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– with special reference to malignancy

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To my family.

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ABSTRACT

The human body harbours a diverse microbiota, which may have beneficial or detrimental effects on health. Bacteria can protect the body against viral infection or predispose the host to it. In addition, viruses and bacteria may induce cancer development, either alone or synergistically. The interactions between the indigenous bacteria and viruses remain poorly characterised.

This thesis focused on the composition of the bacterial microbiota in the oral mucosa, cervix, placenta and breast milk of pregnant women and the oral cavity of their infants. Furthermore, the associations between human papillomavirus (HPV) infection and the bacterial microbiota were investigated. Similarities between the neonatal mouth microbiota directly after birth and the microbiota in the maternal mouth, cervix and placenta were assessed. In addition, the invasion of HPV positive cervical carcinoma cells to benign leiomyoma derived matrices and the impact of HPV viral load and ionizing radiation were evaluated in vitro.

This study was based on 39 mother-infant pairs. The maternal mouth, cervix, placenta and breast milk, as well as the infant mouth were discovered to harbour a distinct bacterial microbiota, which varied between the anatomical regions. The bacterial microbiota of HPV-positive samples was significantly different from that of HPV-negative samples in both the mother's and child's mouth as well as in the cervix and placenta. It is of note that HPV-positive samples were found to exhibit more potentially pathogenic bacteria as compared to HPV-negative samples. The bacterial microbial community in the neonatal mouth immediately after birth shared features with that detected in the placenta. Similarity to the maternal birth canal or oral microbiota was less pronounced. In the experimental model, HPV-positive cervical carcinoma cells were able to invade myoma-based tissue models. The carcinoma cell line containing less HPV was slightly more invasive than the cell line containing more HPV, but there was no difference in the area of invasion. Exposure to ionizing radiation reduced carcinoma cell invasion.

To conclude, there is a relationship between the bacterial microbiota composition and HPV infection in the maternal and infant mouth, uterine cervix and placenta. Infant oral colonization may begin before birth. In the experimental myoma tissue-model, the HPV viral load in carcinoma cell lines had only minor effect on cell invasion. In contrast, exposure to ionizing radiation reduced the depth and area of invasion of cervical carcinoma cells.

KEYWORDS: human papillomavirus, HPV, bacterium, bacteria, microbiota, microbiome, woman, infant, neonate, oral, mouth, cervix, placenta, breast milk

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TIIVISTELMÄ

Ihmiskehon eri osien mikrobistolla voi olla suotuisia tai haitallisia vaikutuksia terveyteen. Bakteerit voivat suojata kehoa virusinfektioilta tai edesauttaa niiden tarttumista. Lisäksi virukset ja bakteerit voivat altistaa syövän kehittymiselle joko yksinään tai yhteisvaikutuksellisesti. Bakteerien ja virusten vuorovaikutukset ovat huonosti tunnettuja.

Väitöskirjatutkimus keskittyi selvittämään raskaana olevien naisten ja heidän vastasyntyneiden lastensa suun limakalvojen, kohdunkaulan, istukan sekä äidinmaidon bakteerimikrobiston koostumusta ja sen yhteyttä ihmisen papilloomavirusinfektioon (HPV). Tutkittiin myös sitä, vallitseeko äidin suun, kohdunkaulan ja istukan bakteerimikrobiston ja lapsen suussa heti syntymän jälkeen todetun mikrobiston välillä yhteys. Lisäksi arvioitiin HPV positiivisten kohdunkaulasolulinjojen invaasiokykyä hyvänlaatuisista myoomakasvaimista kehitettyihin matrikseihin sekä HPV virusmäärän ja ionisoivan säteilyn vaikutusta tähän invaasioon.

Tutkimus perustui 39 äiti-lapsiparin näytteisiin. Äidin suussa, kohdunkaulassa, istukassa ja äidin maidossa sekä lapsen suussa todettiin niille ominainen bakteerimikrobisto, joka vaihteli anatomisten alueiden välillä. HPV-positiivisten näytteiden bakteerimikrobisto oli erilainen kuin HPV-negatiivisten näytteiden sekä äidin että lapsen suussa, kohdunkaulassa ja istukassa. Merkille pantavaa on, että HPV-positiivisissa näytteissä todettiin enemmän tautiprosesseihin liitettyjä bakteereita. Lapsen suun bakteerimikrobisto heti syntymän jälkeen muistutti eniten istukassa todettua mikrobistoa. Samankaltaisuus äidin synnytyskanavan tai suun bakteeriston kanssa oli vähäisempi. Kokeellisissa tutkimuksissa HPV-positiiviset kohdunkaulan syöpäsolut kykenivät invasoimaan myoomapohjaisiin kudosmalleihin. Vähemmän HPV:tä sisältävä solulinja invasoi hieman voimakkaammin kuin suuremman virusmäärän sisältävä solulinja, mutta invaasion pinta-alalla ei ollut eroa. Altistus ionisoivalle säteilylle vähensi syöpäsolujen invaasiota.

Yhteenvetona voidaan todeta, että tutkittujen anatomisten alueiden bakteerimikrobiston koostumuksen ja HPV-infektion välillä on yhteys. Vastasyntyneen lapsen suun mikrobisto voi alkaa muodostua kohdussa jo ennen syntymää. Kokeellisessa mallissa HPV:n virusmäärällä syöpäsolussa oli vain vähäinen vaikutus solujen invaasioon. Sen sijaan altistus ionisoivalle säteilylle merkittävästi vähensi syöpäsolujen invaasion syvyyttä ja pinta-alaa.

AVAINSANAT: ihmisen papilloomavirus, HPV, bakteeri, bakteeristo, mikrobisto, nainen, lapsi, vastasyntynyt, suu, kohdunkaula, istukka, äidinmaito

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Abbreviations

ANOSIM	Analysis of similarities
BWA	Burrows-Wheeler aligner
CSCC	Cervical squamous cell carcinoma
CSS	Cumulative sum scaling normalization
DMEM	Dulbecco's modified Eagle's medium (minimal essential medium)
DMSO	Dimethyl sulfoxide
EBV	Epstein-Barr virus
FISH	Fluorescence *In Situ* Hybridization
FBS	Foetal bovine serum
HNSCC	Head-and-neck squamous cell carcinoma
HOMD	Human oral microbiome database
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HPV	Human papillomavirus
HR-HPV	High-risk human papillomavirus
HSPG	Heparan sulphate proteoglycan
IgA	Immunoglobulin A
ISH	*In Situ* Hybridisation
IHC	Immunohistochemistry
LCR	Long control region
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
LR-HPV	Low-risk human papillomavirus
MFI	Median fluorescence intensity
NGS	Next generation sequencing
OPSCC	Oropharyngeal squamous cell cancer
ORF	Open reading frame
OTU	Operational taxonomical unit
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
pRb	Retinoblastoma protein

RDA	Redundancy discriminant analysis
RRP	Recurrent respiratory papillomatosis
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulphate
TGS	Third generation sequencing
TSS	Total sum normalization
WGS	Whole genome sequencing

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV):

I Tuominen H, Rautava S, Syrjänen S, Collado MC, Rautava J. HPV infection and bacterial microbiota in the placenta, uterine cervix and oral mucosa. *Scientific Reports*, 2018; 8:9787.

II Tuominen H, Rautava S, Collado MC, Syrjänen S, Rautava J. HPV infection and bacterial microbiota in breast milk and infant oral mucosa. *PLoS ONE*, 2018;13(11):e0207016.

III Tuominen H, Collado MC, Rautava J, Syrjänen S, Rautava S. Composition and maternal origin of the neonatal oral cavity microbiota. *Journal of Oral Microbiology*, 2019;11:1.

IV Tuominen H, Al-Samadi A, Salo T, Rautava J. Human myoma tissue-based extracellular matrix models for testing the effects of irradiation on the HPV positive cells. *Submitted.*

1 Introduction

The microbiome is defined as the microbiota and its environment, namely the human host. Microbiota is composed of bacteria, archaea, viruses and fungi. Host interactions shape the size, species composition and biogeography of microbial communities and the microbiota influences human health. Viruses are obligate intracellular pathogens. They require the host cell protein synthesis machinery to replicate. The interaction between the commensal microbiota and viruses is not clear. The microbiota may protect against or help to acquire viral infection, or it may promote viral evasion by direct or indirect mechanisms. The host's microbiota may in addition increase or diminish cancer susceptibility. The causal relationship between viral-bacterial interactions is complex and its role in specific (pathological) events is difficult to demonstrate.

Human papillomavirus (HPV) is relatively common human virus and around 80% of the population will get the infection at some point of their life (Braaten and Laufer 2008; Chesson *et al.* 2014). Infection is usually acquired via horizontal transmission during sexual intercourse or in another close skin and/or mucosal contact. HPV can also be transmitted via vertical transmission and this has been observed mainly in the mother-infant context. A mother with present HPV infection can, for example, infect her infant during pregnancy or after delivery while taking care of the infant. Other possible infectious routes include haematogenous spread via circulating blood and ascending infection straight from vagina. (Syrjänen and Puranen 2000; Deng *et al.* 2005; Syrjänen 2010; Koskimaa *et al.* 2012; Park *et al.* 2012; Freitas *et al.* 2013; Hahn *et al.* 2013; Liu, Rashid and Nyitray 2015; Trottier *et al.* 2016).

HPV infects the epithelium and most of the infections remain asymptomatic. HPV can cause small epithelial lesions such as verruca, papilloma or condyloma. Within months, most of these infections are cleared by individuals own immune defence (Louvanto *et al.* 2010; Hibma 2012; Rautava *et al.* 2012a). The impacts of HPV infections in infants are currently not well known. It has been speculated that most of the infections in infants clear similarly to adults. HPV infection may also be latent, in which case HPV is preserved in the body, but it hides from immune defence system and it cannot be detected with traditional HPV detection methods (Gravitt

and Winer 2017). Some HPV genotypes, called high-risk HPVs, may remain and cause a persistent infection. These persistent infections can, in the duration of years and decades, develop into squamous cell carcinoma (SCC) (Schlecht *et al.* 2001; Louvanto *et al.* 2010, 2011a). HPV causes nearly 100% of uterine cervix SCCs (Durst *et al.* 1983; Boshart *et al.* 1984; zur Hausen 2002, 2009). During the past 20 years, it has been discovered that the HPV causes a major part of oropharyngeal SCCs (OPSCC) as well (Kreimer *et al.* 2005; Arbyn *et al.* 2012; Mehanna *et al.* 2013). The natural history of oral HPV infection remains unclear.

The impact of bacteria to human health and disease is well known. Commensal bacteria are present on body surfaces covered by epithelial cells and exceed the number of cells forming human body. Commensal bacteria co-evolve and protect their host. Under specific conditions they may overcome protective human responses and exert pathologic effects. Bacterial dysbiosis depicts a situation where the bacterial microbiota becomes instable and pathogenic conditions may occur. This dysbiosis alone may have an effect to e.g. inflammatory bowel disease, periodontitis and even to cancer or Alzheimer's disease (Turnbaugh *et al.* 2009; Bäckhed *et al.* 2012; Hsiao *et al.* 2013; Huttenhower, Kostic and Xavier 2014; Petersen and Round 2014; Trompette *et al.* 2014; Garrett 2015; Wang *et al.* 2017; Karpiński 2019; Kowalski and Mulak 2019).

The indigenous bacterial microbiota is established during early life. The mother is the most important source of colonizing bacteria (Yassour *et al.* 2018). For decades it has been believed that foetus develops in a sterile environment and encounters bacteria and viruses only after the delivery. In recent years, it has been suggested that placenta may harbour specific bacterial microbiota that could possibly have an effect on the developing foetus (Satokari *et al.* 2009; Rautava *et al.* 2012b; Stout *et al.* 2013; Aagaard *et al.* 2014; Collado *et al.* 2016; Gomez-Arango *et al.* 2017a; Parnell *et al.* 2017; Benny *et al.* 2019).

This thesis focuses on the interaction between HPV and bacteria in the oral cavity of mothers and infants as well as uterine cervix, placenta and mother's breast milk. The presence of HPV infection and the composition of bacterial microbiota were investigated from samples obtained from healthy Finnish pregnant women and their newborns before, during and after delivery. In addition, this work aimed to reveal the impact of mothers' microbiota to the development of neonate's oral microbiota during and directly after delivery. Further on the effect of different delivery modes to neonatal oral microbiota composition was also investigated. Another aspect of this study was to evaluate *in vitro*, how HPV positive cervical SCC cells can be cultivated in novel human extracellular matrices and additionally, to investigate the impact of different HPV copy numbers and irradiation to cell invasion.

2 Review of the Literature

2.1 Human papillomavirus

2.1.1 The virus structure

Human papillomavirus (HPV) is a non-enveloped double-stranded circular DNA virus that has the potential to infect human epithelial cells. Over 200 HPV types have been identified today, and the different genera are divided to Alpha-, Beta-, Gamma-, Mu- and Nu -groups according to the nucleotide sequence of the L1 protein (de Villiers 2013; Bzhalava, Eklund and Dillner 2015). Furthermore, alpha HPVs are further divided into groups according to predilection to mucosa or skin surface and to high-risk (HR) or low-risk (LR) types according to their oncogenic potential (de Villiers *et al.* 2004; de Villiers 2013; Bzhalava, Eklund and Dillner 2015)

The virus itself is composed of a double-stranded DNA structure and is about 8.000 base pairs in length. The DNA genome can be divided into three functional domains; the long control region (LCR) and to early (E1-E7) and late (L1-L2) open reading frames (ORF's) which have different functions to cell survival and viral production (Harden and Munger 2017) (**Figure 1**).

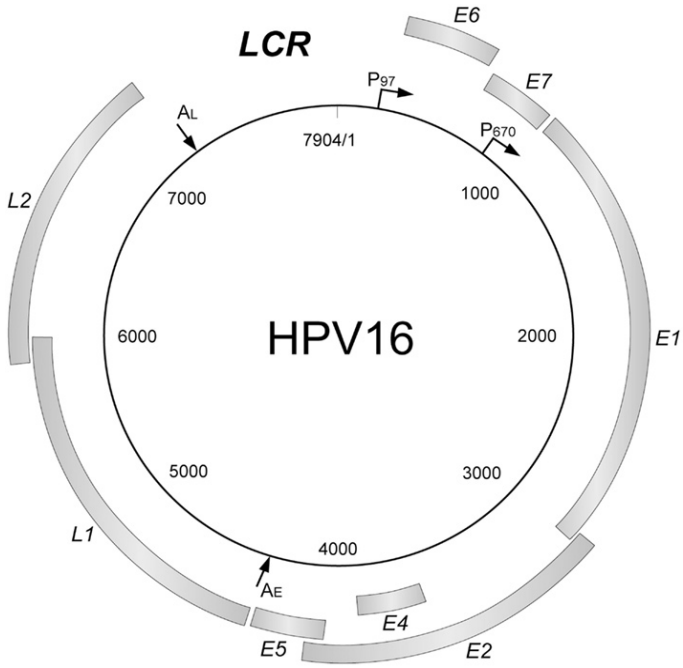

Figure 1. HPV16 genome. (Kajitani *et al.* 2012).

The E1, E2, E4, L1 and L2 are so called core genes which all have an essential function in cell viral genome replication and genome packaging and these proteins are somewhat conserved between different HPV types. The classification of HPVs is based on L1 nucleotide sequence. On the other hand, there are the accessory genes E5, E6 and E7. They modify the cellular environment to support and tolerate viral genome replication and to help the cell to survive in order to complete the viral life cycle in the site of infection. (Egawa and Doorbar 2017). The LCR controls the function of oncoprotein E6 and E7 and includes the transcriptional regulatory elements (**Table 1**).

Classification of HPV according to biological behaviour

The division to HR- and LR-HPVs present clinically significant differences. At first, the division was made after detecting certain genotypes more frequently in carcinoma specimens (Durst *et al.* 1983; Boshart *et al.* 1984; Schiffman *et al.* 1993). It was later noticed that LR-HPV types does not usually use their E6/E7 oncogenes as what HR-HPVs do in order to reach excessive cell proliferation (Schwarz *et al.* 1985; Yee *et al.* 1985). HR-HPV oncogenes E6 and E7 have a direct effect to cell differentiation process and have the ability to immortalize epithelial cells (Moody and Laimins 2010). The two groups (HR and LR) have different cell transcriptional

regulation of these and other specific genes which make the LR-HPV less malignant (Egawa and Doorbar 2017). Consequently, HR-HPVs are more pathogenic and have the ability to develop precancerous lesions and even carcinoma after long persistence. The most often encountered HR-HPVs are 16 and 18, whereas 6 and 11 in LR-HPVs. (de Villiers *et al.* 2004; de Villiers 2013; Egawa and Doorbar 2017). (**Table 2**)

Table 1. HPV DNA protein functions. Modified from Egawa & Doorbar, 2017b.

HPV protein	Function
E1	- required for origin of **replication** of viral DNA - maintains viral DNA as episomal - mediates the cell growth arrest
E2	- initiates viral DNA **replication**; directs the E1 to the viral replication origin - regulates viral and cellular gene expression - induces cell growth arrest - serves as negative regulator of expression of E6/E7 oncogenes - maintains of viral DNA as episomal - encapsidates virion
E4	- replicates viral DNA - arrests cell cycle - encapsidates viral genome - **releases virion**
E5	- enhances the cell transforming activity of E6 and E7 - controls cell growth and differentiation - prevents **immune response** against infected cell
E6	- inhibits cell growth arrest, differentiation and apoptosis by **degradation of p53** (major tumour suppressor protein) reducing the cell's ability to respond to DNA damage - prevents telomere shortening during cell proliferation - induces loss of cell polarity
E7	- induces cell hyperproliferation and productive viral life cycle by **inhibition of retinoblastoma proteins** (pRb, another tumour suppressor protein) - alters cell cycle control and gene expression - prevent apoptosis together with E6 and activates cellular telomerase
L1	Major capsid protein - encapsidates the viral genome - is responsible of **cell attachment** during infectious entry
L2	Minor capsid protein - encapsidates the viral genome - is responsible of **viral cell entry** into the host cell and viral genome transfer into the cell nucleus

Table 2. **Differences of LR- and HR-HPV.** Some of the HPVs are listed with clinical significance. Furthermore, main protein function differences are described. Modified from IARC 2012; Egawa and Doorbar 2017.

	e.g. of HPV types	The main differences in protein function	The main differences in infection	Clinical significance
LR-HPV	**HPV 6** **HPV 11** HPV 83 HPV 70	**E6**: no degradation of p53 protein + no activation of telomerase **E7**: no destabilizing of pRb	Little or no expression of E6/E7	Benign lesions: (genital) warts condyloma papilloma
HR-HPV	**HPV 16** **HPV 18** HPV 58 HPV 66	**E6**: degradation of p53 and inhibition of p53 transactivation + activation of telomerase **E7**: destabilizing pRb	E6/E7 inhibit immune response to tolerate viral gene expression	Carcinogenic potential

2.1.2 HPV transmission and infection

HPV transmission

Horizontal transmission (e.g. sexual interaction or other close skin and/or mucosa contact between individuals) or vertical transmission (e.g. from mother to infant) are thought to be the main HPV transmission methods (Oriel 1971; Schneider and Koutsky 1992; Burchell *et al.* 2006; Syrjänen 2010; Freitas *et al.* 2013). Today, HPV infection is mainly considered to be a sexually transmitted infection, and the main way of transmitting the virus (Oriel 1971; Burchell *et al.* 2006; Rautava and Syrjänen 2011). Nevertheless, in addition vertical transmission has been observed. This has been reported to occur mainly between mother and her infant, either by prenatal transmission (via circulating peripheral blood, or as an ascending infection from cervix/vagina or as an infection via placenta/amniotic fluid), during delivery from cervix/vagina or during care-taking after the delivery (Syrjänen and Puranen 2000; Bodaghi *et al.* 2005; Deng *et al.* 2005; Rombaldi *et al.* 2008; Sarkola *et al.* 2008a; Chen *et al.* 2009; Syrjänen 2010; Park *et al.* 2012; Koskimaa *et al.* 2012, 2014; Freitas *et al.* 2013; Hahn *et al.* 2013; Hong *et al.* 2013; Merckx *et al.* 2013; Liu, Rashid and Nyitray 2015). HPV positive breast milk is not thought to be a risk factor for infant to acquire oral HPV infection (Sarkola *et al.* 2008b; Yoshida *et al.* 2011; Louvanto *et al.* 2017).

Vaginal delivery has been detected to slightly increase the risk of HPV vertical transmission, but HPV have been observed to transmit even with caesarean section, supporting the concept of prenatal transmission (Wang, Zhu and Rao 1998; Xu *et al.* 1998; Medeiros *et al.* 2005; Mammas *et al.* 2012; Chatzistamatiou, Sotiriadis and Agorastos 2016). HPV has been detected in epithelial cells of the placenta with *In situ* hybridisation (Sarkola *et al.* 2008a). A recent systematic review did not detect difference in HPV transmission between vaginally or caesarean section delivered infants (Zouridis *et al.* 2018) The risk of perinatal transmission of HPV infection has been estimated to be around 4%-24.5% (Medeiros *et al.* 2005; Castellsagué *et al.* 2009; Rombaldi *et al.* 2009; Park *et al.* 2012; Hahn *et al.* 2013). HPV has been considered to be a threat to the pregnancy but there are several studies showing infection with normal, healthy births as well (Sarkola *et al.* 2008a; Koskimaa *et al.* 2012; Trottier *et al.* 2016).

