TIINA PETÄJÄ

Human Papillomavirus Vaccine
Immune Response and
Protectivity

ACADEMIC DISSERTATION
To be presented, with the permission of
the Board of the School of Health Sciences
of the University of Tampere,
for public discussion in the Auditorium 2 of Frami B,
Kampusranta 9, Seinäjoki,
on March 7th, 2014, at 12 o’clock.

UNIVERSITY OF TAMPERE
To my family
Abstract

The human papillomavirus (HPV) infection is the most common sexually transmitted disease in the world. Most sexually active individuals of both sexes will acquire an HPV infection at some point in their lives. Although most HPV infections clear within two years, approximately 10% persist, possibly resulting in the development of precancerous lesions and cancer.

The most common high-risk (hr) HPV types 16 and 18 cause up to 70% of cervical cancers, and all hrHPVs are responsible for 5% and 10% of all cancers worldwide in males and females, respectively. HrHPVs cause up to 100% of cancers in the cervix, 90% in the anus, 35-50% in the oropharynx, 50% in the vulva, 64-91% in the vagina and 40-45% in the penis. Decreasing the transmission of prevalent undetected, asymptomatic, and long-term HPV infections by vaccination is therefore of utmost interest.

The licensed vaccines - the bivalent vaccine comprising HPV types 16 and 18 and the quadrivalent vaccine comprising HPV types 6, 11, 16 and 18 are safe and 90-100% efficacious against persistent infections and precancerous cervical lesions associated with HPV types 16 and 18. Both vaccines have also shown 97-100% efficacy against cervical intraepithelial neoplasia (CIN) 2+, CIN3, and adenocarcinoma in situ (AIS) associated with the vaccine types. Both vaccines protect against anogenital infections with the non-vaccine HPV type 31, and the bivalent vaccine additionally against the non-vaccine types 33, 45 and 51. Some vaccine efficacy against oropharyngeal infection has been shown for the bivalent vaccine and against anal HPV infection for both vaccines.

The purpose of this thesis is to evaluate the antibody responses following HPV 16/18 vaccination in adolescent girls and boys and young women and men. The antibody responses up to four years following the HPV 16/18 vaccination were evaluated in adolescent girls and young women. Also, antibody levels of HPV 16/18 in serum samples and cervical secretions were compared in young women. The prevalence of HPV deoxyribonucleic acid (DNA) was compared in HPV 16/18 vaccinated and non-vaccinated young men.
The University of Tampere, School of Health Sciences, conducted these prospective clinical studies in collaboration with GlaxoSmithKline at several study sites in Finland and abroad during 2004-2013.

The immune responses in adolescent girls and boys aged 10-14, and young women and men aged 15 to 25, were excellent, with approximately 100- and 40-fold (HPV 16 and 18) higher antibody titres as compared to those resulting from natural HPV infections. The antibody titres were higher in younger age groups of both genders than in young women. Sustainability of the high antibody levels following vaccination with the HPV 16/18 vaccine was demonstrated in adolescent girls and young women up to four years. The level of mucosal antibodies correlated with the serum antibody levels in young women four years post vaccination. HPV 16/18 antibodies, transudated through the epithelium to mucosal surfaces, provide protection at sites where infection occurs. Vaccination also reduced the prevalence of vaccine HPV types HPV 16 and 18 in males, ensuring reduced transmissibility.

High antibody levels will most likely persist for a long time. As HPV infections are usually acquired within three years of sexual debut, the HPV vaccination should be administered prior to this to both girls and boys, to achieve the desired herd immunity with a reduction in the global HPV burden.
Ihmisen papillomaviruksen (HPV) aiheuttama infektio on maailman yleisin sukupuoliteitse tarttuva tauti. Suurin osa seksuaalisesti aktiivisista ihmisistä saa HPV-infektion jossakin vaiheessa. Valtaosalla HPV-infektio paranee kahden vuoden sisällä, mutta noin 10 % infektiosta pitkittyvyyt, mikä mahdollistaa syövän esiasteiden ja syövän kehittymisen.

Yleisimmät suuren riskin HPV-tyypit 16 ja 18 aiheuttavat jopa 70 % kohdunkaulan syövistä. Yhteensä kaikki suuren riskin HPV-tyypit aiheuttavat maailmanlaajuisesti 5 % miesten ja 10 % naisten syövistä. Näistä 100 % kohdunkaulan, 90 % peräaukon, 35–50 % suun ja nielun, 50 % ulkosynnyttimien, 64–91 % emäntä ja 40–45 % peniksen syövistä on suuren riskin HPV:n aiheuttamia. Tavallisen mutta yleensä oireettoman HPV-infektion esiintyvyyden vähentäminen rokottamalla on tärkeää syöpien ehkäisemiseksi.

Nykyään saatavilla olevat kaksi HPV-rokotetta, kaksivaikutteinen rokote joka sisältää HPV-typit 16 ja 18, ja nelivaikutteinen rokote joka sisältää HPV-typit 6, 11, 16 ja 18, ovat turvallisia. Molemmat suojaavat tehokkaasti (90–100 %) pitkittynneeltä, kuusi kuukautta kestävältä kohdunkaulan HPV 16/18 infektiolta ja syövän esiasteilta. Rokotteet antavat jopa 97–100 % suojan vakavampia (CIN 2+, CIN3 ja AIS) kohdunkaulan solumuutoksia vastaan. Molemmat rokotteet vähentävät tehokkaasti peräaukon seudun HPV-infektiota ja kaksivaikutteinen rokote myös suun- ja nielualueen HPV-infektiota. Molemmat rokotteet vähentävät HPV-typin 31 ja kaksivaikutteinen rokote lisäksi HPV-tyyppeen 33, 45 ja 51 esiintyvyyttä peräaukonseudulla ja genitaalialueella immunologisen ristisuojan kautta.


On todennäköistä, että vasta-ainetasot pysyvät korkeina vuosikymmeniä. HPV-rokote tulisi antaa tytöille ja pojille ennen seksuaalisesti aktiivisen elämän alkamista mahdollisimman pitkäikäisen suojan ja laumaimmuniteetin saavuttamiseksi sekä yleisen HPV-taustitaakan vähentämiseksi.
List of original publications

This dissertation is based on the following papers, which are referred to in the text by their Roman numerals.


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Abbreviations

ADC  cervical adenocarcinoma
AIS  adenocarcinoma in situ
AIN  anal intraepithelial neoplasia
APC  antigen presenting cell
ATP  according-to-protocol
CI   confidence interval
CIN  cervical intraepithelial neoplasia
CIS  carcinoma in situ
CVS  cervicovaginal sample
CRPV cottontail rabbit papillomavirus
DC   dendritic cell
DNA  deoxyribonucleic acid
E    early protein
ECM  extracellular matrix
EGL  external genital lesion
EGW  external anogenital warts
ELISA enzyme-linked immunosorbent assay
EU/ml ELISA units per milliliter
FVU  first void urine
FUTURE Females United to Unilaterally Reduce Endo/Ectocervical disease
GMT  geometric mean titre
HC   Hybrid Capture
HIM-study HPV In Men study
HIV  human immunodeficiency virus
HNSCC head and neck squamous cell carcinoma
HPV  human papillomavirus
hr   high-risk
HSIL high-grade squamous intraepithelial lesion
IARC International Agency for Research on Cancer
ICTV International Committee on the Taxonomy of Viruses
IFN  interferon
IL   interleukin
ITT  intention-to-treat population
IUD  intrauterine device
L    late protein
LBC  liquid based cytology
LC   Langerhans cell
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>LCR</td>
<td>long control region</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>lr</td>
<td>low-risk</td>
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<tr>
<td>LSIL</td>
<td>low-grade squamous intraepithelial lesion</td>
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<tr>
<td>MGP</td>
<td>modified general primer</td>
</tr>
<tr>
<td>MPL</td>
<td>monophosphoryl lipid A</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSM</td>
<td>men having sex with men</td>
</tr>
<tr>
<td>NASBA</td>
<td>nucleic acid sequence-based amplification</td>
</tr>
<tr>
<td>NK cell</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frames</td>
</tr>
<tr>
<td>Pap smear</td>
<td>Papanicolaou smear</td>
</tr>
<tr>
<td>PATRICIA</td>
<td>PApilloma TRIal against Cancer In young Adults</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PIN</td>
<td>penile intraepithelial neoplasia</td>
</tr>
<tr>
<td>PPP</td>
<td>per-protocol population</td>
</tr>
<tr>
<td>PV</td>
<td>papillomavirus</td>
</tr>
<tr>
<td>Rb</td>
<td>retinoblastoma</td>
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<tr>
<td>RFLP</td>
<td>restriction-fragment length polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RRP</td>
<td>recurrent respiratory papillomatosis</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SCC</td>
<td>squamous cell carcinoma</td>
</tr>
<tr>
<td>STI</td>
<td>sexually transmitted infection</td>
</tr>
<tr>
<td>Tc cell</td>
<td>cytotoxic T cell</td>
</tr>
<tr>
<td>Th cell</td>
<td>helper T cell</td>
</tr>
<tr>
<td>TMA</td>
<td>transcription-mediated amplification</td>
</tr>
<tr>
<td>TVC</td>
<td>total vaccinated cohort</td>
</tr>
<tr>
<td>VAIN</td>
<td>vaginal intraepithelial neoplasia</td>
</tr>
<tr>
<td>VE</td>
<td>vaccine efficacy</td>
</tr>
<tr>
<td>VIN</td>
<td>vulvar intraepithelial neoplasia</td>
</tr>
<tr>
<td>VLP</td>
<td>virus-like particle</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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1 INTRODUCTION

Human papillomavirus (HPV) infection is the most common sexually transmitted disease worldwide with the highest prevalence in young women and men. Most sexually active individuals of both sexes will acquire a genital HPV infection at some point in life. While most HPV infections resolve spontaneously within two years, the persistence of cervical high-risk (hr) HPV infection combined with certain risk factors like smoking and Chlamydia trachomatis infection increase the risk of progression of cervical precancerous lesions and cancer. The prevalence of other HPV-related diseases like head and neck or anal cancer is increasing among both women and men. The most common oncogenic HPV types 16 and 18 are responsible for approximately 70% of all cervical cancers (Bosch et al. 2008), 75-80% of anal cancers (Hoots et al. 2009) and 86% of HPV positive oropharyngeal cancers (Kreimer et al. 2005). Up to 5.2% of cancers worldwide are estimated to be associated with HPV infection (Parkin 2006).

Papillomaviruses infect epithelial cells and depend on their differentiation to complete their lifecycle. HPV infection causes no cell death or viremia to trigger the host’s immune system, and it can stay undetected for long times. Natural HPV infection induces only low levels of serum antibodies resulting in insufficient protection against reinfection or infection by other HPV types (Palmroth et al. 2010).

Vaccination is a powerful tool in protecting vulnerable populations against specific infections. Prophylactic vaccines are effective in preventing HPV infection and cervical lesions in females negative for vaccine type HPVs (Lehtinen & Dillner et al. 2013). As HPV infection is sexually transmitted, the vaccine has to be administered before sexual debut to achieve high efficacy and protection against HPV types covered by the vaccine.

