	13 (50.0%)	12 (46.2%)	1 (3.8%)	0 (0%)	
Urethra HPV species at baseline* **	26					0.26
Species 10 (HPV 6, 11)	6	1	5	0	0	
Species 9 (HPV 16, 33)	8	5	3	0	0	
Species 8 (HPV 43)	1	1	0	0	0	
Species 7 (HPV 70)	2	2	0	0	0	
Species 6 (HPV 53, 56)	2	1	1	0	0	
Species 5 (HPV 51)	2	1	1	0	0	
Multiple HPV types	5	2	2	1	0	
Urethra high-risk HPV at baseline	115	55	52	6	2	**0.001**
No	95 (82.6%)	39 (41.1%)	48 (50.5%)	6 (6.3%)	2 (2.1%)	
Yes	20 (17.4%)	16 (80.0%)	4 (20.0%)	0 (0%)	0 (0%)	
History of oral warts	105 (100%)	49	47	7	2	0.15
No	98 (93.3%)	46 (46.9%)	45 (45.9%)	5 (5.1%)	2 (2.0%)	
Yes	7 (6.7%)	3 (42.9%)	2 (28.6%)	2 (28.6%)	0 (0%)	
History of genital warts	105	48	48	7	2	0.94
No	88 (83.8%)	39 (44.3%)	41 (46.6%)	6 (6.8%)	2 (2.3%)	
Yes	17 (16.2%)	9 (52.9%)	7 (41.2%)	1 (5.9%)	0 (0%)	
History of cutaneous warts	107	50	48	7	2	0.60
No	59 (55.1%)	31 (52.5%)	22 (37.3%)	4 (6.8%)	2 (3.4%)	
Yes	48 (44.9%)	19 (39.6%)	26 (54.2%)	3 (6.3%)	0 (0%)	

**n=Number of women at each variable group according to HSV serology status. In total, there were 120 male spouses available for HSV serology analysis, but the total n is variable among different variables, as not all participants had answered the individual questions.*

***p-values were calculated separately for each variable. The statistically significant difference between HSV serology groups according to each variable has been calculated.*

****Detected oral/urethral HPV types at baseline are given in parenthesis.*

6 DISCUSSION

6.1 Oral HSV

The current study assessed intraoral HSV shedding by detecting HSV-1 and HSV-2 DNA in oral epithelial brush specimens of young healthy Finnish pregnant women and their male spouses in the Finnish Family HPV Study cohort during a six-year follow-up during years 1998-2008. The Finnish Family HPV Study was previously planned to study HPV infections and thus, for the current study of HSV, in the same cohort, symptoms of HSV disease have not been recorded. The study hypothesis was that HSV DNA can be found in oral epithelial cells of healthy mucosa in adults near the delivery of their offspring. Oral epithelial HSV-1 DNA was found in 2.2% (42/1873) of the women's samples and in 2.8% (20/722) of their spouses' samples. The detected prevalence of oral HSV-1 DNA is similar to that found in previous studies on asymptomatic individuals, ranging between 2% to 9% on average among the healthy adult population (Wheeler, 1988; Miller and Danaher, 2008; Roizman et al., 2013). However, similar long-term follow-up studies among pregnant women and their spouses have not been done before. It is possible, however, to assume that the samplings in current study missed some of the short HSV shedding episodes and the prevalence of both HSV-1 and HSV-2 DNA would have been higher if the sampling could have been performed more often (in this study, the timespan between two subsequent samplings varied between 2 months and 3 years), as shedding of oral HSV is likely also to happen between the follow-up visits, and the HSV shedding episodes are short in duration, ranging individually between 4 hours to 12 days in healthy individuals (Mark et al., 2008).

