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Review of potential entry and replication of human papillomaviruses in reproductive cells and possible correlations to health conditions

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Emilie Pisani Ekeli

Abstrakt

Bakgrunn: Humant Papillomavirus (HPV) infiserer basale epitelceller i hud og slimhinner og i det siste er det fremmet hypotese om at HPV også kan smitte i ikke-epitelceller. Denne oversiktsstudien belyser omfanget av rapporterte funn av HPV i reproduktive celler og diskuterer mulige inngangsmekanismer og om replikasjon er mulig i disse cellene, og om infeksjonen kan assosieres med andre helsetilstander enn kreft.

Metoder: Et systematisk litteratursøk ble gjort for å diskutere om HPV finnes i sædceller og eggceller, ved å følge bestemte inkludering- og ekskluderingskriterier. Inkluderte studier ble videre analysert for relevante funn.

Resultater: Alle inkluderte studier fant HPV i eller på sædceller, og 8 av 14 studier mistenker at sædceller transporterer HPV til kvinnelige reproduktive celler. Ni studier assosierer HPV med sterilitet og/eller spontanaborter, og 2 studier finner ikke assosiasjon.

Konklusjon: HPV kan feste seg til hoved reseptor (syndecan-1) på hodet på sædceller, og på den måten transporteres til kvinnelige celler ved befruktningen. Om HPV entrer sædceller fullstendig, eller forblir på utsiden, er fortsatt uavklart. Tegn på aktiv viralt genuttrykk har blitt observert i mannlige og kvinnelige reproduktive celler, men disse funnene trenger videre bekreftelse. HPV kan stanse fosterutvikling og celledeling i ny-befruktet egg, og kan assosieres med sterilitet og spontanaborter.

Abstract

Background: Human Papillomaviruses (HPV) infect the basal epithelial cells in skin and mucous membranes. Recently, new concerns regarding HPV in non-epithelial cells have been raised. This review elucidates HPV presence in reproductive cells and investigate possible entry mechanism and replication status in these cells, and if infection can be associated with any other health conditions than cancer.

Methods: A systematic literature search was conducted to assess HPV presence in sperm cells and oocytes, by following a set of inclusion and exclusion criteria. Included studies were further analyzed for relevant findings.

Results: All included studies found HPV in or on sperm cells, and 8 of 14 studies suggest that sperm function as a carrier for HPV into female reproductive cells. Nine studies associate HPV to infertility and/or miscarriages, and 2 studies do not find such an association.

Conclusion: HPV may specifically attach to their primary receptor (syndecan-1) on the sperm head and be transported to female cells upon fertilization. If HPV enters the sperm cells completely remains unclear. Active HPV genome transcription has been observed in both male and female reproductive cells, but this needs further confirmation. HPV may cause stage arrest of fertilized oocyte and embryo development and can be associated with infertility and miscarriages.

1 Introduction

1.1 Human Papillomavirus Epidemiology

Human papillomavirus (HPV) are DNA viruses belonging to the virus family *Papillomaviridae*, that infect basal epithelial cells in skin and mucous membranes through small cuts and wounds. HPV is the most common virus infection in the reproductive tract and it is estimated that more than 70% of the population worldwide will have a genital HPV infection during life (Tønjum, 2018). Recently, it has been reported more than 200 different types of the virus, where around 40 of them are sexually transmitted (Latsuzbaia et al., 2018). Most HPV infections are asymptomatic and will disappear within a few years, but persistence of some types can cause warts on skin and genitalia, and others can cause malignant tumours (World Health Organization, 2019).

HPVs are classified within five different genera in the *Papillomaviridae* family, (alpha, beta, gamma, mu and nu-papillomaviruses), and further into species and subtypes based on percentage of similarity in their DNA sequence. The most disease related HPVs have evolved from the apes and belong to the alphapapillomaviruses. Within this group “high-risk” and “low-risk” HPVs are recognized infecting the human genital tract, where high-risk types are cancer related. Low-risk types are associated with genital warts and respiratory papillomatosis (benign outgrowths in air passages), where HPV-6 and HPV-11 are responsible for 90%. According to International Agency for Research on Cancer (IARC), 12 high-risk types are defined as group 1 carcinogenic (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59), and HPV16 and 18 are responsible for 70% of all cervical and pre-cancerous cervical lesions (World Health Organization, 2019). In addition, cancers in anus, vulva, vagina, penis and oropharynx have been associated with HPV infection (Center for DIsease Control and Prevention, 2019).

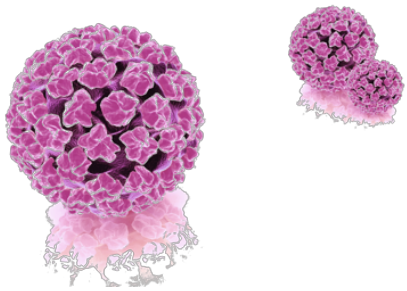


Figure 1 - Human Papillomavirus. Figure taken from SiBe, IMGBIN (2017). License: Non-commercial use.

In other words, HPV infections has a big impact on human health worldwide and many young adults will be affected directly or indirectly. Recently, new concerns have been raised regarding HPV in non-epithelial cells in the reproductive tract, which may be associated with other health conditions such as infertility (Foresta *et al*., 2013), miscarriage (Garolla *et al*., 2016), epilepsy (Chen *et al*., 2012) or autism (Godar & Merrill, 2017). Review work summarizing all aspects of HPV presence in or on reproductive cells and possible entry mechanisms will help shed further light on other HPV-associated health conditions than cancer.

1.2 Virus structure and gene expression

The human papillomaviruses are small, non-enveloped viruses with circular dsDNA and a size of 55 nm in diameter. The viral particle has an icosahedral structure made of capsid proteins that protect the genome, as can be seen in figure 2. The HPV genome is about 8000 base pairs long and consists of six early genes, two late genes and a non-coding region called the long control region (LCR). Early genes are E1, E2, E4, E5, E6 and E7, which all have a specific purpose in the virus replication, transcription, cell cycle, modification of host cell and production of new viral particles. The proteins encoded by the early genes are not part of the mature viral particle, but the early genes are transcribed during replication. Presence of E6/E7 proteins or RNA in an infected cell is therefore sign of active transcription of HPV genome. HPVs do not code their own DNA polymerase to perform genome replication and are dependent on the host cell's polymerase to replicate and produce new viruses. The polymerase is synthesized during Synthesis Phase in the cell cycle (S-phase), which is the phase where DNA replication takes place in the nucleus, and HPV must enter the nucleus of the infected cell to get access to the DNA polymerase. L1 and L2 are the late genes and code for two structural proteins of the viral particle; L1 being the major, and L2 the minor capsid proteins. The capsid structure is required to spread and protect the viral genome in the environment. Late

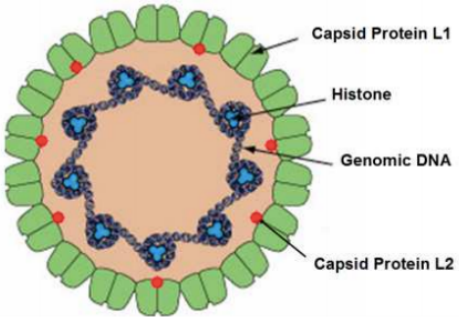


Figure 2 - The structure of HPV with capsid proteins, histones and circular DNA. Figure taken from SWISS Institute of Bioinformatics (2010). License: Creative Commons Non-Commercial Use

proteins are also responsible for viral connection to target cells through attachment to host cell receptors for new infections. Figure 3 illustrates the orientation of the genes in HPV-16 with numbers describing base pair position.

Early gene E1 codes for the DNA helicase that untangle the helical double-stranded genome structure and form the replication fork to give access to the DNA polymerase. E2 is involved in replication initiation and is the main regulator for viral gene expression and controls the level of E6 and E7 gene expression. Upregulated gene expression of E6/E7 may lead to uncontrolled cell growth and development of malignant tumours. E4 is the last gene that is expressed before the late genes, and is important in HPV genome amplification, cell cycle arrest and virion assembly. E5 plays an important role in the cell cycle and differentiation, and helps the virus evading the host cells immune system (Graham, 2017). E6 and E7 are oncogenes, that target important host cell tumour suppressor proteins, protein 53 (p53) and protein Retinoblastoma (pRb), respectively. P53 is the main sensor for DNA damage and regulate cellular processes. During infection, E6 protein binds to p53 and supresses its function. PRb inhibits uncontrolled cell growth by controlling G1 (gap 1 phase) and entry to S-phase in the cell cycle and is supressed by binding of E7. Since S-phase is the stage in cellular division where host DNA polymerase is synthesized, this stage is crucial for the HPV replication. Through its binding to pRb, E7 leads the host cell to re-enter the S-phase, and HPV thereby gain access to the polymerase.

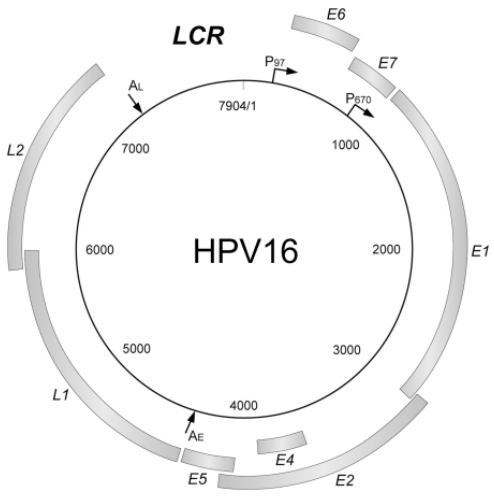
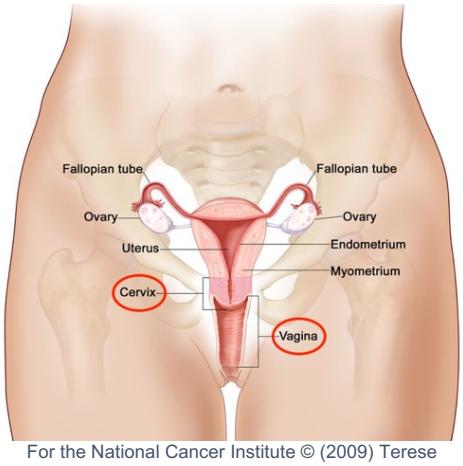


Figure 3 – Genome organization of HPV-16. Early genes (E), late genes (L) and long control region (LCR). Obtained from Kajitani, N (2012). License: Creative Commons Non-Commercial use.

The virus must infect basal cells, as these are the only epithelial cells that undergo division where HPV can enter the nucleus upon new infection, for expression of its early genes and first round of genome replication (Knipe & Howley, 2013). After this initial infection step, the combined function of E6 and E7 allows for viral replication in the suprabasal cell layers, by inducing further cell division.

1.3 HPV entry and replication in epithelial cells

The cell types that are most prone for HPV infection are the basal epithelial cells in cervix, oropharynx, anus, penis foreskin, vulva and vagina and (World Health Organization, 2019). Interpersonal interaction during sexual intercourse makes this an easy transmission route for HPV. The virus can replicate successfully in these tracts, and sometimes cause malignant tumours. Figure 4 illustrates the male and female reproductive tract, where HPV prone areas are marked in red. Penile infection is most common in the foreskin, but cancer rarely occurs here (Bodily & Laimins, 2011). In the female tract, vagina and cervix are most exposed for infection, where the cervix is highly prone to cancer.



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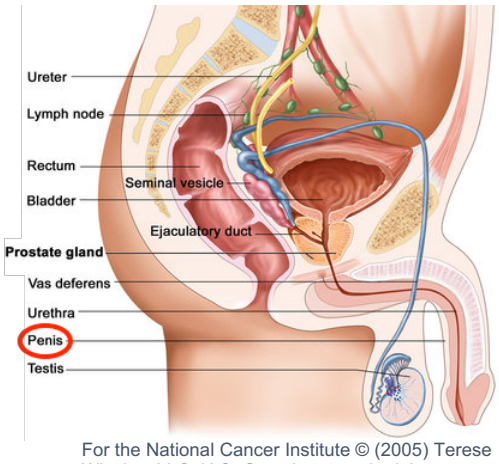

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Figure 4 – Human reproductive tract. This figure illustrates the male and female reproductive system where the areas marked in red are most prone to HPV infection. Figures are obtained from Terese Winslow LLC, with permission from Winslow, T.

The virus enters the basal cells through a complex cascade of receptor binding with capsid proteins as shown in figure 5. L1 major capsid proteins of HPV-16 attach to a first receptor before attachment to several co-receptors that trigger conformational changes and allow entry to the cell (Raff *et al*., 2013). The first receptor, which is also considered the main HPV receptor, is the heparin sulphate proteoglycans (HSPGs). From this group of receptors, syndecan-1 is the most abundant in epithelial cells and the receptor mainly used by HPV (Surviladze *et al*., 2015). After attachment to syndecan-1, Raff *et al* (2013) suggest that growth factors get activated and an intracellular signalling cascade begins, leading to further activation of internal events. Convertase causes conformational changes in the virus

structure so that L2 minor capsid proteins are partly exposed on the surface (Graham, 2017) and bind to the co-receptors α6-integrin and A2t. Following these reactions and signalling events, endocytosis of the HPV-16 takes place as demonstrated in figure 5. Other HPV types and host cells may require another strategy for entry, but HPV-16 is most common in the research articles and therefore used as an example for viral entry in this review (Graham, 2017).