In this thesis the HPV 16/18 vaccine-induced serum antibody levels were studied in adolescent girls aged 10-14 years and young women aged 15-25 years up to four years post vaccination. In addition, the presence of vaccine induced anti-
HPV 16/18 antibodies in cervicovaginal secretions of young women was evaluated. The HPV 16/18 vaccine-induced antibody levels were also studied in boys aged 10-18 years one month after completing the three-dose (0, 1 and 6 months) vaccination schedule. The presence of HPV DNA in urine samples was compared in vaccinated and unvaccinated young men aged 18-19 years four years post vaccination.
2 REVIEW OF THE LITERATURE

2.1 History of human papillomaviruses (HPV)

Papillomaviruses (PV) are highly species-specific and cause epithelial infections in most mammals and birds (de Villiers et al. 2004). The first PV was identified in the warts of cottontail rabbits (Shope & Hurst et al. 1933). Later, in 1935, Francis Peyton Rous discovered that the cottontail rabbit papillomavirus (CRPV) can induce malignant transformation and neoplastic growth in the skin of affected rabbits. This was the first direct proof that a virus could cause cancer in mammals (Rous & Beard 1935). Rous received the Nobel Prize for his work on tumour viruses in 1966.

In 1975 Harald zur Hausen postulated, after failing to find herpes simplex virus type 2 DNA in cervical cancer samples, the connection between HPV and cervical cancer (zur Hausen et al. 1975, zur Hausen 1976). Some years later his research group identified two novel HPV types, 16 and 18, in squamous cell carcinoma and adenocarcinoma of the uterine cervix, respectively (Dürst et al. 1983, Boshart et al. 1984). The discovery of human papillomaviruses as causes of human cancers resulted in the Nobel Prize being given to zur Hausen in 2008.

The causal association of HPV 16 and HPV 18 with cervical cancer was taken a step further in Nordic longitudinal epidemiologic studies, indicating a 10- to 20-fold risk to develop cervical cancer in women infected with these viruses 10 to 20 years earlier (Lehtinen et al. 1996, Wallin et al. 1999). Final proof of causal association, i.e. demonstration that prophylactic HPV 16/18 vaccination protects against cervical cancer, is emerging.

Like the hepatitis B-virus surface antigen, the major structural L1 protein of HPV was found to form virus-like particles (VLP) (Zhou et al. 1991, Kirnbauer et al. 1992). This discovery led to the improvement of HPV serology and, most importantly, to the development of prophylactic HPV vaccines (Schiller & Heldesheim 2000, Koutsky et al. 2002), the first two of which were licensed in 2006/2007.
2.2 Structure, classification and replication of papillomaviruses

Papillomaviruses are circular nonenveloped double-stranded DNA-viruses with an icosahedral capsid structure. The virus is about 55 nm in diameter and its DNA contains approximately 8000 base-pairs. Its protein shell or capsid is composed of 72 capsomeres comprising of two structural or late proteins L1 and L2. L1 represents 80% of the capsid and is called the major structural protein of the virus.

The ICTV (International Committee on the Taxonomy of Viruses) has accepted that papillomaviruses are a distinct family of viruses. Human papillomaviruses belong to this *Papillomaviridae* family. By comparing the L1 gene, papillomaviruses have been divided into genera, species (clades) and types. The genera, which are identified by Greek letters, include species that are phylogenetically related but often biologically quite diverse. There are 16 genera that include all known papillomaviruses. The clinically most significant genus is that of alpha-papillomaviruses, which contains all HPV types associated with mucosal and genital lesions. Papillomaviruses that have a 60-70% homology are classified as being part of the same species (de Villiers et al. 2004, Bernard 2005).

All HPV types of the alpha-papillomavirus genus form 15 species, based on their genomic sequences. Twelve of the 15 HPV types, which are classified as high-risk (hr) HPV types due to their association with cervical cancer, are members of two species: A7 and A9. HPV 16 of the A9 species and HPV 18 of the A7 species are the most common representatives of their species and also the most common hrHPV types.

The genome of about 120 different HPV types have been isolated and completely sequenced. The HPVs are divided into different types based on the most conserved gene in the L1 ORF (open reading frame). An HPV type is defined as a complete genome, whose L1 gene sequence is at least 10% dissimilar to that of another HPV type. A difference of 2-10% in the L1 region defines a subtype, and less than 2% difference a variant (de Villiers et al. 2004, Bernard 2005). Of the 120 HPV types isolated, approximately 40 types infect mucosal epithelia. These anogenital HPVs are divided into high-risk and low-risk (lr) types, depending on their capacity of causing malignant transformation in cervical cells.

HPV types have a genome consisting of eight ORFs, which encodes two late, structural proteins (L1 and L2) and six early proteins (E1, E2, E4, E5, E6 and E7). In addition, the genome contains a long control region (LCR) devoid of any protein-coding function. The early genes cover about 50% of the genome, and the early proteins are responsible for viral DNA replication (E1), transcription (E2),
transformation (E5, E6, E7) and structural changes of the host cell following viral replication (E4) (Figure 1).

![HPV genome](image)

**Figure 1.** HPV genome (Doorbar 2006). Permission from Portland Press.

The regulatory proteins E1 and E2 are necessary for viral DNA replication and transcription in all HPV infected cells. HPV E4 protein is expressed at high level in cells that support viral genome amplification (Griffin et al. 2012). E5, E6 and E7 proteins encoded by hrHPVs are called oncoproteins and are responsible for initiation and progression of cellular transformation leading to cancer.

### 2.2.1 Viral life cycle

Papillomaviruses are perfectly adapted to their natural host tissues, mucosa and skin, the differentiation of which sets the stage for the viral life cycle (Doorbar 2005).

The proximal epithelia at each of the body openings are stratified squamous structures, while the internal epithelia are columnar. The lining between these structures is called the squamo-columnar junction, a metaplastic transformation zone comprising rapidly cycling and dividing keratinocytes. Such zones can be found in the larynx, nasal sinuses, urethra, cervix and anal/rectal junction. Metaplastic epithelial cells are particularly susceptible to papillomavirus infection as there is no protective overlay of differentiated or desquamating cells (Chow et al. 2010).
Papillomavirus life cycle begins when the virus infects the basal layer cells of the epithelium. This has been suggested to require a microwound in the stratified epithelium, allowing the virus to enter the basal cells. Basal cells, which are the only proliferating cells in normal epithelium, consist mainly of stem cells and parabasal cells or transit amplifying cells (Conway & Meyers 2009). It has also been suggested that a papillomavirus must infect an epithelial stem cell to establish persistent infection (Doorbar 2005, Maglennon et al. 2011).

There are cell surface receptors that allow initial attachment of the virus to the host cell, depending on the presence of heparan sulphate on the extracellular matrix (ECM) (Giroglou et al. 2001). The interaction between HPV 16 L1 and the ECM is very specific and mediated by a few amino acids, e.g. lysine in the FG- and HI-loops of the protein (Bishop et al. 2007). Inhibition of this interaction may be important in the antibody-mediated neutralization of the virus. The attached virus enters the cell slowly through endocytosis of clathrin-coated vesicles or a caveolin-mediated pathway, depending on the HPV type (Selinka et al. 2002, Bousarghin et al. 2003, Smith JL et al. 2007).

Inside the cell, the viral genome migrates into the nucleus where it is established as extrachromosomal elements or episomes. Episomal viral DNA is amplified to about 100 copies per cell soon after infection. E1 and E2 proteins are involved in viral DNA replication in basal and parabasal cells. In HPV 16 infection, E1 is needed at replication origins in initial replication, but not for the maintenance of productive replication (Egawa et al. 2012).

Mitotic activity of stem cells is a prerequisite for nuclear import of the viral genome. The HPV genome does not encode enzymes necessary for viral replication, and therefore HPVs must rely on the host cell replication proteins to provide viral DNA synthesis. Different genes are activated in the different epithelial cell layers, allowing different functions of the cells. The transit-amplifying cells both divide and differentiate. The division of transit-amplifying cells takes several months after which they reach terminal differentiation. Constant upward movement of transit-amplifying daughter cells, which form the full thickness of the differentiating epithelium, sets the stage for HPV replication. In the differentiating cells of the middle to upper epithelial layers, the production of structural viral L1 and L2 proteins and E4 protein takes place, and viral DNA replicates to a high copy number (Chow et al. 2010).
The structural proteins L1 and L2 are expressed in highly differentiated epithelial cells after the replication of the viral genome is completed. The genomes are packaged into self-assembling capsids in terminally differentiated cells. The infection causes no cytolysis and the progeny virions are not released until the infected cells reach the epithelial surface and die naturally (Figure 2).

**Figure 2.** The main stages of the papillomavirus life cycle. In normal epithelium (right side), the daughter cells of the epithelial stem cells divide along the basement membrane and then mature vertically through the epithelium without further division. After introduction of HPV into stem cells (left side) in the basal layer of the epithelium, expression of viral non-structural proteins occurs. Under the regulation of these proteins, the dividing-cell population expands vertically and epithelial cell differentiation is delayed and is less complete. Viral proteins are expressed sequentially with differentiation, and mature virions are produced only in the most superficial layers of the epithelium. Intraepithelial APCs are depleted in the HPV infected epithelium (Frazer 2004). Permission from Nature Publishing Group.

The life cycle of papillomaviruses takes 2-3 weeks, which corresponds to the time of a normal epithelial cell to undergo differentiation from a basal cell to a keratinized cell (Stanley 2010).
Persistent HPV infection and the development of cancer are possible because of immune evasion that enables the virus to stay undetected for a long time. In the absence of viremia and inflammation, the host’s immune response is not activated.

Viral genomes are not always cleared following regression of the lesions, but can remain at the site of infection, probably in the basal cells, in a silent state resembling latency. Reactivation of silent HPV infection is thought to occur in immnosuppressed individuals, as well as during pregnancy and during aging (Gonzalez et al. 2010, Maglennon et al. 2011, Rositch et al. 2012). Immunosuppression has, however, been shown to cause activation of the silent infection only in animal models (Doorbar 2011), and whether papillomaviruses cause latent infections is an open question.

Figure 3. A model of persistent papillomavirus infection and escape from immune surveillance. 1. An active infection follows the entry of papillomavirus into an epithelial stem cell in the basal layer of the epithelium. The cells in the basal layer and above are driven into a cell cycle allowing viral replication and production of new virions to occur in the intermediate and upper cell layers. Low-level protein production in the lower epithelial layers assists the virus in evading immune detection. 2. The triggering of an effective immune response leads to a regression of the lesion, accompanied by an infiltration of T cells. Viral gene expression is shut off and lesion regression occurs. 3. Viral persistence may ensue with viral genomes restricted to stem cells in the basal layer of the epithelium. Productive infection is prevented by host immune surveillance. 4. Certain conditions, such as immune suppression, may allow reactivation to occur. Completion of the virus life cycle may or may not be associated with the reappearance of a visible lesion. (Amended from Virology 2011, Maglennon et al.) Permission from Elsevier.
2.2.2 Malignant transformation

High-risk HPV's can expand the life span of epithelial cells, and thus also the persistence of an infection. Compared to high-risk HPV-types, low risk types are not efficient in inducing cellular immortalization and transformation (Münger & Howley 2002). In HPV infected cells the HPV genome is usually found as episomes in the nucleus, whereas in most high-grade lesions the genome is integrated into the host chromosome. The integration of HPV genome is considered to contribute to malignant transformation of the epithelial cells.

The HPV genome integration into the host cell genome results in a disruption of the E1-E2 region. The disruption of E2 leads to a deregulated expression of early viral genes and an increased proliferative capacity. This event increases the expression and stabilization of the E6 and E7 transcripts.