No oral specimens positive for HSV-2 DNA were found, which was expected, as oral HSV-2 shedding is rare among healthy individuals (Wald et al., 2004). Based on the previous literature, the estimation was that HSV-2 shedding would have been common in genital mucosa (Tronstein et al., 2011; Wald et al., 2000) of these healthy individuals, while oral HSV-2 shedding would have been frequent in immunocompromised individuals, only (Aggarwal et al., 2014; Kim et al., 2006). In addition, according to a meta-analysis, asymptomatic HSV-1 shedding was 7.5 times more likely than HSV-2 shedding to be found from the oral cavity among healthy individuals ($p<0.0001$) (Miller and Danaher, 2008).

In the Finnish Family HPV Study, oral epithelial brush specimens were collected from the upper and lower vestibular area and from the buccal mucosa of both cheeks with a small brush. The contamination of the epithelial brush specimens with saliva cannot be totally eliminated, and some of the HSV shedding detected

in this study might have also come from other oral areas than those which were sampled via rinsing with saliva. It is expected that the frequency of HSV shedding in the present studies would have been higher, if the whole saliva would have been used for HSV DNA testing instead of the epithelial brush specimens. It is known that HSV sheds intraorally into the saliva from still partly unknown intraoral shedding sites. Only a little is known to date of the distribution of the intraoral HSV shedding sites, as there are only a few previous studies available. However, in recent studies, HSV-1 shedding was similar at different oral sites (da Silva et al., 2005; Ramchandani et al., 2016) and only minor differences between the left and right sides of the oral cavity were found (Ramchandani et al., 2016). Due to the small number of previous studies, and because in the current study no comparisons between different oral sites were made, it is impossible to make conclusions about the most frequent sites of intraoral HSV shedding. However, one can speculate that in the oral cavity HSV shedding occurs near the primary infection site and within the neuronal innervating area of the trigeminal nerve of the same side (left or right) (Sawtell, 1998).

In this study, oral HSV-1 DNA prevalence and viral load were compared between genders before and after the birth of their offspring. In order to assess the possible effect of pregnancy on oral HSV-1 shedding. The oral HSV-1 DNA positivity in epithelial specimens was found to be nearly the same among the men (2.8%, 20/722) as in their female spouses (3.0%, 25/834), which confirms earlier results. The previous meta-analysis showed that there are no clear gender differences in oral shedding of HSV (Miller and Danaher, 2008), even though e.g. Kaufman et al. 2005 showed that HSV-1 shedding was more prevalent in men (Kaufman et al., 2005). However, these studies did not include any pregnant women (Kaufman et al., 2005, Miller and Danaher, 2008). Interestingly in the current study, both the frequency and viral load of HSV-1 DNA were higher in the men after the birth of their offspring. At the baseline visit, the men had low HSV-1 DNA prevalence and viral load but at the 2-month follow-up visit HSV-1 DNA was detected in 7.2% of the men, of which was distinguishable high. This result is difficult to explain. One possible explanation could be the increased number of sexual activity between the spouses (e.g. oral sex) two months after delivery. However, this would lead to increased number of HSV seroconversions, which however, probably was not the case as none of the seroconverted men had oral epithelial HSV-1 DNA detectable during the follow-up, but they could have had HSV-DNA in their whole saliva. Van Anders et al. 2013 made a study on postpartum sexuality by investigating physical and psychosocial influences on the experiences of partners (n= 95 men, 18 women, 1 unspecified) of parous women during three months following their child's birth. The results showed that the partners reported most frequent engagement in intercourse in the postpartum period, earliest engagement in masturbation, and highest enjoyment of receiving

oral sex compared with other sexual activities (van Anders et al., 2013). Partners' sexual desire was not correlated with the psychosocial variables. Findings for partners' sexuality were similar by gender, except for perceptions of social support and likelihood to engage in intercourse (van Anders et al., 2013). Another explanation could be that the men (i.e. fathers) probably experienced more stress after the birth of their infant (e.g. staying awake during nights), which could result in higher amount of oral HSV-1 recurrences. This is supported by the fact that the proportion of oral HSV-1 DNA positive men was the largest during the 2-month follow-up point followed by that of 6-month follow-up point. One technical point has also to be considered. Among men the standard deviation of HSV viral load was also most high at the 2-month follow-up visit. However, as the total number of HSV-1 DNA positive men is relative low, it is premature to make any conclusions on trends in viral loads of the men.