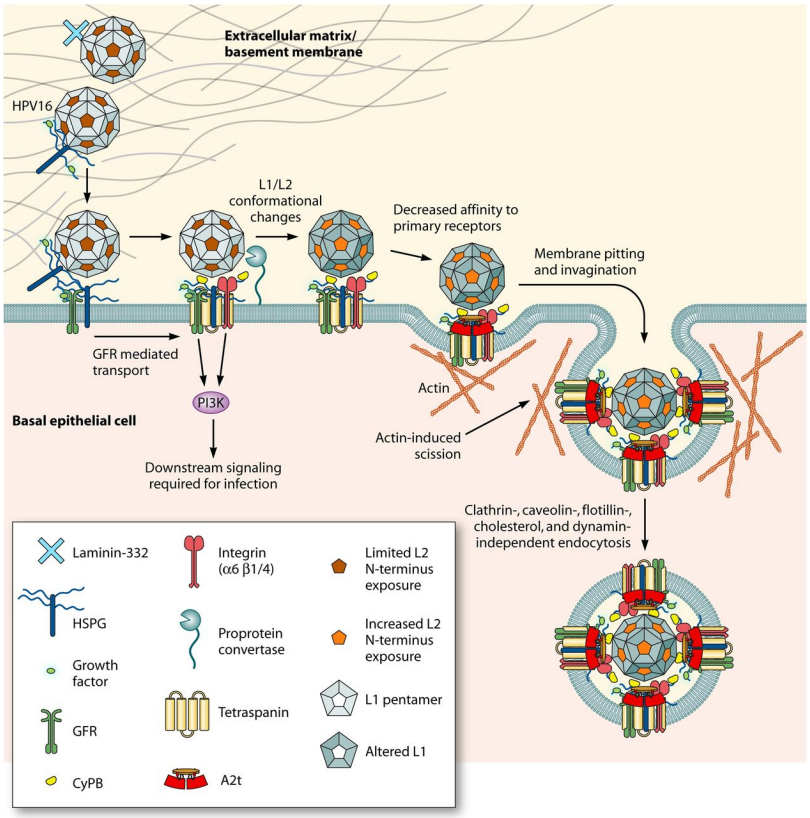

Figure 5 - Binding and entry of HPV16. The figure shows the complex internalization of HPV16 into basal cells. The virus binds to receptor, co-receptors and different growth factors, as presented in the white box. The complex cascade reactions activate an endocytosis of the virus. Figure taken from Raff *et al*. (2013) with permission from Woodham, A.

When the virus has entered the cell, low pH values in the late endosome will cause degradation of the virus particle, releasing a complex formed by the viral genome and the minor capsid protein L2. L2 mediates escape of the complex from the endosome and further trafficking towards the nucleus. Entry into the nucleus is not fully elucidated, but it is hypothesized that the L2/HPV DNA complex gains access to the nucleus through breakdown of the nuclear membrane upon cell division that takes place in basal cells of epithelial tissues (Aksoy *et al*., 2017). The HPV genome is normally kept as an episome in the nucleus, but integration of viral DNA

into host cell genome does happen, a process that potentially leads to carcinogenesis.

The first biosynthetic step after nuclear entry is expression of the early genes to prepare for replication. E1 and E2 bind to the replication origin to initiate replication and recruit host cell DNA machinery. The host will complete viral episome DNA replication and produce about 50-100 copies of the viral DNA (Graham, 2017). The virus can stay latent in the basal cell layer and produce a low level of E1 and E2 proteins as shown in figure 6, just enough to maintain as an episome upon cell division, but low enough to avoid activation of the immune system.

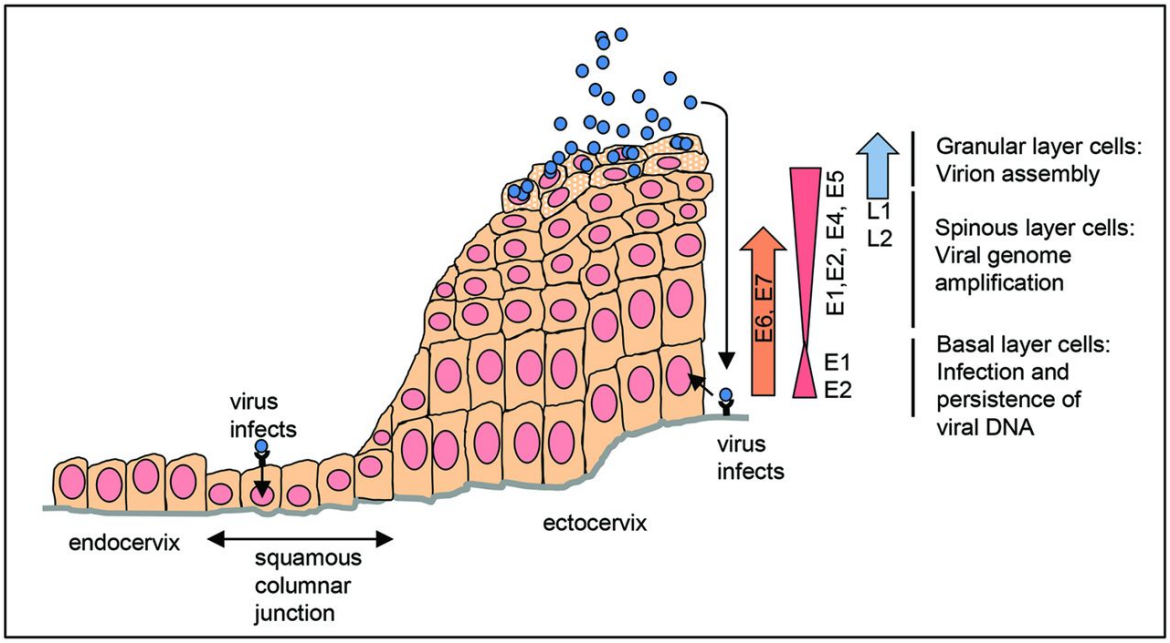

Figure 6 – HPV replication cycle in epithelium. Virus infects basal epithelial cells where it can enter the nucleus and stay persistent with expression of E1 and E2. The virus follows the epithelial cell cycle and move with the dividing cells to the spinous layers where the rest of the early genes are expressed. In the outer layer, the late proteins (L1 and L2) are expressed and the virion assembles to exit the epithelium to infect new cells. Figure taken from Graham, S. (2017). License: Creative Commons Attribution License.

When the infected basal cells have divided, they migrate towards the outer layers of the epithelium. E2 stimulates expression of E6 and E7 in this stage and the spread of infected cells will start. E5 will be expressed at this point, preventing antigens from being exposed to immune cells. Amplification of virus particles starts when all early genes are expressed, and the infected cells spread to the squamous layer. In the last stage, late genes get expressed and the virion assembles with all its components, ready to leave the mature epithelium to infect new cells.

HPV and the human cells both contain dsDNA, which allow the virus to use the different components from the host cell machinery for transcription of mature mRNA (including splicing, capping, and polyadenylation), translation and DNA replication. Figure 7 presents an overview of the cycle from viral dsDNA → mRNA → viral proteins and maintenance of the dsDNA. Cellular DNA dependent RNA polymerase II performs synthesis of viral mRNA. The viral mRNA strands migrate to the ribosomes in the cytosol and get translated to viral proteins. DNA dependent DNA polymerase copy both strands of the dsDNA and generate genome that assemble with viral proteins into new virus particles, ready to infect other cells.

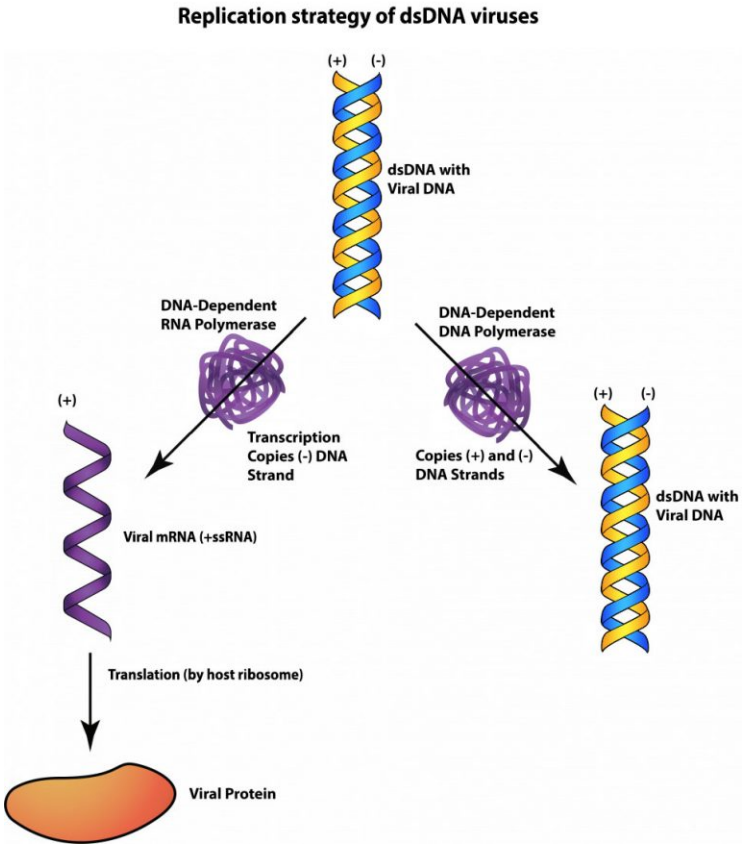

Figure 7 – Replication strategy of dsDNA viruses. Replication of the genome is performed by the DNA Dependent DNA Polymerase, that makes between 50-100 copies of the genome. The DNA Dependent RNA Polymerase transcribes the viral mRNA (+ssRNA), that is translated to viral proteins in the ribosomes. Figure taken from Bruslind and Open Textbook (2017). License: Creative Commons Non-Commercial Use

1.4 Reproductive cells and system

The semen consists of semen fluid and sperm cells. About 70% of the semen fluid is produced in seminal vesicles and tubules while around 30% is produced in the prostate (Svendsen & Nesheim, 2019). The sperm cells develop in the testicles from spermatogonia, a type of stem cell, which through mitosis, meiosis and differentiation complete the spermatogenesis (Rye *et al*., 2013). Stimulation by hormones trigger the production of sperm cells and these mature further in the epididymis on each testicle. Mature sperm cells are stored in the seminal vesicles together with the semen fluid, and these are released together during sexual intercourse (Norsk Helseinformatikk (NHI), 2017). The sperm cell consists of a head, body with mitochondria and tail for moving. The head includes a nucleus where the male genetic material is and an acrosome cap at the end as shown in figure 8. Their purpose is to fertilize the female oocyte in the fallopian tubes by swimming up through the vagina, cervix and uterus.

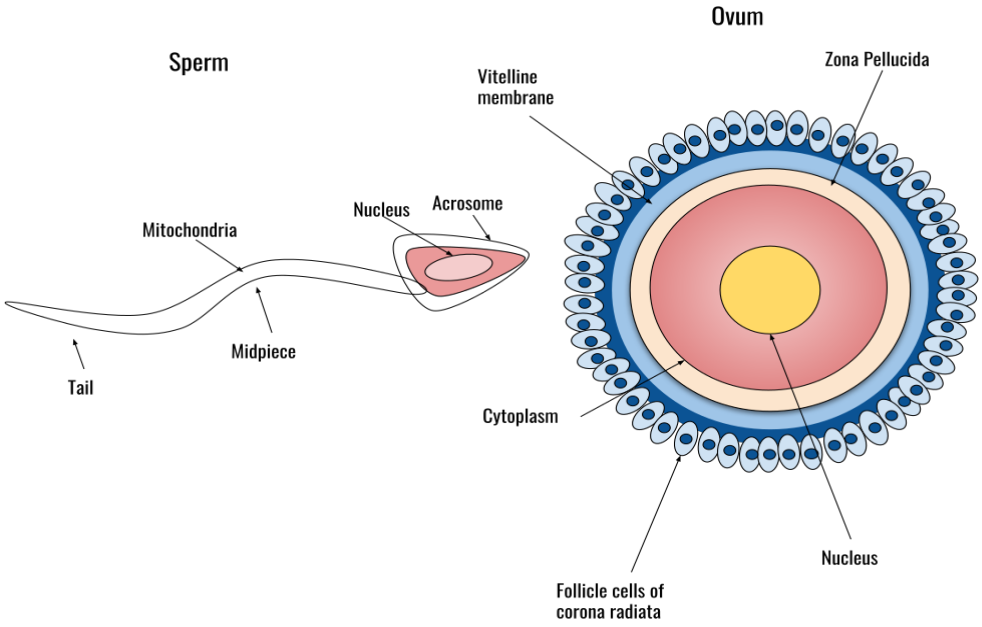

Figure 8 - Structure of the two reproductive cells, sperm and oocyte. Figure taken from Wikimedia Commons, Atdoan0 (2018). License: Creative Commons Attribution-Share Alike International

When females are born, their ovaries possess a limited number of oocytes. These also develop from a stem cell through oogenesis, called oogonium, and through mitosis before birth and meiosis I during puberty, the oocyte is mature and stored in the ovaries. Matured oocytes migrate to the fallopian tubes when the female

ovulates. During this ovulation, the oocyte is surrounded by a sticky membrane called *zona pellucida*, which sperm cells may attach to (Holck & Heiberg, 2018). The oocyte consists of *cumulus cells (*also referred to as *corona radiata)*, which is cell cluster that surrounds and protect the oocyte, zona pellucida, cytoplasm and nucleus as shown in figure 8.