One possible way of acquiring HPV infection is autoinoculation. In this case, individual infects itself again to another body site with already existing HPV infection (Brouwer, Meza and Eisenberg 2015). Furthermore, haematogenous spread of HPV may occur, since HPV has also been detected from amniotic fluid and cord blood samples (Armbruster-Moraes *et al.* 1994; Wang, Zhu and Rao 1998; Xu *et al.* 1998; Sarkola *et al.* 2008a; Syrjänen 2010). The exact mechanism behind the possible HPV transmission via blood is so far unclear.

HPV infection

In order to infect a host, HPV requires some kind of wound, microtrauma or abrasion on the mucosa or skin surface in order to reach to the basal cells. The basal cells are the dividing epithelial cells, and thus essential for the virus to produce new viral copies. Furthermore, HPV can only infect and replicate in fully differentiated squamous epithelial cells. (Reinson *et al.* 2015).

After reaching the target (basal) cell, the virus attaches to heparan sulphate proteoglycans (HSPG) with the L1 viral protein. After complex mechanisms, which are not yet fully understood, the virus enters the cell nucleus (Aksoy, Gottschalk and Meneses 2017). Thereafter, the viral early transcription starts by the activation of E1 and E2 (Sanders and Stenlund 2000). The first round of replication results in total of 50-100 episomal copies of HPV per nucleus (Maglennon, McIntosh and Doorbar 2011). It has been observed, that in most of the HPV related carcinoma or its precursors, the HPV genome can be found integrated in the host cell genome (zur Hausen 2002). This allows greater expression of oncogenes E6 and E7 (Taberna *et al.* 2017).

After the first round of viral DNA replication, the copy numbers are kept low and the function of the gene minimal in the dividing epithelial cells. There is a tight

control of viral proteins at this stage. The virus activates all its genes in a massive upregulation only after the cell stops dividing and starts to proliferate. At this stage, the number of HPV DNA copies can go up to thousands per cell. In the most superficial layer of the skin, the late proteins L1 and L2 are activated to encapsidate the viral genomes and the virions are released along with the shedding of the cells to infect a new host. (Stanley 2010). Schematic figure of viral life is presented in **Figure 2.**

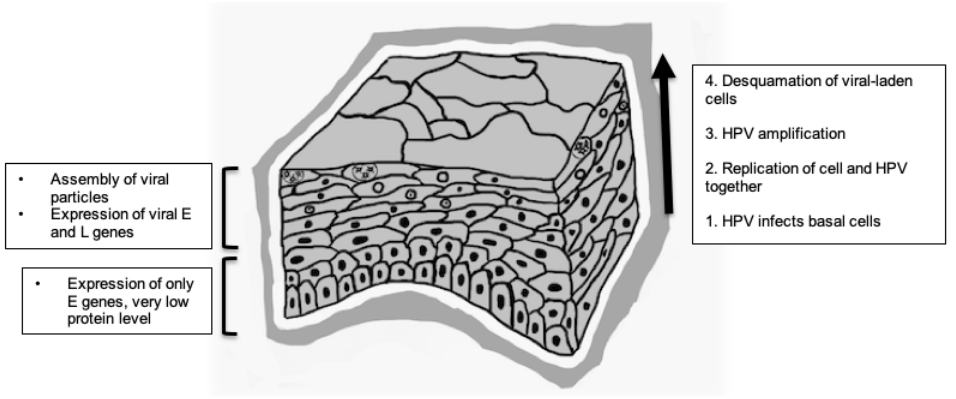

Figure 2. HR-HPV viral life cycle in epithelial cells. The average time from infection to desquamation is around six weeks.

2.1.3 Natural history, prevalence and clinical significance

Around 80% of adult population have an HPV infection some time over the course of their life (Chesson *et al.* 2014). Around 11%-21% of small infants (0-3 months old), have been detected to be positive for HPV (Rintala *et al.* 2005a; Trottier *et al.* 2016). Of HPV infected, only a few percent will ever develop visually present symptoms of a disease such as verruca, papilloma or condyloma (Syrjänen *et al.* 1990). These benign lesions are mainly caused by low risk (LR) HPVs (mainly genotypes 6 or 11). Even benign lesions can sometimes cause serious problems if they are located for example in the respiratory tract (known as infants recurrent respiratory papillomatosis, RRP) (Bauman and Smith 1996; Blackledge and Anand 2000; Silverberg *et al.* 2003; Egawa and Doorbar 2017).

Most of the HPV infections are cleared within few months after infection, but in some cases, HPV can persist (Louvanto *et al.* 2010; Hibma 2012; Rautava *et al.* 2012a). Only a fraction of HPV infected individuals develop a persistent infection, which has the potential to proceed to malignancy. Especially, high-risk (HR) HPVs, such as HPV16 or HPV18, have a potential to cause persistent infections (Schlecht *et al.* 2001; Louvanto *et al.* 2010, 2011a; Rautava *et al.* 2012a) and HR-HPVs can

predispose to the development of squamous cell carcinoma (SCC). Still, HPV infection progression to carcinoma is relatively rare event taking into account the number of HPV infected individuals (Stanley 2010).

2.1.4 HPV induced squamous cell carcinoma in cervix and in head-and-neck region (HNSCC)

The hypothesis of the cervical carcinoma association to HPV was first introduced in the 1970s (Hausen *et al.* 1974) and first HPVs were isolated in cervical carcinoma biopsies in the 1980s (Durst *et al.* 1983). The role of HPV in cervical carcinoma has since been extensively studied, and HPV can be found in nearly all of the cervical SCC specimens (IARC 2004). Cervical carcinoma screening has been introduced already decades ago to detect HPV induced premalignant changes in the female cervical region to help prevent carcinoma development (Franceschi *et al.* 2011; Arbyn *et al.* 2012). In addition to cervical SCC, HPV has the potential to cause disease in the anogenital region (zur Hausen 2009; Stanley 2010).

A growing body of evidence shows that HPV infection is partially responsible for the rapidly growing numbers of the head-and-neck squamous cell carcinomas (HNSCC) and especially SCCs in the oropharyngeal region (OPSCC) (Kreimer *et al.* 2005; Arbyn *et al.* 2012; Mehanna *et al.* 2013). Around 35% - 87 % OPSCC have been detected to be HPV positive (Hansson *et al.* 2005; Gillison *et al.* 2015; Ducatman 2018). The most often detected genotype is HPV16 (Herrero *et al.* 2003; Hansson *et al.* 2005; Kreimer *et al.* 2005; Rautava *et al.* 2012a).

HPV DNA detection in carcinoma does not prove a causal relationship; HPV may be a bystander, not causing the disease. To detect active HPV infection, the most often used biomarker is p16 immunochemistry (IHC) by the E7 oncoprotein. (Ducatman 2018; Ruuskanen *et al.* 2018). The p16 is a common tumour suppressor gene found in almost all cancers. It's main function is to inhibit cell growth and p16 is often overexpressed in HPV associated carcinomas. (Serra and Chetty 2018). However, p16 method is indirect and not specific to only HPV infection and there is a risk of misclassification (Rayess, Wang and Srivatsan 2012; Salles *et al.* 2014; Jouhi *et al.* 2017; Belobrov *et al.* 2018). The disease burden of HPV induced oropharyngeal carcinoma has been increasing in recent years especially to otherwise healthy, young patients who generally do not smoke or consume large amounts of alcohol (IARC 1985; Chaturvedi *et al.* 2011; Gillison *et al.* 2012, 2014; Young *et al.* 2015; Taberna *et al.* 2017).

The cervix and oropharyngeal sites have been speculated to be the predominant target of HPV infection because of the so-called transformation zone (Gillison *et al.* 2014; Chen and Zhou 2015; Mallen-St Clair *et al.* 2016). This area in the cervix and in the oropharyngeal region consists of the interface between the squamous and

columnar epithelial cells. The basal cells are more exposed at this site where there is constant changing of cell morphology. **Figures 3 and 4** present the gross anatomy of the oral cavity and oropharynx, respectively.

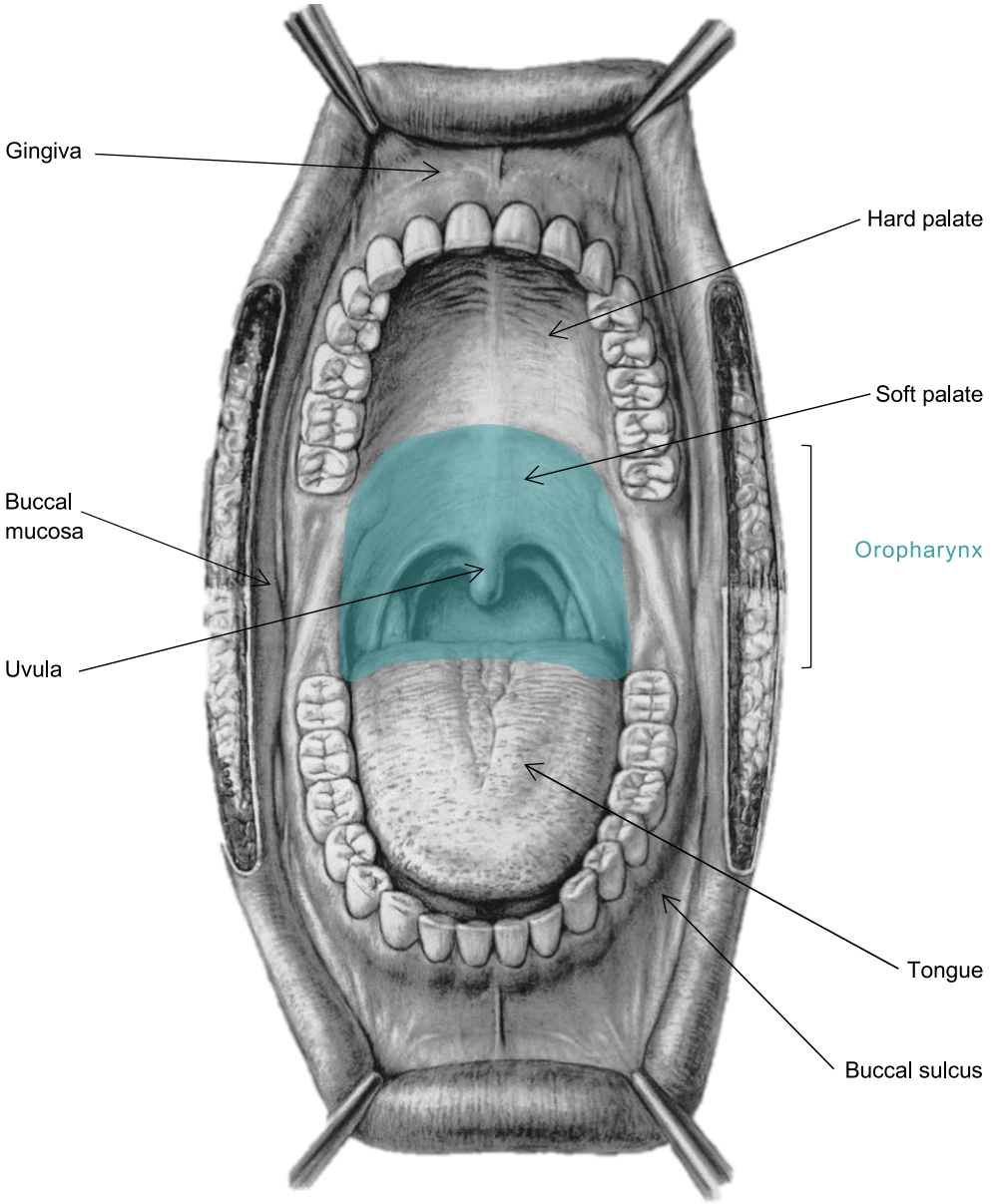

Figure 3. The oral cavity. Figure modified from MIS 65-5390-1, National Museum of Health and Medicine, Otis Histological Archives.

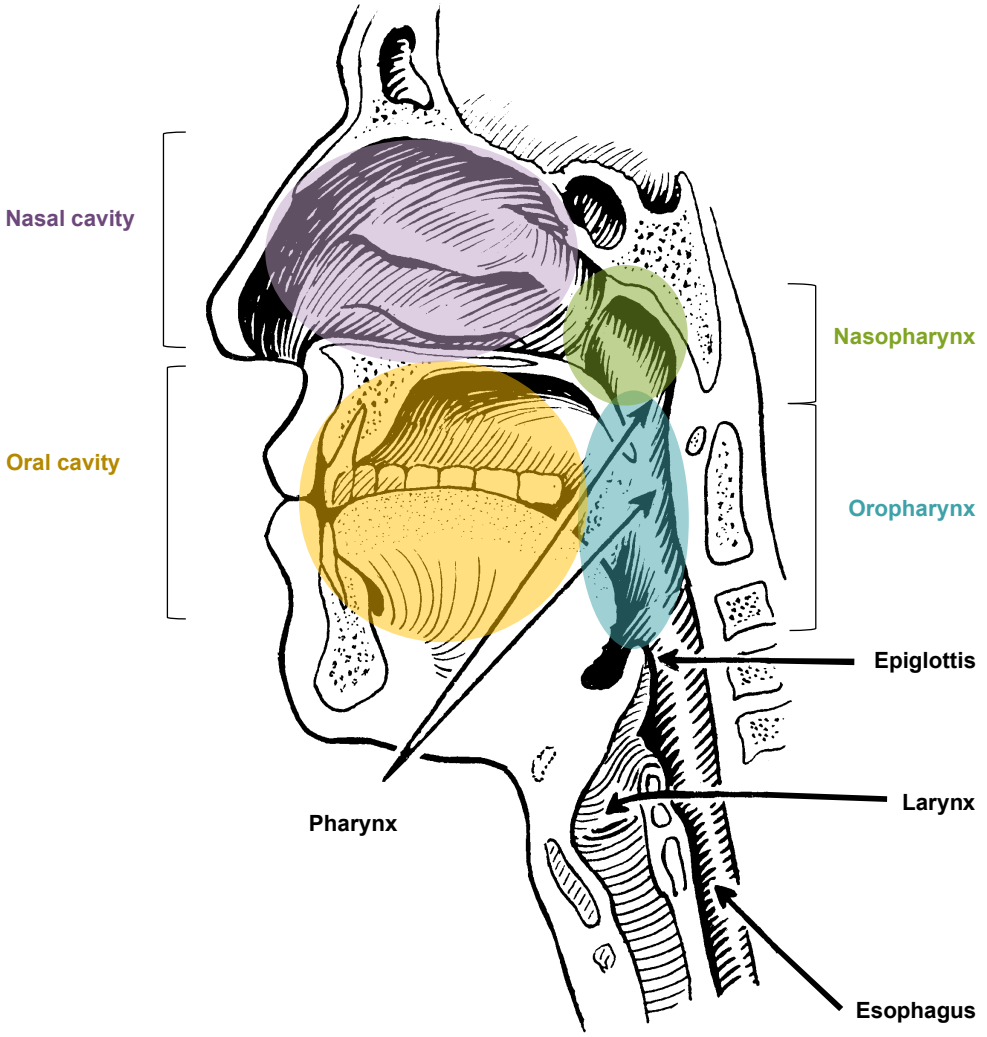

Figure 4. Cross-section of oral, nasal and pharyngeal regions. Soft palate and base of tongue are part of oropharynx. Figure modified from Wikimedia Commons by Pearson Scott Foresman.

HPV positive OPSCC exhibits a more favourable response to treatment and especially radiation treatment than HPV negative OPSCCs which are mainly caused by tobacco and alcohol use (IARC 1985; Ang *et al.* 2010; Chaturvedi *et al.* 2011). The latest WHOs classification even separates HPV positive and HPV negative OPSCC into different diseases since the behaviour of the tumour is significantly different (El-Naggar *et al.* 2017). This difference according to HPV status has not been detected in all studies (Nagel *et al.* 2013; Schneider, Bol and Grégoire 2017; Taberna *et al.* 2017). Furthermore, the new TNM staging system classification takes

into account the HPV status of the OPSCC (Brierley, Gospodarowicz and Wittekind 2010).

2.1.5 HPV detection methods

There is no golden standard method to detect HPV infection (Abreu *et al.* 2012). For research purposes, HPV DNA detection is the main laboratory analysis method to detect HPV in a sample.

One of the most common HPV detection methods in research today is based on HPV viral DNA nucleic acids detection and amplification by polymerase chain reaction (PCR). It can be used in both swab samples and with tissue biopsies. PCR is based on primers that target specific conserved regions in the HPV genome, often in either L1 or E1, which enables detection of multiple different HPV genotypes in one assay run. The end-results are visualized with a scanner after washing steps. This technique is flexible, specific and very sensitive but possible contamination by previous material may result in false positive samples. Additionally, PCR is not ideal for detecting multiple simultaneous HPV infections with low copy numbers. Another issue regarding PCR HPV detection is that the method only reveals if HPV is present in a sample, but no conclusions can be drawn regarding whether the infection is active or if the HPV has induced carcinoma. Therefore, more precise detection methods of HPVs clinical relevance are used such as HR-HPV E6/E7 mRNA detection, ISH and IHC to detect the expression of the p16 protein. However, p16 is not specific to only HPV and there is a genuine risk for misclassification. (Brink, Snijders and Meijer 2007; Snijders, Heideman and Meijer 2010; Abreu *et al.* 2012; Rayess, Wang and Srivatsan 2012; Ndiaye *et al.* 2014; Salles *et al.* 2014; Jouhi *et al.* 2017; Belobrov *et al.* 2018; de Souza *et al.* 2018; Ducatman 2018; Serra and Chetty 2018). A recent review concluded HPV p16 sensitivity to be 90.5 % and specificity 83.3 % (Jouhi *et al.* 2017).

2.2 The human microbiota

A vast number of microbes including bacteria, viruses, fungi and archaea colonize and inhabit the skin and mucosal surfaces of the human body. Germ free mice have been widely used in research in decades and it has been proven that without microbiota the mice will not behave or develop normally (Round and Mazmanian 2009; Heijtz *et al.* 2011). Healthy interaction and homeostasis between the host and the microbiome is essential for the function of the human body and, consequently, life without microbiota is not possible (Gilbert and Neufeld 2014). In this thesis, the word microbiota is used to describe mainly the bacterial microbiota.

The microbiota starts to develop gradually before and after the delivery (Jiménez *et al.* 2008; Collado *et al.* 2016). Full term or preterm pregnancy and the delivery mode (vaginal or caesarean section) have been shown to shape the structure of the early microbiota composition in newborns, as well as possible perinatal antibiotic treatments and neonates feeding methods (breast milk of formula) (Dominguez-Bello *et al.* 2010, 2016; Lif Holgerson *et al.* 2011; Aujoulat *et al.* 2014; Jakobsson *et al.* 2014; Mueller *et al.* 2015; Blaser and Dominguez-Bello 2016; Nuriel-Ohayon, Neuman and Koren 2016; Chong, Bloomfield and O'Sullivan 2018). Most of these initial changes in the bacterial microbiota composition starts to settle after some months of life. However, absence or short duration of breast feeding or infant antibiotic treatment leave a trace in the bacterial microbiota composition even to later in life. (Dzidic *et al.* 2018b). Six to 12 months of age, after the end of breast feeding, the infant gut microbiota starts to resemble more adult gut microbiota (Bäckhed *et al.* 2015). Similarly, in the oral cavity, the microbiota matures during the first years of life (Lif Holgerson *et al.* 2015; Nuriel-Ohayon, Neuman and Koren 2016; Dzidic *et al.* 2018b).

The adult gut microbiota is one of the most studied microbiota niches in human body. Generally, it is regarded that more diverse microbiota with a large number of beneficial species is better for health (Turnbaugh *et al.* 2007). The amount of the overall bacterial volume differs dramatically between regions. Estimated amounts of different taxa and volume of bacteria in selected body sites are presented at **Figure 5**.