In normal cells, two main tumour suppressor proteins p53 and retinoblastoma (Rb) inhibit formation of tumours. Both p53 and Rb have an important role in cell cycle control. p53 controls apoptosis, and Rb prevents excessive cell growth. The E6 protein of hrHPVs binds cellular p53 protein, which promotes the degradation of p53 by the cellular ubiquitin proteolysis pathway. The E7 protein inactivates the cellular retinoblastoma protein (pRb), which leads to transcriptional activation of several genes involved in cell proliferation. This eventually interferes with the control of the cell cycle and DNA repair (Villa 2006a, Moody & Laimins 2010).

Both E6 and E7 are required for the immortalization of human keratinocytes. Binding of Rb by E7 can lead to inhibited cell growth and apoptosis through a tumour suppressor p53-dependent pathway, and to increased levels of p53. E6 in turn is able to bind p53, which leads to degradation of p53 and hence prevention of cell growth inhibition (de Villiers et al. 2004, Conway & Meyers 2009). The resulting genomic instability induces chromosomal rearrangements and mutations in cellular genes that contribute to the events (Moody & Laimins 2010). Furthermore, the E5-protein cooperates with E6 and E7 and enhances cell proliferation that contributes to transformation. The high-risk E7 protein binds Rb with higher affinity than low-risk E7. This factor may partly explain why low-risk HPV types do not cause high-grade cervical lesions or cancer.
2.3 Natural history of HPV infection

2.3.1 Immune response

The protective immune system, which is needed to resist and tackle infections, comprises the innate (non-specific) and adaptive (specific) immune system. Each of these has both cellular and humoral components by which they carry out protective functions. The innate immune system is the first-line defence in which immediate response to infectious or foreign agents is executed independent of the antigen. An inflammatory process, caused by cell injury or cell death, activates and recruits phagocytic and natural killer (NK) cells to the site of infection. The innate immune system has no specific memory but activates the adaptive system, which in turn is antigen-dependent and results in the development of immunologic memory. Antigen-presenting cells (APC), including dendritic cells (DC) which in the epithelium are known as Langerhans cells (LC), and macrophages, are important in innate immunity, and they also act as a link to evoke the adaptive immune system. On the epithelial surfaces, Langerhans cells are the main APCs that present the endocytosed HPV antigens to CD4+ T helper cells (Th), using the major histocompatibility complex class II molecules.

The cells of the adaptive immune system include the B and T lymphocytes. After exposure to an antigen, B cells differentiate into plasma cells, which are responsible for antibody production. T cells either differentiate into cytotoxic (Tc or CD8+) or T helper (Th or CD4+) cells of which there are two types, Th1 and Th2 cells.

Th1 cells secrete interferon-gamma (IFN-γ) as the main cytokine, which activates, generates and reinforces specific and non-specific cytotoxic cell-mediated immunity through CD8+ cells, macrophages and, NK cells, respectively. Th2 cells secrete interleukin 10 (IL-10) as the main cytokine and IL-4, and help antigen-primed B lymphocytes differentiate into plasma cells capable of secreting antibodies (Stanley 2006a).

In HPV infection, progeny virions are released through normal desquamation of the epithelium, which causes no cell death or destruction to activate the innate immune system. There is no viremic phase in the HPV life cycle, thus only minimal amounts of replicating virus are exposed to the immune system (Stanley 2006a).
There are only a little or no proinflammatory cytokines in HPV infection to activate and accomplish migration of APCs (Stanley 2008). The DC-line antigen-presenting cells usually detect virus capsid entry, but there is evidence that LCs are depleted from sites of HPV infections and are not activated by the uptake of HPV capsids. When incubated with HPV 16 L1 VLPs, LCs do not initiate epitope-specific immune response against L1 derived antigens (Fausch et al. 2002). Furthermore, non-professional antigen presenting cells (e.g. keratinocytes) can be induced by concomitant C.trachomatis infection to express MCH molecules without the accessory molecules (Lehtinen et al. 1995). In effect, the innate (and adaptive) immune system may be tolerized by VLP/HPV uptake (Fausch et al. 2002). Thus, the virus can be practically invisible to the immune system and can therefore remain undetected for a long period.

On the other hand, HPV-infected keratinocytes should activate the production of type-1 interferons that act as immunostimulatory agents and activate immature DCs. HrHPV types are able to inhibit interferon synthesis and the HPV 16 E6 and E7 oncoproteins can directly interfere with the interferon-signalling pathway (Kanodia et al. 2007).

Most HPV infections, however, resolve with time, despite of the viral effort to evade host defences. Once aroused, the cytotoxic T cells are the most important in the clearance of the virus. This has been demonstrated in the clearance of anogenital warts and low-grade intraepithelial lesions (Coleman et al.1994). The importance of T helper cell augmentation of the specific cell-mediated immune response in the immune surveillance of HPV infections is especially illustrated in immunosuppressed individuals, in whom both the incidence of HPV infections and the progression of HPV-associated lesions are increased (Palefsky & Gillison 2006).

Following genital HPV infection, antibodies specific for the HPV L1 major capsid protein have been demonstrated, but there is no correlation between HPV DNA detection and seroreactivity, or concordance between cervical DNA detection and co-existent seropositivity (Paaso et al. 2011). The sensitivity of HPV serology for past type-specific infection by HPV types 16, 18 and 33 is 65-75% (Kjellberg et al. 1999), but some women simply do not develop detectable HPV-type-specific responses (Dillner 1999a, Coseo et al. 2010). In most individuals with persistent HPV infection, however, seroconversion usually occurs between 6 and 18 months (Carter et al. 1996), but more rarely following incident HPV infections (Carter et al. 2000, Paaso et al. 2011). With higher natural infection-derived antibody titres the risk of subsequent re-infection is reduced (Safaeian et al. 2010). In general
the titres of neutralizing antibody levels induced by natural HPV infection immediately after seroconversion are low, and hence protection against reinfection is poor (Viscidi et al. 2004, Olsson et al. 2009). HIV-positive women show significantly less (66.7%) antibody response to HPV infection as compared with HIV-negative women (89.3%), due to impaired immunological functions associated with HIV (Giorgi et al. 2008).

In the community-based HPV 16/18 Costa Rica vaccine trial, about 37% of HPV 16 DNA-positive and 42% of HPV 18 DNA-positive individuals were seronegative. Seroprevalence increased with time since sexual debut, whereas HPV DNA prevalence did not (Coseo et al. 2010). The correlates of HPV 16 and/or HPV 18 seropositivity are strongly related to sexual behaviour, particularly to an increased number of lifetime sexual partners (Dillner et al. 1996).

Factors associated with sustained HPV exposure, such as an elevated HPV viral load, increasing lifetime partners, or use of hormonal contraception, seem to predict HPV 16 but not HPV 18 seropositivity (Porras et al. 2010). Cigarette smoking seems to impair serological reactivity following natural HPV infection (Simen-Kapeu et al. 2008).

Seroconversions among HPV-infected men are infrequent, which may be related to the genital location (limited mucosa) of infection and smoking (Edelstein et al. 2011).

2.3.2 HPV oncogenesis

HPV is a necessary, but not sufficient, cause of cervical cancer (Walboomers et al. 1999). Cervical cancer develops through premalignant phases; from mild dysplasia or cervical intraepithelial neoplasia (CIN1) to moderate dysplasia (CIN2) or severe dysplasia (CIN3). Prolonged or persistent hrHPV infection, with a high viral load of especially hrHPV types 16 and 18, increases the risk of developing CIN3, the immediate precursor of cervical cancer (Schlecht et al. 2001). Serial measurement of type-specific viral load could predict whether the infection is transient or leading to CIN3+ (Depuydt et al. 2012). Longitudinal studies based on seroepidemiology and HPV DNA detection have also shown an increased risk of developing cervical cancer following hrHPV infection (Lehtinen et al. 1996, Wallin et al. 1999). The time between HPV infection and CIN3 has been estimated to be 7-15 years when primary infection occurs in the late teens or early twenties, and CIN3 diagnosis peaks around 25-30 years of age (Bosch & de Sanjose 2003).
Prospective cohort studies have, however, documented more rapid development of CIN2-3 among women with incident HPV 16 or HPV 18 infection (Winer et al. 2005). The risk of incident CIN (CIN3) increases with an increasing number of deliveries and decreases with a history of genital warts (Rintala et al. 2012) or after an HPV 6/11 infection (Luostarinen et al. 1999, Luostarinen et al. 2013).

Oncogenic HPV types have been found in cervical cancer tissues in several studies (Bosch et al. 1995, de Sanjose et al. 2010) with a prevalence ranging from 95-100% in cervical squamous cell cancer (SCC) (Bosch & de Sanjose 2003), and 76 – 82% in cervical adenocarcinoma (ADC) (Clifford et al. 2003, Li et al. 2011). All HPV genotypes that are known to be associated with cervical cancer belong to a few genetically related species of the alpha genus (A9, A7, A6 and A5). According to a worldwide study of HPV genotype distribution in cervical cancer including over 10,000 cervical cancer cases, the most common HPV types in invasive cervical cancer are HPV 16, 18, 31, 33, 35, 45, 52 and 58. HPV types 16, 18 and 45 are the most common types found in both cervical squamous cell carcinoma (46-63%, 10-14% and 2-8%, respectively) and adenocarcinoma (26-36%, 37-41%, 5-7%, respectively) (Clifford et al. 2003, de Sanjose et al. 2010). HPV18 is the most predominant type found in cervical adenocarcinomas.

In 2005 a group of scientists from 13 countries met at the International Agency for Research on Cancer (IARC) to reassess the carcinogenicity of human papillomavirus. They concluded that HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 are carcinogenic to humans (Cogliano et al. 2005). Later the carcinogenicity of HPV 66 has been re-evaluated and classified as non-carcinogenic and HPV types 67, 68, 73 and 82 as being probably carcinogenic (Schiffman et al. 2009, Clifford et al. 2011).

### 2.4 Epidemiology

#### 2.4.1 HPV incidence and prevalence in women

HPV is the most common sexually transmitted infection (STI), infecting mucosa and adjacent skin. A majority of HPV infections are asymptomatic or subclinical and regress without ever being recognized. The lifetime acquisition of cervical HPV infection has been estimated to occur in up to 80% of females, and
most infections take place by the age of 50 years (Syrjanen et al. 1990, Trottier & Burchell 2009). Half of the women who acquire genital HPV infection are infected within three years of sexual debut (Collins et al. 2002), but the infection is usually transient and resolves spontaneously within two years in 90% of young women (Franco et al. 1999). In young women, most cellular changes caused by HPV up to CIN2 spontaneously resolve within three years (Moore et al. 2007).

Based on data from multiple international studies, the estimates of cervical oncogenic HPV DNA prevalence among women around the world range from 2% to 44%, with a median prevalence cervical HPV DNA of 15.1% among all women and 9.2% among women over 30 years. The variation is explained by the differences in the age range studied and the sensitivity of the DNA assay used for HPV detection (Bosch & de Sanjose 2003). HPV 16 is the most common genotype and has the longest persistence (Louvanto et al. 2010). Clearance of HPV 16 is slow, 18-23 months. HPV types 18, 31, 33 and 52 form the intermediary clearance group (12-18 months), and other hrHPV types have even considerably shorter clearance-time of 6-7 months (Bulkmans et al. 2007, Lehtinen & Dillner 2013).