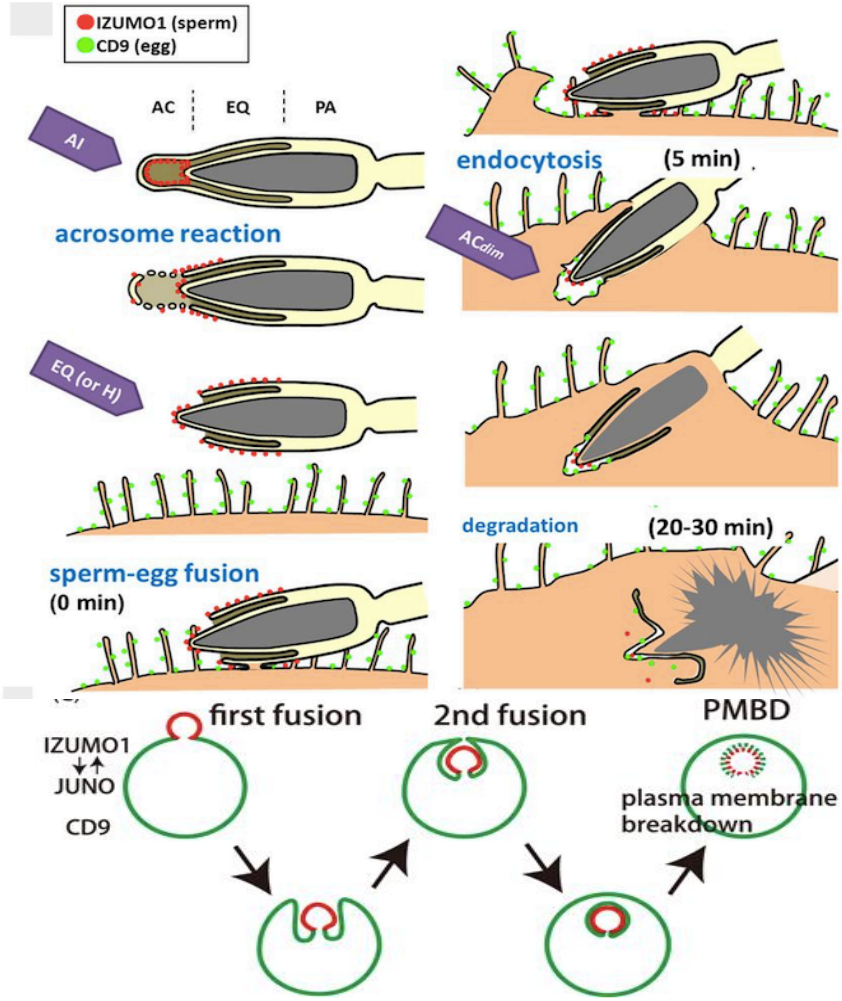

Figure 9 - Sperm-egg fusion and fertilization. The figure illustrates how the fusion and fertilization happens, step by step. IZUMO1 (sperm) and CD9 (egg) are the two proteins essential in sperm-egg fusion. Internalization of sperm happens through acrosome reaction, sperm-egg fusion with the equatorial region, endocytosis and degradation of the sperm nucleus to release the DNA. Figures taken from Satouh *et al* (2012) and Okabe (2018) with permission from Okabe, M for both.

In order to fertilize the oocyte, the sperm must pass through the cumulus cells. After passage through this barrier, top head of sperm cells (*acrosome*) attaches to receptors on zona pellucida. This attachment activates enzymes that enable sperm to fertilize the oocyte. Their membranes will fuse together, making it possible for the male and female genetic material to interact. As can be seen in figure 9, fusing of the

two cells cause an endocytosis, where part of the sperm membrane is internalized with the sperm nucleus (Okabe, 2018; Satouh et al., 2012). Both sperm and oocyte nuclear membranes will break down to expose their genetic material. The fertilized oocyte will complete meiosis II, including the genetic material from sperm, resulting in a fertilized egg with 46 chromosomes for a human being, 23 from the male reproductive cell and 23 from the female (Rye *et al*., 2013). Rapid division of this egg will increase number of cells and form a ball constituting the *blastocyst.* The blastocyst development from fertilized oocyte is illustrated in figure 10 with respective timeline.

In the blastocyst, the cells rearrange into the *inner cell mass (ICM)*, and an outer layer called *trophoblast*. ICM possesses stem cells for the development of the embryo, while trophoblast develop into the placenta that nourishes the fetus.

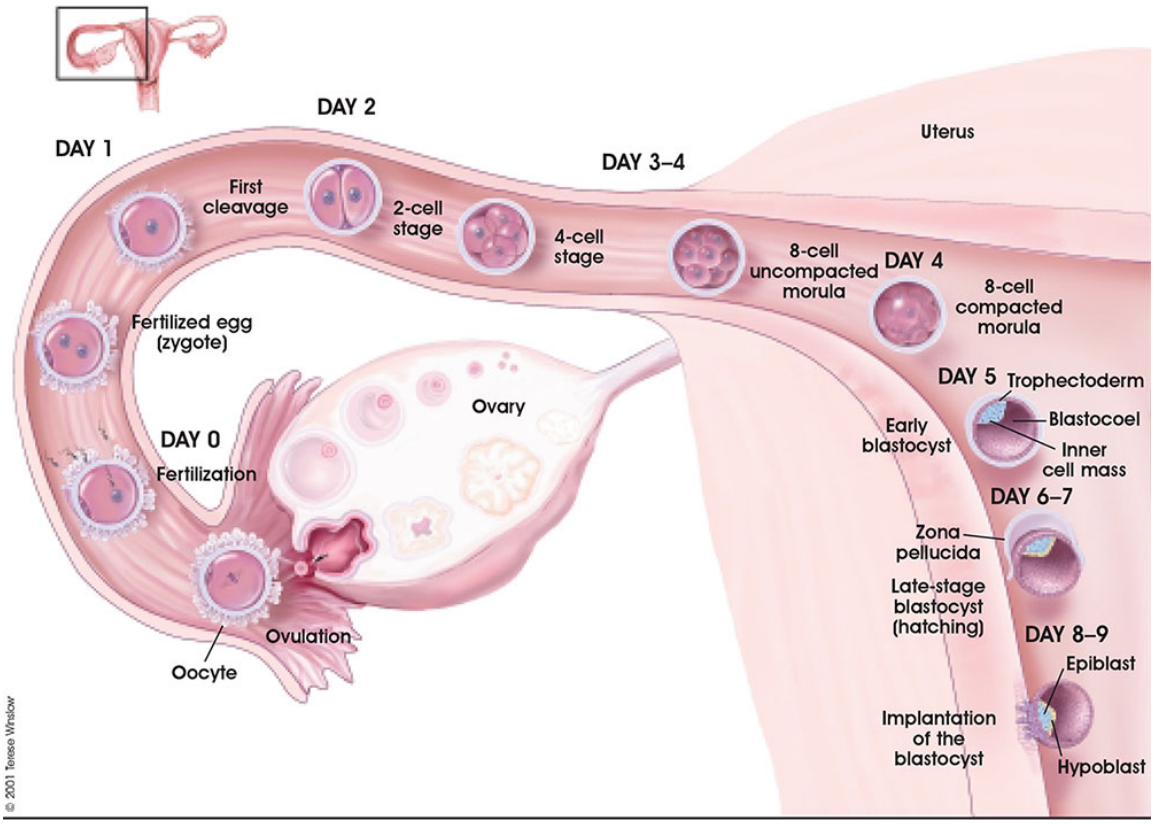

Figure 10 - Timeline of the development from oocyte to blastocyst. The oocyte is surrounded by zona pellucida and fertilized in the fallopian tubes before transfer to uterus. After fertilization, the cell division starts – becoming the early blastocyst. The early blastocyst consists of trophectoderm (trophoblast) that becomes the placenta, inner cell mass (ICM) that becomes the fetus and blastocoel which is the blastocyst cavity. In the late-stage, zona pellucida releases the blastocyst to prepare for implantation. The blastocyst is implanted in the uterus, initiating the embryo development. Figure taken from Terese Winslow LLC, with permission from Winslow, T. For the National Cancer Institute © (2001) Terese Winslow LLC, U.S. Govt. has certain rights.

Oocyte and cumulus cells will from now be referred to as female cells, while blastocyst, ICM and trophoblast will be referred to as fetal cells. Placenta contains blood from the mother and is connected to the fetus by the umbilical cord. All nutrition and oxygen are transferred from mother to fetus through this conduit (Nesheim, 2019). The placenta is made up of different cell types and layers. In addition to blood, it consists of different trophoblastic cells which it derived from, *Hofbauer cells* which are non-epithelial macrophages, fibroblasts between trophoblast and fetal vessels, and fetal vascular cells that include muscle, perivascular and endothelial cells (Wang & Zhai, 2010). All these cells constitute the placenta that protects and help maturation of the fetus.

1.5 Potential HPV infection in reproductive cells

A considerable amount of literature regarding HPV in the reproductive tract has been published on PubMed and Web of Science. From that volume of published studies, some describe the role of HPV in sperm cells and oocytes. Previously, it has been thought that HPVs only infect epithelial cells in skin and mucous membranes. Sperm cells and oocytes are the male and female reproductive cells, and concerns have been raised regarding HPV infection in these cells as well. Epithelial cells are *susceptible* and *permissive* for HPV, meaning they possess the right receptors for internalization and allow the virus to replicate inside, respectively, allowing production of progeny viral particles. As previously mentioned, the productive viral replication cycle is tightly associated to the differentiation status of the layered squamous epithelium. HPV infections in other epithelial cells is thought to lead to abortive, latent or transforming infections. In the past three decades, researchers have shown an increased interest in determining whether a range of non-epithelial cells also are susceptible and/or permissive for this virus. In addition, little research has been done to investigate alternative entry mechanisms for HPV at non-epithelial sites, and in particular in other cells in the reproductive tract than cells lining the cervix or in the penis, with possible transmission to the fetus.

For example, Zika virus (ZIKV), that is a vector borne virus in the *Flaviviridae* family is suspected to use alternative routes to infect hosts (Noronha *et al*., 2016). ZIKV is transmitted from mosquitos to humans and has been shown to cause a serious health condition in the fetus of pregnant women, called *microcephaly*. This

condition gives the fetus an underdeveloped brain and head, leading to a range of functional disabilities. Researchers believe that this devastating viral disease is transmitted from mother to fetus through damage of the placental barrier. Noronha *et al* (2016) detected ZIKV in Hofbauer cells, the placenta macrophages as previously mentioned. ZIKV is hypothesized to use these macrophages as a way to cross the placental barrier and infect the fetal brain. This mechanism has been proposed for how HPV may spread to fetal brain cells, and potentially cause epilepsy (Chen *et al*., 2012) or autism (Godar & Merrill, 2017) in children as a consequence of *in utero* HPV infection. There is a chance that hitchhiking is an unrevealed transmission route for HPV as well. Different approaches have been used to investigate this aspect both *in vitro* with HPV capsid or DNA fragments and *in vivo* on patient semen samples. On the other hand, HPV infecting sperm cells might bring HPV infection directly into the oocyte, similarly to what has been shown for rhabdoviruses in salmon, where virus attached to spermatozoa gains access to oocytes (Øvergård *et al*., 2017).

2 Thesis Issue

The purpose of this thesis is to give an overview of current knowledge on how HPV interact with the reproductive cells and which medical conditions this interaction possibly can be related to. This thesis systematically reviews the findings from different published studies on this issue.

3 Methods

3.1 Search strategy and study design

The study design of this thesis is similar to meta-analysis with a systematic approach. In meta-analysis several studies with a comparable issue are analyzed to be a part of the systematic search. The purpose of a systematic search is to "summarize findings from several studies in order to conclude with a higher degree of safety" (Norsk Helseinformatikk (NHI), 2018). This review is based on articles from two different reliable searching engines, where researchers have investigated similar issues with different techniques.

By systematically search through PubMed and Web of Science, articles were analyzed and considered for inclusion in this review. Choice of searching engines was discussed with supervisors and librarians at Norwegian University of Life Science, Ås. PubMed and Web of science are considered as good engines for trustworthy scientific papers that cover the most relevant studies. No limitations concerning the date for publishing were used in the search, and publications from all years were analyzed in order to get a more comprehensive view of the issue. Words included in the search for HPV in different cells in the reproductive tract was:

1. human papillomavirus
2. sperm cells
3. oocyte
4. egg cell
5. semen
6. reproductive tract
7. placenta
8. non-epithelial cells
9. Hofbauer cells

Different combinations of these words were used for testing the extent of results and to get a feeling of how specific the searches should be. When a preferable word combination was chosen, the systematic approach started. Inclusion and exclusion criteria were made and are described in the following chapter.

3.2 Inclusion and exclusion criteria

To be able to include relevant and exclude irrelevant articles, inclusion and exclusion criteria were made beforehand. For including an article, the first criterion was that it should be written at a cellular level to understand the connection between HPV and the different cell types investigated and how this may trigger health conditions. This can be revealed by finding comments regarding the virus genome, structure and receptors, or results related to altered cell functions when infected with HPV. The second criterion required original studies, not review articles. Relevant review articles would be included later in the discussion section, but not in the results from the systematic approach.

A strategy to meet these criteria in each study is crucial to avoid systematic bias and to include the correct and eligible papers. To find articles meeting these inclusion criteria, relevant words were searched for in full text version by using CMD + F (Mac). Relevant articles which included some of these words were read in more detail. To organize relevant and irrelevant articles from the search, a list for each website with number for each result was used. **INCLUDE** or **EXCLUDE** was written for each result with an additional description on the reason for the article to be included or excluded. Period of search was from 10th of March 2019 to 5th of May 2019. Further details for every search are described in the following chapters, respectively.