Contrary to the men, the delivery seems to have a decreasing effect on oral HSV-1 DNA prevalence and on viral loads in women. The highest HSV-1 DNA prevalence and viral load among women were observed at the baseline visit, when they all were pregnant in their 3rd trimester, and the lowest HSV-1 DNA prevalence and viral load after delivery at 2-month follow-up visit. This could be explained by hormonal and immunological changes during pregnancy and postpartum as follows: during pregnancy, women are more prone to HSV infections (Sappenfield et al., 2013) or/and there is a low-level immunosuppression (Mellor and Munn, 2001). However, studies based on questionnaires have shown that women reported less symptomatic HSV recurrences in the oral area during pregnancy than when not being pregnant (Scott et al., 2003). The mechanism is unclear, but interestingly, a similar pattern in HPV 16 copy numbers was found in the same cohort during and after pregnancy (Lorenzi et al., 2017), as found here with HSV. Women from the same cohort with HPV 16 infection had relatively high HPV viral loads during pregnancy compared to after delivery at the 2-month follow-up visit, and the male spouses with HPV 16 infection had low HPV viral loads before delivery and high viral loads after delivery of his child (Lorenzi et al., 2017). These findings of HSV-1 and HPV 16 infection suggest that pregnancy and delivery might have an effect on viral loads and reactivation due to the immunology while in men the results could be explained by behavioral habits. Further studies are needed to understand the interesting gender differences.

In this study, it was possible to compare oral HSV-1 shedding among HSV-seropositive, -seronegative and -seroconverted individuals. Among HSV-seropositive individuals alone, the oral HSV-1 shedding was more common than among all participants, as 19% (31/168) of HSV-seropositive women and 20% (13/64) of their HSV-seropositive spouses tested oral HSV-1 DNA positive by qPCR. Our finding is in accordance with previous studies, which showed that

approximately 20% to 30% of HSV-1-seropositive persons have recurrent intraoral HSV-1 infections 1-4 times annually, especially after stressful events (Miller and Danaher, 2008). The present and the previous results allow the conclusion that oral HSV-1 shedding is common among HSV-seropositive individuals. However, only two women out of all the seroconverted individuals showed also HSV-1 DNA in their oral specimens during the six-year follow-up, even though HSV shedding episodes occur most frequently near primary infections, as shown in genital infections (Benedetti et al., 1999). A possible explanation for the low number of detected HSV recurrences among the seroconverted might be that HSV recurrences did not have enough time to occur before the end of the follow-up, because most of these seroconversions might have occurred late during the follow-up, at 36-month and 72-month follow-up visits. However, it is not certain when the seroconversions took place, as detection of HSV antibodies was done only at the baseline visit and at the latest follow-up visit of each individual. In addition, the longest timespan between subsequent samplings during this study occurred between the last two follow-up visits (approximately 3 years), and HSV recurrences and shedding had probably also occurred between these sampling points (Mark et al., 2008; Tronstein et al., 2011).

In addition, a small fraction of the women and their spouses who remained HSV seronegative during the follow-up tested HSV-1 DNA positive in their oral specimens. This finding was expected, as previous studies have also reported that seronegative individuals shed HSV intraorally (Hatherley et al., 1980; Kaufman et al., 2005; Miller and Danaher, 2008) or to the genital mucosa (Posavad et al., 2010). Posavad and coworkers found that six immunocompetent individuals had T-cell immunity to HSV, but were seronegative to both HSV-1 and HSV-2. It should be considered that among seronegative individuals in the current study, the oral HSV-1 DNA has most probably come from some external source e.g. from the saliva or genital mucosa of another individual, as the viral load was found low among most of these HSV-seronegative individuals. However, based on previous studies, the HSV-1 DNA found in the oral cavity of seronegative individuals could represent either a newly acquired undetected HSV infection or an acquired cell-mediated immunity in the absence of HSV (Kaufman et al., 2005; Posavad et al., 2010). In addition to these hypotheses, it has also been theorized that a fraction of the latency-associated HSV shedding might also be due to a low-level chronic HSV infection in epithelial cells or in the autonomous nervous system, rather than a real latent HSV infection accompanied by HSV IgG production, although viral persistence in non-neuronal tissues remains controversial (Openshaw et al., 1995; Schiffer and Corey, 2009). However, a previous *in vitro* study conducted in keratinocyte culture supports these findings, as expression of the LAT gene was seen by hybridization and HSV DNA was observed by PCR, even though no cytopathic effects of HSV infection were ob-