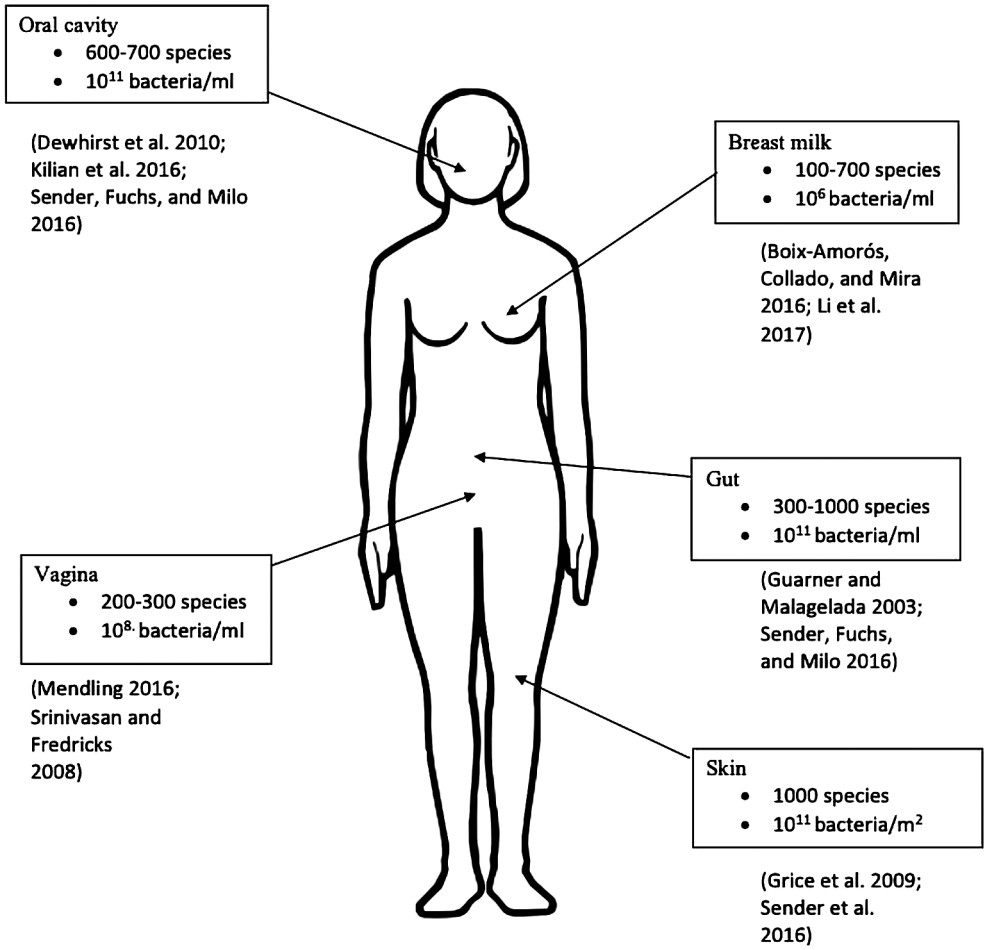

Figure 5. The number of different bacterial species and an average number of bacteria in different body sites. Human figure from ClipArtMag.com

2.2.1 Microbiota in health and disease

The microbiota interacts with multiple human body functions. It can e.g. modulate innate and adaptive immune physiology, modify the food intake, affect appetite signalling pathways, take part in multiple vitamin biosynthesis, protect from exogenous pathogens and produce antimicrobial substances (Wang *et al.* 2017). These effects are considered to be beneficial in everyday life. However, if something distorts microbiota homeostasis and/or some pathogenic bacteria become more abundant, disease can also occur as a result of the changed bacterial microbiota composition.

Dysbiosis is used to describe a condition where abnormalities occur in otherwise balanced system (Petersen and Round 2014). Increasing evidence has emerged suggesting that early bacterial colonization may have a significant impact to health

later in life. Several diseases have been connected to alterations in microbiota composition. Inflammatory bowel disease, type I and II diabetes, obesity, allergies, asthma, autism, Alzheimer's disease and cancer are some of the most thoroughly studied diseases where a certain bacterial microbiota composition has been suggested to predispose the individual to disease development (Turnbaugh *et al.* 2009; Bäckhed *et al.* 2012; Hsiao *et al.* 2013; Huttenhower, Kostic and Xavier 2014; Petersen and Round 2014; Trompette *et al.* 2014; Garrett 2015; Wang *et al.* 2017; Karpiński 2019; Kowalski and Mulak 2019).

The dilemma lies in the extremely complex nature of these disease and therefore, it is difficult to isolate only one cause to a disease, especially in humans where there are numerous everchanging variables which may affect simultaneously. Especially abnormal microbiota composition in the gut in infancy has been shown to have an impact to individuals' health in the following years to come, as it may precede e.g. allergies and asthma (Kalliomäki *et al.* 2008; Vael and Desager 2009; Abrahamsson *et al.* 2014; Nylund *et al.* 2014; Pärtty *et al.* 2015; Rodríguez *et al.* 2015; Saari *et al.* 2015; Isolauri, Salminen and Rautava 2016; Tanaka and Nakayama 2017; Zhuang *et al.* 2019).

Interaction between an individuals' microbiota and the environment is dynamic. However, individually there are usually no major fluctuations of the microbiota composition over time (Ursell *et al.* 2012). On the other hand, between individuals, however, the microbiota composition may vary widely according to age, sex, race and diets within health and disease (Huttenhower *et al.* 2012). Furthermore, vast geographical variation of the microbiota composition has been observed (Lloyd-Price, Abu-Ali and Huttenhower 2016). Generally, a diverse microbiota is considered beneficial for human health, but not all body regions benefit from diverse microbiota composition. For example, the vaginal microbiota in a healthy situation is predominantly dominated by different *Lactobacillus* species, which are thought to be necessary for maintaining the overall health (Gupta, Kakkar and Bhushan 2019; Rinninella *et al.* 2019). In the vagina, shifts in the balance to more diverse microbiota can trigger the onset of bacterial vaginosis or even preterm birth in pregnant women (Fredricks, Fiedler and Marrazzo 2005; DiGiulio *et al.* 2015; Wang *et al.* 2017).

The bacterial microbiota may be modulated intentionally with prebiotics or probiotics or the modulation can be unintentional (Rautava *et al.* 2012b; Sánchez *et al.* 2017). The International Scientific Association for Probiotics and Prebiotics (ISAPP) defines probiotics as "live micro-organisms which when administered in adequate amounts confer a health benefit on the host" (Hill *et al.* 2014). Probiotics are living non-pathogenic bacteria which often have a positive effect on human microbiota, e.g. lactic acid bacteria, such as Lactobacillus and Bifidobacterium, and these have been encouraged to use alongside antibiotics treatment to hep to restore bacterial microbiota in the gut and to avoid adverse side-effects of antibiotic treatment (Williams 2010). Prebiotics, on the other hand, defined by ISAPP as "a

substrate that is selectively utilized by host micro-organisms conferring a health benefit" (Gibson *et al.* 2017). Generally, these are considered as non-digestible food ingredients which beneficial gut bacteria (especially Lactobacillus and Bifidobacterium) can utilize as food and therefore succeed (Nicolucci *et al.* 2017).

In recent years, the effect of vaccination, and the immune responses it generates, has been connected to an individuals' bacterial microbiota composition. Antibiotic exposure may induce drastic changes in the composition of gut microbiota for up to six months in otherwise healthy adults (Palleja *et al.* 2018). The gut microbiota is known to play a key role in human immune system development and maturation (Lazar *et al.* 2018). Recent studies suggest that different microbiota composition during vaccination, with or without antibiotics, soon after the course of antibiotics or during antibiotic treatment, could decrease the development of sufficient response to vaccination (Jamieson 2015; Zimmermann and Curtis 2018).

2.2.2 Bacterial 16S rRNA

The indigenous bacteria generally represent a wide variety of different species and their structure and other features have significant differences. In order to study bacteria and to categorize them, a highly conserved part of the ribosomal subunit, a 16S rRNA gene, has been used (Clarridge 2004). 16S is a part of the small subunit of bacterial ribosome. The ribosome is responsible of protein synthesis via mRNA for cell function (Wilson and Cate 2012).

There are different regions in the 16S rRNA and some of these regions are conserved while others more variable (Clarridge 2004; Chakravorty *et al.* 2007). The conserved regions of the 16S rRNA gene have been observed to be very similar between different taxa and basically no mutation has been occurred in its structure over the duration of millions of years of bacteria evolution (Woese 1987). This makes it possible to design universal primers to target 16S rRNA for vide variety of different bacteria (Woese *et al.* 1975). The general approach of 16S-based microbiota analysis is to use a primer that can target the conserved region and then amplify the variable region. Different bacterial taxa can then be distinguished from each other based on the variable regions. In total, there are nine conserved and another nine variable regions in one 16S rRNA gene (**Figure 6**). (Petrosino *et al.* 2009).

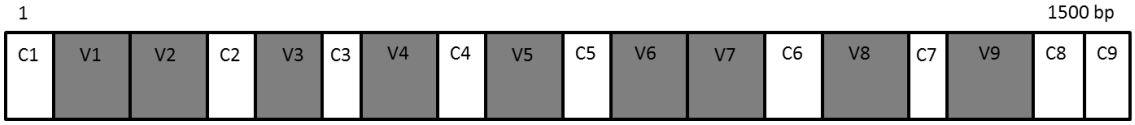

Figure 6. The highly conserved (C1-C9) and highly variable (V1-V9) regions of the 16S rRNA gene.

2.2.3 Bacterial taxonomy

As with HPVs, bacteria have their own classification designed to help categorizing different species by their structure. At first, the classification was based on the appearance of bacteria under light microscopy (Palleroni 2010). Nowadays, more specific identification method is used based on the 16S rRNA gene sequences. The unknown 16S rRNA gene, which is being investigated, is being amplified and compared to a database with the existing taxonomical hierarchy being known (Cole *et al.* 2009; Rajendhran and Gunasekaran 2011). In **Figure 7** some oral bacteria are being categorized by their bacterial taxonomy.

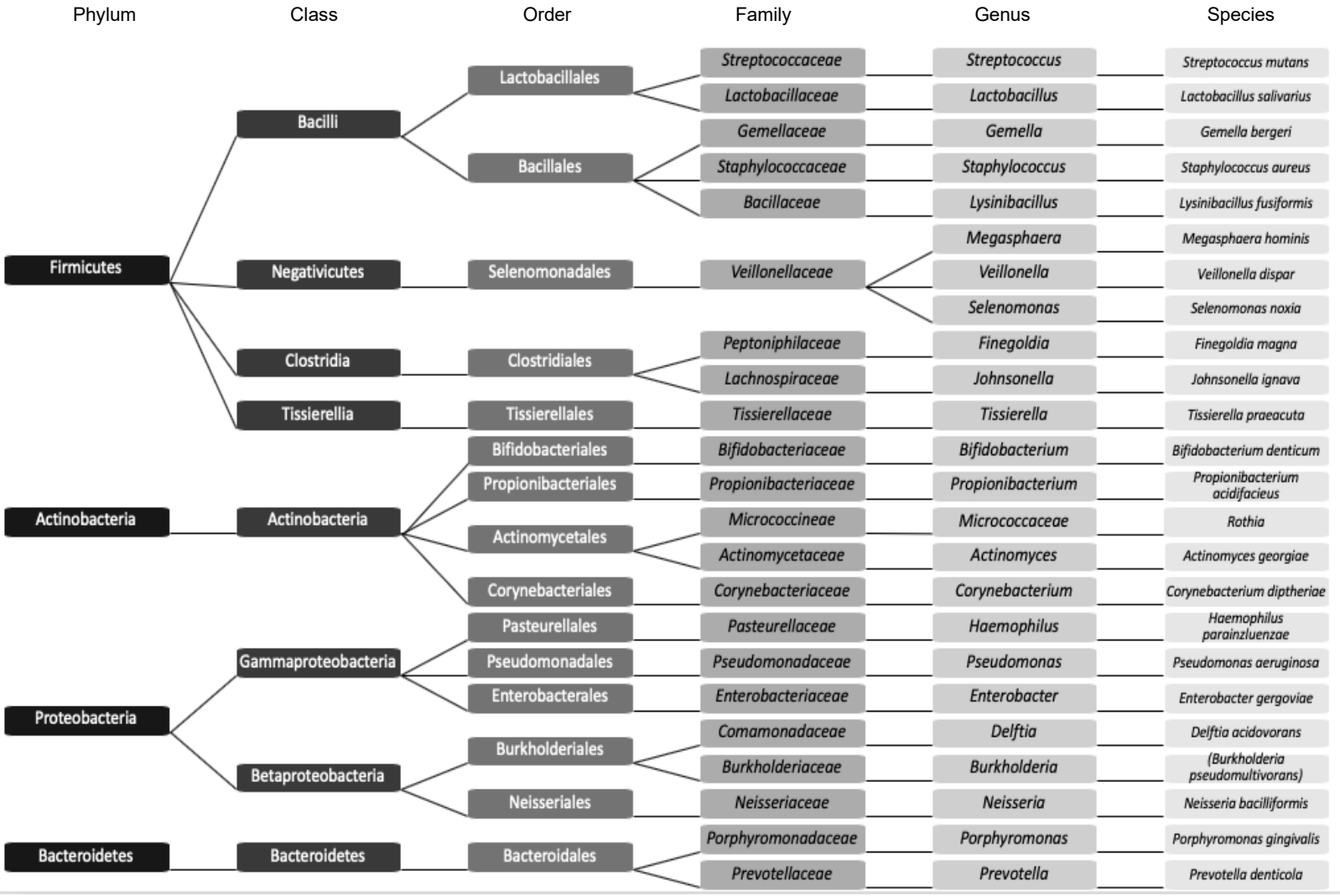

Figure 7. Examples of taxonomic microbiota composition in the oral cavity.

2.2.4 Microbiota analysing methods

Previously, culturing was the only available method to analyse the bacteria by their shape and primitive function. Non-cultivation methods, such as Fluorescence In Situ Hybridization (FISH) emerged in the 90's. Since the achievements of computational technology and molecular techniques, the possibilities of microbiota analysing have grown exponentially.

The 454 sequencing method was used to detect bacterial DNA in the beginning of 21st century. The 454 was considered the first next-generation DNA sequencing method that became commercially successful. It utilized pyrosequencing and was considered as the cutting edge at that time due to its speed but it was somewhat prone to errors and has later subsided for faster and cheaper methods. (Liu *et al*., 2012; Mardis, 2008).

Today, the microbiota is often analysed by next-generation sequencing (NGS) of the 16S rRNA gene. Each bacterium can have several 16S rRNA genes and by amplifying that gene by a specific target region, it is possible to detect present microbiota (Liu *et al.* 2012). The amplified sequences are grouped together to form operational taxonomic units (OTUs) and selected groups are then compared to already existing databases to generate microbiota data composition in a selected area. Strict quality control is necessary in order to clean the generated bacterial data from possible contaminants and, for example, to remove possible chimeras generated during amplification (Edgar *et al.* 2011; Ursell *et al.* 2012).

A new emerging way of analysing microbiota is so called whole genome sequencing (WGS), in which the entire bacterial genome is sequenced at a single time. One of the main advantages is that it provides more accurate bacterial sequences and might reveal even totally new species. Furthermore, WGS provides information regarding microbiota functions beyond mere identification of taxa. On the downside, it is still somewhat too expensive for extensive analyses and takes a relatively long time to process. Nevertheless, efforts are being made to reduce the cost of whole genome sequencing dramatically in order to produce more accurate science and a drastic reduction in sequencing costs has already been experienced (Ursell *et al.* 2012).

Third generation sequencing (TGS) is under development for the future. The main advantages include that it reads nucleotides at single molecular level and does not require DNA strand splicing, amplification and synthesis. Also, the speed of analysing and simple sample preparation offers promising prospects for future research. Still, the rate of errors is higher than with NGS preventing scientist for developing commercial derivates. (Bleidorn 2016).

2.3 HPV and bacterial interactions

Bacteria and viruses constitute a complex that is continually changing in response to external and internal stimuli. Since bacteria and viruses interact with each other constantly and one could impact to other's function, it is meaningful to study the interactions between these two. The objective of studying viral-bacterial interactions is to understand these sufficiently to recognise their impact on human health. Furthermore, we need to understand when dysbiosis occur and how to remedy it when necessary. The ultimate goal in the future is to discover new therapeutic targets for the treatment of disease. (Almand, Moore and Jaykus 2017).

In lung diseases, simultaneous co-infections with a virus and bacterium can result in higher severity of illness (Deng 2013). In the respiratory tract, it is believed, that present viral infection predisposes the individual to bacterial infection as well as a result of alterations in epithelial and immune cells (Deng 2013; Bellinghausen *et al.* 2016). The opposite situation may occur as well, when bacteria infect the subject first and establish an environment prone to viral co-infections (Bellinghausen *et al.* 2016; Almand, Moore and Jaykus 2017). Similar results have been shown in the gut as well (Monedero, Buesa and Rodríguez-Díaz 2018). On the other hand, there is evidence that the microbiota may improve the influence of immune defence against viral infection (Ichinohe *et al.* 2011).

Below, the microbiota composition in specific body sites of women and infants are described in greater detail. Furthermore, the HPV prevalence is explained as it is currently known as well as interactions between HPV and bacterial microbiota.

2.3.1 Women

Oral cavity

The oral cavity harbours around 600-700 different bacterial species and the total volume of bacteria is around 10^{11} bacteria/ml (Dewhirst *et al.* 2010; Kilian *et al.* 2016; Sender, Fuchs and Milo 2016) (**Figure 5**). The oral cavity is the only area in the human body where hard tissue, teeth, reaches outside the soft tissue, the gingival mucosa. This offers different tissues and surfaces for bacteria colonization in the mouth; teeth, three different types of mucosa, the gingival sulcus and furthermore the tonsils in the oropharynx. They all present unique characteristics for bacterial biofilm formation (**Figures 3 and 4**). Most studies investigating the oral microbiota have obtained their samples with mucosal swabs or as saliva samples and it is therefore difficult to dissect which oral environment the samples represent.

Almost all oral diseases are infections: caries, gingivitis and periodontitis as well as endodontic infections and alveolitis (**Table 3**). The oral microbiota has been

associated with cardiovascular diseases, preterm birth, pneumonia and various gastrointestinal cancer (Offenbacher *et al.* 1998; Joshipura *et al.* 2003; Beck and Offenbacher 2005; Seymour *et al.* 2007; Awano *et al.* 2008; Michaud and Izard 2014; Karpiński 2019).

Table 3. The main pathogenic bacteria in periodontal disease and in dental caries, respectively. Modified from Awadh Al-Shahrani 2019; Könönen, Gursoy and Gursoy 2019.

Periodontal disease	Dental caries
Aggregatibacter actinomycetemcomitans	*Streptococcus mutans*
Porphyromonas gingvalis	
Tannerella forsythia	*Lactobacilli spp.*
Treponema denticola	*Actinomyces spp.*
Prevotella intermedia	
Prevotella nigrescens	
Parvinomas micra	
Campylobacter rectus	
Fusobacteium nucleatum	

The Human Oral Microbiome Database (HOMD) lists the most prevalent phyla in adult human oral cavity to be composed by Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Fusobacteria (Dewhirst *et al.* 2010; Huse *et al.* 2012; Wade 2013) (**Figure 7**). Since the oral cavity is under a constant exposure to exogenous micro-organisms while eating, drinking and breathing, it is not easy to determinate which specific species are indigenous and which are only transient (Dewhirst *et al.* 2010).

The prevalence of adult oral HPV infection has been reported to range from 3.6% - 34%. HPV infection in the oral cavity has previously been connected with risky sexual behaviour (several partners, practice of oral sex), simultaneous infection by human immunodeficiency virus (HIV infection), the presence of genital warts, older age and smoking. (Rintala *et al.* 2005b; Kreimer *et al.* 2010; Gillison *et al.* 2012). Spousal persistent oral HPV infection is a risk-factor for persistent oral HPV infection in the another spouse (Rintala *et al.* 2006). We have previously shown, that oral HPV infection prevalence in the mother is lower before delivery (16%) than two years after (27%) (Rintala *et al.* 2005b). This might indicate that pregnancy may enhance immune defence and result in not acquiring HPV or clearing the present HPV infection. Around 7% of mothers cleared the infection and another 7% persisted in the 24-month follow-up (Rintala *et al.* 2006).

Our current understanding of the interactions between HPV infection and the oral microbiota in adults is very limited. Guerrero-Preston an colleagues (Guerrero-Preston *et al.* 2016, 2017) presented higher amount of *Lactobacillus gasseri/johnsonii*, *Lactobacillus vaginalis* and *Gemellaceae* genus in saliva samples from HPV positive HNSCC patients. These bacterial species have previously been connected to higher caries levels in another study (Caufield *et al.* 2015).

Cervix

The uterine cervix and vaginal microbiota are mainly dominated by *Lactobacillus* species, the main function of which is to regulate the acid pH by lactic acid production. The vaginal/cervical microbiota are often divided into specific groups according to which *Lactobacillus* species is the dominant one; *L. crispatus, L. gasseri*, *L. iners* or *L. jensenii.* (DiGiulio *et al.* 2015; Lloyd-Price, Abu-Ali and Huttenhower 2016; Di Paola *et al.* 2017). Hormonal changes during life (normal menstrual cycle, hormonal contraceptive use and pregnancy) all have effect to the vaginal microbiota composition in addition to geographical differences and race (Ravel *et al.* 2011; Gajer *et al.* 2012; Anahtar *et al.* 2015; Nuriel-Ohayon, Neuman and Koren 2016; Brooks *et al.* 2017). Shifts in this balance to more diverse microbiota and lower levels of *Lactobacillus* spp. have been linked to bacterial vaginosis, cervical intraepithelial neoplasia and its severity and even to pre-term birth in pregnant women (DiGiulio *et al.* 2015; Mitra *et al.* 2015a, 2015b, 2016). Bacterial vaginosis is characterized by lower levels of *Lactobacillus* spp. and higher levels of *Preveotell*a species, *Mycoplasma hominis* and *Atopobium vaginae* (Gillet *et al.* 2011; Di Paola *et al.* 2017).

Cervical HPV infection is observed in around 18.9% - 75% of adult women and is thus a fairly common finding (Eppel *et al.* 2000; Worda *et al.* 2005; Paaso *et al.* 2011; Koskimaa *et al.* 2012; Park *et al.* 2012; Uribarren-Berrueta *et al.* 2012; Brotman *et al.* 2014; Steinau *et al.* 2014; Mitra *et al.* 2015b). HPV16 infection is the most prevalent HPV genotype in cervical region (Louvanto *et al.* 2011a; Zhao *et al.* 2014; Santos Filho *et al.* 2016; Hooi *et al.* 2018; Ge *et al.* 2019). Risk-factors for cervical HPV infection include multiple life-time sexual partners, initiation of oral contraceptive use after 20 years of age and young (<13 years) age at initiation of smoking, multiple sexual partners and sexual activity (Louvanto *et al.* 2011a; Shi *et al.* 2017; Hooi *et al.* 2018).