Multiple HPV infections are common among young women. Up to 50% of individuals, who have acquired genital HPV infection, will acquire another HPV type within a few years (Trottier et al. 2006). The risk for an hrHPV seropositive woman who probably has or has had a persistent infection of acquiring another hrHPV infection, as indicated by antibody positivity or seroconversion, is 3-6 times higher than for an hrHPV seronegative woman to acquire the first HPV infection (Lehtinen et al. 2006, Kaasila et al. 2009).

The prevalence of HPV infections peaks in women under 25 years of age (Franceschi et al. 2006), and in some geographical areas a second peak appears in older women, 50-55 years of age, close to the age of cervical cancer peak incidence. These two age-associated peaks have been observed in many studies around the world, except for Asia, where the age-specific HPV prevalence decreases with increasing age (Franceschi et al. 2006, de Sanjose 2007, Bosch et al. 2008).

In a global review of age-specific HPV from 0 to over 90 years of age, HPV 16 seroprevalence peaked in women aged 25-40 years, and decreased or plateaued in older ages. HPV 18 prevalence was lower than HPV 16 prevalence, and peaked slightly later in life. In altogether 23 studies in which both HPV antibody- and HPV DNA -prevalences were measured, the age-stratified DNA-prevalence of HPV 16 and 18 peaked at about 20-30 years and declined with age, whereas the corresponding seroprevalence peaks appeared later, at 35-55 years (Tiggelaar et al. 2012).
The prevalence of genital HPV infection in men varies widely between 1.3 - 72.9% according to a systematic review of 40 publications. This is explained by different HPV DNA detection methods used, different anatomical sites sampled, and by differences in populations studied (Dunne et al. 2006). Prevalence of HPV DNA collected from different anatomical sites in young adult males lies between 32-53% (Weaver et al. 2004, Kjaer et al. 2005). The HPV prevalence was even higher, 65.2%, in a multinational HPV in Men (HIM) study conducted in Brazil, Mexico and USA, among altogether 1160 asymptomatic males between 18 and 70 years of age (Giuliano et al. 2007b). The most common hrHPV types detected were 16, 51 and 52 and the most common lrHPV types were 84, 62 and 6 (Giuliano et al. 2008a).

The prevalence of HPV DNA sampled from different genital sites in men varies between 24 - 50% in the penile shaft, 16 - 36% in the glans, and 17 - 34% in the scrotum (Weaver et al. 2004, Giuliano et al. 2007b). The median time to clearance of any HPV infection is 5.9 months, with 75% of infections clearing within 12 months (Giuliano et al. 2010). Age is not significantly associated with HPV incidence or duration of infection in men (Giuliano et al. 2008b), but clearance of hrHPV is more rapid with increasing age (Giuliano et al. 2011a), probably due to a reduced number of re-infections.

Compared to the two-peaked HPV prevalence age-curve found in women, the age-specific prevalence in men seems to peak at slightly older ages and remains constant or decreases slightly with increasing age, across all geographic regions. In men, the peak HPV prevalence spans over a wide age range and is not concentrated in the younger age groups (Smith JS et al. 2011).

Modifiable factors associated with single and multiple HPV infections in men are: lifetime number of sexual partners, smoking (Kjaer et al. 2005, Partridge et al. 2007) and condom use (Nielson et al. 2007). HrHPV infection is significantly associated with a high number of lifetime female sexual partners or the number of male (anal) sex partners (Giuliano et al. 2011a). Men having sex with men (MSM) infected with human immunodeficiency virus (HIV) have a high prevalence of anal HPV, especially of HPV 16 (Palefsky et al. 1998), and are at increased risk of persistent HPV infection, which is associated with a higher risk of anal neoplasia (AIN). The prevalence of AIN in MSM with HIV is 16%, but the use of highly active antiretroviral therapy reduces the risk on AIN significantly (van der Snoek et al. 2003).
Mucosal HPV infections are transmitted through direct skin or mucosal/skin-to-skin/mucosal-mucosal contact, and the most common transmission route is through penetrative sex, which is often assumed to cause microtrauma on the epithelial surfaces (Stanley 2010). The estimated probability of HPV 16 transmission per sexual partnership has been estimated to be as high as 60% in a mathematical modelling analysis (Barnabas et al. 2006), but probably varies according to HPV type. In discordant couples, the transmission rates from female-to-male (4 per 100 person-months CI 95% 3.0-5.5) and male-to-female (3.5 per 100 person-months CI 95% 2.7-4.5) are similar (Burchell et al. 2011).

Transmission implies that an infection has been initiated with expression of viral mRNA, production of viral proteins, and release of new infectious virions, which are detectable by viral DNA testing. Furthermore, seroconversion should occur (Dillner et al. 1999b). Seroconversion takes place in not more than 65-75% of HPV DNA positive individuals, however (Kjellberg et al. 1999), and HPV DNA detection is mostly used as an estimate of transmission, even if the method may suffer from mere contamination being misclassified as acquired infection.

In cross-sectional studies, the HPV type-concordance among couples ranges from 3% to 37%. The wide range can be explained by different sampling techniques and HPV assays used, or by different definitions (either group-specific or type-specific) of concordance (Burchell et al. 2006). By increasing the threshold of viral load detection, the concordance of specific human papillomavirus types is higher among sex partners (Bleeker et al. 2005). In newly formed couples, the type-specific concordance is as high as 42% in the beginning of the relationships up to 6 months (Burhell et al. 2010a). In couples examined 24 hours after intercourse, HPV-concordance can be up to 95% but in most cases the detected HPV DNA is contamination and does not represent established HPV infections (Widdice et al. 2013).

Detection of HPV in infants and children suggests a possible vertical transmission of HPV from mother to infant (Smith EM et al. 2010, Syrjänen 2010, Koskimaa et al. 2012). Here, the transmission occurs most likely during vaginal delivery (Rintala et al. 2005a, Rintala et al. 2005b, Koskimaa et al. 2012). The concordance of HPV-types between mother and infant is almost 100% at delivery, suggesting that oral HPV in the newborn is acquired from the mother. However, the concordance is lost within two months, indicating rather a transient infection.
or mucosal contamination than true transmission of HPV (Koskimaa et al. 2012).
Caesarean section is associated with reduced risk of HPV exposure as compared to
vaginal delivery (Tseng et al. 1998), but in most studies significant association
between the mode of delivery and the transmission rate was not found (Sarkola et
al. 2008). Data on prenatal transmission of HPV from women with subclinical
HPV infection to the child is controversial. Positive HPV DNA findings in
placenta and umbilical cord blood in some studies suggest possible transmission
(Rombaldi et al. 2008, Sarkola et al. 2008), whereas other studies have not detected
HPV in amniotic fluid, placenta or cord blood (Eppel et al. 2000, Worda et al. 2005,
Ruffin et al. 2006).

2.4.3.1 Promotion of HPV transmission

Persistent hrHPV infection is associated with a risk of progression to cervical
precancerous lesions and to cancer if left untreated (McCredie et al. 2008). Cigarette
smoking (McIntyre-Seltman et al. 2005, Vaccarella et al. 2008), long-term oral
contraceptive use (Moreno et al. 2002, International Collaboration of
Epidemiological Studies of Cervical Cancer 2007), multiple live births (Munoz et al.
2002) and C. trachomatis infection (Silins et al. 2005) have been shown to be risk
factors for persistence and progression of HPV infection. Only smoking and
C. trachomatis infection have, however, been proven to be independent co-factors
for increasing the future risk of neoplasia (Simen-Kapeu et al. 2009b, Lehtinen et al.
2011, Luostarinen et al. 2013). Cigarette smoking seems to impair the antibody
response following natural infection, especially in young women (Giorgi et al. 2008,
Simen-Kapeu et al. 2008).

2.4.3.2 Protection of HPV transmission

In a meta-analysis of 20 studies there was no coherent evidence that condom
use would decrease the risk of becoming HPV-positive (Manhart & Koutsky 2002).
A lower prevalence of HPV infection in men has, however, been proven in studies
where condom use was consistent (Nielsen et al. 2010, Pierce Campbell et al. 2013).
The risk of cervical and vulvovaginal HPV infection appears to be reduced in
women only if their partners used a condom consistently (Winer et al. 2006).
Overall, condom usage seems to have a stronger protective effect against HPV infection among men than among women (Burchell et al. 2010b).

Intrauterine device (IUD) use might act as a protective cofactor in cervical carcinogenesis (Castellsague et al. 2011). This may be explained by cellular immunity triggered by the device. In current users of oral contraceptives, the risk of cervical cancer increases with increasing duration of use but declines after discontinuation of medication (International Collaboration of Epidemiological Studies of Cervical Cancer 2007).

Higher viral load in the male genital area implies a higher risk of transmission to a sex partner (Bleeker et al. 2005). The evidence of male circumcision being protective against HPV infection and its long-term sequelae in men and women is, however, contradictory. Circumcision protects against STIs by preventing accumulation of infected vaginal secretions. Another possible explanation is the reduced surface of non-keratinized mucosal epithelium as compared to keratinized skin, as mucosa is a more favourable environment for HPV than skin. The rate of HPV acquisition is not reduced among circumcised males, thus the protective effect on cervical cancer can also be due to a lower risk of transmission to their female partners. Among circumcised males, the prevalence and incidence of urethral hrHPV infection is reduced (Auvert et al. 2009), as is overall genital HPV prevalence (Albero et al. 2012). This might explain why women with circumcised partners have a reduced risk of cervical cancer. The increased clearance of HPV infection in circumcised men also fits into this picture (Lu et al. 2009, Gray et al. 2010, Hernandez et al. 2010).

### 2.5 HPV-associated diseases

It is estimated that 5.2% of all cancers worldwide are associated with HPV infection (Parkin 2006). The prevalence of HPV in cervical carcinoma varies in different studies between 87% and 99%. HPVs cause approximately 85% of anal squamous cell cancers (Daling et al. 2004), 40-50% of penile cancers (Parkin 2006), 33-60% of oropharyngeal cancers, and 23% of oral cancers (Mork et.al 2001, Kreimer et al. 2005). However, in addition, genital HPV types cause a number of benign, yet important, clinical conditions.
2.5.1 Anogenital warts

External anogenital warts (EGW) or condylomas occur anywhere on the external genitalia and are the most commonly recognized clinical manifestation of genital HPV infection in both women and men. They are generally considered as a benign condition but they can be difficult to treat, and recurrence is frequent. HPV is found in approximately 90% of condylomas. The most frequently recognized type is HPV 6 followed by HPV 11 (Aubin et al. 2008, Garland et al. 2009b). The incubation time from HPV infection to the development of EGW is between 3 weeks to 8 months, and the majority of warts develop around 2-3 months after infection. Approximately 65% of individuals who have sex with an infected partner will develop warts (Oriel 1971). About 20-30% of genital warts regress spontaneously (Coleman et al. 1994).

Patients with condylomas might have an increased risk of HPV-related anogenital and certain head and neck cancers, probably due to immunologic or behavioural reasons (Blomberg et al. 2012). HrHPVs often coexist in genital warts, which may lead to premalignant lesions (Brown et al. 1999). On the other hand, no increased risk of cervical cancer has been found to be associated with EGW (Sigurgeirsson et al. 1991). Furthermore, it has been shown in independent longitudinal studies that previous infections by HPV types 6 or 11 protect against development of hrHPV associated cervical cancer (Luostarinen et al. 1999, Arnheim Dahlstrom et al. 2011).