served in light microscopy (Syrjänen et al., 1996). HSV-1 DNA found among the seronegative participants in this study could be due to low-level chronic epithelial infection in the gingival sulcus, as it has been implicated as a reservoir for viral replication and latency within the oral cavity (Amit et al., 1992). New studies on anatomical locations of HSV shedding in the oral cavity are needed to establish this finding. In the present study, one of the HSV-seronegative women had an exceptionally high viral load of HSV-1 DNA (copy number of 13 4054) at one follow-up visit, although the viral load is usually low during asymptomatic shedding. The high copy number found here might indicate an active lytic HSV-1 infection.

6.1.1 HSV-1 and HPV co-infection in oral epithelial cells

In this study, previously published presence of HPV DNA in the oral cavity (Louvanto et al., 2010; Rautava et al., 2012b) were evaluated together with the presence of HSV-1 DNA in the same oral epithelial specimens, in order to gain further insights into the frequency of coincident oral HSV-1 and HPV infection among young Finnish adults. As far as the author knows this is the first study to report the prevalence of HSV-1 and HPV coinfection in healthy oral epithelium during a long-term follow-up providing thus new information in the field. In this study it was found that only 0.2% (4/1873) of women and 1.0% (7/722) of men had HSV-1 and HPV DNA co-carriage in oral epithelial specimens. Thus, simultaneous HSV-1 and HPV coinfection seems to be rare in the oral epithelium of young healthy women and their male spouses in Finland. There are only a few previous cross-sectional studies on the prevalence of HPV and HSV co-carriage in healthy tissues, and most of these studies have focus on squamous intraepithelial neoplasias or carcinomas. Thus, none of them are comparable with the current study, because this study is a long-term follow-up study on oral clinically healthy mucosa. Therefore, further studies are needed to confirm the present findings on oral co-carriage of HPV and HSV-1 at population level. One previous study reported that HSV-2 and HPV 16 or 18 co-infection is common in cervical dysplasias and in cervical carcinomas, but rare (0-4%) in normal cervical tissues (Zhao et al., 2012). Even though these results represent findings from genital mucosa, the prevalence of co-infection in healthy tissue is in agreement with the present results from oral mucosa. A possible explanation for the lack of dual infections in the present cohort could be that epithelial HSV infection is often lytic, leading to rapid disappearance of the HSV infected cells (Roizman et al., 2013). On the contrary, HPV infection is not lytic and is immunologically more silent, allowing persistence in the epithelium for longer intervals (Harden and Munger, 2017). The rare co-infections may thus represent either a state of

dual infection where HSV infection has not yet led to elimination of the cells, or a rare long-term quiescent HSV infection in epithelial cells (Openshaw et al., 1995; Stanberry et al., 2000). However, it is not possible to assess whether the HSV-1 and HPV infected the same oral epithelial cell or existed in separate cells close to each other.