3.3 HPV in sperm cells

For the systematic search involving HPV and sperm cells, the following combination was used “human papillomavirus sperm cells,” in both search engines to cover the same type of articles and to make a comparison possible. Combination with “semen” instead of “sperm cells” was also considered but gave too many results dealing with non-spermatozoa cells in semen. “Sperm cells” gave a smaller extent of results than “sperm” and also included papers written on a more cellular level, than paper results with only “sperm”. Due to this aspect “human papillomavirus sperm cells” was considered the best option to find relevant papers.

All articles retrieved in the search were analyzed for fitting the inclusion criteria. Each article was opened in full text if available, the abstract was read, and a

further search in each article for specific words was done. These words were “E1”, “E2”, “E6”, “E7”, “L1”, “RNA”, “DNA”, “mRNA”, “receptor”, “entry”, “binding”, “replication”, “transcription”, “capsid”, “protein.” The first eight words refer to the most relevant genes and HPV genome and the following words are relevant for virus and host cell interaction and replication. Unavailable papers and papers without any of these words and no cellular description connected to the issue, were excluded. If any words were found, the context around it, results and conclusion were read. Studies with relevant topics were read in more detail. Relevant review papers were excluded from this search but may be relevant in the discussion section.

3.4 HPV in oocyte

Since the sperm cells penetrate the female oocyte, this may be a relevant place to investigate for HPV. With regard to embryo development from fertilized oocyte, HPV infection in this cell type may have crucial consequences. Word combination for this search was: human papillomavirus oocyte. Different word combinations were tried out, such as “human papillomavirus reproductive tract” and “human papillomavirus egg cells” but resulted in either too many results, cancer related results or the same results (egg cells). “Oocyte” was therefore considered the best word solution, although few studies have been conducted with respect to this issue.

The same inclusion strategy as for sperm cells was used and all papers were opened in full text if available. To find out if the article met the criteria, these following words were searched for in full text; “E1”, “E2”, “E6”, “E7”, “L1”, “RNA”, “DNA”, “mRNA”, “receptor”, “entry”, “binding”, “replication”, “transcription”, “capsid”, “protein”, “sperm”, “semen”. With no hits on these words, the article was considered irrelevant to this review and excluded.

4 Results

4.1 Systematic search results

Search for “human papillomavirus sperm cells” gave 72 results in PubMed and 57 results in Web of Science. After considering all 72 + 57 papers, 14 + 11 original studies were included as described in figure 11, with an overlap of 11 articles.

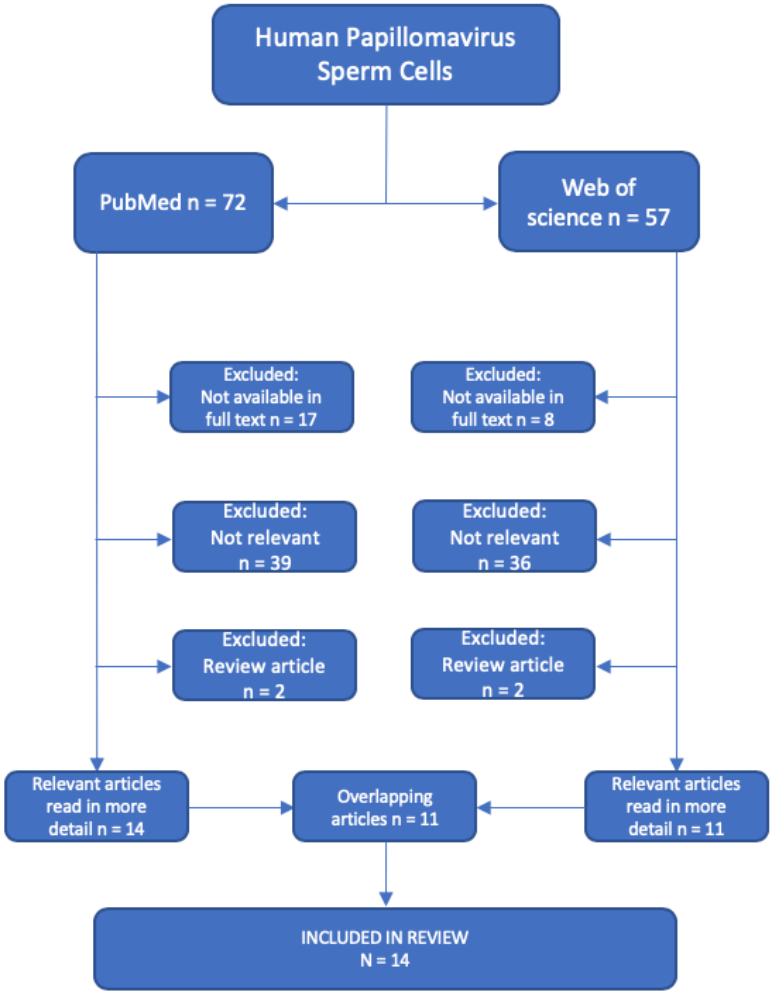

Figure 11 - Flow chart describing searching strategy for "human papillomavirus sperm cells" where n = number of papers, resulting in a total of 14 relevant articles from this search.

The oocyte search gave 18 results in PubMed and 17 results in Web of Science. All 18 + 17 papers were considered, and 2 + 3 original studies were included, with an overlap of 2 articles as shown in figure 12. From these results, a total of 3 papers were relevant for HPV in oocytes. All 3 papers overlapped with the sperm cell search, meaning no papers from the oocyte search were exclusive.

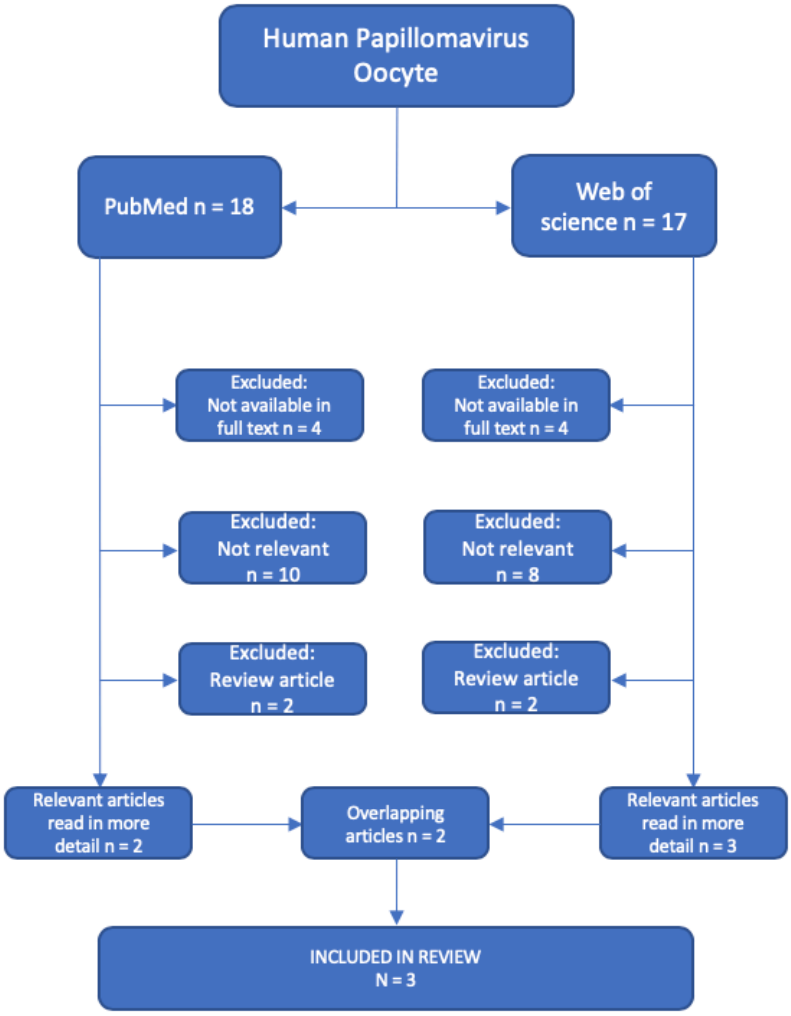

Figure 12 - Flow chart describing searching strategy for "human papillomavirus oocyte" where n = number of papers, resulting in a total of 3 relevant studies from this search.

The library of all 14 papers conducted from both sperm cell and oocyte search was read one more time, to elect main characteristics and findings. A table divided into reference, method, findings and conclusion for each study was made, sorted alphabetically in the respect of authors (supplementary table 1). The table provides an overview of experimental data and is placed at the end of this document because it would be too extensive in the result section. Further investigation of this literature was done by comparing study findings and conclusions to observe any intercorrelation.

4.2 Overview of the results

The search resulted in 14 + 3 relevant papers for HPV in sperm and oocytes, respectively. All 3 papers from the oocyte search were overlapping with the sperm search, resulting in a total of 14 papers included in this thesis. Short versions of the supplementary table 1 are shown in table 1 and 2.

As shown in figure 13 most studies were *in vivo* patient studies, while some were *in vitro* studies. One study had both patients and *in vitro* experiments, marked in grey on the figure (Garolla *et al*., 2012a). Subjects from *in vivo* studies were either sperm donors, HPV-positive, selected randomly to analyze presence of HPV or from infertile couples to evaluate presence of HPV in infertility cases. *In vitro* studies used either incubation of reproductive cells with HPV DNA, either unspecified DNA fragments, DNA fragments from L1 gene or E6/E7 genes, or directly transfection with HPV DNA (plasmid containing E6/E7), or incubation of reproductive cells with L1 protein or virus-like particles.

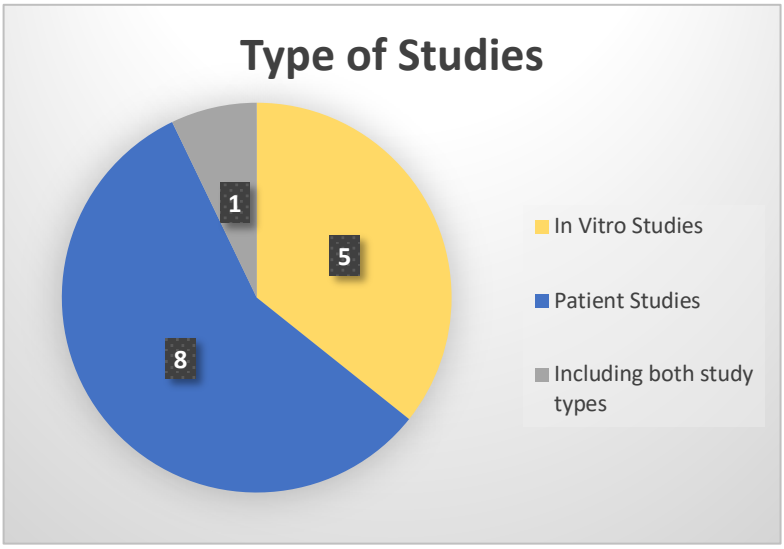

Figure 13 – Distribution of study types for the 14 papers.

Type of study is elementary due to different approaches and findings. The main characteristics obtained from analysis of all studies are summarized in figure 14. As can be seen from the figure, all studies detected HPV either inside or on sperm cells. Figure 15 and 16 separate *in vitro/in vivo* studies and provide further details regarding findings based on methods.

From figure 14, it can be seen that 8 studies suggest sperm as a carrier for HPV into female or fetal cells, being oocytes, cumulus cells, blastocysts, trophoblast and

ICM. One of these 8 studies suspect further transmission of HPV through placenta and into embryo brain (Foresta *et al* 2013). Six studies did not comment on this issue and no studies disagreed.

Correlation between HPV infection and health conditions as infertility, miscarriage, epilepsy and autism are highly controversial topics, and 9/14 studies suspect correlation to infertility and miscarriages. Two studies claim that this intercorrelation is inconclusive due to lack of sufficient evidence (Golob *et al*., 2014; Schillaci *et al*., 2013). However, they both detected HPV in or on sperm, and one of them even suggested sperm as carrier into oocyte. The last 3 studies did not comment HPVs impact on health condition. The authors had different goals for their research and all issues are therefore not the same, but the main characteristics are further elucidated. The next chapters are separated by *in vitro* and *in vivo* studies with respective tables 1 and 2, and the figures (15 and 16) present different findings based on experimental approach.