2.5.2 Recurrent respiratory papillomatosis

Recurrent respiratory papillomatosis (RRP) is a rare condition in which wart-like lesions or papillomas can occur anywhere in the respiratory tract, but most commonly in the larynx. Although histologically benign, epithelial proliferations may cause permanent laryngeal pathology and result in voice disturbances, progressive hoarseness, stridor, airway obstruction, and respiratory distress (Ilmarinen et al. 2011). The clinical course of RRP is unpredictable and varies from mild to serious and potentially life threatening disease (Tasca & Clarke 2006). In juvenile-onset form before the age of 4 years the most probable transmission mode is from the mother during birth. Condyloma during pregnancy increases the risk of RRP in the offspring more than 200 fold, but still less than 1% of children born to mothers with condylomas develop RRP. Caesarean section does not seem to protect against RRP (Silverberg et al. 2003). In adult-onset of RRP the median age
is 34 years, and it is more common among males than among females. The transmission mode can be either sexual or non-sexual (Omland et al. 2012). Patients with adult-onset laryngeal papillomatosis seem to be prone to other HPV infections, such as genital and skin warts as well (Aaltonen et al. 2005).

2.5.3 Cancer of the cervix

Cervical cancer is the third most common cancer and the fourth leading cause of cancer deaths among women worldwide, with 530 000 new cases and 275 000 deaths in 2008 (Jemal et al. 2011). The majority of cervical cancers are squamous cell carcinomas. Approximately 20% of all cervical cancers are classified as adenocarcinoma and their prevalence depends on the screening practice of the country.

More than 85% of new cervical cancers are found in developing countries with particularly high incidences in parts of South-America, Eastern, Western and Southern Africa, and South-Central Asia. The incidence rates are lowest in Scandinavia, Western Asia, Australia and New Zealand, and North America. The high burden of cervical cancer in developing countries is largely due to lack of screening (Jemal et al. 2011). Organized cytological screening can decrease the incidence and mortality rates of squamous cervical carcinoma up to 80% but depends on screening coverage and attendance rate. However, several studies have shown that cytological screening is not efficacious in reducing the risk of cervical adenocarcinoma, because the transformed cells are within the cervical canal and out of reach for sampling (Ault et al. 2011).

2.5.4 Cancer of the vulva

Vulvar cancer is rare but the incidence increases with age. Most vulvar cancers are squamous cell carcinomas, which can be classified according to their association with HPV infection. The term VIN includes only high-grade squamous vulvar lesions and is subdivided into usual type VIN and differentiated VIN.

Usual type VIN includes warty, basaloid and mixed VIN and is associated with hrHPV and other HPV risk factors, such as cigarette smoking and immunocompromised condition (Committee on Gynecologic Practice of American College Obstetricians and Gynecologists 2011). HPV 16 is the predominant genotype found in vulvar neoplasia (Smith JS et al. 2009), followed by HPV18
Prospective seroepidemiologic studies show that subjects seropositive for HPV 16 have an increased risk of developing vulvar cancer (Bjorge et al. 1997). The prevalence of HPV in warty and basaloid vulvar cancers is 75-100%. Overall HPV prevalence is about 40% in vulvar carcinomas and 80-85% in usual type VIN (De Vuyst et al. 2009, Smith JS et al. 2009). Differentiated VIN is not associated with HPV, but instead with dermatologic conditions, such as lichen sclerosus, and occurs mostly in older women. Notably, differentiated VIN associated with lichen sclerosus is more likely to be associated with vulvar SCC than usual-type VIN (Committee on Gynecologic Practice of American College Obstetricians and Gynecologists 2011).

2.5.5 Cancer of the vagina

Cancer of the vagina is very rare although the natural history of vaginal HPV and cancer is similar to that of cervical carcinogenesis. The premalignant lesions of the vagina, vaginal intraepithelial neoplasia (VAIN) 1-3, range from mild to severe before development of cancer. The upper third of the vagina is the most common site of VAIN and the lesions are usually multifocal. The mean age of women with VAIN is 35 years (range 18-52 years), and VAIN often occurs in women with previous VIN or CIN (Dodge et al. 2001). The prevalence of HPV in VAIN 1, VAIN 2/3 and vaginal cancer is about 98-100%, 90-93% and 65-70%, respectively (De Vuyst et al. 2009, Smith JS et al. 2009). Risk factors for vaginal SCC are hrHPV infection, tobacco smoking, and alcohol consumption (Madsen et al. 2008a). HPV 16 is the most common type in vaginal cancers (55.4%) and VAIN2/3 (65.8%) (Smith JS et al. 2009). Seropositivity for HPV 16 is associated with an increased risk of developing vaginal cancer (Bjorge et al. 1997).

2.5.6 Cancer of the penis

Cancer of the penis is a rare cancer accounting for less than 0.5% of cancers of men (Parkin 2006, Hernandez et al. 2008). Almost 90% of all invasive penile cancers are squamous cell carcinomas. Other less common penile cancers include basal and transitional cell carcinomas, adenocarcinomas, melanoma, and Kaposi’s sarcoma.

Penile SCC and precancerous lesions can be classified into HPV related and HPV unrelated groups. Similarly to vulvar cancer, HPV related types include warty
and basaloid penile SCC. HPV DNA is found in about 50% of all penile tumours (Chaux & Cubilla 2012). Basaloid and warty SCC may be preceded by premalignant penile intraepithelial neoplasia (PIN1-3). About half of the penile tumours are associated with HPV 16 and 18 (Miralles-Guri et al. 2009), and these genotypes are the most commonly found in penile SCC as well (Daling et al. 2005, Heideman et al. 2007). Again, seropositivity to HPV 16 is associated with an increased risk of developing penile cancer (Bjorge et al. 1997). The 5–year survival of patients with hrHPV-positive penile cancer seems to be better than that of patients with hrHPV-negative penile cancer (Lont et al. 2006).

HPV unrelated penile SCCs include usual, papillary, and verrucous types, and they are associated with factors like phimosis, chronic inflammation, and lichen sclerosus. Neonatal circumcision seems to reduce the risk of penile cancer threefold as compared to uncircumcised men (Maden et al. 1993). Phimosis at least 5 years before diagnosis of penile cancer, early and high sexual activity, a high number of female sex partners before age 20, first intercourse at a low age, penile-oral sex, a history of anogenital warts, never having used condoms, and smoking, are all significant risk factors for penile cancer (Daling et al. 2005, Madsen et al. 2008b).

2.5.7 Cancer of the anus

The transformation zone between colorectal-type mucosa and squamous epithelium in the anal canal is similar to that between the columnar and squamous epithelia in the cervical canal. It is particularly susceptible to HPV infection, and it is the site where anal squamous cell carcinoma develops. HPV infection causes most anal cancers, and 75-80% of anal cancers are associated with HPV 16 or 18 (Hoots et al. 2009). Most anal cancers are squamous cell carcinomas, adenocarcinomas, or basaloid and cloacogenic carcinomas. Anal SCC is preceded by AIN and is twice as common in females as in males (Parkin & Bray 2006). HPV prevalence in anal carcinoma is also higher among women (90.8%) than among men (74.9%). The overall HPV prevalence is 91.5%, 93.9% and 84.3% in AIN1, AIN2/3 and anal carcinomas, respectively (De Vuyst et al. 2009).

The incidence and distribution of anal cancer worldwide have increased appreciably since 1960, especially among women and homosexual men. The reasons for this could be changes in sexual behaviour that facilitate the spread of
HPV (Frisch et al. 1993). Sexual promiscuity increases the risk of anal cancer in both men and women (Frisch et al. 1997). Other risk factors for anal cancer are cigarette smoking, anal intercourse, and high number (≥15) of lifetime sexual partners (Daling et al. 2004).

2.5.8 Head and neck cancers

HPV is etiologically linked to a subset of head and neck squamous cell carcinomas (HNSCC), such as oropharyngeal, oral and tonsillar cancers (Mork et al. 2001, Kreimer et al. 2005). The incidence rates for oral cancers associated with HPV infections, such as cancer of the oropharynx, tonsils and base of the tongue, have been increasing among young adults in the United States, Australia and some countries in Europe, which might be associated with increased practice of oral sex (Hocking et al. 2011, Jemal et al. 2011). Overall the incidence of head and neck cancer has increased rapidly, especially in women (Curado et al. 2009).

HPV prevalence is 35.6% in oropharyngeal SCC, 23.5% in oral SCC, and 24% in laryngeal SCC. Of all HPV-positive oropharyngeal, oral and laryngeal SCC, HPV 16 is the most prevalent type and detected in 86.7%, 68.2% and 69.2% of HPV positive cases, respectively (Kreimer et al. 2005). The relative risk of developing oropharyngeal cancer in HPV 16 seropositive individuals is 14, i.e. equal to the risk of developing cervical cancer (Lehtinen et al. 1996, Mork et al. 2001). Incident oral HPV infection is most frequently caused by HPV 16 (Louvanto et al. 2013). HPV 16 and 6 are the most common genotypes in oral HPV infections and they are also the most likely to persist. Use of oral contraceptives and a second pregnancy protect against oral HPV persistence (Rautava et al. 2012). The prevalence of oral HPV in men and women is ten to twenty times lower than the prevalence of HPV in genital sites. It remains unclear whether this reflects reduced exposure or that the oral region is more resistant to HPV infection compared with anogenital sites (Kreimer et al. 2011b).

Risk factors for HNSCC are tobacco smoking and chewing, alcohol consumption, and poor oral hygiene (Hashibe et al. 2009, Kreimer et al. 2011b). HPV 16-positive HNSCCs have a different risk factor profile, including active sexual behaviour and exposure to marijuana, as compared to HPV 16-negative HNSCCs (Gillison et al. 2008). Patients with HPV-positive HNSCC have a better prognosis, including overall improved survival and a lower risk of progression and death, than patients with HPV-negative tumours. HPV-positive HNSCC tumours
also respond better to chemotherapy and chemoradiation treatment (Fakhry et al. 2008, Ang et al. 2010).

2.5.9 Other cancers

HPV infection has been found in both squamous cell carcinoma of the skin and its precursor actinic keratosis. Individuals with a rare hereditary condition, epidermodysplasia verruciformis, and transplant organ recipients, have an increased risk for developing skin cancer (Arnold & Hofbauer 2012). Longitudinal seroepidemiological studies indicate increased risk for future development of SCC or basal cell carcinoma of skin in individuals seropositive for some HPV types in genus beta species (Andersson et al. 2012).

The role of HPV in lung cancers, oesophageal cancers, colorectal cancers and breast cancers is controversial but mostly remains negative and inconclusive (IARC 2008).

2.6 Screening of HPV-associated lesions

2.6.1 Papanicolaou (pap) test

In a conventional pap test, cervical samples obtained by a brush and spatula are fixed on a microscope slide. Liquid-based cytology (LBC) is an alternative to conventional sampling. In LBC, samples collected by brush are transferred into a liquid fixative solution, after which the cells aimed for cytology are filtered from the solution until they form a monolayer on a glass slide.

The presence of a perinuclear halo in the so-called koilocytotic cells stemming from the differentiated layers of the squamous epithelium represents the morphological change that is pathognomonic to HPV infection (Purola & Savia 1977). The E5 and E6 proteins of both low- and high-risk HPVs contribute to the koilocyte formation in infected cervical cells (Krawczyk et al. 2008). Not all persistently infected cells, however, present such changes.