Even though HPV and HSV-1 co-carriage was found to be very rare in this study, persistent HPV 16 infection was found in three women and six men among these cases. Furthermore, the women also showed at least two HSV-1 shedding episodes during follow-up. Persistent HPV 16 infection is a key risk factor of HPV-associated carcinogenesis (zur Hausen, 2009; Rautava and Syrjänen, 2012; Mehanna et al., 2013). However, only a minority of HPV 16 infections or other high-risk HPV infections will progress toward malignancy. In HPV induced malignant transformation additional co-factors are needed, such as hormones, smoking and/or other carcinogens (Shigeishi and Sugiyama, 2016). The role of infections with other pathogens such as HSV, EBV or HIV have been discussed as possible co-factors of HPV induced carcinogenesis (Castellsagué et al., 2002; Guidry and Scott, 2017). HPV/EBV and HSV/HPV coinfections have been shown to be associated with a shorter survival (Rautava et al., 2012a; Turunen et al., 2017). Thus, these HPV 16 and HSV-1 co-infected women and men identified here could theoretically be at higher risk for HPV-associated oral high risk squamous intraepithelial lesions or carcinomas.

In the present studies it was also possible to investigate the contribution of HSV in the persistence of oral HPV infection. Previous studies suggest that coincident HSV-1 infection in oral mucosa could be one of the cofactors in HPV persistence (Hara et al., 1997; Guidry and Scott, 2017). However, this hypothesis has not been previously studied in long-term follow-up. In the present studies persistent oral HPV 16 was detected at similar frequency among oral HSV-1 DNA positive and negative individuals, and between genders. This result indicates that oral HSV-1 infection seems not to be the primary cause for HPV persistence in oral mucosa. However, no firm conclusions can be made due to the low number of HSV and HPV co-infections in the cohort. Thus, population-based studies are needed to confirm the results shown here.

6.1.2 The effect of detection assay sensitivity

In the current study, oral HSV shedding has been detected by quantitative PCR (qPCR), which is a sensitive and quantitative method to detect viral DNA. In older studies, HSV detection in oral samples was performed using (less sensitive) cell cultures, and the detection rate among healthy individuals varied between

0.1% and 7.4% (Douglas and Couch, 1970; Spruance, 1984; Kameyama et al., 1988). With more sensitive methods such as PCR, the detection rate of intraoral HSV is higher (Wald et al., 2003; Miller and Danaher, 2008). In a meta-analysis, asymptomatic shedding of oral HSV-1 was detected in 6.3% of specimens in a single day by viral culture studies, and in 33% of specimens by PCR-based studies (Miller and Danaher, 2008). A previous study showed that the ratio of PCR positive to culture positive results increased from winter (3.8:1) to summer months (8.8:1), as the viral culture specimens are vulnerable during transport in warm weather (Wald et al., 2003). There are also other advantages when using PCR. First, the DNA specimens can be stored for long periods before analyses, as the quality of viral DNA remains stable (Jerome et al., 2002), and second, PCR is faster to perform than a cell culture. However, in clinical practice, viral cultures from blisters or ulcers of lytic HSV infection are sensitive enough to detect HSV (Välimaa et al., 2013), but viral culture cannot detect HSV in asymptomatic individuals reliably due to low viral load. In the current study, the HSV DNA detection rates have supposedly not been affected by the long storage (approximately 20 years) of oral DNA samples at -70°C, as repeated freezing and thawing was not done. Repeated freezing and thawing of samples might deteriorate the quantification of PCR results. It has also been suggested that the sensitivity of standard PCR is sufficient to detect HSV-1 DNA soon after the onset of clinical signs, but asymptomatic shedding of HSV-1 DNA could be detected by the more sensitive nested PCR (Knaup et al., 2000; da Silva et al., 2005). However, nested PCR is prone to contamination. qPCR was chosen instead of nested PCR (Rodrigues et al., 2013) to detect HSV DNA in the present studies, because an assay with sufficient sensitivity and specificity even for cerebrospinal fluid specimens was available in the laboratory (Hukkanen et al., 2000). In this assay the HSV DNA findings are confirmed by melting-temperature analysis. Verification of correct amplification e.g. by melting-temperature analysis or by hybridization excludes incorrect amplification. It has also been verified earlier that the primer pairs used in present qPCR detect the clinical HSV strains circulating in Finland (Hukkanen et al., 2000). Errors in PCR studies can occur if reagents are handled incorrectly. For example, the DNA polymerase needs to be kept at a low temperature and protected from light. Also, because of the high sensitivity of the PCR method precautions have to be taken to avoid contamination of samples with exogenous viral DNA.