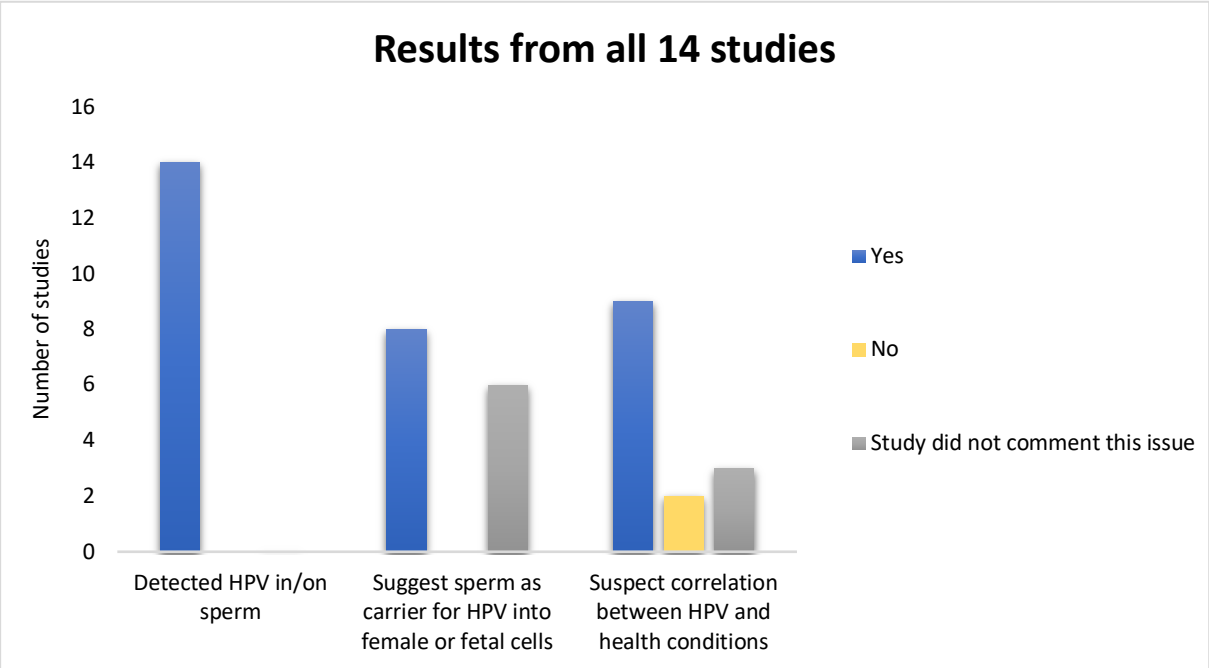

Figure 14 – Bar plot with main characteristics and common features from all papers where n = number of papers. It is apparent from this figure that all studies found HPV in or on sperm cells. Eight studies suggested sperm as a carrier of HPV into female or fetal cells as oocyte, cumulus cells, blastocyst, trophoblast, ICM or placenta. Nine studies suspect correlation between HPV and health conditions and 2 studies disagree on this correlation. The bars in grey did not comment on the respective issue.

4.3 *In Vitro* Results

All aspects discussed in this *in vitro* result section, are shown in figure 15 and table 1. Most *in vitro* studies (5/6) incubated sperm with HPV L1 gene or protein. Four of them detected L1 protein outside sperm cells, mostly located at the equatorial region of sperm head (Dona *et al*., 2018; Foresta *et al*., 2011; Garolla *et al*., 2012a; Perez-Andino *et al*., 2009), while the last one, looking at L1 DNA, found L1 inside sperm cells (Chan, 2000).

Two out of 6 studies incubated sperm with HPV E6/E7 gene fragments and exposed the sperm to female or fetal cells to analyze HPV transmission. One of the studies detected the E6/E7 gene fragments in blastocyst, trophoblast and ICM (Chan, 2000), and the other study found E6/E7 in nucleus of cumulus cells that surrounds the oocyte (Kadze *et al*., 2002)

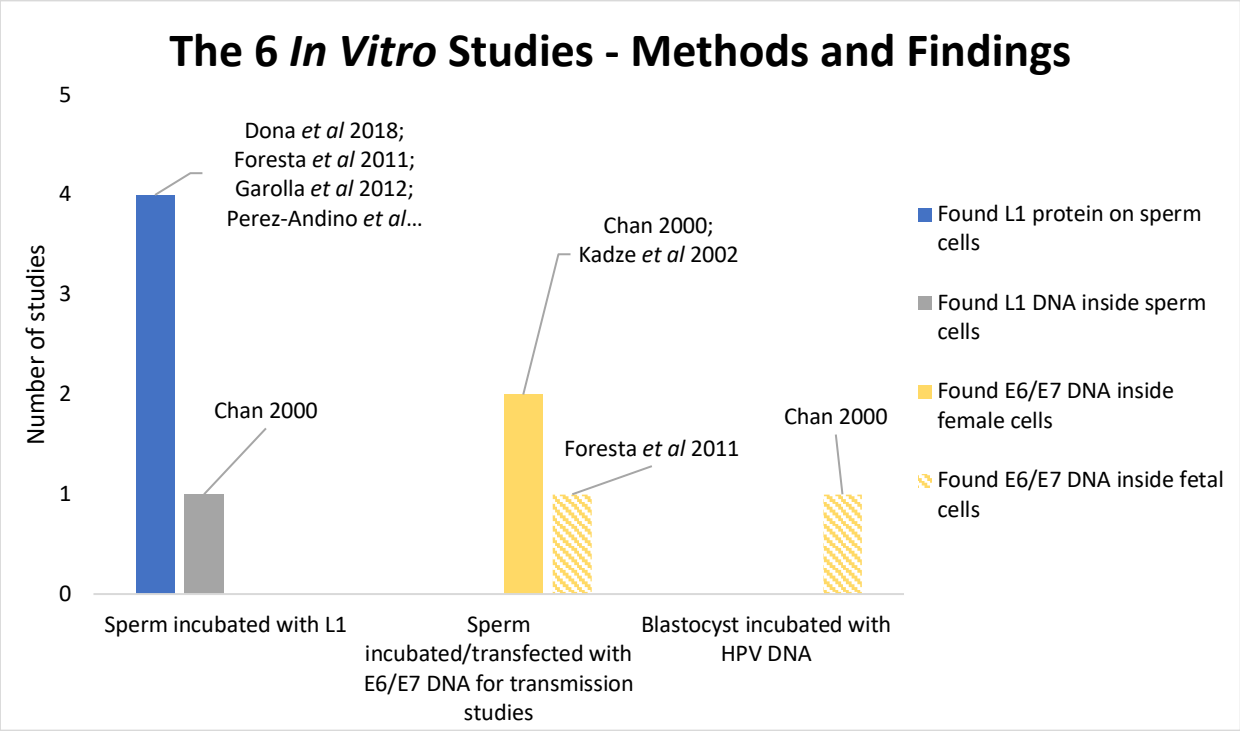

Figure 15 - Overview of experimental approach in 6 *in vitro* studies and results from each approach. References attached to each bar. Four studies that incubated sperm with L1 detected L1 on surface of sperm cells. One study that incubated with L1 detected L1 inside the sperm cell. Two studies that incubated sperm with E6/E7 DNA detected the HPV DNA inside blastocyst, trophoblasts, ICM and cumulus cells. One study that transfected sperm with E6/E7 DNA detected HPV DNA inside blastocyst. One study also incubated blastocyst with HPV DNA and detected the DNA inside.

One study transfected sperm with E6/E7 plasmid and fertilized oocyte with the transfected sperm. Researchers later detected transcripts of E6/E7 in cytoplasm of oocyte where they suggested active viral transcription (Foresta *et al*., 2011).

Chan *et al* (2000) presented three study parts with different authors in one study, summarizing all three experiments. As seen in figure 15, Chan *et al* (2000) incubated sperm with both L1 and E6/E7 DNA. L1 DNA was found inside the sperm cell, while the E6/E7 gene fragments were detected in blastocysts. The last experiment in this study was incubation of blastocyst with unspecified HPV DNA. Chan *et al* (2000) managed to detect the HPV DNA inside the blastocyst but did not comment the location in the blastocyst.

Table 1 – Overview of the *in vitro* studies. The table contain reference and main characteristics connected to each study.

In vitro studies	**Main findings**
(Chan 2000)	Sperm incubated with HPV L1 gene. Found L1 in sperm cells and suggest sperm as HPV carrier to female cells. Blastocyst incubated with unspecified HPV DNA. Detected HPV DNA inside blastocyst. Sperm incubated with E6/E7 genes to analyze sperm carrying. Found E6/E7 in blastocyst, trophoblast and ICM.
(Dona *et al*. 2018)	Sperm incubated with HPV L1 protein. Found L1 on sperm head, suggesting infertility.
(Foresta *et al*. 2011)	Sperm incubation with HPV L1 protein and transfection with E6/E7 genes. Confirm HPV L1 binding to syndecan-1 receptor on sperm head. Found transcription of E6/E7 in oocyte penetrated by the sperm. Suggest infertility as sperm may carry HPV into oocyte.
(Garolla *et al*. 2012)	Sperm incubation with HPV L1 proteins. Detected HPV L1 on sperm cells. Reduced motility in naturally infected sperm (L1) and suggest HPV as cause of infertility and miscarriages.
(Kadze *et al*. 2002)	Sperm incubated with E6/E7 genes. Found transmission of HPV E6/E7 from sperm cells to nucleus of cumulus cells and suggest a further infection to oocyte causing infertility.
(Perez-Andino *et al*. 2009)	Sperm incubated with HPV L1 protein. Detected HPV L1 on sperm cells. Suggest sperm as carrier for HPV into oocyte. Recommend blocking of sperm-HPV receptor.

4.4 *In Vivo* Results

All results in this *in vivo* result section are presented in figure 16 and table 2. All *in vivo* studies have analyzed semen samples from male patients, either from sperm donors, infertile couples (undergoing *In vitro* fertilization (IVF)), patients with HPV infection, infected partner or randomly selected.

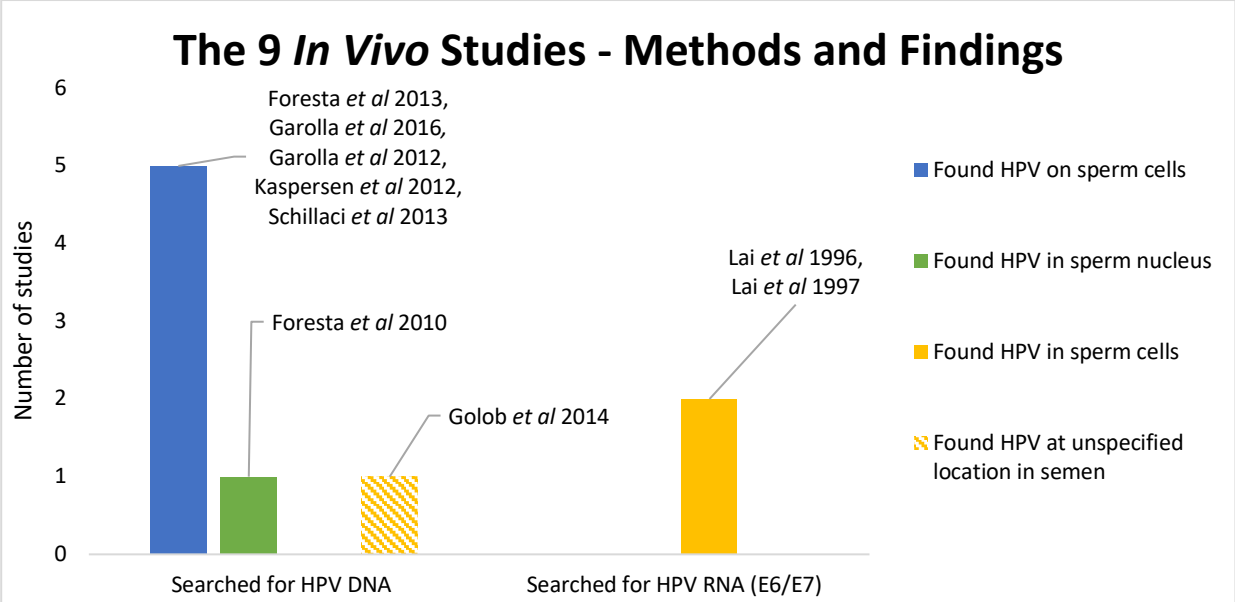

Figure 16 - Overview of findings from 9 *in vivo* studies and results from each experiment approach. References attached to each bar. Five studies found HPV DNA on sperm cells and, and one found HPV DNA inside sperm nucleus. Two studies detected HPV RNA inside sperm cells, and the last study detected HPV DNA in semen, but with unspecified cell type location.

Five studies searched for HPV DNA in semen samples from infected patients, sperm donors and infertile couples, and detected HPV DNA (L1) on sperm cells (Foresta *et al*., 2013; Garolla *et al*., 2012a; Garolla *et al*., 2016; Kaspersen *et al*., 2011; Schillaci *et al*., 2013). Garolla *et al* (2012) did not specify where the HPV was located in semen but used the same procedure as searching for HPV on sperm cells, indicating that HPV was attached to sperm surface. Garolla *et al* (2016) did not comment if the viral DNA was detected inside or bound to sperm cell surface, but figures and comments in the study might indicate that it was detected on the outside.

One study searched for E7 DNA in semen from infertile males and detected HPV DNA inside the sperm nucleus (Foresta *et al*., 2010). Two studies searched for E6/E7 DNA and RNA in semen from randomly selected patients at fertility clinic, and detected DNA and mRNA inside sperm cells (Lai *et al*., 1996; Lai *et al*., 1997). The last *in vivo* study detected HPV DNA but at unspecified location in semen, which means it might be present in non-spermatozoa cells (Golob *et al*., 2014).

Table 2 – Overview of the *in vivo* studies. The table contain reference and main characteristics connected to each study.

In vivo studies	**Main findings**
(Foresta *et al*. 2010)	Semen samples FISH analyzed for HPV E7 gene. Detected HPV DNA in sperm cell nucleus of infertile men and reduced motility. Suggest HPV as cause of infertility.
(Foresta *et al*. 2013)	FISH analysis in semen for HPV markers (L1, E6/E7). Detected HPV DNA on sperm cells and reduced motility, causing infertility. Suspect HPV in fetus brain through placenta.
(Garolla *et al*. 2012)	FISH analysis on semen samples, showed reduced motility in naturally infected sperm and suggest HPV as cause of infertility and miscarriages.
(Garolla *et al*. 2016)	Semen FISH analyzed for HPV DNA. Found HPV DNA on sperm cells. Higher rate of adverse pregnancy outcomes in HPV infected couples. Suggest sperm as carrier for HPV into oocyte, causing infertility.
(Golob *et al*. 2014)	Semen analyzed for HPV DNA with Linear Array. Detected HPV DNA in semen but unspecified location. Concludes with no intercorrelation between HPV and infertility.
(Kaspersen *et al*. 2011)	Semen analyzed with *in situ* hybridization for HPV L1. Detected HPV DNA on sperm cells after washing, recommend screening of sperm banks due to infertility factors.
(Lai *et al*. 1996)	Semen analyzed with PCR/RT-PCR for HPV E6 region. Detected mRNA E6 in sperm cells, suggesting viral transcription. Sperm may carry HPV to cells where replication is allowed.
(Lai *et al*. 1997)	Semen analyzed with PCR/RT-PCR for HPV DNA/RNA (E6/E7). Detected HPV DNA/RNA in sperm cells, and in oocyte with unclear entrance. Associate HPV with poor sperm movement.
(Schillaci *et al*. 2013)	Semen analyzed for HPV DNA. Detected HPV DNA (L1) at sperm head and suggest sperm as carrier into oocyte, but not enough evidence to intercorrelate HPV to infertility.