Decreased levels of *Lactobacillus* and increased diversity in cervical microbiota have been shown to have a positive correlation with present HPV infection and its persistence and severity of disease (Gillet *et al.* 2011; Gao *et al.* 2013; Mitra *et al.* 2016; Di Paola *et al.* 2017; Kero *et al.* 2017; Mortaki *et al.* 2019). Levels of *Gardnerella vaginalis* and *Atopobium vaginae* have been reposted to be significantly higher in HPV positive cervical samples (Gao *et al.* 2013; Di Paola *et al.* 2017). It is

already relatively clear that the vaginal microbiota is associated with HPV infection but the direction of causality between the two remains unclear (Mortaki *et al.* 2019).

Placenta

The schematic illustration of the placenta structure is presented in **Figure 8**. The main function of the placenta is to provide nutrients and oxygen to the developing foetus while clearing waste products. In addition, the placenta secretes hormones which are necessary for the continuation of the pregnancy. (Gude *et al.* 2004).

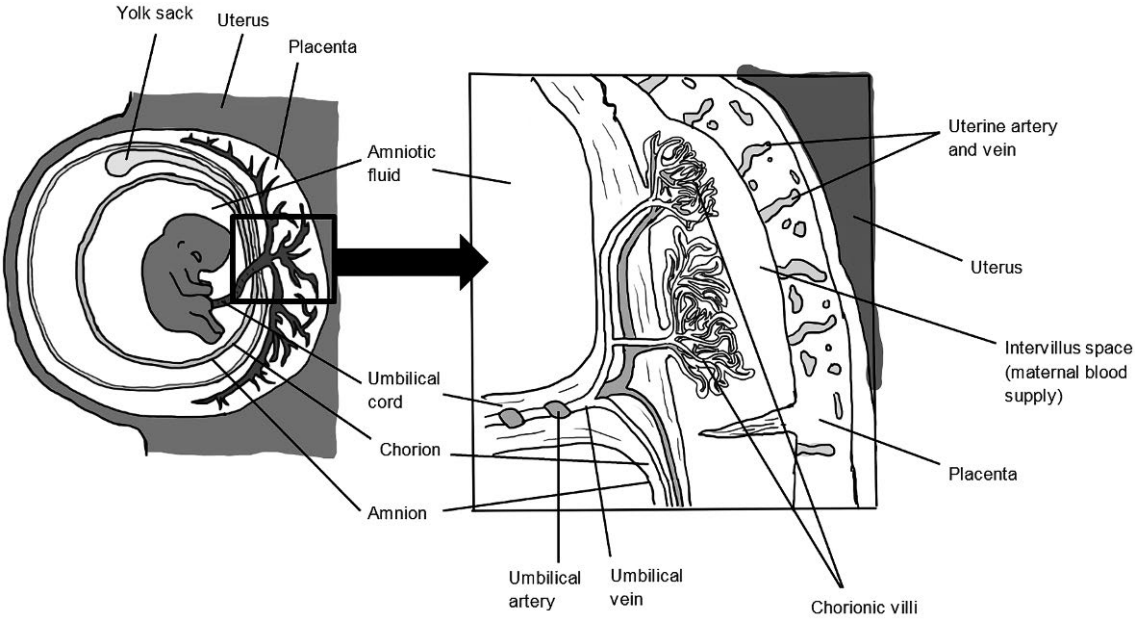

Figure 8. The gross anatomy of the placenta. The embryo is about two months old.

The presence of microbes in the placenta was suggested a few years ago. The placenta reportedly harbours mainly Proteobacteria, *Prevotella, Streptococcus* and *Veillonella* (Satokari *et al.* 2009; Rautava *et al.* 2012b; Stout *et al.* 2013; Aagaard *et al.* 2014; Collado *et al.* 2016; Gomez-Arango *et al.* 2017a; Parnell *et al.* 2017; Benny *et al.* 2019). The placenta microbiota has been suggested to resemble the adult oral microbiota (Aagaard *et al.* 2014). Though, not all studies have been able to corroborate these findings (Lauder *et al.* 2016; de Goffau *et al.* 2019; Theis *et al.* 2019). The problem with determination of placenta microbiota lies in the small number of detectable bacteria. This makes it difficult to distinguish true bacterial colonisation from contamination. Furthermore, researchers have not been able to consistently cultivate the bacteria detected in placenta to show their viability. (Perez-Muñoz *et al.* 2017).

Nevertheless, statistically significant differences in the placenta microbiota have been observed in term versus preterm deliveries, in mothers who gain excess weight during pregnancy, in mothers with gestational diabetes, in case of pre-eclampsia, chorioamnionitis and according to neonate's birth weight, when compared placenta samples from a healthy pregnancy. Increasing evidence suggests that some bacteria might be present in the healthy placenta. (Queiros da Mota *et al.* 2013; Amarasekara *et al.* 2015; Antony *et al.* 2015; Zheng *et al.* 2015, 2017a, 2017b; Lloyd-Price, Abu-Ali and Huttenhower 2016; Doyle *et al.* 2017; Benny *et al.* 2019; Younge *et al.* 2019).

In the placenta, HPV is reportedly detected in 5.7% - 85% of the cases studied. HPV has been localized into the trophoblast (epithelial) cells. (Hermonat *et al.* 1998; Liu *et al.* 2001; Gomez *et al.* 2008; Rombaldi *et al.* 2008; Sarkola *et al.* 2008a; Weyn *et al.* 2011; Skoczyński, Goździcka-Józefiak and Kwaśniewska 2011; Uribarren-Berrueta *et al.* 2012; Chisanga *et al.* 2015; Slatter *et al.* 2015; Ambühl *et al.* 2016; Trottier *et al.* 2016; Bober *et al.* 2019). The presence of HPV in the placenta has been observed to increase the risk of spontaneous abortion and chorioamnionitis (Liu *et al.* 2001; Gomez *et al.* 2008; Slatter *et al.* 2015; Ambühl *et al.* 2016; Bober *et al.* 2019). However, HPV positivity in placenta has also been detected with healthy pregnancy and birth in several studies (Sarkola *et al.* 2008a; Koskimaa *et al.* 2012; Lee *et al.* 2013b; Trottier *et al.* 2016).

Bacterial inflammation and infection of the placenta, even without the presence of HPV, is a known risk-factor for extremely preterm deliveries (Trivedi *et al.* 2012). Furthermore, specific bacterial such as *Ureaplasma* species, in the vagina, cervix and placenta, have been linked with chorioamnionitis and preterm deliveries (Kwak *et al.* 2014; Cox *et al.* 2016; Sweeney *et al.* 2016). There is no information about present HPV infection interactions to microbes in the placenta.

Breast milk

The microbiota of human breast milk is composed mainly of *Streptococceae*, *Staphylococcaceae*, *Pseudomonadaceae* and *Bifidobacteriaceae*. The milk microbiota composition has been observed to be unique to each mother showing also geographical differences (Hunt *et al.* 2011; Urbaniak *et al.* 2014; Boix-Amorós, Collado and Mira 2016; Biagi *et al.* 2017; Li *et al.* 2017b). The delivery mode affects the bacterial composition of breast milk, as well as the mothers' weight. Fluctuation in breast milk bacterial composition is observed over the course of lactation (Cabrera-Rubio *et al.* 2012; Li *et al.* 2017b; Hermansson *et al.* 2019). Besides bacteria, breast milk contains oligosaccharides, proteins, immunoglobulins (such as IgA) and lipids and provides all the essential ingredients for infants' nutrition (Nuriel-Ohayon, Neuman and Koren 2016; Aakko *et al.* 2017; Mosca and Giannì 2017).

Around 2.5% - 21% of breast milk have been observed to harbour HPV, which is thought to shed from ductal epithelial cells (Sarkola *et al.* 2008b; Yoshida *et al.* 2011; Glenn, Whitaker and Lawson 2012; Diaz *et al.* 2018). Not all studies have been able to detect any HR-HPVs in breast milk (Mammas *et al.* 2011). HPV in breast milk has been observed to be a risk-factor for the spouse to acquire oral HPV infection (Louvanto *et al.* 2017). There are currently no reports on interactions between HPV and bacterial composition in breast milk.

2.3.2 Infant

The development of the oral cavity microbiota

Neonatal gut microbiota has been suggested to start developing already in utero. The same has not yet been observed in the oral cavity despite the close connection of the two via the gastrointestinal tract. (Collado *et al.* 2016). The neonatal oral cavity microbiota is known to be very delicate and easily affected by outer factors such as delivery mode, antibiotics given to mother during delivery and by the mode of feeding (Dominguez-Bello *et al.* 2010; Lif Holgerson *et al.* 2011; Al-Shehri *et al.* 2015; Drell *et al.* 2017; Gomez-Arango *et al.* 2017b; Timby *et al.* 2017; Dzidic *et al.* 2018b; Li *et al.* 2018).

Directly after delivery and during the first months of life, the main oral bacterial taxa consist mainly by *Streptococcus* and *Staphylococcus*. In the following months the *Gemella, Actinomyces* and *Veillonella* becomes more abundant. (Sampaio-Maia and Monteiro-Silva 2014; Biagi *et al.* 2017; Dzidic *et al.* 2018b). Changes detected in the oral microbiota during the first few weeks of life caused by differences in delivery mode are no longer detectable after six weeks of life (Chu *et al.* 2017). Some changes in the bacterial microbiota composition emerge again when deciduous teeth erupt (Cephas *et al.* 2011; Sampaio-Maia and Monteiro-Silva 2014). The oral microbiota maturation continues during the entire childhood (Crielaard *et al.* 2011; Dzidic *et al.* 2018b). It has recently been suggested that maternal microbiota might influence to the neonatal oral microbiota development, and that especially bacteria detected in the placenta might contribute significantly to neonatal oral microbiota development (Younge *et al.* 2019).

HPV and microbiota in the infant oral cavity

HPV infection in healthy newborns oral cavity has been observed to fluctuate between 0.9% - 14.3 % (Rintala *et al.* 2005b; Castellsagué *et al.* 2009; Rombaldi *et al.* 2009; Smith *et al.* 2010; Martinelli *et al.* 2012; Park *et al.* 2012; Hahn *et al.* 2013; Trottier *et al.* 2016). The main studies from the past ten years are listed in **Table 4**.

Table 4. The prevalence of infant oral HPV infection. Study group, country, number of participants, sample, type, HPV detection method, the oral HPV presence and the most prevalent HPV genotypes are listed.

Country study population	Number of subjects	Sample	HPV detectlon method	Oral HPV +ve% Basellne (at blrth)	OralHPV +ve% 1–2 months	OralHPV +ve% 6 months	Most prevalent HPV genotypes
Brazll	63	buccal swab	multlplex PCR and nested multlplex PCR	12.2%	10.2%	2%	HPV 6 & 11 (53.3%)
Spaln	117	oral swab	PCR	6.0%	12.7%	9.1%	HPV 16 (30.8%)
USA	333	oral swab	PCR	0.9% (wlthln 24 hours after dellvery)	N.A.	N.A.	HPV 16 (0.9%)
Italy	14 to 42	oral-pharyngeal swab	nested PCR	7.7%	9.4%	14.3%	HPV 81 (20%)
South Korea	291	oral mucosal scrape	PCR	3.4%	N.A.	0%	HPV 16 (20%)
Republlc of Korea	469	oral swab	PCR	3.2%	N.A.	N.A.	HPV 53 (12.5%)
Canada	67	buccal swab	PCR	8.1%	N.A.	N.A.	HPV 89 (40%)

The risk-factors in parent related transmission of HPV infection include HPV positive mother (with multiple HPV genotypes) during the time of delivery and vaginal delivery (Medeiros *et al.* 2005; Park *et al.* 2012; Hahn *et al.* 2013; Trottier *et al.* 2016). Still, not all studies have been able to identify specific parental risk-factors for infant HPV transmission (Smith *et al.* 2007). There have been no studies examining the interactions between bacterial microbiota and oral HPV infection in the neonatal or infant oral cavity. The impact of neonatal oral HPV infection to later life also still remains to be clarified.

2.4 Microbiome and cancer

Cancer development is a complex process and numerous risk-factors have been detected to increase the risk for malignant transformation. The most important recognised risk-factors include carcinogens in various substances, such as in tobacco smoke and alcohol use. Ionising irradiation, genetic susceptibility and everyday mutations during normal cell division processes are all possible mechanisms for

acquiring severe mutations in a single cell. If the mutated cell is then able to pass all body's regulators, which function to monitor normal cell division processes, there is a chance that it could proceed to cancer. Furthermore, viruses, such as HPV, Epstein-Barr virus (EBV) and hepatitis B and C viruses (HBV and HCV) may induce cancer development. It has been estimated that viruses and bacteria cause 10-20% of all cancer (Zur Hausen 1991; De Martel *et al.* 2012; Khoury *et al.* 2013).

Our knowledge regarding the impact of the bacterial microbiota to cancer development is increasing. Alterations in microbiota composition have been detected in various human cancer types, including colorectal cancer, pancreatic cancer, breast cancer and oral cancer. Especially the oral and gut microbiota have been detected to be implicated in this complicated issue. (Lu *et al.*; Schwabe and Jobin 2013; Wang and Ganly 2014; Michaud and Izard 2014; Zitvogel *et al.* 2015; Garrett 2015; Chen, Domingue and Sears 2017; Zhao *et al.* 2017; Lee *et al.* 2017; Mani 2017; Rajagopala *et al.* 2017; Flemer *et al.* 2018; Healy and Moran 2019).

One possible mechanism behind this is that certain pathogenic bacteria and general dysbiosis of the microbiota might induce an inflammatory response and the formation of toxic metabolites which, after a significant duration, could induce carcinogenesis and cancer development (Brennan and Garrett 2016; Lucas, Barnich and Nguyen 2017; Nieminen *et al.* 2018). The underlying mechanisms are very complex and difficult to study, given the complexity of cancer development in general and the fact that multiple simultaneous mechanisms are often required in order for the cancer to develop. Research have mainly been able to make cross-sectional studies in human study populations, while the causal relationship remains unclear. Furthermore, cancer itself might be able to cause dysbiosis. (Steed and Stappenbeck 2014; Healy and Moran 2019).

When treating patients with cancer, surgery, radiotherapy and cytostatic drugs are the main choices of treatment. Previously, there was suggestions that HPV positive carcinomas, especially in the head-and-neck region, could become more aggressive after radiation treatment (Majoros, Devine and Parkhill 1963; Zehnder and Lyons 1975; Bauman and Smith 1996; Blackledge and Anand 2000). On the contrary, more recent studies have detected higher radiosensitivity in HPV positive carcinoma patients with HNSCC and cervical squamous cell carcinoma (CSCC) (Harima *et al.* 2002; Kimple *et al.* 2013; Zhang *et al.* 2015). It is shown that HPV positive OPSCC patients have longer life-expectancy and better prognosis than patients with HPV negative OPSCCs (Ang *et al.* 2010; Kimple *et al.* 2013; Zhang *et al.* 2015; Li *et al.* 2017a). Still, not all the studies have corroborated these findings (Nagel *et al.* 2013; Schneider, Bol and Grégoire 2017). On the other hand, CSCC patients positive with multiple simultaneous HPV genotypes have poorer overall prognosis compared to CSCC with single HPV type (Bachtiary *et al.* 2002).

3 Aims of the Study

The present study focuses on understanding how the microbiota and present HPV infection interact with each other in the maternal oral cavity and uterine cervix, placenta, and breast milk and in the infant oral cavity. Furthermore, we aimed to investigate the presence of bacterial DNA from placenta samples to evaluate whether the placenta might harbour a bacterial microbiota. Finally, the HPV positive cell behaviour and invasion into the human uterine extracellular leiomyoma-based matrix was evaluated.

The specific study aims were:

1. To investigate the bacterial microbiota composition in the maternal oral cavity, uterine cervix, placenta, and breast milk as well as in the infant oral cavity.
2. To study the associations between HPV and microbiota composition at the same body sites.
3. To understand how maternal microbiota from the oral cavity, cervix and placenta influence the initial neonatal oral cavity microbiota.
4. To see how HPV positive cell lines (SiHa and CaSki) behave in novel cultures of myoma invasion assay and Myogel Transwell invasion assay and how irradiation and different HPV copy numbers affect to cell invasion.

4 Materials and Methods

4.1 Subjects and materials

4.1.1 The Finnish Family HPV Study (I, II, III)

The samples in the studies I-III are derived from the longitudinal Finnish Family HPV study (Syrjänen *et al.* 2009; Rautava *et al.* 2012a). The samples were collected during the years 1998-2001 at the Department of Oral Pathology, Institute of Dentistry, University of Turku and the Department of Obstetrics and Gynaecology, Turku University Hospital, Finland. The original study was designed to evaluate the interactions of HPV infection between families; mothers, fathers and their infants.

In total, 329 mothers were enrolled in their last trimester of their first pregnancy. The participating women gave birth to 331 infants (two sets of twins were born). In addition, 131 fathers were enrolled. The study protocol and its amendments were approved by the Research Ethics Committee of the Intermunicipal Hospital District of Southwest Finland (#3/1998, #2/2006, 45/180/2010). Written informed consent was obtained from all participants of the original Finnish Family HPV Study.

4.1.2 Biological samples (I, II, III)

A total of 39 mothers were selected for the studies (I-III) based on the placental HPV status. Altogether, from the entire Finnish family HPV study, 13 placentas out of 329 were detected positive for HPV DNA and were selected as cases. The HPV infection in the placenta was earlier confirmed with *In Situ* Hybridisation (Sarkola *et al.* 2008a). The placenta controls were selected based on the availability of samples and the mode of delivery (26 placenta controls; 13 from vaginal deliveries and 13 from caesarean section). In total we worked with 39 placenta samples from the original Finnish Family HPV study. In the bacterial microbiota analyses, however, we were forced to exclude a number of placenta samples because low number of reads. At the end 49.0% (n=19/39) of high-quality placenta samples (of which vaginally delivered 52.6%, n=10/19) were included in the microbial analyses.

In study I, in addition to the placenta samples, the corresponding oral and cervical samples (n=39) from these women were analysed. For the second (II) study,

the corresponding neonates' oral samples (n=40) were analysed in addition to breast milk samples (n=39) from the mothers. Five samples from infant oral and four breast milk samples had to be excluded from bacterial analyses, because of poor DNA quality of the original sample.

For the third study (III), we continued to work with the same mother and neonate pairs but selected the subjects from whom all four following samples were available: maternal oral cavity, uterine cervix, placenta and neonatal oral samples collected directly after delivery. Altogether eight mother-infant pairs were selected for the third study.

The samples used in studies I-III are presented in **Table 5**. Also, the infant sex, the duration of the gestation and the delivery mode are listed.

Table 5. The samples used in the microbiota studies (I, II, III).

No/family	Infant sex (B boy, G girl)	Delivery mode (V vaginal, C caesarean section)	Gestation weeks	Study number
1	G	V	42.0	I, II**
2	G	V	41.0	I, II, III
3	G	C	35.4	I, II**
4	G	V	40.1	I, II
5	B	V	37.6	I, II
6	G	V	40.1	I, II
7	B	V	42.0	I, II
8	G	V	39.4	I, II
9	G	V	38.3	I, II***
10*	B	V	38.0	I, II
11*	B	V	38.0	I, II
12	B	C	38.5	I, II
13	B	C	42.2	I, II
14	G	V	41.5	I, II
15	B	V	41.0	I, II
16	B	V	39.0	I, II
17	G	C	40.0	I, II, III
18	B	C	42.1	I, II
19	G	C	42.4	I, II
20	G	C	41.1	I, II
21	G	V	39.6	I, II
22	B	C	38.4	I, II***, III
23	G	V	39.0	I, II**
24	B	V	42.0	I, II, III
25	G	V	40.4	I, II, III
26	G	C	39.0	I, II
27	B	V	40.4	I, II, III
28	G	V	41.0	I, II
29	G	C	39.1	I, II
30	G	V	41.6	I, II
31	G	C	40.6	I, II
32	B	V	38.3	I, II, III
33	G	V	40.6	I, II
34	G	V	40.0	I, II, III
35	G	C	39.0	I, II, III
36	G	C	41.4	I, II***, III
37	B	V	39.6	I, II**
38	B	C	35.3	I, II***, III
39	B	V	36.6	I, II**
40	B	C	42.0	I, II, III

* twins, **infant oral sample not included, ***breast milk sample not included

4.1.3 Collection of the samples (I, II, III)

Oral samples

Oral samples from the mothers and neonates were collected as mucosal scrapings with a small brush (Cytobrush, MedScan, Malmö, Sweden). A swab was taken from both cheeks and the superior and interior buccal vestibule with three to four gentle back-and-forth scrapes. Maternal oral sample was obtained during the third trimester of the pregnancy. Neonatal oral samples were collected directly after delivery or at two months of age. Brushes were placed in a tube containing 70% ethanol and frozen to -80°C until used.