6.2 HSV seroepidemiology

One of the hypotheses of current study was that, in Finland, only 50% to 60% of young adults near reproductive age would now be HSV seropositive, as especially HSV-1 seroprevalence has decreased in developed countries, including in Finland, during recent decades (Pebody et al., 2004; Bradley et al., 2014; Puhakka et al., 2016). As expected, the results were according to the hypothesis as the total HSV seropositivity was 57% (232/405) among women and their spouses. In addition, as HSV seroconversions were detected in 12% (23/196) of the participants, these results could support the earlier findings that primary HSV infections in Finland are currently acquired during young adulthood near reproductive age, average age being 25 years (Välimaa et al., 2013), and not as before, during early childhood. The primary HSV infection can still be acquired during early childhood, but in fewer individuals than decades ago (Aarnisalo et al., 2003; Puhakka et al., 2016). The prevalence of seroconversion in this study cohort is also in accordance with the estimated overall annual rate of HSV transmission, 4-5% annually (Roizman et al., 2013). However, the current study cohort comprised only Finnish pregnant women and their spouses and thus does not represent the general population. In the current study, the long storage of serum specimens at -70°C does supposedly not impact HSV IgG detection, as the specimens have not been thawed before.

This study evaluated HSV seroprevalence and seroconversion in Finland among young Finnish women and men before and after delivery of their child in a longitudinal setting. There are only a few earlier studies for comparison. Previous cross-sectional studies among pregnant women in Finland are available, and they represent average HSV seroprevalence among pregnant women during a given year, but no long-term follow-up studies of these women are available. These previous cross-sectional studies report a statistically significant decline in HSV seroprevalence among pregnant women in Finland, from 70% (in 1992) to 54% (in 2000) and to 45% (in 2012) (Alanen et al., 2005; Puhakka et al., 2016). The higher HSV seroprevalence of the current study sampled between years 1998-2008 compared to lower seroprevalence found in the more recent study in the year 2012 (Puhakka et al., 2016) indicate that the HSV seroprevalence among Finnish pregnant women and their male spouses may still have decreased. Based on this estimation the current HSV seroprevalence could be even lower in a cohort similar to Finnish Family HPV Study.

In this study, it was possible to compare HSV seroprevalence and its predictors among pregnant women and their male spouses. The current study showed that women (59%) were slightly more often HSV seropositive than their spouses (53%) at the end of the follow-up (*p=non-significant*). Previous studies have also

shown that women could be on average more HSV seropositive, as HSV-1 seroprevalence was slightly more common in women than men in the USA, in Northern Europe (including Finland) and in Australia (Bradley et al., 2014; Cunningham et al., 2006; Pebody et al., 2004; Smith et al., 2006), but not in all countries (Pebody et al., 2004). However, similar cohort studies to detect HSV in both women and their corresponding male spouses before and after delivery of their child have not been conducted before. Thus, comparison between average differences in HSV seroprevalence and this study cohort is problematic, as pregnancy and delivery might have affected HSV seroprevalence, as discussed in more detail in the previous chapter on oral HSV-1 shedding in women and men. The slightly higher HSV seroprevalence among women might be due to unknown biological differences in HSV seroprevalence among genders, as also showed in previous studies (Cunningham et al., 2006, Bradley et al. 2014), or it might be related to low-level immunosuppression during pregnancy (Mellor and Munn, 2001, Sappenfield et al., 2013).