Cervical infections are manifested, as detected by conventional or liquid-based cytology, as low-grade squamous intraepithelial lesions (LSIL) or high-grade squamous intraepithelial lesions (HSIL). An abnormal pap test results in either a
control pap test or in a referral to colposcopy. In histopathological diagnostics, which is the golden standard for disease classification, cervical lesions are graded from CIN1 to CIN2 and CIN3, which is a true precancer.

The sensitivity of the pap test for detection of CIN2+ (CIN2 or worse) is low, 70% at best (Cuzick et al. 2006). Furthermore, the sensitivity of the pap smear for adenocarcinoma in situ or cervical adenocarcinoma is considerably lower than for lesions of the squamous epithelium (Scheiden et al. 2004). The pap test also has a lower specificity for high-grade lesions than low-grade ones, which easily leads to overtreatment (Nanda et al. 2000). Further limitations of pap testing include interobserver variability, misinterpretations, and the mere fact that young women prefer opportunistic screening to organized screening (Salo et al. 2013).

A cytological specimen might be inadequate or unsatisfactory due to inflammation, blood or lubricant contamination, or scant cellularity (Rosa et al. 2013). Up to 8% of pap smears are reported as unsatisfactory (Islam et al. 2004). The use of LBC might reduce the proportion of unsatisfactory slides (Castle et al. 2010). On the other hand, there is no clear evidence that LBC would increase the sensitivity of cytology and even less that its introduction would increase the efficacy of cervical screening in preventing invasive cancers (Ronco et al. 2012).

2.6.2 Detection of HPV

The introduction of organized cytological screening for cervical cancer using the pap test has decreased the incidence of, and mortality from, cervical squamous cell cancer by 80% in Finland between the mid-1960s and 1990s (Louhivuori 1991, Anttila & Pukkala 1999) and in the UK since 1988 (Peto et al. 2004). Similar reports have appeared from many countries worldwide in which organized pap screening has been introduced (Mathew & George 2009). Cytological screening for cervical cancer has, however, never been evaluated in a randomized controlled trial.

When HPV testing is incorporated into a well-established organized screening programme, primary HPV screening with cytology triage is more sensitive than conventional cytology in detecting high-grade (CIN3+) lesions (Naucler et al. 2007, Anttila et al. 2010, Dillner 2013). Among women aged 35 years or older, primary HPV DNA screening with cytology triage is more sensitive and also more specific than conventional screening, and decreases the number of colposcopy referrals (Naucler et al. 2007, Leinonen et al. 2009). The addition of an HPV test to the pap test reduces the incidence of CIN2-3 or cancer by 40-50% in women aged 32-38
years (Naucler et al. 2007). Primary HPV based screening with cytology triage detects more CIN lesions than conventional screening (Kotaniemi-Talonen et al. 2008). Most of the lesions are mild and might result in overdiagnosis, since most mild lesions are regressive.

The Finnish Current Care Guidelines recommend screening by cytology every five years for women aged 30 to 60 years, but allows screening in women aged 25 to 65 years. Screening should not start before the age of 25 years, but cytology testing outside the organized screening is allowed in women over 20 years, if they have been sexually active for over three years. HPV testing has also been accepted beside cytology for women over 30 years (Käypä-hoito 2010).

According to the American recommendations regarding screening for early detection of cervical precancer and cancer, women younger than 21 years of age should not be screened, regardless of sexual initiation at an early age or other risk factors. For women aged 21 to 29, screening with cytology alone every 3 years is recommended. HPV testing should not be used in this age group (Saslow et al. 2012). For women between 30 and 65 years of age, screening by cytology and HPV testing every 5 years, or by cytology alone every 3 years, is recommended (Saslow et al. 2012).

HPV testing is advantageous both in the triage of women with equivocal abnormal cytology, in follow-up after treatment of CIN lesions, and in primary screening of women aged 30 years or older (Arbyn et al. 2012). According to the ARTISTIC trial a negative HPV test is more protective than normal cytology, suggesting that HPV negative individuals could be screened with a six year interval (Kitchener et al. 2011).

2.6.3 Immunohistochemistry

Immunohistochemical staining for p16INK4a has provided significant improvement in the diagnosis of high-grade CIN in histopathological specimens (Wang et al. 2004, Carozzi et al. 2013). It is a highly sensitive, but not 100% specific, marker for oncogenic HPV infection. Overexpression of p16 occurs when pRB is inactivated by the HPV E7 oncoprotein, and it has been shown to take place in the vast majority of cervical precancers and cancers (Klaes et al. 2001). As recommended, a p16 positive CIN2 specimen should be interpreted as a high-grade lesion with potential transformation capability, whereas a p16 negative CIN2
specimen remains a low-grade lesion associated with small transformation capability (Waxman et al. 2012).

By cutting the rate of false-negative histological results in half, the use of p16 staining increases the sensitivity for detecting high-grade CIN. Reproducibility of p16 staining has been shown to be excellent (Bergeron et al. 2010).

2.7 Molecular HPV detection

There are at least 148 commercially available HPV tests with different performance characteristics. The selection of an HPV test is based on the available sample material and the purpose of testing, diagnosis, genotyping, epidemiology, or research (Dillner 2013). In clinical practice, the choice of the HPV test is important for distinguishing between relevant and irrelevant hrHPV infections. This is particularly important in HPV screening and in follow-up after treatment of high-grade lesions (Snijders et al. 2003). The most commonly used and clinically well validated tests are presented in the following chapter.

2.7.1 Hybrid Capture II

The most commonly used assay in HPV detection is the second-generation Hybrid Capture II (HCII) assay, which is based on liquid hybridization. It is easy to perform and relatively fast. The technology is based on liquid hybridization, where a probe cocktail is used to recognize 13 hrHPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and 5 lrHPV types (6, 11, 42, 43 and 44). The targeted HPV DNA is hybridized to labelled HPV RNA–probes covering all the HPV genomes. These DNA-RNA hybrids are captured onto microtitre wells by antibodies to RNA-DNA hybrids, detected by alkaline phosphatase-labelled antibodies to these hybrids, and after a substrate reaction visualized by chemiluminescence (Moliën et al. 2005, Brink et al. 2007). The intensity of the emitted light is proportional to the amount of target DNA present in the specimen and provides a semiquantitative measure of the viral load (Iftner & Villa 2003). The HC II test is relatively sensitive and has a high negative predictive value.
2.7.2 PCR-assays to detect HPV DNA

In polymerase chain reaction-based assays, double-stranded DNA in the specimen is denaturated through heating into single-stranded DNA. After this, a single-stranded primer with a complementary sequence is annealed to target the DNA. DNA polymerase then starts to synthesize a new DNA strand starting from the primer. After 30 cycles of amplification, PCR can theoretically produce 1,000,000 copies from one double-stranded DNA molecule in a process that takes about 90 minutes (Cuzick et al. 1999). Since PCR can be performed on very small amounts of DNA, it is ideal for use on specimens with low DNA content (Zaravinos et al. 2009).

In PCR, the DNA of a number of HPV types can be amplified by consensus primers, and the amplimers can be detected by type-specific probes (Brink et al. 2007). The mostly used consensus primers are GP5/6 and its extended version GP5+/6+, the MY09/11 degenerate primers and its modified version PGMY09/11, and the SPF10 primer set. These primers are all directed to the highly conserved regions of the L1 gene and have the potential of detecting all mucosal HPV types (Iftner & Villa 2003).

The MY09/11 primer set comprises forward and reverse primers that contain one or more degenerate bases to compensate for the sequence variation between the different HPV types at the priming sites. MY09/11 amplifies a fragment of 450bp of the L1 gene.

The GP5+/6+ version incorporates one forward and one reverse primer aimed at a conserved region of 150bp within the L1 gene, but fully complements only one or a few HPV genotypes.

The third design of PCR primers, such as the SPF primer set, is a combination of a number of distinct forward and reverse primers, aimed at the same position of the viral genome. These may contain inosine, which matches with a nucleotide. This method has the advantage of being highly reproducible. The SPF-PCR primers amplify a small region of 65bp of the L1 gene (Molijn et al. 2005).

One commonly used read-out system involves an enzyme immunoassay (EIA) staining procedure. This is used in GP5+/6+ and SPF10 PCR. These EIA-based read-out assays are useful for high-throughput analyses but less suited for genotyping. They are also laborious and require automation and large amounts of PCR product.
Compared to non-amplifying hybridization methods, PCR is substantially more sensitive and specific, and also reproducible. While the analytical sensitivity of HC2 assay has been adjusted to 5000 copies of HPV DNA, the sensitivity of PCR is between 10-400 copies of HPV DNA. In clinical practice, PCR might therefore detect low levels of clinically irrelevant viral DNA with a negligible risk of cervical cancer (Ylitalo et al. 2000). Low amounts of HPV PCR may be present due to contamination or insignificant transient infection cleared by the immune response. On the other hand, despite of its high sensitivity, PCR can miss severe precancer or cancer cases because of viral integration and loss of the L1 or E1 regions targeted by the assays (Gravitt et al. 1991, Kuypers et al. 1993). For these reasons, the amplified region, as well as the sensitivity and cut-off of PCR based tests, should be carefully considered to ensure that they fit the purpose of testing.

2.7.3 mRNA detection methods

While HPV DNA may be present in clinically irrelevant conditions, the expression of E6/7 mRNA represents an active HPV infection in which malignant transformation might occur (Brink et al. 2007). The presence of viral mRNA as an indicator of a true, established HPV infection has great potential as a basis for molecular testing. In clinical specimens, HPV mRNA can be detected by a reverse-transcriptase reaction followed by PCR (RT-PCR), nucleic acid sequence-based amplification (NASBA) or transcription-mediated amplification (TMA). RT-PCR assays can be applied to fresh-frozen specimens or to samples in which RNA is well preserved, such as LBC-samples of cervical scrapings. The PreTect HPV Proofer assay that detects E6/E7 mRNA from five hrHPV (16, 18, 31, 33, and 45) is a NASBA assay. The Aptima HPV assay uses TMA technology and detects E6/E7 mRNA of 14 hrHPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) (Snijders et al. 2010, Abreu et al. 2012). The prognostic or triage value of HPV mRNA testing can be improved by concomitant p16 immunohistochemistry (Cuschieri et al. 2008).
2.7.4 HPV genotyping

HPV genotyping is needed in epidemiological studies and in studies to determine vaccine efficacy. Reverse hybridization techniques have been introduced for genotyping purposes. HPV genotypes can be determined separately; using techniques such as Linear probe assays, direct sequencing, restriction-fragment length polymorphism (RFLP), or genotype-specific primers (Abreu et al. 2012). These methods use strips, filters, microarrays and microbeads that detect the hybridized PCR product by chemiluminescence or colorimetric reaction. Examples of strip formats are Linear Array HPV genotyping for the PGMY products, LiPa for the SPF10 products, and Digene HPV genotyping RH test for the GP5+/6+ PCR products. For the GP5+/6+ amplimers, a reverse line blot called filter-based assay can be used (Snijders et al. 2010).

Real-time PCR is used to detect and genotype HPV in tissue specimens and cellular samples. The assay can amplify different nucleic-acid targets and measure viral loads in very small concentrations. The read-out system for real-time PCR uses type-specific probes labelled with fluorescent dyes or SYBR green. Real-time PCR tests such as Cobas 4800 and AbbottRealTime PCR, which detect 14 hrHPV types but genotype only HPV 16 and 18, are highly reproducible, rapid and readily applicable to clinical samples (Abreu et al. 2012).