Cervical samples

The cervical scrapings were taken with a brush (Cytobrush, MedScan, Malmö, Sweden) during the third trimester and the brush was then placed in a tube containing 0.05 M PBS (phosphate buffered saline) with 100 µg of gentamycin and stored at -80°C until analysed.

Placenta samples

Placenta samples were tissue samples obtained directly after the delivery in the delivery room and included all the tissue layers from the central part of maternal side of the placenta. The samples were around 1 cm in diameter and were stored at -80°C until analyses.

Breast milk samples

The breast milk samples were obtained by the mothers themselves manually separately from feeding and after careful hand disinfection. No nipple disinfectant was used before sample collection and the procedure was done apart from feeding the infant. Samples were collected after the delivery or two months postpartum from the first proportion of the breast milk. Time when the samples were collected varied between individuals. The milk was collected into 3 ml sterile plastic containers, sealed and stored at -70°C until used.

4.1.4 Cell lines in 3D extracellular matrix cultures (IV)

Commercially available uterine cervix HPV positive SCC cell lines SiHa (ATCC® HTB-35™) (LGC Standards GmbH, Wesel, Germany) and CaSki (ATCC® CRL-

1550™) (LGC Standards GmbH, Wesel, Germany) were used in addition with HPV negative human oral tongue SCC cell line HSC-3 (JCRB 0623; Osaka National Institute of Health Sciences, Osaka, Japan) for cell cultivations. CaSki contains approximately 600 HPV viral copies per cell, while SiHa only one to two HPV copies (Yee *et al.* 1985; Raybould *et al.* 2014). HSC-3 cells were used as a control.

4.2 Methods

4.2.1 DNA extraction (I, II, III)

Oral, cervical and placenta samples

DNA was extracted from the brush samples (oral and cervical scrapings) and from placenta tissue with the high-salt method (Miller et al. 1988). A piece of frozen placenta was grounded into pieces with a cutter on ice before starting the lysis. A lysis buffer (10 mM Tris, 400 mM NaCl, 100 mM EDTA, 1% SDS) was used to lyse the samples. This was followed by digestion with proteinase K (10 µl/ml) in +37°C overnight. Proteins were precipitated with saturated NaCl and ethanol. DNA was then dissolved in 50 µl water, mixed for 30 minutes and stored at -70°C until used.

Breast milk samples

DNA was extracted from milk samples by using high pure PCR template preparation kit (Roche Diagnostics GmbH, Penzberg, Germany). The samples were centrifuged at 12.000 rpm for 5 minutes for the pellet to format, followed by lysis with proteinase K in Tissue lysis buffer at 55°C for 1 hour. The lysis was then applied to a filter tube and passaged through a glass fleece with centrifugation. Residual impurities were removed with wash-and-spin steps. All purified DNA samples were stored at -70°C.

4.2.2 HPV detection (I, II)

DNA extraction

Nested PCR with external (MY09/MY11) and internal (GP05+/GP06+) primers were used to detect HPV DNA from oral, placental and breast milk samples. For cervical samples, only single PCR with GP05+/GP06+ primers were used. A 50 µg reaction mixture was used with Amplitag Gold DNA polymerase (Perkin Elmer, NJ, USA). The sensitivity of the PCR method was around 20 copies of HPV.

HPV genotyping

Genotyping was performed by using the fluorescent bead array of the Multimetrix assay (Progen Biotechnik GmbH, Heidelberg, Germany), which detects 24 HPV types (LR-HPV6, 11, 42, 43, 44, and 70; HR-HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82 types). The test was performed according to manufacturers' instructions except the nested PCR products were re-amplified for biotinylation using GP05+/GP06+ primers and only half of the recommended volume, but in the final step, 100 µl of the blocking buffer was used. Hybridized beads were measuring with a Luminex LX-100 analyser (Bio-Plex 200 system, Bio-Rad Laboratories, Hercules, USA) (**Figure 9**). The median fluorescence intensity (MFI) of at least 100 beads was computed for each bead in the sample. The cut-off value for each HPV probe was defined individually; 1.5x negative control MFI (background) +5 MFI.

The samples detected positive for HPV16 were re-analysed from the original sample with nested PCR and with internal and external primers, in addition with bead based HPV16 genotyping assay. This additional step was done in order to control any possible contamination of HPV16 during previous testing. Other possible contamination of tested samples was controlled by the simultaneous analysis of HPV negative control samples with each run.

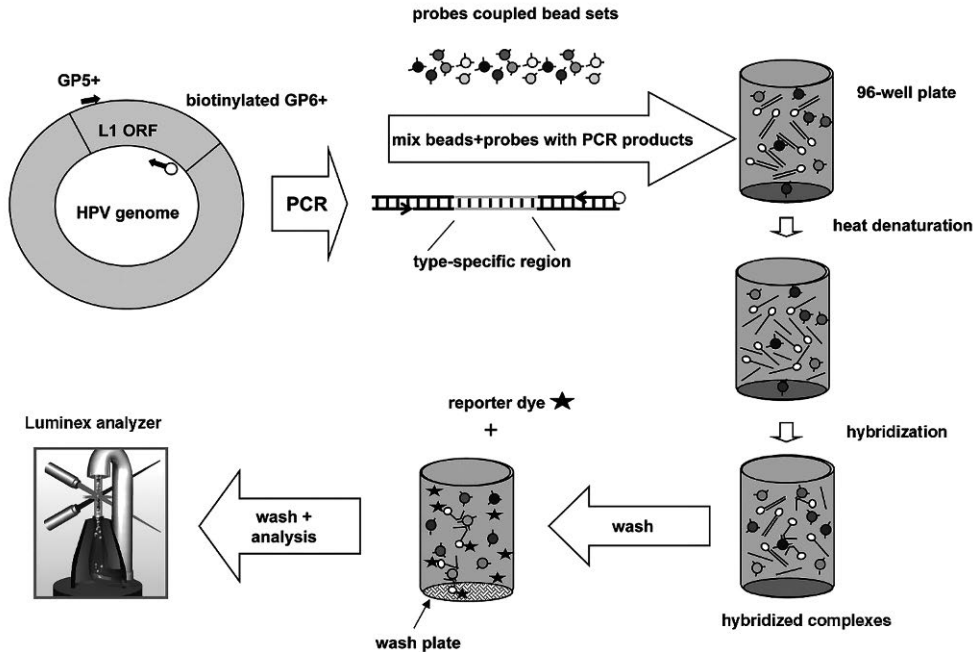

Figure 9. The bead-based multiplex HPV genotyping (Luminex) assay. (Schmitt *et al.* 2006)

4.2.3 Microbiota analysis (I, II, III)

After the total DNA extraction, the isolated double-stranded DNA concentrations were evaluated and quantified with Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, USA). Working solution was prepared mixing Qubit® reagent 1:200 to Qubit® buffer. All samples were normalized to 10 ng/µl prior to 16S rRNA gene sequencing with Illumina MiSeq Platform.

The V3-V4 region of the 16S rDNA bacterial gene was amplified with PCR using Illumina adapter overhang nucleotide sequences following the Illumina protocols. After the 16S rDNA gene amplification, the multiplexing step was conducted using Nextera XT Index Kit (Illumina, San Diego, CA, USA) to construct DNA libraries. PCR products were checked in Bioanalyzer DNA 1.000 chip (Agilent Technologies, Santa Clara, CA, USA). Libraries were sequenced using 2x300 bp pair-end run (MiSeq Reagent Kit V3) on a MiSeq Illumina platform (Lifesequencing sequencing service, Valencia, Spain). The schematic illustration of the V3-V4 detection is presented in **Figure 10**.

To rule out any possible contaminants, PCR amplifications and library kit controls were sequenced along each run as negative controls. Strict quality control was carried out when working with low bacterial biomass, placenta and infant oral samples, in particular. Before microbiota analyses, all possible contaminants were removed: single OTUs, OTUs with relative frequency below 0.01, sequences that could not be classified to domain level and *Cyanobacteria*, *Chloroplasts* and *Rhizobiales* were all removed.

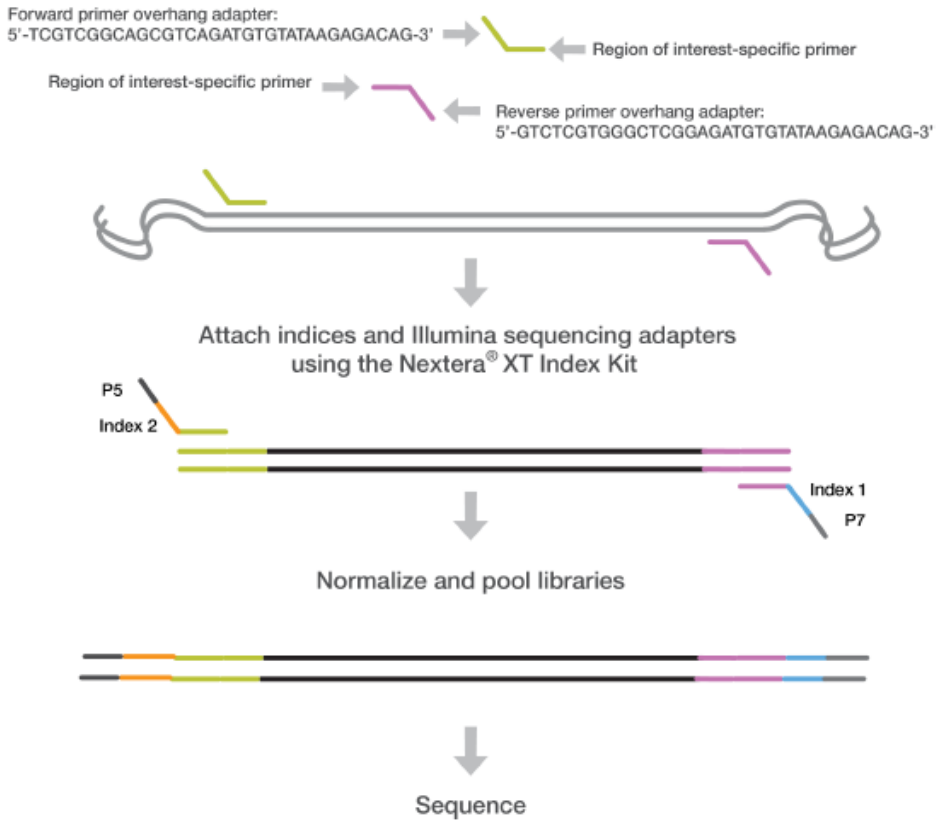

Figure 10. Schematic view of Illumina platform workflow. Libraries are normalized, pooled and sequenced on the MiSeq system by using v3 reagents. Figure from the Illumina 16S Metagenomic Sequencing Library Preparation protocol.

4.2.4 Myoma and Myogel (IV)

Three-dimensional cancer cell behaviour modelling has been used for some ten years. Myoma invasion assay and Myogel Transwell assay have been developed in the University of Oulu by Professor Salo's research group (Nurmenniemi *et al.* 2009; Salo *et al.* 2015).

Myoma is a benign tumour derived from monoclonal smooth muscle cells and arises in the uterine wall. These tumours are mainly composed of extracellular matrix, including collagen, fibronectin and proteoglycan. Myomas are quite common affecting more than half of women worldwide and they do not have potential to develop into malignant tumours over time. In most of the cases, they do not present any symptoms. If symptoms such as abdominal pain or over-excessive (menstrual) bleeding occur, the myomas may be removed surgically (Parker 2007; Tinelli *et al.* 2016). The myoma specimens for the assays have been obtained during routine

surgical procedures and the studies have been approved by the Northern Ostrobothnia Hospital District Ethics Committee (statement #8/2006, amendment 19/10/2006 and statement #35/2014, 28.4.2014). Myogel has been derived from myomas.

With the myoma model, all the normal extracellular components are maintained (e.g. vessels, collagen fibres and soluble factors, including cytokines and growth factors) and the cell behaviour is not influenced by foreign materials (Nurmenniemi *et al.* 2009; Dayan *et al.* 2012; Teppo *et al.* 2013; Alahuhta *et al.* 2015; Al-Samadi *et al.* 2017; Naakka *et al.* 2019). Even though the myoma discs provide an excellent hypoxic, human tumour derived three-dimensional solid platform to study cancer cells behaviour *in vitro*, it is essential to keep in mind that human body has several other factors, such as blood derived cells and cytokines, that affect cancer cell aggressiveness *in vivo*.

Another myoma derived matrix, Myogel, has been designed for gel-based attachment, transwell and spheroid invasion assays (Nurmenniemi *et al.* 2009; Salo *et al.* 2015, 2018; Sundquist *et al.* 2016; Åström *et al.* 2018). These novel matrices help us to extend our knowledge of carcinoma cell invasion *in vitro*, in order to understand carcinoma cell behaviour *in vivo*.

Myoma discs preparation

Myoma tissue preparation has been described in detail previously (Nurmenniemi *et al.* 2009). Briefly, all myomas were visually evaluated to discard any defected myomas. The samples were cut out from 3 mm thick slices with 8 mm biopsy punch (Kai Industries Co., Gifu, Japan) and placed in media with 10% DMSO solution in -70°C for later use.

Myogel preparation

Myogel has been derived from myoma (Salo *et al.* 2015). The liquid nitrogen frozen myoma section were grounded to powder with CryoMill (Retsch, Haan, Germany) and a 10 g of tissue powder was then suspended to 20 ml of cold NaCl buffer (3.4 M, pH 7.4) followed by centrifugation. To homogenize the pellet, 20 ml of the same NaCl buffer with T18 Ultra-Turrax (IKA®-Werke GmbH & Co. KG, Staufen, Germany) was added. Protein concentration was measured with DC Protein Assay (Bio-Rad) and the absorbance at 590 nm with Victor3V 1420 Multilabel Counter and Wallac 1420 Manager (Perkin Elmer Life and Analytical Sciences, Turku, Finland). The Myogel solution was stored in -20°C for later use.

4.2.5 Cell cultures (IV)

Cell culture

The SiHa and CaSki were cultivated in DMEM with 10% heat-inactivated foetal bovine serum (FBS), 100 U/ml penicillin, 100 µl/ml streptomycin and 1% non-essential amino acids (all from Gibco, Life Technologies, Paisley, UK). HSC-3 cells had a different media, which consisted of DMEM/F-12 (1:1) with 10% FBS, 100 U/ml penicillin, 100 µl/ml streptomycin (Gibco, Life Technologies, Paisley, UK) in addition with 4 mg/ml Hydrocortisone, 50 µg/ml Ascorbic Acid (vitamin C) and 250 ng/ml Fungizone (Sigma-Aldrich, Ayrshire, UK). SiHa and CaSki were cultured in normal DMEM-media. For HSC-3 cells 1:1 DMEM/F-12 was used.

Myoma invasion assay

To prepare the cultures, the myomas were thawed to room temperature and washed with appropriate media. Myoma discs were placed in sterile tubes with 10 ml media to wash overnight. After 24 h myomas were carefully placed in a 24-well Transwell inserts (Corning #3422, membrane diameter, 6.5 mm, pore size 8.0 µm; Corning, Inc., Corning, NY) avoiding breaking the myoma tissue. Around 750.000 cells in 50 µl media were added on top of each myoma disc. Lower chamber of the well was filled with 500 µl appropriate cell culture media. Cells were incubated and allowed to attach and grow for 24 h in +37°C.

The myoma discs were carefully removed from the inserts and placed in 12-well culture plate on top of metal grid and uncoated nylon membrane with 1.000 µl media in the bottom of the well. It was important to notice, that the grid and the membrane were in contact with the media and no air bubbles were left under the grid. The cells were allowed to invade altogether for 14 days. The cell culture media was changed in every three to four days. **Figure 11** presents the Myoma invasion model in schematic overview.

The culture was finished after day 14 and the tissues were prepared for histology by fixing with 4% formalin overnight. The specimens were then dehydrated, cut in half and embedded in paraffin. Six µm sections were deparaffinised and stained with haematoxylin-eosin (HE) to estimate the overall success of the cell cultures and then with cytokeratin (Cytokeratin High Molecular Weight, Clone 34BE12 or Pancytokeratin, Clone AE1/AE3, Dako, Clostrup, Denmark) to detect epithelial cells and to analyse cell invasion depth and invasion area. The results, performed in triplicate, represent an average of two to six independent cultures.

Irradiation of myomas

A part of the myomas (one 12-well plate) were irradiated with Faxitron MultiRad350 (DatekMedíco, Aarhus, Denmark) irradiator according to the manufacturer's instructions in order to give the cells ionized radiation equivalent to 4 Gy on the third day of cultivation. The 4 Gy radiation dose is the smallest amount of ionised radiation given in a single dose with visible effects to cell invasion (Väyrynen *et al.* 2018). Also, one 12-well plate was mock-irradiated simultaneously and carried along but did not receive any radiation.

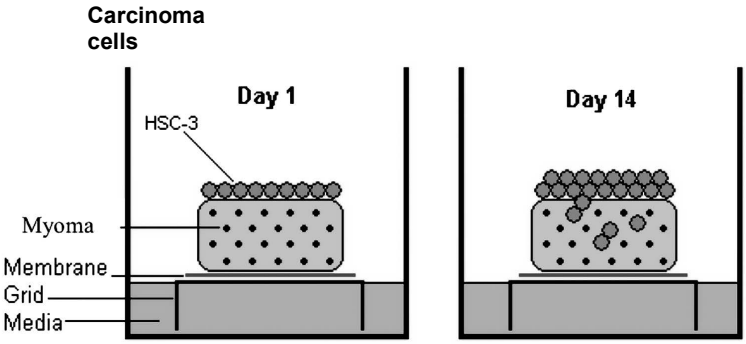

Figure 11. Schematic overview of the Myoma invasion model. Figure modified from the myoma Transwell invasion protocol manual.

Myogel Transwell invasion assay

Myogel mix of 0.8 mg/ml rat collagen and 2.4 mg/ml Myogel was prepared on ice. A volume of 50 µl of this Myogel mix was used to coat 24-well Transwell inserts, followed by 30 minutes to 1-hour incubation at +37°C for the gel to develop. To the bottom of each well, 500 µl of normal cell culture media (according to the cell line used) was added. Total of 70.000 cells in 200 µl lactalbumin media were added on top of Myogel. In lactalbumin media, the FBS was replaced by 0.5% lactalbumin (Sigma-Aldrich, Ayrshire, UK).

The cells were allowed to grow and invade for 72 h and then fixated with 4% paraformaldehyde for 1 h. Paraformaldehyde was collected to waist container and cells were washed once with 500 µl PBS. The bottom of the well and the Myogel from the insert were drawn empty. Invaded cells were stained with 1% Toluidine blue + 1% Borax at room temperature for 10 minutes. After staining, the cells were washed several times with distilled water and non-invaded cells from the upper chamber of the inserts were carefully removed with wet cotton swabs.

Staining was eluted with 500 µl 1% SDS. The eluted solution was transferred to three wells on 96-well plate, 150 µl to each well. The absorbance was measured at

650 nm using MTT assay with Synergy™ HT (BioTek Instruments Inc., Winooski, Vermont, USA) according to the manufacturer's instructions. The results represent an average of three cultures, performed in triplicate.

4.2.6 Bioinformatics and statistical analyses

Microbiota studies (I, II, III)

After primal removal, FASTQ files with sequences less than 300 nucleotides read length or with quality less than Q20 were trimmed and quality filtered by using the Fastx tool kit version 0.0134. Once the sequences were cleaned based on quality scores, we trimmed traces of the 16S rRNA primers and sequencing adapters using cutadapt version 1.2.5. After primer removal, sequences with <300 nucleotides read length were trimmed using pearl scripting. Sequences were mapped against the human genome BWA version 0.7.1 (Li and Durbin 2010) and filtered with samtools version 1.3.1–50. The clean FASTQ files were converted to FASTA files and chimeric sequences were removed by using UCHIME program version 4.2. (Edgar *et al.* 2011).

An open reference Operational Taxonomical Units (OTU) picking method using 97% (I) -99% (II, III) identity to the Greengenes 13_8 database was performed using QIIME pipeline (version 1.9.0) (Caporaso *et al.* 2010). Singletons and OTUs with a relative abundance below 0.01 were removed. Also, sequences that could not be classified to domain level, or were classified as *Cyanobacteria, Chloroplasts* (as they likely represent cellulose and cotton material) and *Rhizobiales* (as potential environmental contaminants) were removed from the dataset.

Alpha diversity indices for Chao1 (species richness) and Shannon (species diversity) in addition with beta diversity using UNIFRAC (phylogenetic) and Bray Curtis distance (non-phylogenetic) were used for microbiota analyses. The analysis of Similarities (ANOSIM) test was used to detect statistically significant differences between the different groups. Calypso software version 8.10 (I) or 8.24 (II, III) (http://cgenome.net/calypso/) was used with total sum normalization (TSS) for the statistical analyses and Cumulative Sum Scaling normalization (CSS) for multivariate tests (Redundancy Analysis - RDA). Linear discriminant analysis effect size (LEfSe) was used to detect unique biomarkers (linear discriminant analysis (LDA) score > 3.0) in relative abundance of bacterial taxonomy. Inter- and intraindividual variability was detected by pair-level analyses (paired t-test). SourceTracker 1.0.1 with QIIME1 was used to analyse the contribution of microbiota from different body sites to neonatal oral cavity microbiota. PcoA and RDA analyses were used to depict interactions between different bacterial microbiota groups. PCoA analysis is a mathematical method, which detects

similarities or dissimilarities in a given data set with multiple different groups studied at the same time. PCoA utilises a distance matrix, takes into account the composition and abundance and provides a three-dimensional figure to visualise the interactions. On the other hand, RDA is a multivariate analysis and assumes linear relationships and provides p-values in addition to visualisation. P-values ≤ 0.05 were considered statistically significant.