Among the women of the current study, but not among the men, the HSV seropositivity increased statistically significantly along with age. Younger age increased the probability of HSV seroconversion in women but not among men. In previous studies, age has been the strongest predictor of HSV seropositivity globally among both women and men (Pebody et al., 2004; Bradley et al., 2014). The adults who acquire primary HSV infections (seroconversions) are usually young, near the reproductive age (Bernstein et al., 2013). The mean age of participating men (28.7 years) in this study is higher than of the women (25.5 years) (*p=non-significant*). The average age of primary HSV infection in Finland is 25 years, hypothetically suggesting that the men in the present study had acquired HSV infection and seroconverted earlier and thus age was not found to be associated with seroconversion. Furthermore, the lack of statistical association between age and HSV seroprevalence among the men is most probably due to low total number of men in this study cohort.

In this study, oral HSV-1 DNA and serum HSV antibodies were analyzed in couples with sexual relationship, in order to assess the possible relationship to the transmission of HSV between sexual partners. However, it cannot be demonstrated that a certain HSV infection has been transmitted from a certain individual, as genotyping of the oral strains was not performed. However, it is known that HSV is transmitted in close contact and thereby HSV transmission happens most probably within families. Asymptomatic HSV shedding is considered to be the major source of HSV transmission between individuals. In the current study, only four couples (2.9%, 4/139) were found concordantly HSV-1 DNA positive in their oral mucosa and only in one couple oral HSV-1 DNA was found at a same time, and only once. This means that there was no partner synchronization of oral

HSV reactivations, which could reinforce transmission of oral HSV-1 between couples. However, seven of the eight seroconverted men were the spouses of HSV-seropositive women while only 4/15 of the seroconverted women were spouses of seropositive men. This finding suggests that transmission of HSV may be more frequent from women to men than from men to women. Similarly, oral sexual habits seemed to influence HSV seroprevalence among the men but not among the women, as a high frequency of oral sex practiced (or experienced) by the men was associated with their HSV seropositivity. Together, these findings could implicate that HSV could transmit between the spouses during oral sex, between oral and genital mucosa/skin, and mostly from women to men. Women are more prone to genital HSV infections due to a wider mucosal surface area, as HSV infects mucosa more easily than skin, and female genitals mostly represent mucosa while male genitals are mostly covered by skin. Earlier studies also support the oro-genital transmission route, as an increasing number of primary genital HSV-1 infections (Löwhagen et al., 2000; Kortekangas-Savolainen and Vuorinen, 2007; Välimaa et al., 2013) were connected to oral sex practices (Docherty et al., 1985; Scott et al., 1997b; Lafferty et al., 2000). There is also some limited evidence suggesting that the transmission of oral HSV-1 to the genital mucosa is much more common than the transmission of genital HSV-2 to the oro-labial mucosa (Docherty et al., 1985). Transmission between partners through asymptomatic genital HSV-2 shedding has also been demonstrated (Rooney et al., 1986; Barton et al., 1987). However, more studies, larger cohorts and characterization of HSV clinical strains are needed to demonstrate the transmission (route) through asymptomatic oral HSV shedding between sexual partners.

In this study, other behavioral factors were also explored through questionnaire, but many of the factors that were expected to be associated with HSV seroprevalence, such as the number of earlier sexual partners, frequency of sex practices, age at sexual debut and a history of other sexually transmitted diseases, showed no association. In earlier studies, however, it was shown that HSV seroprevalence was higher among sexual risk behaviour groups (Nahmias et al., 1990; Smith and Robinson, 2002; Roizman et al., 2013), increasing along with the number of sexual partners (Alanen et al., 2005; Xu et al., 2007; Bradley et al., 2014). The present cohort represented couples with stable marital status, where risk behavior is not supposed to be common. Also an early age at first sexual intercourse has been associated with HSV-1 seropositivity among young individuals (Stock et al., 2001). The reason for the lack of significant association between these behavioural factors and HSV seropositivity in the present study is most probably due to the low number of respondents to some of these questions, or due to under- or overrating in their answers.