4.5 Reference related results

The themes identified in these responses are obtained from table 1 and 2. The year of publication vary in a range from 1996 to 2018. It is apparent from the tables that year of publication does not impact the findings drastically. The main characteristic findings seem to be spread throughout the years. Interestingly, the oldest studies suspect sperm as a carrier to other cells, and that HPV in oocyte have unclear entrance (Lai *et al*., 1996; Lai *et al*., 1997). This suspicion seems to be more understood in later years. Later studies suggest that HPV in oocyte and other reproductive cell types, may hitchhike with the sperm cell to enter these cells (Chan, 2000; Foresta *et al*., 2011; Garolla *et al*., 2016; Kadze *et al*., 2002; Lai *et al*., 1996; Lai *et al*., 1997; Perez-Andino *et al*., 2009; Schillaci *et al*., 2013).

The latest studies, and especially *in vivo* studies, have focused on medical conditions related to HPV infection and some of them have suggested treatment with heparinase III and astaxanthin to eliminate the HPV from sperm (Dona *et al*., 2018; Foresta *et al*., 2013; Garolla *et al*., 2012a; Garolla *et al*., 2016). These results are provided in greater detail, including key points on experimental design, in the supplementary table 1.

Several authors in the included studies have participated in more than one study. Foresta, C. have participated in 5 of 14 studies, Garolla, A. have participated in 4 of 14 studies, Chan, J. and Lai, Y.M. have both participated in 2 of 14 studies. The authors have collaborated and based their research on each other, as some of the same names appear in many studies and literature lists.

5 Discussion

The main purpose of this thesis was to develop an understanding of how HPV interact with reproductive cells and which health conditions and outcomes this interaction may lead to. Discussion of results takes into account the experimental design of the different studies, presented in the supplementary table 1. The discussion chapter will first interpret *in* vitro and then *in vivo* results, both in greater detail with respect to findings, and the last sections presents an overall literature assessment and considerations based on other studies.

5.1 Interpretation of *In Vitro* Results

In vitro studies proved attachment to sperm surface and uptake of HPV DNA to reproductive cells. Infectious HPV viral particles cannot be cultivated in cell cultures, and *in vitro* parts of the virus such as proteins and DNA are the closest researchers can investigate a natural infection. Although these studies do not exactly reproduce natural conditions, they provide insight in viral mechanisms at molecular level compared to *in vivo* studies.

L1 proteins were mostly detected on sperm surface (Dona *et al*., 2018; Foresta *et al*., 2011; Garolla *et al*., 2012a; Perez-Andino *et al*., 2009), but Chan *et al* (2000) detected L1 DNA inside the sperm (Chan, 2000). These findings clearly indicate connection between HPV and sperm cells.

Interestingly, some studies confirmed the transmission from sperm cells to female and fetal cells. As shown in figure 15, E6/E7 genes incubated or transfected into sperm, were detected in female and fetal cells after fertilization, confirming the transmission route (Chan, 2000; Foresta *et al*., 2011; Kadze *et al*., 2002). Kadze *et al* (2012) alone confirms nuclear location of HPV-16 E6/E7 in cumulus cells and suggest stage arrest of embryo development. These evidences indicate that HPV DNA, if present in sperm cells will potentially transmit to the oocyte and even to the fertilized blastocytes. A drawback with the evidence is that Kadze *et al* (2012) does not elucidate how the virus would transmit from cumulus nucleus to oocyte.

Finding of HPV L1 protein bound to sperm cells suggest, on the other hand, that HPV may hitchhike outside the sperm and somehow enter the oocyte during

fertilization. If hitchhiking on the sperm surface is the strategy, HPV must be internalized during fusion. As demonstrated in figure 9, part of the sperm membrane is internalized during fusion (Satouh *et al*., 2012). This part of the membrane is the equatorial region at the sperm head, which is the location where many studies detected HPV (Dona *et al*., 2018; Foresta *et al*., 2011; Kadze *et al*., 2002; Kaspersen *et al*., 2011; Schillaci *et al*., 2013).

Foresta *et al* (2011) found the main HPV-16 receptor, syndecan-1, present at the equatorial region of the sperm head. This would explain the high rate of HPV presence on sperm cells and the entry of HPV into oocytes, as the equatorial region seem to be internalized as well. This hitchhiking transmission method is used by ZIKV (in Hofbauer) and Rhabdoviruses (on sperm) and could also be a strategy for HPV (Noronha *et al*., 2016). Other HPV genotypes may use different receptors but syndecan-1 is considered the main receptor in this thesis due to evidence on HPV-16 (Raff *et al*., 2013). As previously mentioned, and confirmed by Raff *et al* (2013), HPVs require multiple receptors for internalization into basal epithelial cells. While other endocytic processes may happen in sperm cells, HPV might otherwise most likely hitchhike on the sperm surface through its strong attachment to syndecan-1. Either way, this may be an effective transmission route for HPV into oocytes.

From the previously presented figure from Raff *et al.,* (2013), the following receptors have been detected on sperm cells: syndecan-1 (Foresta *et al.,* 2011), $\alpha 6\beta 1$ (Barraud-Lange et al., 2007), tetraspanin (CD63 on immature sperm cells) (Ramakrishna & Surani, 2018), tetraspanin (CD81 on acrosome of sperm cells) (Jankovicova et al., 2016). Evidences on the remaining HPV co-receptors are still missing on sperm cells and needs further investigation. CD81 was detected on the acrosome, and these different locations of receptors would explain the complications regarding internalization. CD63 was detected in immature sperm cells, which indicate that sperm may possess different receptors dependent on the development stages and internalization of HPV may thereby depend on sperm cell cycle.

Foresta *et al* (2011) presented evidence of viral transcripts in cytoplasm of oocytes by detection of E6/E7 mRNAs, following transmission by transfected sperm. It is possible that conditions for HPV replication are better in fertilized oocytes, as these are rapidly dividing. Viral transcripts in cytoplasm is a sign of active transcription in nucleus, as the transcripts will be translated in ribosomes in the cytoplasm. Differently, Kadze *et al* (2012) detected E6/E7 in nucleus of cumulus

cells, suggesting that HPV has a strategy to enter nucleus of several female cell types. However, the only possibility for independent transcription of E6/E7 in oocytes, is if the virus has entered the nucleus and the host cell possesses all necessary transcription factors for HPV. Foresta *et al* (2011) was the only group performing transfection of plasmid containing HPV genes, which possess its own promotor sequences that may be recognized by cellular transcription factors, and the reported E6/E7 mRNA findings do not necessarily reflect what would normally take place during HPV life cycle, as E2 in particular, is needed for transcription of E6/E7 *in vivo* (Graham, 2017).

HPV follow a cell layer dependent replication with a strict differentiation program, thus making *in vitro* production of infective viruses challenging. A detailed internalization mechanism into nucleus is therefore not elucidated, which would be an essential key aspect of this thesis. Based on the *in vitro* evidences, it is shown that sperm cells when carrying HPV DNA are able to transmit it to female cells, both under and after fertilization. It is also shown that transcription may take place in fertilized oocyte from the plasmid constructs, suggesting that not only chromosomal, but also episomal DNA may gain access from the sperm cell to the oocytes upon fertilization. However, it cannot be confirmed that HPV would be able to transcribe and replicate in fertilized oocytes in a natural infection event, even though the findings serve as leading threads that require more research.

5.2 Interpretation of *In Vivo* Results

Since *in vitro* studies does not represent natural infection, *in vivo* studies need to confirm discoveries. The *in vivo* studies analyzed semen samples from different male patients by targeting HPV DNA and RNA sequences.

Five studies detected HPV DNA on sperm cells in semen from infertile couples undergoing IVF (Schillaci *et al*., 2013), HPV infected patients (Foresta *et al*., 2013; Garolla *et al*., 2012a; Garolla *et al*., 2016) and sperm donors (Kaspersen *et al*., 2011). The first study suspect sperm as HPV carrier into oocyte but claim that correlation to infertility is inconclusive based on these evidences. Schillaci *et al* (2013) analyzed semen from 308 males from couples undergoing IVF treatment. From this group of patients, HPV DNA was only detected in 8% and with that perspective, this study is considered less informative on this issue. However, this

indicates that multiple factors may cause infertility, and in this case, HPV was not the main cause.

The 4 remaining studies suspect correlation between HPV and infertility, and Foresta *et al* (2013) even suggest further transmission through placenta and to fetal brain causing disabilities. In theory, this transmission route is possible, given that placenta develop inside the blastocyst (Younes *et al*., 2009) and HPV has been found in blastocyst (Chan, 2000). This hypothesis is mutually exclusive with two other studies, *in vitro* and *in vivo*, which suggest stage arrest during embryo development with HPV infection (Dona *et al*., 2018; Garolla *et al*., 2016). However, Foresta *et al* (2013) do not discuss at what time point the mother gets infected in her reproductive organs. If infection is at early embryonic stage, concomitant with fertilization, it is unlikely that embryo development will succeed, and placenta will not develop. If infection of placental tissue happens at a later stage, through ascending infection from the cervix, for example, transplacental infection further to fetal brain could be possible. It has been hypothesized that Hofbauer cells, the placental macrophages, transport HPV virus to fetal brain as ZIKV does (Jurado *et al*., 2016). However, transplacental infection could affect the pregnancy outcome and embryo development.

Unlike Schillaci *et al* (2013), Foresta *et al* (2013) focused on HPV infected patients and found reduced motility in sperm cells of infected males, a theory confirmed by several studies presented in this thesis (Dona *et al*., 2018; Foresta *et al*., 2010; Foresta *et al*., 2011; Foresta *et al*., 2013; Garolla *et al*., 2012a; Lai *et al*., 1997).

Three research groups searched for E6/E7 in semen from infertile males (Foresta *et al*., 2010) and randomly selected patients (Lai *et al*., 1996; Lai *et al*., 1997). The two latter research groups suggested active viral transcription in sperm cells, and Foresta *et al* (2010) found HPV DNA in sperm nucleus in 73% of infertile patients (108 subjects). Interestingly, other patient groups in Foresta's study with genital warts and infected partners also had HPV infection, but in non-spermatozoa cells. This might indicate that HPV has a critical role on sperm function and infertility, due to the high rate of sperm infection in infertile males.

Both studies conducted by Garolla *et al* detected HPV DNA in/on sperm cell surface, *in vitro* in 2012 and *in vivo* in 2016. The study from 2012 also had an *in vivo* experiment where HPV DNA was detected in semen, most likely at the sperm cell

surface as the same procedure as for sperm cells was used. This is not stated as such in the study, but due to clearance of HPV following treatment of sperm cells with heparinase III it seems likely that HPV is present on sperm surface. Moreover, they reported reduced sperm motility in naturally infected patients, which might indicate spermatozoa HPV location and attachment of HPV to the sperm cells. Garolla *et al* (2016) do not specify if the HPV DNA is present inside or outside sperm cells. Due to figures and comments in the study, it can be postulated that HPV was present on the surface of sperm cells, but this is not for sure. Higher rate of miscarriages and lower pregnancy rate was reported in infected couples. Garolla *et al* (2016) also suggested sperm as a carrier of HPV viral particles into oocytes, but with reduced penetration rate and they postulate that viral replication is possible in female cells.

Golob *et al* (2014) used 340 males from infertile couples to analyze for HPV infection, and 40% of the males were positive in their external genitalia, while 13% had HPV positive semen samples. No significant difference was observed between the different patient groups and the researchers claim that HPV has no correlation to health outcomes. Golob *et al* (2014) did not comment on what location HPV DNA was detected, and it is possible that HPV was present in non-spermatozoa cells in semen. This was evidenced by Foresta *et al* (2013) where infertility rate was higher in males with sperm infection, while the non-spermatozoa HPV infection was related to other conditions than infertility. This study is therefore less reliable than other studies, as there was no description of HPV cell-type location, while fluorescence *in situ* hybridization (FISH) is considered the best way to analyze and map viral infection (Garolla *et al*., 2018). It is apparent from *in vivo* studies that clinical outcomes have been more essential than the molecular mechanism and replication, but HPV seems definitely present in reproductive cells with possible correlation to health conditions.

5.3 Overall literature assessment

From the *in vitro* studies, it has been evidenced that HPV is able to attach to syndecan-1 at the equatorial region on sperm head and thus may transmit to female and fetal cells. In addition to HPV detection on sperm surface, *in vivo* studies have detected early genes in sperm cells, suggesting that sperm is susceptible for HPV despite the lack of the co-receptors HPV normally needs for entry into basal epithelial

cells. These studies have not detected HPV in female or fetal cells as they only analyzed semen samples, but the authors suspect transmission to female and fetal cells where replication may be possible.