HC II and GP5+/6+ PCR-EIA -assays have shown an optimal balance between clinical sensitivity and specificity in large randomized controlled screening trials (Snijders et al. 2010), and so has Cobas 4800 when compared to HC II (Heideman et al. 2011). HC II, GP5+/6+ PCR, Cobas 4800 PCR, AbbottRealTime PCR and Aptima HPV test are considered clinically validated for use in primary screening, as they fulfill the criteria formulated in the international guidelines of HPV test requirements (Meijer et al. 2009, Arbyn et al. 2012, Heideman et al. 2013).

2.8 Primary prevention of HPV

2.8.1 HPV vaccine immunology

Prophylactic HPV vaccines are based on virus-like particles (VLPs) that are formed when the major capsid protein L1 is expressed in yeast or insect cells. The
L1 protein alone has the capacity to assemble into empty capsid-like structures, whose immunogenicity resembles that of infectious virions. The VLPs do not contain oncogenic viral genetic material and thus there is no risk of infection or oncogenic progression associated with vaccination (Zhou et al. 1991, Kirnbauer et al. 1992). Early animal papillomavirus research by Shope et al. demonstrated that injection of CRPV generated neutralizing antibodies in rabbits, and that the rabbits became completely resistant to subsequent viral exposure (Shope 1937).

HPV VLP vaccines are administered intra-muscularly with a high antigen dose, and the rapid and direct access to lymph nodes and spleen initiates adaptive immune responses. The licensed prophylactic vaccines induce neutralizing antibodies that prevent the development of HPV-associated precancerous lesions and hence the development of cervical cancer and other HPV-related cancers. The minimum level of antibodies needed for protection or the role of B cell memory in cases of waning antibodies have not yet been established (Stanley et al. 2012).

The short-lived plasma cells maintain high antibody levels for a few weeks after which the antibody titres decline. Long-lived plasma cells continue to produce antigen-specific antibodies, which decline with slower kinetics. This is how conventionally adjuvanted vaccines trigger long-term IgG antibody sustainability (MacLennan et al. 2003).

### 2.8.2 Prophylactic HPV vaccines

Two prophylactic HPV vaccines have been licensed. The bivalent vaccine (Cervarix™) contains 20 μg of HPV 16 L1 VLP and 20 μg of HPV 18 L1 VLP. Both VLPs are produced in a recombinant baculovirus expression vector system, using *Trichoplusia ni* Hi-5 insect cells. The vaccine preparation is formulated with an AS04-adjuvant containing 500 μg aluminium hydroxide and 50 μg 3-desacylated monophosphoryl lipid A (MPL) (Harper et al. 2004, Stanley et al. 2006b). MPL is derived from a chemical modification of the potent immunomodulator lipopolysaccharide of *Salmonella Minnesota* (Einstein et al. 2009). The AS04-adjuvant elicits an increased frequency of HPV V1 VLP specific memory B cells when compared to aluminium salt only formulations. This attributes to an enhanced immune response inducing long-lasting protection (Giannini et al. 2006).

The quadrivalent vaccine (Gardasil™) contains 20 μg of HPV 6 VLP L1, 40 μg of HPV 11 VLP L1, 40 μg of HPV 16 VLP L1, and 20 μg of HPV 18 VLP L1, synthesized in *Saccharomyces cerevisiae* using recombinant DNA technique. The
relative doses of the four VLPs purified and adsorbed onto 225μg amorphous aluminium hydroxyphosphate sulphate adjuvant were determined in a phase II trial that had 100 Finnish participants in 2000 (Villa et al. 2005). The aluminium-adjuvant has been commonly used for stimulation of both a variety of antigen presenting cells and B/T-cells in prophylactic vaccination for decades (Exley et al. 2010), and it is well documented and safe.

2.8.3 Immunogenicity and efficacy trials

2.8.3.1 Monovalent vaccine

Several phase I and II studies proved the immunogenicity and safety of monovalent VLP vaccines. They generated high levels of neutralizing antibodies and were generally well tolerated irrespective of the vector used for their expression (Harro et al. 2001). Vaccination with HPV 16 L1 VLP induced cellular immunity to phylogenetically related HPV types like 31, which suggested the possibility of cross-protection (Pinto et al. 2006).

Koutsky et al. (2002) were the first to prove that systemically administrated monovalent yeast-derived HPV 16 L1 VLP vaccine adjuvanted with aluminium salt vaccine could prevent mucosal infection. In this double-blinded placebo-controlled study, the HPV 16 vaccine given in three inoculations at 0, 2 and 6 months reduced the incidence of both HPV 16 infection and HPV 16-related cervical intraepithelial neoplasia (CIN1/2), with an efficacy of 90-100% (Koutsky et al. 2002). In long-term follow-up, the monovalent HPV 16 vaccine has remained efficacious and has provided high-level protection against persistent HPV 16 infection and HPV 16-related CIN2-3 (Mao et al. 2006, Rowhani-Rahbar et al. 2009).

2.8.3.2 Bivalent vaccine

The bivalent vaccine (Cervarix™) adjuvanted with AS04, given with a three-dose regimen (0, 1 and 6 months), is highly immunogenic and induces seroconversion to both antigens in all vaccinated women aged 15-25 after the third
dose. The bivalent vaccine is not therapeutic, nor does it enhance clearance of (pre-)existing HPV 16/18 infection (Hildesheim et al. 2007).

The bivalent vaccine induces high and sustained immune responses in women aged 15-55 years, with antibody levels remaining several-fold higher than after natural infection levels, for at least 4 years after the first vaccine dose (Schwarz et al. 2011). In young girls and boys aged 10-14 or 12-15 years, the antibody levels are considerably higher than those in the young women aged 15-25 years: more than 4-fold and more than 2-fold higher for anti-HPV 16 and anti-HPV 18 antibodies, respectively (Medina et al. 2010).

The antibody levels induced by the bivalent vaccine in 15-25 year old young women sustain up to 8.4 years, as does the efficacy against incident infections (95-97%) or infections persisting for 6 (94-100%) or 12 months (100%), cytological abnormalities and histopathological lesions (CIN2+ 100%) caused by HPV 16 and/or 18 (GlaxoSmithKline Vaccine HPV 007 Study Group 2009, Roteli-Martins et al. 2012). Mathematic modelling based on data on young adult women suggests that anti-HPV 16 and anti-HPV 18 antibody levels following AS04-adjuvanted vaccination will persist for at least 20 years and will stay substantially above levels produced after natural infection (David et al. 2009). However, retrieving biobanked sera from the vaccinees up to 20 years post vaccination will finally settle this issue (Lehtinen et al. 2012).

In the end-of-study analysis of the PATRICIA (PApilloma TRIal against Cancer In young Adults) trial, the vaccine efficacy (VE) increased with higher severity of the developed lesion in women of the total vaccinated cohort-naïve (TVC-naïve included women with no evidence of hrHPV infection at baseline), irrespective of the HPV type in the lesion; 50.3% for CIN1, 64.9% for CIN2 and 93.2% for CIN3. Vaccine efficacy in the total vaccinated cohort (TVC included women that received at least one vaccine dose regardless of their baseline HPV status) was lower; 27.7% for CIN1, 33.1% for CIN2, and 45.6% for CIN3 (Table 1).

The bivalent vaccine showed excellent VE against CIN3+ and AIS, irrespective of the HPV type. The efficacy was 100% in TVC-naïve and 45.7% in TVC for HPV 16/18-associated CIN3+ lesions, and 93.2% in TVC-naïve and 45.6% in TVC for all CIN3 lesions (Table 1). Vaccine efficacy against all AIS was 100% and 77% in the TVC-naïve and TVC, respectively (Lehtinen et al. 2012). The vaccine efficacy remained high in HPV DNA negative subjects, regardless of their baseline serological HPV status (Szarewski et al. 2012b). Consistent cross-protective efficacy
of the bivalent vaccine was demonstrated against specific non-vaccine HPV types 31, 33, 45 and 51 (Malagon et al. 2012, Szarewski et al. 2012a, Wheeler et al. 2012).

Vaccine efficacy against prevalent anal HPV 16/18 infection in women, as measured four years post vaccination, was lower (62%) compared to that for cervical infection (76.4%) in a full cohort. Compared to a restricted cohort including HPV-naïve subjects (i.e. negative for cervical HPV 16 and HPV 18 DNA, and HPV 16 and HPV 18 seronegative before enrolment), vaccine efficacy against anal HPV 16/18 infection was 83.6%, which was similar to vaccine efficacy against cervical HPV 16/18 infection 87.9% (Kreimer et al. 2011a). Estimated VE against prevalent oral HPV 16/18 infection four years after vaccination was 93.3% indicating a probable protection against HPV 16/18-associated oropharyngeal cancers (Herrero et al. 2013). So far vaccine efficacy trials have not been conducted among men.

2.8.3.3 Quadrivalent vaccine

The quadrivalent HPV VLP vaccine (Gardasil™) given in a three-dose regimen (day 1, 2 and 6 months) is highly immunogenic in inducing antibody levels that are 27-145-fold higher than HPV antibody levels detected in HPV infected women. All vaccinated subjects seroconvert after the third dose (Villa et al. 2005, Villa et al. 2006b). Yet, three years after immunization the vaccine-induced antibody levels declined to the levels induced after natural infection for HPV 6, 11 and especially 18. In a study in over 17,600 women, the correlation of vaccine-induced serum antibody responses and efficacy was evaluated. Despite reduction of HPV 18 antibody levels during three years post vaccination, with 40% of the vaccinees turning anti-HPV 18 seronegative at the end of the study, the efficacy against HPV 18-related disease remained high (98.4%) (Joura et al. 2008, Olsson et al. 2009). Furthermore, a booster dose administered five years after the initial immunization induced an anamnestic response with even higher vaccine type antibody levels than after the initial three-dose immunization (Olsson et al. 2007). Age at vaccination initiation seems to be inversely proportional to the vaccine-induced anti-HPV response, i.e. the younger the subject, the higher the response to the vaccine. Interestingly, subjects who are seropositive at day 1 to a vaccine HPV type have more robust anti-HPV responses to that type than subjects who are seronegative at baseline (Giuliano et al. 2007a).
The FUTURE II study (Females United to Unilaterally Reduce Endo/Ectocervical Disease) is an international, double-blind, placebo-controlled, randomized efficacy trial of the quadrivalent vaccine. The study with over 12,000 15-26 year old young women demonstrated a vaccine efficacy of 98% against HPV 16/18-associated high-grade cervical disease (CIN2+) in the per-protocol group (PPP), and of 44% in the intention-to-treat group (ITT) three years after initial immunization. In the ITT group, vaccine efficacy was 17% against all high-grade cervical lesions, regardless of HPV type, and 34% against vulvar or vaginal perianal warts, intraepithelial neoplasia grades 1 to 3, or cancer (Garland et al. 2007). Vaccine efficacy against HPV 6/11/16/18-related VAIN2/3 and VIN2/3 was 100% and 100% in baseline negative subjects, and 86% and 73%, respectively, in all subjects (Kjaer et al. 2009).

Vaccine efficacy was 91% against disease or infection related to HPV 6, 11, 16 and 18, in subjects aged 24-45 years not infected with respective HPV types at enrolment. The efficacy against vaccine-type infection in baseline HPV infected women was 31% against infection or disease related to HPV 6, 11, 16 or 18. The efficacy against infection or disease related to HPV 16 was 83% and for HPV 18 the efficacy was 23% in the same populations (Munoz et al. 2009).