Cell cultivation study (IV)

Analyses were performed with Fiji software ImageJ 1.52a (National Institute of Health, USA) (Åström *et al.* 2018). The statistical analyses were all performed with IBM SPSS (SPSS for Windows, version 22.0.0.1, SPSS Inc., Chicago, IL, USA). After checking the normality distribution of all the samples, either the independent samples Student's T-test or Mann-Whitney U-test was used for unrelated samples. If the samples were related, paired samples T-test or Wilcoxon signed-rank test was used. Again, p-values ≤ 0.05 were considered statistically significant.

5 Results

5.1 HPV DNA detection (I, II)

From the entire Finnish Family HPV Study, a total of thirteen HPV positive placentas were found (3.9% = 13/330). These subjects were selected as the basis of our work and 26 subjects with HPV negative placentas were selected as controls. When the HPV status of the samples from other anatomical sites and the infants' oral cavity in the study population were examined, HPV was detected in 33.3% maternal oral cavity samples, 23.1% of the cervical samples, 8.6% of the breast milk samples and 40.0% of the infant oral cavity samples. The most predominant genotypes were HPV16 (17.9%) in the maternal oral cavity, multiple genotypes (12.8%) in the cervix, HPV16 (17.9%) in the placenta, HPV6 (5.7%) in breast milk and HPV16 (14.3%) in the infant's oral cavity. Multiple simultaneous HPV infections were discovered in 7.7% maternal oral, 12.8% cervical and 2.9% infant oral cavity samples. Only the placenta and breast milk did not present with simultaneous multiple HPV infections. Detailed information of HPV positive samples, in addition with the detected HPV genotypes, is presented at **Table 6**.

Table 6. HPV genotype distribution among all of the samples studied.

	HPV genotype/s	Mother oral (n=39)	Cervix (n=39)	Placenta (n=39)	Breast milk (n=35)	Infant oral (n=35)
HR- HPV	HPV 16	7 (17.9%)	2 (5.1%)	7 (17.9%)	1 (2.9%)	5 (14.3%)
	HPV 18	-	-	-	-	2 (5.7%)
	HPV 58	-	-	-	-	1 (2.9%)
	HPV 66	1 (2.6%)	-	-	-	2 (5.7%)
LR-HPV	HPV 6	1 (2.6%)	2 (5.1%)	5 (12.8%)	2 (5.7%)	1 (2.9%)
	HPV 11	1 (2.6%)	-	-	-	-
	HPV 83	-	-	1 (2.6%)	-	-
	HPV 70	-	-	-	-	1 (2.9%)
	multiple types	3 (7.7%)	5 (12.8%)	-	-	1 (2.9%)
	Total HPV +ve	13/39 (33.3%)	9/39 (23.1%)	13/39 (33.3%)	3/35 (8.6%)	13/35 (37.1%)

None of the mother-infant pairs were HPV positive in all of the five body sites simultaneously. One mother-infant pair was HPV16 positive at the same time in four locations (mother oral cavity, cervix, placenta and infant oral cavity). One additional pair was HP16 positive in three locations simultaneously (mother cervix, placenta and infant oral cavity) and one mother had HPV6 in her oral cavity, cervix and placenta at the same time, but her infant was born HPV negative in his oral cavity. Altogether, 13 mother-infant pairs were completely HPV negative from all the different body sites investigated at the time of the sample collection.

5.2 Bacterial DNA detection (I, II, III)

We detected different amounts of bacterial DNA in the different anatomic sites studied. The cervical samples had the highest amount of DNA, on average 86.609 reads per sample, followed by the maternal oral cavity (80.470 reads), infant oral cavity (62.312 reads), breast milk (46.356 reads) and placenta with 36.268 reads. The proportional volume of the 16S rRNA gene copies per ng of DNA is presented in **Figure 12**.

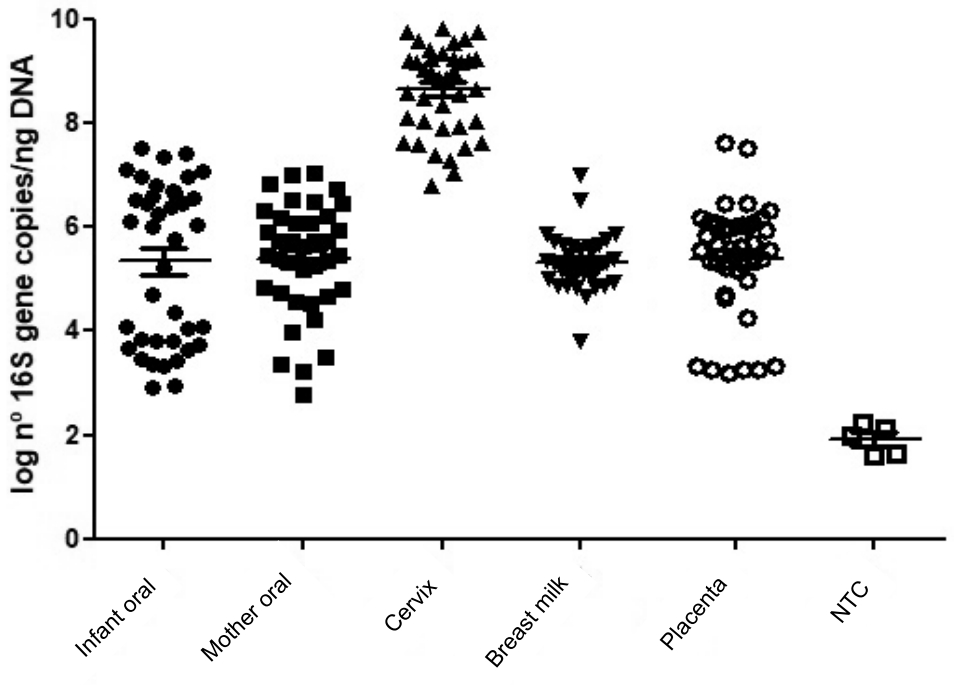

Figure 12. The copy numbers per nanograms (ng) DNA from all of the samples studied. NTC=negative PCR controls from the study.

5.3 The bacterial microbiota composition at different body sites (I, II, III)

The bacterial microbiota compositions from all the anatomical sites irrespective of HPV status are presented at phylum level in **Figure 13**. The most abundant phyla were similar at all the studied anatomical sites, with Firmicutes exhibiting highest relative abundance, followed by Proteobacteria, Actinobacteria and Bacteroidetes. The different body sites represented unique composition when examined at family level (**Figure 14)**.

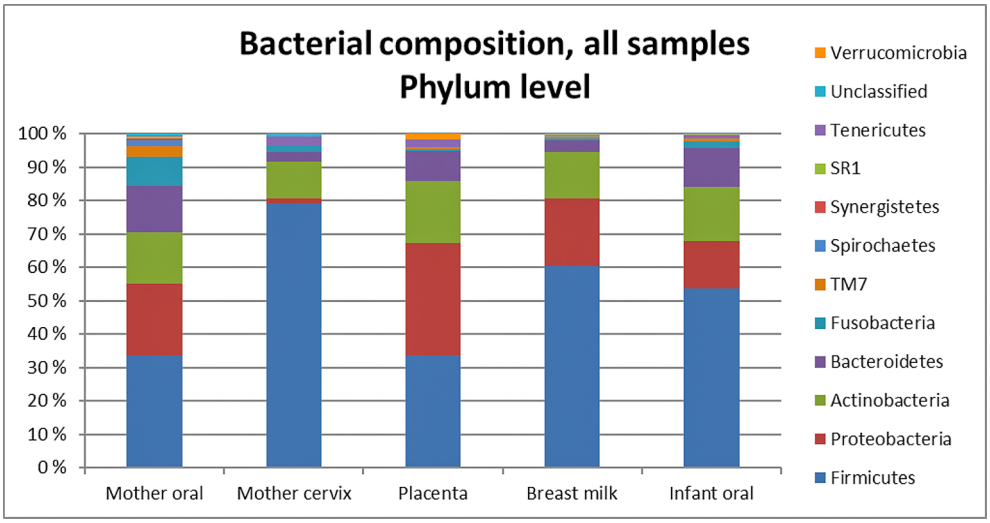

Figure 13. The bacterial microbiota compositions at phylum level. Mother oral, cervix, placenta, breast milk and infant oral cavity were consistent with each other as Firmicutes presented as the most abundant family followed by Proteobacteria and Actinobacteria. The results on the placenta microbiota from both delivery modes have been merged together.

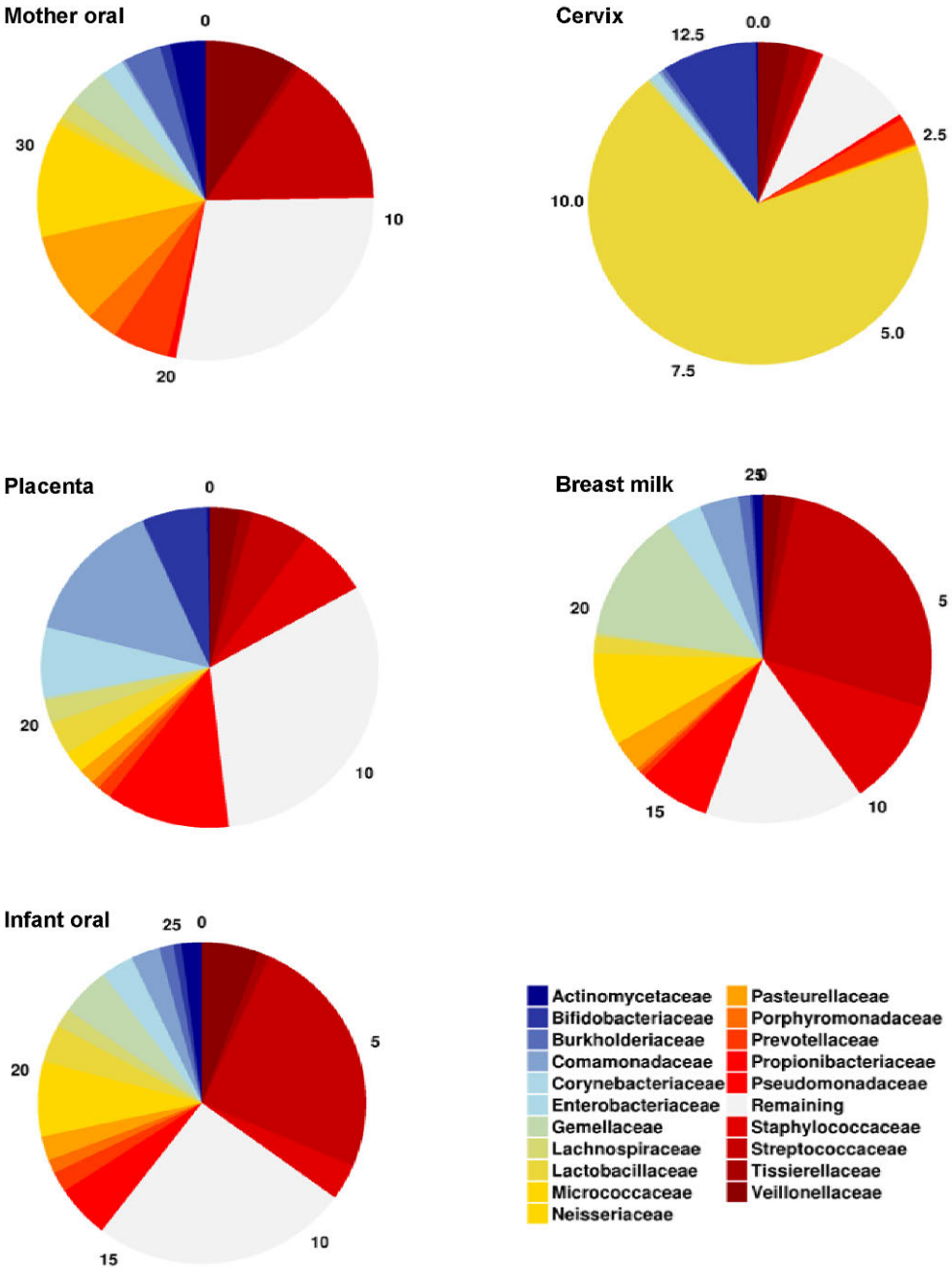

Figure 14. The bacterial distribution at the different groups according to family level. The top 21 most abundant families are presented in each group. The results on the placenta microbiota from both delivery modes have been merged together.

Maternal oral cavity

At phylum level, the maternal oral cavity harboured Firmicutes (33.7%), Proteobacteria (21.4%), Actinobacteria (15.4%) and Bacteroidetes (13.7%). The most abundant families in the maternal oral cavity were *Streptococcaceae* (15.0%), *Pasteurellaceae* (9.2%) and *Veillonellaceae* (8.8%).

Cervix

The most abundant phylum in the uterine cervix was Firmicutes (79.3%), followed by Bacteroidetes (13.0%), Actinobacteria (10.9%) and Proteobacteria (1.4%). *Lactobacillaceae* (69.2%) were the predominant family in cervical samples, followed by small amounts of *Bifidobacteriaceae* (8.9%) and *Veillonellaceae* (3.0%).

Placenta

The most abundant phyla in the placenta were Proteobacteria (33.9%), Firmicutes (33.5%), Actinobacteria (18.4%) and Bacteroidetes (9.0%). The main families were *Comamonadaceae* (14.4%), *Staphylococcaceae* (6.9%) and *Bifidobacteriaceae* (6.2%).

Breast milk

The most abundant phyla in breast milk were Firmicutes (60.3%), Proteobacteria (20.3%), Actinobacteria (14.0%) and Bacteroidetes (3.5%), which constituted more than 90% of the bacteria detected in milk. More than 50% of the families were composed from *Streptococcaceae* (26.7%), *Gemellaceae* (12.8%) and *Staphylococcaceae* (10.4%).

Infant oral cavity

Infant oral cavity also harboured Firmicutes (53.7%), as the most abundant phylum, followed by Actinobacteria (16.4%), Proteobacteria (14.0%) and Bacteroidetes (11.7%). The predominant families were *Streptococcaceae* (24.7%), *Micrococcaceae* (6.2%) and *Veillonellaceae* (5.6%).

At genus level, eight genera we detected to be shared between the groups (**Figure 15**). The core microbiota consisted of *Streptococcus, Staphylococcus, Pseudomonas, Prevotella, Lactobacillus, Delftia, Corynebacterium* and Unclassified.

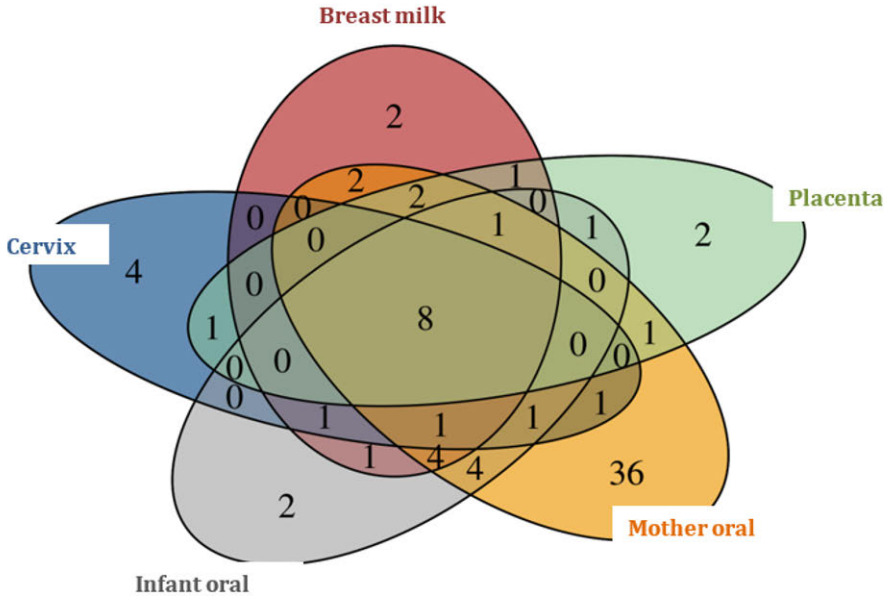

Figure 15. Core microbiota at genus level. A total of eight genera were detected to be shared between the five different groups studied.

5.4 The impact of delivery mode to microbiota composition (I, II, III)

We did not detect any association between the mode of delivery and the bacterial composition at any of the anatomical sites. As per PCoA and RDA (p-values shown) analyses, the maternal oral cavity (p=0.685), cervix (p=0.880), placenta (p=0.316), breast milk (p=0.942) and infant oral cavity (p=0.565) bacterial microbiota were all did not affect by the delivery mode in this study.

5.5 HPV and microbiota interactions (I, II)

Maternal oral cavity

Bacterial microbiota richness (Chao1, p=0.0319) was higher in HPV positive (33.3%, n=13/39) maternal oral samples as compared to HPV negative samples (publication I, Figure 3A). No differences in microbiota diversity were observed between the groups as assessed by the Shannon index (publication I, Figure 3B). Saccharibacteria (formerly known as TM7) was more abundant (p=0.026) at phylum level and *TM73* (p=0.011), *Selenomonas* spp. (p=0.0032) and *Megasphaera* spp. (p=0.026) were more abundant at family level in the HPV positive samples. Furthermore, *TM73* (p=0.018) at species level was increased in

HPV positive maternal oral samples. In the HPV negative samples, levels of *Haemophilus* spp. (p=0.019) were higher when compared with HPV positive group. With the LEfSe test, the genera unclassified *Bifidobacteriaceae* and *Finegoldia* were enriched in the HPV positive samples (LDA score > 3.36 and 3.32 respectively, p<0.05) and *Haemophilus* genus in the HPV negative group (publication I, Figure 3E).

Cervix

Our samples consisted of 23.1% HPV positive cervical samples (n=9/39). No statistically significant differences were observed in the species richness or diversity according to HPV status in the cervical samples (publication I, Figures 2A and 2B). *Peptostreptococcaceae* (p=0.0065), *Enterococcaceae* (p=0.022), *Haemophilus* (p=0.00058) and *Peptostreptococcus* (p=0.0069) were all significantly more abundant in HPV negative cervical samples. *Coriobacteriaceae* (p=0.083) and unclassified *Coriobacteriaceae* (p=0.07) appeared to be slightly increased in the HPV positive group without statistical correlation. In the LEfSe test, unclassified *Coriobacteriaceae* (LDA score>4.40, p<0.05), and more precisely *Atopobium vaginae*, was enriched in the HPV positive group and *Haemophilus* (LDA score>4.18, p<0.05) in the HPV negative cervical samples as compared to HPV positive samples (publication I, Figure 2E).

Placenta

Placenta microbiota diversity and richness exhibited no differences according to HPV status (31.6% HPV positive placenta samples analysed by bacterial microbiota composition, n=6/19) (publication I, Figures 1A and 1B). However, higher relative abundance of *Lactobacillaceae* (p=0.0036) and *Lactobacillus* genus (p=0.0023) were detected at HPV positive placenta samples (publication I, Figure 1E). The LEfSe test showed increased levels of *Ureaplasma* and *Lactobacillus* genus (LDA score>4.0, p<0.05) in the HPV positive placenta group compared to HPV negative samples (publication I, Figure 1F).

Breast milk

Only three breast milk samples (n=3/35) were positive for HPV (8.6%) and consequently, the bacterial microbiota differences between the HPV positive and negative samples could not be analysed due to the insufficient sample size.

Infant oral cavity

No differences in microbiota diversity or richness were observed between the HPV negative and positive samples (37.1% HPV positive samples, n=13/35; publication II, Figures 5A and 5B). *Veillonella* (p=0.025) and *Veillonella dispar* (p=0.048) were more abundant in HPV negative samples. In HPV positive samples, we observed more *Propionibacteriaceae* (p=0.095), *Propiobacterium* (p=0.095) and *Propionibacterium acnes* (p=0.093) than in HPV negative infant oral samples but the differences did not reach statistical significance. In LEfSe analyses, *Lysinibacillus* and *Ureaplasma* (LDA score>4.0, $p<0.05$) were enriched in the HPV positive samples and *Veillonella* (LDA score>4.0, $p<0.05$) was more abundant in the HPV negative group (publication II, Figure 5F).

5.6 The contribution of maternal microbiota to the initial neonatal oral microbiota (III)

The initial neonatal oral microbiota directly after the delivery, resembled most closely the microbial profile detected in the placenta, followed by the cervical and maternal oral microbiota. As presented in the Network analyses at OTU levels (**Figure 16**), there were mainly connections between the infant oral microbiota and the microbes detected in the placenta. No such interactions were evident with the other maternal sites. Furthermore, the other anatomical body sites tended to cluster more independently and separately from the neonatal oral cavity samples. Furthermore, RDA analyses (**Figure 17**) corroborated these findings as the groups all tended to all cluster separately except for the placental and neonatal oral microbiota profiles (p=0.001).