All discoveries confirm that sperm definitely connects with HPV, as the main receptor is documented on sperm surface and HPV is found in/on sperm cells in all studies. Considering uptake of HPV DNA *in vitro* in sperm cells (Chan 2000), detection of mRNA E6/E7 in sperm cells (Lai *et al*., 1996; Lai *et al*., 1997) and E6/E7 in nucleus of sperm cells (Foresta *et al*. 2010), it is possible that HPV are able to transcribe and replicate in sperm. As previously mentioned, this indicate that sperm possesses the right transcription factors for expression of early viral genes, similar to basal/parabasal epithelial cells. All together, these three *in vivo* studies suggest that sperm is partly permissive for HPV and they confirm Chan's *in vitro* evidence of HPV DNA uptake in sperm (Foresta *et al*., 2010; Lai *et al*., 1996; Lai *et al*., 1997). The virus may be a hazard even though replication is incomplete, with no further viral particles produced, and further research is required to establish this aspect.

Figure 17 presents the two main findings from the included studies, where HPV is presented in yellow. Both theories of HPV infection in/on sperm cells allow the virus to penetrate the oocyte and release its genome. These scenarios require what is known from epithelial infections, that the capsid structure dissolves in the endosome, and that the viral genome can penetrate the host nucleus (oocyte in this case) or be integrated in the sperm genome prior to fertilization. This elucidates the need for further investigation of how HPV exactly interacts with sperm cells.

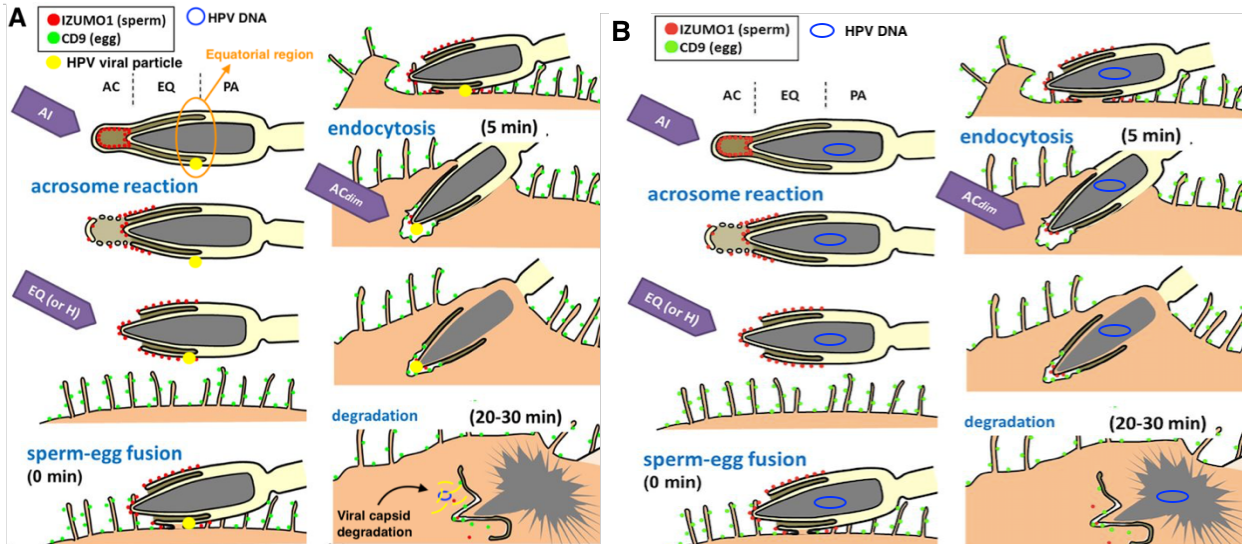

Figure 17 – The two main hypothesis of how HPV interact with sperm cells. A – presents the most common finding where HPV (yellow) is attached to the syndecan-1 receptor at the equatorial region (orange) of sperm cells. The virus is internalized in endosome during the fusion and endocytosis of the sperm cell and oocyte, and it can be speculated that the capsid dissolves in the endosome, releasing the L2/HPV DNA complex as for HPV entry in basal epithelial cells. B – Presents the second hypothesis, where HPV DNA is already internalized and present in the nucleus in the sperm cell upon fusion. In that hypothesis, HPV DNA is simply released together with the sperm nucleus content inside the oocyte. Figure modified from Satouh *et al* (2012).

Whether oocytes are susceptible for HPV is unrevealed and it is uncertain if HPV would have entered the oocyte *in vivo* without hitchhiking with the sperm cell, but HPVs definitely have a strategy to infect female and fetal cells as well. Female cells may be partly permissive for HPV, as fertilized oocytes cells are rapidly dividing. There is still skepticism around this aspect, because evidences from natural infections are still missing and *in vitro* studies have not looked at important genes for early transcription (E1/E2). However, the findings in fertilized oocytes and fetal cells are important, showing that episomal HPV DNA may transmit to oocyte upon fertilization, and to blastocysts. More research on this issue is strongly recommended and whether cases of early miscarriages might be attributable to HPV infections in the initial developmental stages.

The majority of studies suspect HPV as a cause of infertility, due to stage arrest of embryo development and reduced sperm parameters. Researchers recommend caution when it comes to IVF techniques, where sperm may be infected, and they point out the need for more research on this field to support their evidences. Regarding the impact HPV has on reproductive cells, it is likely that miscarriage is a possible outcome of infection.

A major criticism regarding the two studies claiming that HPV and infertility cannot be associated, is that researchers examined males from infertile couples, and not primarily HPV-infected patients, and did not include a control group. Other infertility factors might be present, and these studies do not provide enough evidence to intercorrelate HPV to infertility.

5.4 Considerations based on other studies

Some research groups have done investigations on HPVs role in epilepsy and autism, and have found a connection and suggested correlation (Chen *et al*., 2012; Godar & Merrill, 2017). Although these papers found connections, there is very little scientific understanding of factors involved in these diagnoses. Much uncertainty still exists about the correlation and more extensive research must be done to investigate an eventual role of HPV. It is important to bear in mind the possible publication bias in these findings.

Publication bias is the fact that studies with positive outcome and desired results are more likely to be published and included in review papers than experiments with negative outcome. Publication bias should therefore be taken into account, as unpublished studies could have provided controversial findings and given different results from review papers. In addition, many articles were unavailable in this literature search that could have been important, and there is also a chance for errors when including and excluding papers. The fact that some authors have participated in many of the included studies, might affect the results if they have focused on the desired results, excluding that other factors may affect their findings. It is apparent in the fraction size of author participation in studies that few research groups are working on this field, as the same authors appear in both original studies, review papers and other references related to this issue.

Different review papers that appeared in the systematic search, conclude that HPV plays a role on sperm motility, health conditions and adverse pregnancy outcomes, such as infertility, miscarriages and fetal pathology (Garolla *et al*., 2011; Garolla *et al*., 2018; Gizzo *et al*., 2014; Liu *et al*., 2018; Pereira *et al*., 2015; Souho *et al*., 2015). Both men and women in a couple are at risk for infertility with HPV infection, as the virus reduces sperm motility in men, and prevents embryo development in women (Pereira *et al*., 2015). Authors from these review papers

strongly recommend screening of sperm banks before donating sperm to women, as HPV might explain some of the mysterious infertility rate and unexplained miscarriages (Gizzo *et al*., 2014). As previously mentioned, *in vitro* studies suspect HPV to hitchhike outside the sperm, using it as a vehicle into oocytes, and that viral transcription are activated due to rapid division in these cells (Garolla *et al*., 2011).

As *in vitro* studies provide artificial conditions, authors recommend further *in vivo* research to confirm the *in vitro* speculations and screening of sperm banks to eliminate HPV infections. Another review paper associated HPV infection with apoptosis of sperm cells and embryonic cells, decreased sperm cell count and motility and increased rate of miscarriage and premature rupture of membrane in pregnant females (Souho *et al*., 2015). In addition to this, another group confirmed HPV infection in most parts of the male reproductive tract, where HPV attach to sperm surface and hitchhike to female tract (Liu *et al*., 2018).

Other studies have also reported high prevalence of HPV infection in testicular and prostate cancer, and recommend further research on HPV in these cancer types as the virus may play an important role (Garolla *et al*., 2012b; Glenn *et al*., 2017). If the virus infects these organs, it is likely that transmission to sperm cells happens long before ejaculation which could explain where the sperm cells get infected. It can be postulated that replication may be possible during sperm development, but so far this remains speculative. As HPV have been reported in the semen and sperm production sites, it may be able to replicate during the spermatogenesis. This may be possible in oogenesis as well, as HPV has been reported in endometrial cancer (Olesen *et al*., 2014) and ovarian cancer (Rosa *et al*., 2013) in females. Due to lack of oocytes in research and the limited number of oocytes in females, this aspect is harder to investigate.

In Norway, girls in 7th grade have been vaccinated against HPV types 16 and 18, or HPV types 16, 18, 6 and 11, in the Childhood Immunisation Programme for years. According to Garolla *et al* (2018), the vaccine is proved to be effective in clearing HPV infection from semen of infertile HPV positive men, and from autumn 2018 boys were included in the Norwegian Immunisation Programme (Folkehelseinstituttet, 2018). It is advantageous to vaccinate children, as they get a better level of antibodies and they have not debuted sexually which ensure that infection will not occur, as the virus will be neutralized in the genital tract. If infected men get vaccinated when HPV is present on sperm cells, the binding of antibodies to HPV

can reduce the sperm motility more than it already is. According to Garolla *et al* (2018) this is only during active infection and the vaccine will still be effective, but not at the same level as for uninfected patients.

6 Conlusions

A strong relationship between HPV and sperm cells has been reported in the literature as reviewed in this thesis. Whether HPV hitchhikes on the surface or rather is internalized in sperm cells remains to be established, but hitchhiking appears to be one main plausible mechanism. The fusion between HPV carrying sperm and oocyte permits the virus to enter the oocyte. HPV has been detected in several female or fetal cells, causing health conditions in a range from stage arrest of embryo development to miscarriages. Replication and transcription have been reported in both reproductive cell types, but none of them seem to be 100% permissive for HPV or able to produce new virus particles. Most research on this field connects HPV to infertility and miscarriages, but connection to epilepsy and autism require further research. Numerous researchers recommend screening of sperm banks, as these might contain infected sperm. Vaccination has been reported to be effective against HPV infection for both male and females, and vaccinating young boys, as initiated in Norway, might impact positively on reproductive health in the future.

Finally, further investigation is highly recommended with respect to HPV in reproductive cells. This literature assessment has provided insight on how HPV may bind to sperm cells, or replicate in sperm cells, and on the plausible transmission to oocytes in the fertilization process. Females who had a miscarriage and infertile couples should be tested as a routine to map the prevalence of infection, and the potential therapeutic use of HPV vaccine in infertile HPV positive couple should be investigated further, as HPV may seem to cause more harm to humans than previously recognized.

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Supplementary table 1

Results from systematic search on “Human Papillomavirus Sperm Cells” And “Human Papillomavirus Oocyte” from PubMed and Web of Science

A systematic search has been done for HPV in sperm cells and oocytes, provided in the following table.
The table provides insight in the relevant studies connected to this issue,
what search the article appeared in and what type of study it is (*in vitro* or patient *in vivo* study).

Reference	Method	Results	Conclusion
(Chan, 2000) (Part 1, 2, 3) 1 – Unavailable in full text 2 - (Chan *et al*., 1995) 3 - (Cabrera *et al*., 1997) ▪ ***In vitro* studies** ▪ "Sperm-mediated DNA transfer to cells of the uterus and embryo " ▪ Sperm cell and oocyte search	This article is based on three experiments from the same author. In the first experiment sperm cells were incubated with HPV L1 gene fragment for 30 minutes and washed through three different procedures, to assess HPV DNA uptake in sperm cells. In the second study part, mouse blastocysts got exposed to unspecified HPV DNA fragments from four different HPV types (HPV6, 11, 16 and 18) to examine the uptake of HPV DNA into blastocysts. The third experiment was done with a glass-tube, and repeated with excised mouse reproductive tract, to	In the first experiment L1 DNA was detected in sperm cells after all three washing procedures, suggesting uptake of the HPV L1 gene to sperm cells. Referred to earlier studies who also confirmed uptake of DNA from HPV 16 and 18. The second experiment showed uptake of HPV DNA in blastocyst, but at unequal rates for the different genotypes. Chan *et al* suggest a passive uptake of HPV in blastocysts. In the third experiment, DNA from HPV18 but not from the other genotypes in the glass tube experiments was detected in the	The first two study parts suggest that HPV 16 and 18 DNA is taken up in sperm and HPV DNA taken up in blastocyst. The last study part suggest that the sperm is able to carry HPV DNA and transmit to blastocysts, where the DNA is taken up in the cells. From the last experiment, it is not confirmed if the HPV DNA is integrated in the genome, existing as episomes or destroyed in the blastocyst. Authors refers to other studies that used the same HPV DNA region (E6/E7) where it was detected as integrated in the host genome.

	see if sperm could serve as a self-propelled delivering system to transmit DNA to the blastocyst. Sperm carrying E6/E7 DNA fragments from HPV-16, -18, 31 and -33 (glass-tube experiment), or HPV-16 and 18 (reproductive tract experiment) on one hand and mouse blastocysts on the other hand were placed at each end of tube/mouse uterine tract for 2 hours to examine infection of blastocyst by HPV carrying sperm.	blastocyst. HPV E6/E7 was evidenced in blastocysts in the uterine tract experiment, but only HPV 18 DNA was detected in uterine cells. HPV E6/E7 was detected in inner cell mass and trophoblasts by PCR. DNase I was used to destroy all DNA outside cell, suggesting that the HPV DNA was internalized in the cell.	The evidence presented in this section suggests that sperm is able to take up exogenous HPV DNA and transmit to blastocysts and the reproductive tract.
(Dona *et al*., 2018) ▪ ***In vitro* study** ▪ "Astaxanthin Prevents Human Papillomavirus L1 Protein Binding in Human Sperm Membranes" ▪ Sperm cell search	Sperm from 40 healthy male donors was incubated with 10 μg/mL HPV capsid L1 proteins and analysed with immunocytochemistry.	Shows 100% L1 protein binding on sperm head membrane and detrimental effect on sperm motility. Astaxanthin reduces L1 binding with more than 50%.	Confirm HPV L1 protein binding to sperm head and suggest HPV as a possible cause of infertility and stage arrest of embryo development. Recommend further research and use of astaxanthin due to antiviral effect.