In this study, 45% of the young Finnish women were HSV seronegative at the baseline (before delivery) visit, and 11% (n=15) of them seroconverted after or during pregnancy. This finding is similar to that found previously in other developed countries (Pebody et al., 2004; Bradley et al., 2014) and in Finland, where the prevalence of HSV-seronegative mothers increased from 26% (1992) to 48% (2012) ($p<0.001$) (Puhakka et al., 2016). This indicates that seronegative pregnant women are susceptible to primary HSV infections (seroconversions) near or during pregnancy, as the primary HSV-1 infection is acquired approximately at the age of 25 years in Finland (Välimaa et al., 2013) whereas average age of first-time pregnancy, in Finland, was 29 years in 2017. A population study on Finnish pregnant women, during the years 1987-2013, showed that the odds ratio for high IgM HSV-1/HSV-2 seropositivity (indicating primary HSV infection) in early pregnancy (mean gestation age 11.1 weeks) was 1.94 (95% Cl: 0.74, 5.12) (Werler et al., 2016). Another Finnish study on pregnant women (n=558, median age 30 years) with two serial samplings during the pregnancy, showed no cases with primary HSV infection (Alanen et al., 2005). These two contradictory studies indicate that the risk for primary HSV infection during pregnancy cannot be generalized to all pregnant women. In the present study it cannot be estimated whether any of the 15 women seroconverted during the pregnancy or after it as only the first baseline serum specimen and the last follow-up specimen were analyzed. Primary HSV infection during delivery is a known risk of perinatal HSV infection, which is life-threatening for the infant, if untreated (Corey and Wald, 2009; Kimberlin et al., 2011). In the current study cohort, however, information on neonatal HSV infections were not recorded. However, based on earlier studies (Stephenson-Famy and Gardella, 2014), the examination of signs and symptoms of primary genital HSV infection near the delivery and treatment with antiviral medication (Välimaa et al., 2013) can be recommended, even though the risk of perinatal HSV infection is small.

6.3 HPV among HSV-seropositive and -seronegative individuals

To identify the possible association between HSV serology and previously published results of HPV infection (oral and genital) in the same cohort, statistical comparison has been performed. HSV-seropositive women at baseline visit had more frequently oral HPV infections with HPV genotypes belonging to species 10 (HPV6 and HPV11) and 9 (HPV16 and HPV58) than with other HPV species ($p=0.007$). This finding was expected, as these species include HPV types that are most common in the oral region (Rautava et al., 2012b). It remains unclear why HSV seronegative participants had no statistical association with these HPV species. Women's genital HPV positivity was associated with HSV seropositivity

and seroconversion, and a history of genital warts was associated with women's HSV seropositivity. Also, in men, urethral high-risk HPV positivity was associated with HSV seropositivity. These results might indicate that women and men with genital/urethral HPV infections are more prone to HSV infections or vice versa. Possible explanations include that chronic epithelial HPV infection might favor conditions which aid HSV inoculation to the epithelium (e.g. through small epithelial ulcers), as discussed more in the chapter 6.1.1. Frequency of sexual intercourse has been associated with both HSV and HPV seroprevalence which might indicate the mode of HSV or HPV transmission. Here it was found that frequent oral sex was associated with HSV seropositivity among men. However, further conclusions on HSV transmission route, whether oro-genital or oral-oral, cannot be made as the presence of HSV DNA in genital samples were not assessed here.

7 CONCLUSIONS

The main conclusions of the present study were:

1. Oral HSV-1 infection as detected in epithelial brush specimens was infrequent among young Finnish women and their male spouses during their six-year follow-up time. Co-infection with HSV-1 and HPV was rare and was found in less than 1% of the individuals. No HSV-2 infection was found among the participants.

2. Among the young parents 57% were HSV seropositive, and oral HSV-1 shedding was frequent among them. Sexual habits were associated with HSV seroprevalence in both genders.

3. Approximately 12% of sexually active young married couples seroconverted to HSV during follow-up.

4. Genital or urethral HPV infection is associated with HSV seroprevalence and seroconversion among both young women and men which might reflect the sexual behavior among the couples.

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