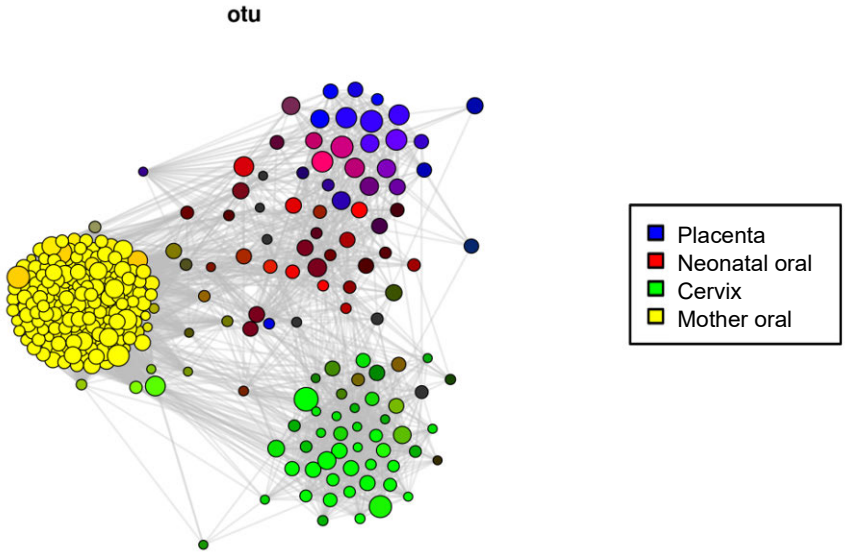

Figure 16. Clustered groups are visible in Network analyses showing the connections between the samples. Infant oral cavity microbiota and microbes detected at placenta shows overlapping, while the other anatomical areas are more clustered separately from these two.

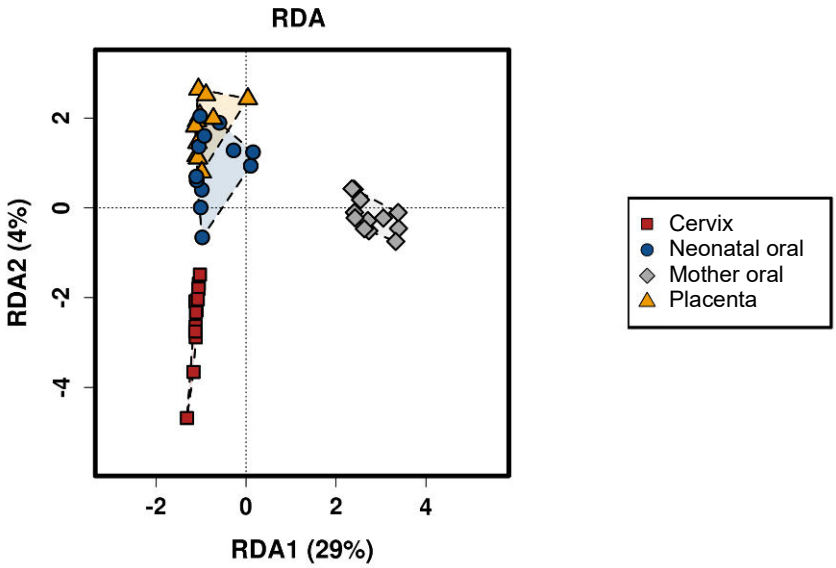

Figure 17. RDA analyses depicting the interaction between the different microbiota profiles. Neonatal oral microbiota and the microbes detected from placenta are clustered together and overlapping, whereas mother oral cavity microbiota and the cervical microbiota are clearly clustered separately.

Although placental and neonatal oral microbiota clustered together, no statistical association was detected (p=0.075) (**Figure 18 A**) was observed in individual mother-infant pairs when compared to unrelated groups. The same was not detected between neonatal and mother oral cavities (B, p=0.83) or neonatal oral cavity and cervical microbiota (C, p=0.79).

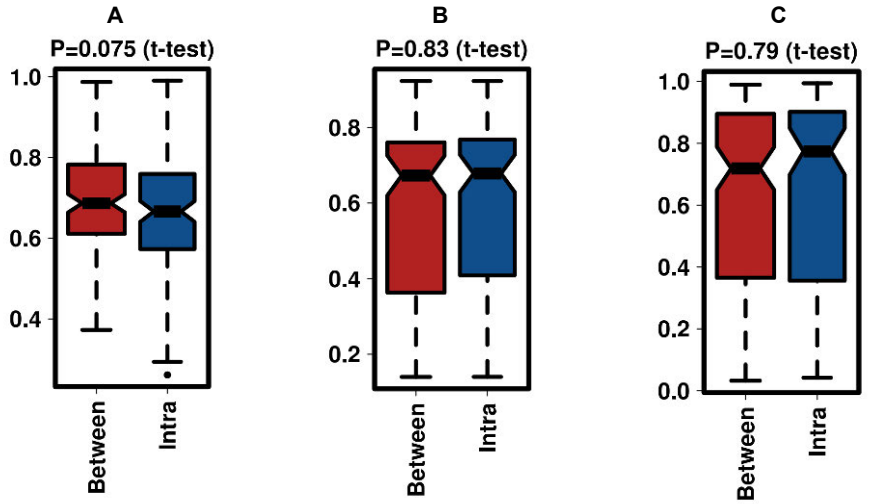

Figure 18. T-tests showing connections between two groups at the time. The microbiota in the placenta and neonatal oral cavity (A) appeared similar but no statistically significant results were observed between the mother-infant pairs compared to unrelated individuals. No connection between the mother-infant pairs were detected in the mother oral cavity and her neonates' oral cavity (B) or the mother cervix and neonatal oral cavity (C).

5.7 HPV positive cell lines SiHa and CaSki invasion in the Myoma and Myogel (IV)

Myoma invasion assay

All three cell lines were observed to invade the myoma model. The average invasion depths were 1.25 µm for HSC-3, 1.28 µm for SiHa and 0.91 µm for CaSki. The difference was significant (p=0.0001) between both the SiHa and HSC-3 (p=0.003) when compared to CaSki invasion depth. SiHa (less HPV) invaded clearly deeper than CaSki cells (more HPV) and HSC-3 invasion was more efficient than in CaSki. No difference between HSC-3 and SiHa invasion depth was observed. When compared, the HPV positive (SiHa and CaSki) cells together to the HPV negative HSC-3 cells, a slight significant difference in the invasion depth (p=0.048) was observed as HPV negative HSC-3 cells invaded better.

The invasion area reached to 86.2 mm^2 HSC-3, 94.3 mm^2 SiHa and 74.9 mm^2 CaSki. No significant differences in the invasion area between the cell lines was detected, nor were there any differences in the invasion area between HPV positive cervical cells and HPV negative oral cells. Furthermore, the invasion indexes were 53.6% HSC-3, 21.3% SiHa and 30.0% CaSki. The invasion index was calculated by dividing the invasion area with the total area of epithelial cells. As seen by the differences between invasion area and invasion index, HSC-3 tend to invade better as the percentage is greater while SiHa produced thicker epithelium and fewer invasion islets.

Cell invasion in Myogel

All the cell lines were detected to invade through the Myogel mixture. The absorbance units for cell invasion were 0.32 HSC-3, 0.30 SiHa and 0.25 CaSki. No statistically significant differences were observed between the absorbance units of the different cell lines.

5.8 The impact of irradiation to cell invasion (IV)

Invasion was significantly reduced in all three cell lines after 4 Gy ionized radiation. The average invasion depths decreased from 1.25 µm to 0.66 µm with HSC-3 ($p=0.008$), from 1.28 µm to 0.42 µm with SiHa ($p=0.001$) and from 0.91 µm to 0.50 µm with CaSki ($p=0.005$) as shown in **Figure 19**. The irradiation affected more to the invasion depth of HPV positive cells (SiHa and CaSki) than HPV negative HSC-3 ($p=0.048$).

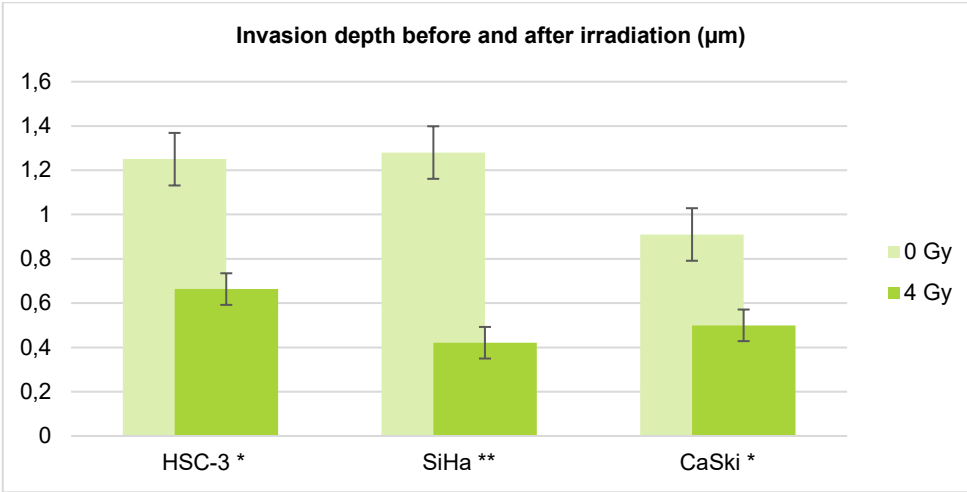

Figure 19. The average invasion depths change after the 4Gy irradiation in HSC-3, SiHa and CaSki cell lines. The mock-radiated samples presented at 0 Gy bar and the 4 Gy ionized radiation administered at day 3 shows a significant reduction in cell invasion in all the cell lines (**p≤0.001, *p≤0.01).

The invasion area was additionally affected by radiation. The average invasion area diminished from 86.2 mm^2 to 32.1 mm^2 with HSC-3, from 94.3 mm^2 to 5.61 mm^2 with SiHa and from 74.9 mm^2 to 3.90 mm^2 with CaSki as depicted in **Figure 20**. No statistically significant differences were observed in the decrease on invasion area after radiation in any of the cell lines studied, nor did we detect any differences between the HPV positive and HPV negative cell invasion decrease after radiation.

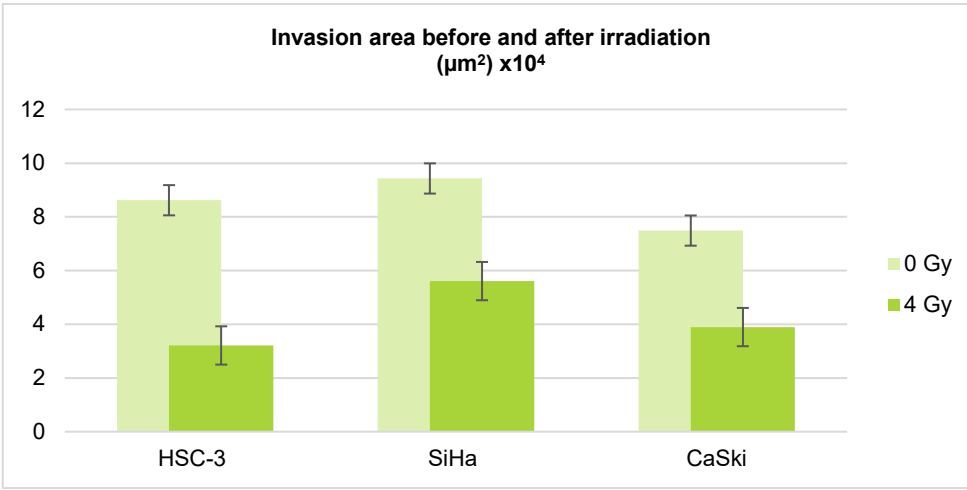

Figure 20. Decrease in the invasion are depicted as μm^2 x10^4 in HSC-3, SiHa and CaSki. The 4Gy ionized radiation administered at day 3 shows a reduction in cell invasion area in HSC-3 and SiHa. Also, slight reduction is observed in CaSki, but not as statistically significant.

The invasion indexes also decreased after the ionized radiation. A reduction from 53.3% to 29.5% was observed in HSC-3, from 21.3 % to 13.7 % in SiHa and from 30.0 % to 26.5% in CaSki.

5.9 HPV copy number impact to cell invasion (IV)

In Myogel invasion assay, no differences between SiHa (less HPV) and CaSki (more HPV) were observed ($p=0.963$) in the invasion though Myogel mixture (publication IV, figure 2).

SiHa invaded into the Myoma significantly better ($p=0.0001$) than CaSki when compared by invasion depths (publication IV, figure 3B). No differences were observed in the invasion area ($p=0.310$) between the two cell lines (publication IV, figure 3C). After ionising radiation, SiHa invasion depth decreased slightly more ($p=0.013$) than CaSki (Figure 19), but neither any differences were observed in the reduction of invasion area ($p=0.222$) (Figure 20).

6 Discussion

In this study, we detected significant changes in the bacterial microbiota composition according to HPV status in the maternal and infant oral cavity, the uterine cervix and in the placenta. In the maternal oral cavity, HPV positivity was also connected to higher bacterial richness than HPV negative samples. The initial neonatal oral microbiota seems not to be affected by delivery mode and we detected the neonatal oral cavity microbiota to resemble mostly the bacteria detected from the placenta. To the authors' best knowledge, we have been the first to show that the bacterial microbiota in the placenta and oral cavity exhibits differences depending on the HPV status. Furthermore, we showed *in vitro* that irradiation reduces cell invasion in a myoma model.

6.1 HPV and microbiota interactions in women

The oral cavity, uterine cervix and placenta exhibited differences in microbiota profiles according to HPV status. Only in breast milk samples, we did not detect any differences, but this may be due to low number of HPV positive samples.

The adult oral cavity microbiota composition has previously been comprehensively investigated (Dewhirst *et al.* 2010; Kilian *et al.* 2016) but no information has been available on the interaction between the microbiota and HPV in the oral cavity. In the present study, adult female oral cavity bacterial microbiota was mainly composed of Actinobacteria, Bacteroidetes, Firmicutes, *Streptococcus* and *Veillonella*, which is in line with the earlier literature (Dewhirst *et al.* 2010; Huse *et al.* 2012; Wade 2013; Verma, Garg and Dubey 2018). The oral specimens positive for HPV presented higher bacterial richness, but no differences in bacterial diversity compared to HPV negative samples. We detected higher levels of *Bifidobacteriaceae, Selenomonas* and *Megasphaera* in HPV positive oral cavity samples and more *Haemophilus* in HPV negative samples.

The homeostasis of different bacterial species is crucial for the overall oral health. *Selenomonas* and *Megasphaera* are well known oral pathogens which have been found in patients with aggressive periodontitis and in endodontic abscesses (Gonçalves *et al.* 2012; Cruz *et al.* 2015; George *et al.* 2016; Johansson *et al.* 2016). *Selenomonas* abundance has been reported to be increased in the oral cavity of

patients suffering from rheumatoid arthritis and systemic lupus erythematosus (Graves, Corrêa and Silva 2019). These changes in microbiota may thus affect oral health in general. It is not known whether HPV lays the foundation for oral microbiota to accumulate these pathogenic bacteria. Furthermore, it is possible that these known periodontal pathogens may, on the other hand, provide a favourable platform for acquiring HPV infection. Interestingly, we did not detect any highly aggressive pathogens linked to periodontal disease to be associated with HPV positivity in oral cavity (**Table 3**). As a limiting factor, we did not have the information about our patients' oral hygiene status (dental caries, periodontal pocket depths, bleeding on probing (BOP) index) while analysing the bacterial microbiota data. When the Finnish Family HPV Study was first designed, factors that could have affected the bacterial microbiota analyses were not considered, since HPV and other viruses were the main focus of the research.

Interactions between HPV and the bacterial microbiota have mainly been studied in the cervical region (Gillet *et al.* 2011; Brotman *et al.* 2014; Mitra *et al.* 2016). *Lactobacillus* is the dominant bacterial genus in the cervix and vagina (Nugent, Krohn and Hillier 1991; Ravel *et al.* 2011; Liu *et al.* 2013; MacIntyre *et al.* 2015). According to our study, *Lactobacillus* contributed up to 86.8% (HPV positive group) to 89.7% (HPV negative group) of the total bacterial community in the cervical samples. Differences according to HPV status were observed in the cervical microbiota. We observed that *Coriobacteriaceae,* mainly *Atopobium vaginae*, was more abundant in the HPV positive cervix. In contradiction to this, *A. vaginae* has previously been associated with HPV persistence and bacterial vaginosis (Jespers *et al.* 2012; Mendes-Soares *et al.* 2015; Mitra *et al.* 2016; Di Paola *et al.* 2017).

HPV infection incidence in the cervical region is highly variable ranging between 18.9% - 75% depending on geographical differences and other risk-factors, such as unprotected sexual intercourse, young age at sexual onset, multiple partners and general bacterial microbiota composition (Eppel *et al.* 2000; Worda *et al.* 2005; Koskimaa *et al.* 2012; Park *et al.* 2012; Uribarren-Berrueta *et al.* 2012; Brotman *et al.* 2014; Steinau *et al.* 2014). Our study detected HPV in 23% of the cervical samples, which is in line with the previous studies (Eppel *et al.* 2000; Koskimaa *et al.* 2012; Park *et al.* 2012).

HPV infection has been linked to higher richness and diversity of bacterial microbiota in the cervix in pregnant and non-pregnant females (da Silva *et al.* 2004; Gao *et al.* 2013; Lee *et al.* 2013a; Mitra *et al.* 2015b, 2016; Dareng *et al.* 2016; Kero *et al.* 2017; Shannon *et al.* 2017). Moreover, higher bacterial diversity increases the risk of bacterial dysbiosis and bacterial vaginosis (Van De Wijgert *et al.* 2014; Mitra *et al.* 2015a). On the other hand, it has been shown, that bacterial vaginosis and high bacterial diversity and richness may predict HPV persistence and even carcinoma development (Mitra *et al.* 2015a; Kero *et al.* 2017). In our samples with pregnant

women, however, no differences in bacterial richness or diversity were seen according to HPV status. Probably due to limited number of samples we were unable to reach statistical significance in the bacterial richness or diversity measures. It is still not completely clear how the bacterial microbiota interacts with HPV and what impact microbiota may have in persistent HPV infection in cervical region (Mitra *et al.* 2016).

Oral and cervical samples were collected by a similar method, by a brush. Our results showed higher amounts of bacterial DNA detected in the cervical samples (average 89.773 reads, range from 34.955 to 138.263 reads) than in the oral cavity samples (average 76.184, range from 32.373 to 128 565 reads). Similar bacterial levels in the uterine cervix have been detected in a recent study when samples were collected with cytobrush as in our study (Mitra *et al.* 2017). This might be considered surprising, since the oral cavity is known to be a rich source of bacteria (**Figure 5**) (Dewhirst *et al.* 2010). It is known that differences in the methodology in DNA extraction process can influence the total number of DNA sequences, but not the microbiota detected (Rosenbaum *et al.* 2019). The brush method may collect more bacterial DNA in the vaginal region but perhaps more human DNA in the oral cavity. Furthermore, in adults, there are normally occurring variations in the bacterial counts in the oral cavity considering the anatomical areas, as well as eating, tooth brushing, sampling method and testing time can influence the results as well (Ghasemi, Mazaheri and Tahmourespour 2017; Coelho *et al.* 2018; Iwawaki *et al.* 2019).

The original Finnish Family HPV Study was designed to evaluate HPV interactions between family members. The sample collection was designed and performed without plans for microbiome studies. Therefore, no restrictions were given to the patients before the sample collection. This may at some extent affect our study results. The hormonal changes associated with pregnancy might have affected the collected bacterial DNA load in this study. In line with this notion, lower levels of bacteria have been detected from pregnant compared to non-pregnant women (Freitas *et al.* 2017). Furthermore, the brush only collects surface epithelial cells and bacteria from the mucosal surfaces. Oral bacteria often form complex biofilms which helps bacteria to attach to surfaces and helps bacteria protect against antimicrobial agents. The dental plaque or biofilm from deeper dental periodontal pocket may not have been included in the oral samples obtained with the brush method. Different methods in sample collection might have altered our results.

In addition, the cervical brush samples were collected into tubes with buffer solution with small amount of gentamycin. Gentamycin is an antibiotic used to treat several different types of bacterial infections in humans. In research purposes, it is often used in cervical sample collection to prevent undesirable bacterial contamination that can occur during sample transportation and storage. Furthermore,

as the original study focused on HPV and on other viruses, gentamycin was not thought to be harmful for the analyses.

Altogether 33% of the placenta samples in this study were positive for HPV. HPV has previously been detected in healthy pregnancies, and, in line with this observation, the pregnancies in our study did not have any adverse complications (Sarkola *et al.* 2008a; Skoczyński, Goździcka-Józefiak and Kwaśniewska 2011; Park *et al.* 2012; Lee *et al.* 2013b; Kim *et al.* 2014). However, there have been some indications that HPV placenta might increase the risk for premature delivery and other pregnancy complications (Gomez *et al.* 2008; Hong, Oshiro and Chan 2013; Slatter *et al.* 2015; Ambühl *et al.* 2016; Bober *et al.* 2019; Caballero *et al.* 2019). In order to distinguish true HPV infection from contamination, mainly from the birth canal, one study obtained placenta samples transabdominally and were still able to detect HPV DNA (Weyn *et al.* 2011). Results of the Finnish Family HPV Study, have shown with tyramide amplified *in situ* hybridisation that the HPV locates inside the syncytiotrophoblast cells (Sarkola *et al.* 2008a). HPV DNA localization in syncytiotrophoblast cells has subsequently been corroborated in other studies (You *et al.* 2008; Bober *et al.* 2019).