(Foresta *et al*., 2010) ▪ **Patient *in vivo* study** ▪ "Clinical and prognostic significance of human papillomavirus DNA in the sperm or exfoliated cells of infertile patients and subjects with risk factors" ▪ Sperm cell search	200 patients: 108 infertile, 66 with HPV partners, 26 genital warts, 90 controls. FISH analysis was done on all 290 subjects to analyse presence of HPV and correlation to infertility, with HPV E7 fragment being the target.	73% of infertile patients had prevalence of HPV DNA in semen, mostly located in nucleus of sperm cells. Other patient groups also had HPV infection, but located in exfoliated cells in semen, and not sperm cells. Either way, HPV was located at nuclear level. All patients (not controls) had reduced sperm motility.	Suggest HPV as a possible cause of infertility due to reduced sperm parameters. Due to this aspect, Foresta *et al* recommend caution when it comes to assisted fertilization, where sperm may be infected by HPV.
(Foresta *et al*., 2011) ▪ ***In vitro* study** ▪ "Mechanism of human papillomavirus binding to human spermatozoa and fertilizing ability of infected spermatozoa" ▪ Sperm cell and oocyte search	Semen from infected patient got analysed with FISH. To study the ability of fertilization, sperm cells got transfected with HPV-16 plasmid containing E6/E7 genes through hamster egg-human sperm penetration test (HEPT) and sperm cells also got exposed to HPV L1 capsid to get a better understanding of how the HPV attaches to sperm.	Semen from infected male patient showed HPV-16 in 25% of sperm cells. Transfection with E6/E7 and HEPT test showed signs of active transcription of E6/E7 with transcripts in cytoplasm of oocyte. Exposure of L1 capsid to sperm cells showed binding to syndecan-1 (HSPG) receptor at the equatorial region of the sperm	Confirm binding of HPV-L1 to syndecan-1 receptor. Sperm may function as a vector for HPV into oocyte, as both transfected sperm and sperm exposed to L1 were able to penetrate the oocyte, where HPV transcription was suspected activated. Foresta *et al* suggest association between

	E6/E7 DNA PCR, RNA RT-PCR and L1-immuncytochemistry were performed on fertilized hamster oocytes.	head. Incubation with sperm and HPV showed possibility to deliver viral genome into the sperm cell.	HPV and infertility, due to reduced motility of HPV carrying sperm cells and active viral transcription in fertilized oocyte with fatal consequences.
(Foresta *et al*., 2013) ▪ **Patient *in vivo* study** ▪ "Human papillomavirus proteins are found in peripheral blood and semen Cd20+ and Cd56+ cells during HPV-16 semen infection" ▪ Sperm cell search	16 patients with HPV-16 semen infection, 16 non-HPV controls from infertile couples. Used fluorescence *in situ* hybridization (FISH), immunofluorescence and different HPV antibodies to detect expression of HPV-16 markers (DNA, L1, E6 proteins) from semen samples and blood samples.	Found an average of HPV DNA (L1) presence on both sperm (26%) and non-spermatozoal cells (19%) in semen. Showed reduced total amount of sperm cells and sperm motility in HPV-16 positive sperm cells. Detected signal for both E6 and L1 proteins in almost all HPV-DNA positive leukocytes, where viral DNA and proteins were found more frequently in cytoplasm than in nucleus. Leukocyte target cells in both semen and PBMC were CD20+ (B cells), and CD56+ cells (Natural Killer cells). HSPG	Concludes widely, that this is the first study finding HPV in PBMC of male patients. Foresta *et al* observed reduced motility in HPV-16 positive sperm cells. HPV DNA was present on sperm cells, and the study suggest HPV and infertility association.

		receptor found on B-cells, which explains attachment, but requires co-receptors α6-integrin and A2t for complete internalization. Mention hypothesis regarding HPV and focal cortical dysplasia (FCD) and HPV in brain during embryo development through transplacental spread.	
(Garolla *et al*., 2012a) ▪ **Patient *in vivo* and *in vitro* study** ▪ "Human papillomavirus sperm infection and assisted reproduction: a dangerous hazard with a possible safe solution" ▪ Sperm cell search	22 HPV infected patients, 13 control subjects. FISH analysis was performed on patients, controls and control sperm samples incubated with HPV-16 L1 capsid proteins – before and after treatment with heparinase III to check if HPV attachment is reduced.	Naturally infected sperm cells showed significantly reduced sperm motility. Males with HPV semen infection had high prevalence (20%) of infected sperm. Garolla *et al* found that treatment with heparinase III eliminated HPV L1 from semen.	Suggest testing of all infertile males as HPV may cause infertility. Recommend couples who had miscarriages to get tested for HPV and to eliminate HPV from sperm through appropriate procedure.

(Garolla *et al*., 2016) ▪ **Patient *in vivo* study** ▪ "Spontaneous fertility and *In vitro* fertilization outcome: new evidence of human papillomavirus sperm infection" ▪ Sperm cell search	226 infertile couples; males were FISH analysed for unspecified HPV in sperm, with use of biotin-labeled HPV DNA probe, containing conserved HPV region. 172 couples were not infected, 54 had HPV in semen. Females got inseminated to look for differences in pregnancy outcome for couples with and without HPV infection.	63% of infected couples had miscarriage vs 17% of the uninfected couples. In addition, pregnancy rate was lower in infected couples. Found HPV DNA in 24% of males on sperm cell surface and suggest that sperm function as a carrier of HPV into the oocyte, but with reduced penetration rate compared to HPV-negative sperm. Garolla *et al* claims consequences of infection in a range from viral replication in fertilized oocytes and trophoblastic cells to early miscarriages. Suggest reduced blastocyst formation, at the 2-cell embryo stage. Authors managed to eliminate HPV in semen	Concludes that HPV use sperm as a carrier into the oocyte. Show a higher rate of adverse pregnancy outcomes in infected couples and suggest further research on this field. Claims that HPV is a cause of infertility due to the increased rate of miscarriages with infection, and the arrest of embryo development.

		through a washing procedure with heparinase III.	
(Golob *et al*., 2014) ▪ **Patient *in vivo* study** ▪ "High HPV infection prevalence in men from infertile couples and lack of relationship between seminal HPV infection and sperm quality" ▪ Sperm cell search	Semen from 340 males from infertile couples, 246 with normal sperm production and 94 with abnormal sperm parameters. Sperm samples and self-collected swabs of the whole penile surface were collected. 299 swab samples, and 316 semen samples had valid results for HPV DNA testing and genotyping, using the Linear Array HPV genotyping test from Roche (37 different HPV types). Semen samples were analysed macroscopic, and through microscope.	Overall presence of HPV infection from external genitalia from swab samples in males were ca 40%. HPV infection was found in 13.61% of semen samples, which is 43 of 316 patients. HPV-53 and HPV-CD6108 were the most prevalent types, with 2.85% and 2.53% respectively. No significant difference was found between patients with normal sperm and patients with abnormal sperm parameters, or between HPV infected patients and uninfected patients.	Golob *et al* concludes that HPV does not impair sperm quality, due to no significant difference between infected and uninfected group.
(Kadze *et al*., 2002) ▪ ***In vitro* study** ▪ "Temperature variable and the efficiency of	Semen sample from HPV-negative donor, was pre-treated at 3 different temperatures (4, 37 and 40 C) and exposed to PCR amplified E6/E7	Found transmission of HPV-16 DNA from head of sperm cells to between 52% and 68% of the cumulus cells (different	Kadze *et al* found sperm cells as vector for HPV to cumulus cells and suggest a possible further infection to oocyte causing

sperm mediated transfection of HPV16 DNA into cells." ▪ Sperm cell and oocyte search	HPV-16 DNA fragments stained with Sybr Gold. Used DNA fragments, instead of oncoproteins due to previous studies showing the sperm ability to deliver exogenous DNA. Cumulus cells, that normally surround the oocyte in the follicles and are released with the oocyte during ovulation, were incubated with the HPV-16 carrying sperm.	temperatures). The HPV-16 DNA was found in both nucleus and cytoplasm of the cumulus cells. Does not state an uptake mechanism to the cumulus cells, but suspect endocytosis or physical membrane contact through CD4 molecules.	failure of blastocyst implantation in the uterus.
(Kaspersen *et al*., 2011) ▪ **Patient *in vivo* study** ▪ "Identification of multiple HPV types on spermatozoa from human sperm donors" ▪ Sperm cell search	267 semen samples from 188 Danish sperm donors was analysed with a sensitive HPV array, targeting the L1 conserved region. In-situ hybridization was performed to examine HPV-sperm association.	HPV L1 DNA was detected in 16% of the donors, where 5% had multiple HPV infections. 67% of these was high-risk HPV types. 5.3% of the donors had two or more HPV types detected.	Concludes that oncogenic HPV types are frequent in men. Authors suspect HPV receptor at the equatorial region of the sperm head, with a strong connection between HPV and sperm cell. Recommend analysis of sperm banks before donating sperm to women.
(Lai *et al*., 1996) ▪ **Patient *in vivo* study**	Semen samples from 24 patients at fertility clinic. Used PCR/RT-PCR to	Provided evidence that HPV infected sperm is able to produce	Study suggest viral transcription in sperm cells due to detection

▪ "Human papillomavirus deoxyribonucleic acid and ribonucleic acid in seminal plasma and sperm cells" ▪ Sperm cell search	examine semen for HPV-16 and -18 DNA, RNA and mRNA (E6).	mRNA (E6 region). HPV16 was found in 25% of patient's sperm cells, with viral DNA-positive sperm indicating active transcription. HPV-18 was found in 11 of 24 patients (45.8%) and 5 of these 11 showed viral-specific transcripts. HPV-18 DNA and RNA found more present in sperm cells and seminal plasma than HPV-16.	of mRNA from E6 genes but are critical due to not enough evidence to conclude that HPV can multiply in sperm cells. Lai *et al* recommend further research on this field and wonder if sperm cells function as a carrier of HPV to other cells which may be a way to transmit the disease and infect epithelial cells where replication is allowed.
(Lai *et al*., 1997) ▪ **Patient *in vivo* study** ▪ "The effect of human papillomavirus infection on sperm cell motility" ▪ Sperm cell search	Specimens of semen from 24 randomly selected patients from fertility clinic. Examined presence of HPV16 DNA and RNA E6/E7 region with PCR/RT-PCR. Quality of semen and sperm function got analysed by computer-aided autoanalyzer.	HPV16 E6/E7 DNA was found in 25% of sperm cells, and HPV-16 RNA was found in 8%. HPV-18 DNA was found in 46% of the sperm cells specimens, and HPV-18 RNA was found in 21%. 75% of HPV infected patients had poor sperm movement, whereas 8% of uninfected patients had the same	Suggest HPV infection into oocyte with unclear entrance, and expression of oncogenic genes (E6/E7) in sperm cells. Showed that HPV DNA and viral-specific RNA can be found in sperm cells, which may affect sperm parameters. Sperm where HPV is detected is

		condition. Refer to another study (Lai *et al.* 1996) where expression of E6 and E7 oncogenes were reported in sperm cells.	associated with poor sperm movement.
(Perez-Andino *et al*., 2009) ▪ ***In vitro* study** ▪ "Adsorption of human papillomavirus 16 to live human sperm." ▪ Sperm cell search	*In vitro* system was used on sperm from four donors to test interaction between HPV L1 capsid protein and sperm cells. Fluorescent HPV-16 capsids were incubated with the sperm samples.	The virus (L1) attached to 72% of the sperm cells in neutral conditions (pH 7.4). Suggest binding to HSPGs, since this is the main receptor for HPV in epithelial cells, and sperm cell surface is covered with same receptors.	Suggest that viruses like HPV, HIV and HSV have a general strategy of hitchhiking with sperm cells to the female oocyte, like rhabdovirus does in salmon (Øvergård *et al*., 2017). Perez-Andino recommend blocking of HPV binding to sperm receptor to prevent sexual transmission of viral diseases.
(Schillaci *et al*., 2013) ▪ **Patient *in vivo* study** ▪ "Detection of oncogenic human papillomavirus	308 male patients from couples undergoing *In vitro* fertilization. *In situ* hybridization was performed to understand the sperm-HPV	HPV L1 DNA was detected in 8% of patients from infertile couples. Study confirm connection between HPV and receptor on the equatorial region on sperm	HPV L1 DNA was detected on sperm cells and located on the equatorial region. Study suggest sperm as carriers for HPV into oocyte, but this was not

genotypes on spermatozoa from male partners of infertile couples” ▪ Sperm cell search	connection, with HPV L1 region as the target for detection.	head. Suggest that sperm may carry HPV to the oocyte, and therefore recommend caution regarding assisted fertilization and screening of sperm banks.	evidence that HPV cause infertility.

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