The possible presence of bacteria in the placenta has been a focus of intensive research in recent years and arguments for and against have been made (Aagaard *et al.* 2014; Wassenaar and Panigrahi 2014; Collado *et al.* 2016; Lauder *et al.* 2016; Doyle *et al.* 2017; Parnell *et al.* 2017; Pelzer *et al.* 2017; Perez-Muñoz *et al.* 2017; Zheng *et al.* 2017a, 2017b). For decades it has been thought that the foetus develops in a sterile environment and first encounters bacteria in birth canal and after the delivery. Germ free animals are known to be bread successfully and the off-springs are germ-free as well after the delivery (Arvidsson, Hallén and Bäckhed 2012). Many of the studies claiming that there are no bacterial traces in the placenta samples have declared that the bacteria detected are a result of contamination either from the analysing kits or from the laboratory (Lauder *et al.* 2016; Perez-Muñoz *et al.* 2017; Leiby *et al.* 2018; de Goffau *et al.* 2019; Theis *et al.* 2019).

Nevertheless, several studies have been able to detect bacterial DNA traces in placenta samples with different methodology (16S rRNA gene sequencing, WGS, culture and staining with microscopy), showing placenta mainly harbouring Proteobacteria, *Prevotella, Streptococcus* and *Veillonella* regardless of delivery mode (Satokari *et al.* 2009; Rautava *et al.* 2012b; Stout *et al.* 2013; Aagaard *et al.* 2014; Cao and Mysorekar 2014; Wassenaar and Panigrahi 2014; Zheng *et al.* 2015; Collado *et al.* 2016; Zheng *et al.* 2017b, 2017a; Gomez-Arango *et al.* 2017a; Parnell *et al.* 2017; Benny *et al.* 2019). Furthermore, differences in the analysed placenta microbiota have been seen in samples obtained from term/preterm deliveries, from mothers with gestational diabetes or excess weight gain during pregnancy or in cases of pre-eclampsia and chorioamnionitis when compared to healthy delivered placenta

samples (Queiros da Mota *et al.* 2013; Amarasekara *et al.* 2015; Antony *et al.* 2015; Bassols *et al.* 2016; Lloyd-Price, Abu-Ali and Huttenhower 2016; Zheng *et al.* 2017b; Doyle *et al.* 2017; Pelzer *et al.* 2017; Zheng *et al.* 2017a; Benny *et al.* 2019; Younge *et al.* 2019). In addition, in our study we detected significant differences in the bacteria detected in the placenta samples according to HPV status. Bacterial composition has also been detected to vary within different sites of placenta (Parnell 2017). There are slowly accumulating data that may interpreted to confirm the novel concept of true microbial colonization in the placenta but this still controversial subject warrants further research for the scientific community to have mutual agreement over the subject.

One of the main findings in our studies was that we were able to detect bacterial DNA from our placental samples, there were no differences in the placenta samples according to delivery mode at family or genus levels. Our samples create a completely new placenta dataset, which has previously not been investigated by any of the bacterial detection methods. The bacteria we detected in the placenta were mainly members off Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes, which is in line with earlier studies that have been able to detect bacteria in placenta samples (Aagaard *et al.* 2014; Collado *et al.* 2016; Doyle *et al.* 2017; Parnell *et al.* 2017; Pelzer *et al.* 2017; Zheng *et al.* 2017a, 2017b; Benny *et al.* 2019).

Even though the research evidence of bacteria in the placenta has been increasing, the topic has created discussion and debate. The dogma of a sterile uterus has prevailed for decades and therefore this novel finding is challenging previous perceptions. The main problem in bacteria identification has been the small number of detectable bacterial DNA traces (Hornef and Penders 2017; Perez-Muñoz *et al.* 2017). In our study, we worked carefully to exclude possible contaminants and excluded some placenta samples from microbiota analyses, because of low number of sequences. The amount of bacterial DNA sequences in some of the placenta samples were relatively small (starting from 100 sequences), but we did have number of samples with very high DNA sequences (up to 20.000 sequences). In addition, our placenta samples presented clearly higher number of reads than our negative controls (**Figure 12**). Nonetheless, when it comes to birth, sample selection is always at risk of contamination.

In addition to the presence of bacterial DNA in the placenta, we detected differences in microbiota composition according to the HPV status of the samples. In HPV positive placenta samples, we detected significantly higher amounts of *Ureaplasma* and *Lactobacillaceae* when compared to HPV negative placenta samples. *Ureaplasma* has previously been connected with pregnancy complications, as it seems to associate with chorioamnionitis and preterm delivery (Sweeney *et al.* 2012, 2016, 2017; Cox *et al.* 2016; Doyle *et al.* 2017; Kikhney *et al.* 2017). The

reason for this was not in the scope of this study and should be evaluated with further studies. All of the pregnancies in our study were carried out without any adverse effects, even the ones with HPV positive placenta and/or cervix.

The placental microbiota has previously been detected to be significantly different in mothers suffering from gestational diabetes (Zheng *et al.* 2017b) or delivering preterm (Antony *et al.* 2015; Prince *et al.* 2016). To the author's best knowledge, this work is the first to describe the differences in the bacteria detected in placenta in association with the HPV status. We interpret our findings to strengthen the novel idea of a true bacterial colonization in the placenta. Further studies are necessary to truly understand the role of bacterial colonization in the placenta and the viral-bacterial interactions.

We further on studied the microbiota composition in breast milk. The milk microbiota included mainly *Streptococcaceae, Staphylococcace*ae and *Proteobacteria* which is in line with other studies, while some geographical differences have been reported to occur (Martín *et al.* 2007; Jeurink *et al.* 2013; Urbaniak *et al.* 2014; Aakko *et al.* 2017; Biagi *et al.* 2017; Cacho *et al.* 2017; Li *et al.* 2017b). HPV has additionally been previously detected in breast milk and it is thought to derive from epithelial cell shedding from the ductal canal or from the nipple epidermis, since HPV requires epithelial cell differentiation for its cell cycle (Cazzaniga *et al.* 2009; Diaz *et al.* 2018). In addition, HPV has been detected from normal breast tissue (without lactating) and from breast tumour specimens (de Villiers *et al.* 2005; Heng *et al.* 2009).

Both mucosal and cutaneous HPV genotypes have been detected in milk (Cazzaniga *et al.* 2009). In general, HPV positivity has shifted between 1.4% - 28.8% in different studies investigating the HPV presence in breast milk (Sarkola *et al.* 2008b; Cazzaniga *et al.* 2009; Yoshida *et al.* 2011; Glenn, Whitaker and Lawson 2012; Louvanto *et al.* 2017; Diaz *et al.* 2018). Contradictory, some investigators have not detected any HR-HPVs in breast milk samples (Mammas *et al.* 2011). In our samples, three breast milk samples were positive for HPV; only one with HR-HPV16 and two others to LR-HPV6. Since the study referred here did not take into account the LR-HPV, they would probably have missed a number of potentially positive samples.

Because only three HPV positive breast milk samples were detected in our study samples no statistical microbiota analyses were performed. Previously, we have shown in the Finnish Family HPV Study (Louvanto *et al.* 2017) that HPV in breast milk could be a risk factor for the spouse to acquire oral HPV infection but transmission of HPV infection to the infant mouth by breast milk positive for HPV has not been observed (Sarkola *et al.* 2008b; Yoshida *et al.* 2011; Louvanto *et al.* 2017). In our study population, all of the infants received breast milk as their primary food source.

6.2 HPV and microbiota interactions in infants

We detected oral HPV infection in 40% of infants, which is somewhat higher than in previous studies, in which oral HPV has been discovered in 3.2% -21% of infants (Rintala *et al.* 2005a; Park *et al.* 2012; Hahn *et al.* 2013; Hong *et al.* 2013; Trottier *et al.* 2016). Our subset population of the Finnish Family HPV Study presented significantly higher levels of HPV DNA in the infant oral cavity, than the entire Finnish Family HPV Study infant population (40% to 10%, respectively) (Rintala *et al.* 2005a). The amount of HPV DNA positive placental samples, which originally identified our sample selection, could have had an impact to our infant data and may distort the results.

Infants may acquire HPV infection by vertical transmission from the mother prenatally via the placenta or during vaginal delivery or from the mother or the father during caregiving (Syrjänen and Puranen 2000; Rintala *et al.* 2005a, 2005b; Koskimaa *et al.* 2012; Park *et al.* 2012; Lee *et al.* 2013b; Merckx *et al.* 2013; Chatzistamatiou, Sotiriadis and Agorastos 2016). HPV positive cord blood reportedly increases the infant's risk of being HPV positive (Rombaldi *et al.* 2008; Sarkola *et al.* 2008a; Teixeira *et al.* 2015).

We detected maturation of the infant oral microbiota from delivery to two months of age. The two-month-old infants had higher richness and *Streptococcus, Veillonella* and *Rothia* species were more abundant in the oral cavity compared to neonates. Our results are consistent with previous studies (Dominguez-Bello *et al.* 2010; Lif Holgerson *et al.* 2011; Rosenblatt *et al.* 2015; Chu *et al.* 2017; Li *et al.* 2018). Firmicutes, *Streptococcus* and *Veillonella* are showed to form the core of infant oral cavity microbiota (Biagi *et al.* 2017; Drell *et al.* 2017; Dzidic *et al.* 2018b). It is of note that neonates are born without teeth and there are only mucosal surfaces in the oral cavity to harbour the microbiota. During the first year of life, the teeth start to erupt and that markedly affects to the microbiota composition with hard tissue surfaces to form biofilms (Caufield, Cutter and Dasanayake 1993; Sampaio-Maia and Monteiro-Silva 2014). It is of note that under these drastic changes, the infant oral microbiota also changes in response to external and internal changes up until two years of age (Dzidic *et al.* 2018b). In contrast to previous studies (Dominguez-Bello *et al.* 2010; Lif Holgerson *et al.* 2011; Rosenblatt *et al.* 2015; Li *et al.* 2018), we did not detect any differences in the initial neonatal oral cavity bacterial microbiota compositions between infants born vaginally (60%, n=21/35) or by Caesarean section delivery (40%, n=14/35).

We detected significant differences in the infant oral cavity microbiota according to HPV status. *Veillonella* and *Veillonella dispar* were more abundant in HPV negative infant oral cavity, while *Propionibacterium* and *Ureaplasma* were significantly enriched in HPV positive infant oral cavity. As far as we know, the connections with bacterial microbiota and HPV in neonates' have not been studied

before. Higher levels of *Propionibacterium* have previously been connected to premature neonates' gut microbiota and *Ureaplasma* has been detected in the uterine cervix in preterm births (Aujoulat *et al.* 2014; Doyle *et al.* 2017). There has not been any connection to HPV in these studies and no information about HPV and microbiota interactions in infants has been available before. Given the possible role of HPV infection in pregnancy complications, discussed above, the interaction between HPV and the bacterial microbiota in the pathogenesis of preterm birth needs further study, since preterm birth may have significant clinical life-long effect to the children (Ruiz *et al.* 2016). It is of note, however, that none of the deliveries in the present study occurred significantly preterm, as even the shortest pregnancy lasted until 35 weeks.

Understanding oral microbiota development in early life is important since the initial oral cavity microbiota during the first year of life has been reported to be associated with children's oral and overall health later in life (Dzidic *et al.* 2018b, 2018a). We investigated the impact of the maternal microbiota to the neonatal oral cavity bacterial microbiota composition. A significant contribution of the bacteria detected in the placenta seemed to contribute significantly to initial neonatal oral colonisation. The samples in our study were collected directly after delivery before any other contact to mother or feeding. A number of different analyses (PCoA, Network and SourceTracker) all gave similar results indicating that the placenta may primarily contribute to the early neonatal oral microbiota. Furthermore, our analyses connected an individual neonate's oral bacteria to his own mother's bacteria detected from the placenta more ($p=0.075$) than to the own mother's oral cavity ($p=0.83$) or to the cervical microbiota ($p=0.79$). The impact of bacteria from the placenta to the developing neonatal oral microbiota has not been studied extensively, but one recent report corroborated our findings and revealed connections between the placenta and neonatal oral cavity microbiota (Younge *et al.* 2019). Furthermore, in the present study, individual mother-neonate pairs resembled more closely each other than unrelated mothers and neonates. Of the 16 individual bacterial genera detected in placenta, 12 genera were in addition present in neonatal oral cavity. All of these findings suggest that neonatal oral cavity might have a prenatal exposure to bacteria before the moment of delivery. Furthermore, we did not observe differences in the neonatal oral bacterial microbiota composition according to delivery mode.

It also seems that HPV infection in the infant oral cavity is a relatively common finding and has an association with the bacterial microbiota composition. Importantly, present HPV infection appears to be connected with well-known pathogens. It is known that infant oral microbiota gradually matures within months and years. Therefore, whether these initial changes observed in infant oral cavity bacterial microbiota composition according to HPV status have an impact to oral health later in life remains to be elucidated in future studies.

Our study is observational, and it is therefore not clear whether the bacterial microbiota alterations and presence of potentially pathogenic bacteria might facilitate HPV transmission or if HPV infection induces these changes in microbiota composition. Furthermore, it is not known how these viral –bacterial interactions might affect HPV persistence and thus further studies are warranted.

6.3 *In vitro* studies

Chronic infection and inflammation, whether derived from bacterial or viral (HPV) infection, may lead to cancer development (Coussens and Werb 2002; Murata 2018). One of the most studied entities in this respect is the role of persistent HPV infection in CSCC and HNSCC development (Schneider and Koutsky 1992; Syrjänen 2005; zur Hausen 2009; Stanley 2010; Louvanto *et al.* 2011b; Rintala *et al.* 2012; Gillison *et al.* 2014). We show here, that HPV positive CSCC lines (SiHa and CaSki) are able to invade to both solid (Myoma) and gelatinous (Myogel) human uterine leiomyoma-based extracellular matrix models. Previously, these models have mainly been studied using the aggressive oral tongue squamous cell carcinoma line, HSC-3 (Teppo *et al.* 2013; Kauppila *et al.* 2015; Pirilä *et al.* 2015; Salo *et al.* 2015, 2018; Mäkinen *et al.* 2016; Sundquist *et al.* 2016; Al-Samadi *et al.* 2017; Korvala *et al.* 2017; Väyrynen *et al.* 2018, 2019).

HPV positive cells were cultivable in Myogel cultures and were able to invade into the prepared Myogel extracellular matrix gel. No information about HPV positive cells behaviour in Myogel has been presented before. Our results show that, SiHa and CaSki cells survive and invade to the Myogel equally. No statistically significant differences were observed between the different cell lines in the Myogel Transwell invasion assay.

The myoma model has been designed for invasion assays as the matrix is of entirely human origin and closely mimics the tumour microenvironment of solid cancers (Teppo *et al.* 2013; Alahuhta *et al.* 2015; Sundquist *et al.* 2016; Salo *et al.* 2018). We observed HPV copy number to be potentially associated with slight differences in invasion to myoma, as SiHa (less HPV) invaded clearly more aggressively than CaSki (more HPV). Nonetheless, no differences were observed in the invasion area, and the detected differences could therefore be explained by the normally occurring differences between the different cell lines. Differences in invasion depth and invasion area may also be explained by different invasion patterns of cellular level; SiHa invaded to the tissue with single cells or tiny cell islands (small buds), whereas CaSki invaded in bigger droplets.

Furthermore, we wanted to investigate whether ionized radiation has an impact on cell invasion. HPV positive HNSCC, OPSCC and CSCC patients have been observed to be more radiosensitive than HPV negative carcinomas (Bachtiary *et al.*

2002; Harima *et al.* 2002; Ang *et al.* 2010; Kimple *et al.* 2013; Zhang *et al.* 2015; Li *et al.* 2017a; Ibragimova *et al.* 2018). HPV negative CSCC has been shown to have greater potential to metastasise, which could explain the poorer prognosis (Riou *et al.* 1990; Crook and Vousden 1992; Li *et al.* 2017a).

Previous studies have shown, that irradiation at 4 Gy significantly decreases cell invasion (Väyrynen *et al.* 2018). Our results are consistent with this notion, as both SiHa and CaSki showed statistically significant decrease in cell invasion depth after irradiation. However, no differences were seen in the invasion area, even though a decrease in numbers was evident and a dramatic drop in the invasion indexes was seen. The invasion depth decreased more in SiHa (less HPV) than in CaSki (more HPV) cells and significant difference ($p=0.048$) between HPV negative cell and HPV positive cell invasion after ionized radiation was observed. This indicates that HPV infection affects radiosensitivity in these cell lines despite the fact that that no similar difference in the invasion area ($p=0.892$) was observed. Our results suggest that ionized radiation impacts overall to cell invasion depth and area even regardless of the HPV status by decreasing the invasive potential of carcinoma cells even after relatively low and only single dose of irradiation.

6.4 Limitations and strengths

There are limitations to this study. First, the relatively small sample size potentially restricts us from detecting significant differences and making definitive conclusions from this study. More than 300 families participated in the original Finnish Family HPV Study. Only 13 women had HPV positive placentas which restricted our final study group in studies I-III. Furthermore, a number of placenta samples, those that had less than 1000 reads per sample, were excluded from the study I, to strengthen the results. In addition, the relatively low number of usable DNA residues from bacteria in placenta samples renders drawing definitive conclusions of the overall composition of microbiota difficult.

Secondly, the small amount of detectable bacterial DNA in both placental and neonatal oral cavity might have some impact on our results. We have used the same methods for collecting oral samples with cytobrush and placenta biopsy samples for bacterial DNA analyses that have previously been used, which therefore should not explain this difference (Rombaldi *et al.* 2008; Lee *et al.* 2013b; Antony *et al.* 2015; Mitra *et al.* 2017; Rosenbaum *et al.* 2019). We would speculate, that sample collection from neonate's oral cavity may have been performed slightly too gently compared to adults.

However, since our study was cross-sectional, we cannot evaluate the causal relationships of HPV and bacterial microbiota interactions. The question whether HPV infection leads to the observed changes in the bacterial microbiota composition

in the mother oral cavity, the cervix, the placenta and in the neonatal oral cavity or whether certain type of microbiota profile predisposes to HPV remains to be determined in future studies.

Thirdly, cell cultivation with HPV positive cell lines is somewhat technique sensitive and naturally occurring differences in the behaviour of different cell lines makes it difficult to compare their behaviour *in vitro.* Furthermore, in these *in vitro* studies we utilized HPV positive CSCC cell lines and compared the results with HPV negative OPSCC cell line. Since several underlying factors contribute to carcinoma cell behaviour, is must be addressed that our results might have been somewhat different had we used carcinoma cell lines from the same anatomical site.

Regardless of these limitations, we have presented evidence for traces of bacterial DNA detected in the placenta samples. We have used the same bacterial DNA method with all of our samples and sequenced the V3-V4 region of the 16S rRNA gene in order to have comparable results. A strict protocol was followed while working with the low DNA quantities obtained from the placenta and infant oral samples, and we included the same strict protocol with PCR amplifications and library kit controls. Before analysing the generated microbiota data, it was cleared from possible contaminants as singletons and OTU's with relative frequency below 0.01 were removed. Furthermore, sequences that could not be classified to domain level were removed. Similarly, *Cyanobacteria*, *Chloroplasts* and *Rhizobiales* as potential environmental contaminants were removed before the microbiota analyses. In addition, we were able to detect differences in the microbiota composition according to HPV status in placenta, mother and infant oral cavity and in cervical samples. These findings corroborate the recently presented novel discovery that the placenta might harbour a specific bacterial microbiota and the foetus might not develop in a sterile environment (Aagaard *et al.* 2014; Collado *et al.* 2016).

7 Conclusions

The main conclusions of the present study were:

1. The oral cavity of pregnant women and their infants as well as the cervix, placenta and breast milk all harbour a unique and distinct microbiota profile. Two-month-old infants' oral microbiota have higher bacterial richness than new-borns oral cavity microbiota. The placental and neonatal oral cavity samples present lower amounts of detectable bacterial DNA than maternal oral cavity, cervix and breast milk.
2. HPV infection is associated with distinct bacterial microbiota in the maternal and infant mouth, uterine cervix and placenta. In particular, potentially pathogenic bacteria are detected to be more abundant in HPV positive samples, such as *Selenomonas* in the maternal oral cavity, *Atopobium vaginae* in the cervix, as well as *Ureaplasma* in the placenta and in the infant oral cavity.
3. The initial neonatal oral microbiota shares features mainly with the microbial profile in the placenta. This observation was consistently made using various analysis methods and the microbiota seems not to be strongly modulated by the birth canal microbiota regardless of the mode of delivery.
4. Both human uterine leiomyoma–based extracellular matrix models, myoma and Myogel, are excellent platforms in investigating the cell invasion with HPV positive cell lines. No differences in the behaviour of the cells, between HPV positive and negative cell lines are apparent in myoma model or in Myogel. Furthermore, there are no drastic differences between SiHa and CaSki, in which the number of viral copies in the genome differ quite dramatically. Ionized radiation results in similar outcomes in all cell lines, as the invasion depth is significantly reduced in all cell lines. We conclude that irradiation affects cells despite the HPV status.

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