Combined final data at 42 months from FUTURE I and FUTURE II studies included 17,622 women aged 15-26 years. The vaccine efficacy in the PPP group against lesions related to the HPV types in the vaccine was 96% for CIN1, 100% for both VIN1 and VAIN1, and 99% for condyloma. Vaccine efficacy against any lesion, regardless of HPV type, in the generally naïve population was 29.7% for CIN1, 75% for VIN1, 48% for VAIN1, and 83% for condyloma (FUTURE I/II Study Group 2010). The vaccine was 49% effective against all VIN2-3 or VAIN2-3, irrespective of whether or not HPV DNA was detected in the lesion (Joura et al. 2007).

The vaccine was up to 100% effective against HPV 16/18-related high-grade cervical, vulvar, and vaginal lesions and against HPV 6/11-related genital warts in a population that was negative to 14 HPV types at baseline. In the ITT group, the vaccine efficacy was 19.3% against all CIN2 and 16.4% against all CIN3. In baseline negatives, the corresponding efficacies were 42.9% and 43.0% (Munoz et al. 2010) (Table 1). Any high-grade vulvar or vaginal lesions were reduced by 51% (Joura et al. 2007) and genital warts by 62%, irrespective of causal HPV type (Munoz et al. 2010).
As proof of cross-protection, vaccination with the quadrivalent vaccine reduced the incidence of all HPV 31 or HPV 45 associated infections by 40% and the incidence of HPV 31 or 45 associated CIN1-3/AIS by 44% (Smith JF et al. 2007, Brown et al. 2009). Vaccine efficacy for CIN1-3/AIS associated with the 10 nonvaccine HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) was 32.5% in sexually naïve women, and 15.1% in sexually active women. The reduction in HPV 31/33/45/52/58 infection and CIN1-3/AIS was 25.0% in sexually naïve, and 18% among sexually active women (Brown et al. 2009, Wheeler et al. 2009). Reductions were, however, consistent only for HPV 31.

<table>
<thead>
<tr>
<th>Baseline negative*</th>
<th>Lesion</th>
<th>Bivalent vaccine (PATRICIA)</th>
<th>Quadrivalent vaccine (FUTURE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV 16/18 associated</td>
<td>CIN 1</td>
<td>96.5 %</td>
<td>97.2 %</td>
</tr>
<tr>
<td></td>
<td>CIN 2</td>
<td>99 %</td>
<td>100 %</td>
</tr>
<tr>
<td></td>
<td>CIN 3</td>
<td>100 %</td>
<td>100 %</td>
</tr>
<tr>
<td>Any HPV</td>
<td>CIN 1</td>
<td>50.3 %</td>
<td>29.7 %</td>
</tr>
<tr>
<td></td>
<td>CIN 2</td>
<td>64.9 %</td>
<td>42.9 %</td>
</tr>
<tr>
<td></td>
<td>CIN 3</td>
<td>93.2 %</td>
<td>43.0 %</td>
</tr>
<tr>
<td>Intention-to-treat</td>
<td>HPV16/18 associated</td>
<td>CIN 1</td>
<td>62.9 %</td>
</tr>
<tr>
<td></td>
<td>CIN 2</td>
<td>60.7 %</td>
<td>53.0 %</td>
</tr>
<tr>
<td></td>
<td>CIN 3</td>
<td>45.7 %</td>
<td>43.5 %</td>
</tr>
<tr>
<td>Any HPV</td>
<td>CIN 1</td>
<td>27.7 %</td>
<td>20.3 %</td>
</tr>
<tr>
<td></td>
<td>CIN 2</td>
<td>33.1 %</td>
<td>19.3 %</td>
</tr>
<tr>
<td></td>
<td>CIN 3</td>
<td>45.6 %</td>
<td>16.4 %</td>
</tr>
</tbody>
</table>

Table 1. Efficacies of the bivalent and quadrivalent vaccines against CIN1-3 lesions in baseline negative and in the intention-to-treat groups according to the final analyses of PATRICIA and FUTURE I/II studies (Lehtinen et al. 2012, Munoz et al. 2010). *Baseline negative defined as negative for HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 in the PATRICIA-study, and negative for HPV types 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 in the FUTURE-study.
In males 16 to 26 years of age, the quadrivalent vaccine efficacy was 65.5% against external genital lesions related to HPV 6, 11, 16, or 18 in the ITT population, and 90.4% in the PPP. Efficacy with respect to persistent infection with HPV 6, 11, 16, or 18 was 47.8% in the ITT population, and 85.6% in the PPP (Giuliano et al. 2011b). Immunologic responses are comparable to those observed in women three years after immunization. Seropositivity was observed in 88.9%, 94.0%, 97.9%, and 57.0% of subjects for HPV 6, 11, 16, and 18, respectively (Hillman et al. 2012). Among MSM, the vaccine efficacy against AIN associated with HPV types 6, 11, 16 and 18 was 50.3% in the ITT population, and 77.5% in the PPP. Vaccination reduced the rate of high-grade AIN (AIN2+) associated with HPV 6, 11, 16 and 18 by 54.2% in ITT, and 74.9% in PPP. The corresponding risk of persistent anal HPV 6, 11, 16 and 18 infections was reduced by 59.4% and 94.9%, respectively (Palefsky et al. 2011). Vaccine efficacy against HPV 6/11/16/18-related external genital lesions was 90.8% in baseline negative subjects, and 66.7% in all subjects. In baseline negative subjects, vaccine efficacies against warts were 89.9% and 85.2% in HPV 6/11/16/18-related and any HPV warts, respectively. The corresponding efficacies against PIN2/3+ lesions were 100% and 100%, respectively (Table 2) (Goldstone et al. 2013).

2.8.3.4 Comparison of the prophylactic vaccines

Both prophylactic vaccines are highly immunogenic and all subjects seroconvert after the third dose. Both vaccines are licensed and recommended for young girls and women aged 9-26. A two-dose schedule appears to be non-inferior in generating antibody levels, compared to the normal three-dose schedule in young girls studied for both vaccines (Romanowski et al. 2011). The third dose of the bivalent vaccine can be administered at any time between 6 and 12 months after the first dose, with no decreasing impact on seroconversion or post-vaccination HPV antibody levels in 15-25 years old young women (Esposito et al. 2011).

The different trial designs of the FUTURE I/II and PATRICIA studies make the direct comparison of the vaccines difficult. Different serological assays were used in the different trials, and the seropositivity statuses are attributed to the parameters and sensitivities of the individual immunoassays (Brown et al. 2011). The trials also differ in how they define HPV negative: participants negative for HPV 6, 11, 16, 18 + 10 non-vaccine types in the FUTURE study vs. negative for
<table>
<thead>
<tr>
<th>Quadrivalent vaccine</th>
<th>Lesion</th>
<th>Vaccine efficacy % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline negative</strong>*</td>
<td>HPV6/11/16/18 related</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EGL</td>
<td>90.8 (70.7, 98.2)</td>
</tr>
<tr>
<td></td>
<td>Warts</td>
<td>89.9 (67.3, 98.0)</td>
</tr>
<tr>
<td></td>
<td>PIN2/3+</td>
<td>100 (-425.5, 100)</td>
</tr>
<tr>
<td></td>
<td>AIN+ cancer**</td>
<td>89.6 (57.2, 98.8)</td>
</tr>
<tr>
<td><strong>Intention-to-treat</strong></td>
<td>Any HPV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EGL</td>
<td>81.5 (58.0, 93.0)</td>
</tr>
<tr>
<td></td>
<td>Warts</td>
<td>85.2 (61.8, 95.5)</td>
</tr>
<tr>
<td></td>
<td>PIN2/3+</td>
<td>100 (-425.5, 100)</td>
</tr>
<tr>
<td></td>
<td>AIN+ cancer**</td>
<td>54.9 (8.4, 79.1)</td>
</tr>
<tr>
<td><strong>Baseline negative</strong>*</td>
<td>HPV6/11/16/18 related</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EGL</td>
<td>66.7 (48.0, 79.3)</td>
</tr>
<tr>
<td></td>
<td>AIN+ cancer**</td>
<td>50.3 (25.7, 67.2)</td>
</tr>
<tr>
<td><strong>Intention-to-treat</strong></td>
<td>Any HPV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EGL</td>
<td>59.3 (40.0, 72.9)</td>
</tr>
<tr>
<td></td>
<td>AIN+ cancer**</td>
<td>25.7 (-1.1, 45.6)</td>
</tr>
</tbody>
</table>

**Table 2.** Efficacies of the quadrivalent vaccine in baseline negative and all males against external genital lesions (EGL), warts, penile intraepithelial neoplasia (PIN) or worse and anal intraepithelial neoplasia (AIN) or worse and anal cancer (Goldstone et al. 2013). PIN2/3+ defined as PIN2/3 or penile/perianal/perineal cancer.*Baseline negative defined as negative for HPV types 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59.**only in men having sex with men.

14 hrHPVs in the PATRICIA study, and in what the inclusion criteria for the study subjects are. In the comparison of cross-protective vaccine efficacy estimates, the bivalent vaccine seems to have higher efficacy against infections and lesions associated with HPV 31 (77.1% vs. 46.2%), 45 (79.0% vs. 7.8%), and against CIN2+ associated with HPV 33 (82.3% vs. 24.0%), compared with the quadrivalent vaccine (Malagon et al. 2012). In a head-to-head comparison of the two vaccines, the geometric mean titres (GMTs) in the according-to-protocol (ATP) cohort were 2.4 - 5.8-fold higher for HPV 16 and 7.7 - 9.4-fold higher for HPV 18 for the bivalent vaccine than in the quadrivalent vaccine. Although the bivalent vaccine induced higher serum antibody levels, both vaccines induced similar mucosal antibody levels. It is important to note that an immunological correlate of protection has not been defined. However, the different magnitudes of immune responses induced by the two vaccines may also influence the duration of protection (Einstein et al. 2011).
2.8.3.5 Safety

Overall, both vaccines seem to be well-tolerated and safe, but vaccine trials are powered to detect only reactions occurring with a frequency over 1/10,000. Very rare vaccine reactions cannot therefore be detected even in large trials (WHO defines very rare as a frequency of <1/10,000) (Agorastos et al. 2009).

The reported local reactions and systemic adverse events are quite similar for both vaccines. Local reactions like pain, redness, and swelling are more common in vaccine than in placebo recipients. Vaccinated girls and boys report significantly more fever than vaccinated women (Block et al. 2006). The rates of systemic adverse events following immunization, including fatigue, headache, fever, nausea, dizziness and myalgia, are higher among vaccine recipients than among placebo recipients. Systemic adverse events within 15 days following immunization with the quadrivalent vaccine are observed in 1% of participants. Serious adverse events are infrequent and occur equally often in vaccine and placebo recipients (Block et al. 2010). The frequency of newly diagnosed chronic diseases, autoimmune disorders and medically severe conditions are similar in the vaccine and the placebo groups (Paavonen et al. 2007, Medina et al. 2010).

A combination of another VLP vaccine or other vaccines with the bivalent or quadrivalent vaccine does not interfere with the immune response to either of the vaccines (Garcia-Sicilia et al. 2010, Vesikari et al. 2010).

Although there is no proved negative effect on pregnancy outcomes, vaccination with HPV VLP is not recommended during pregnancy (Garland et al. 2009a). Increased rate of miscarriages after administration of HPV VLP vaccines has not been observed (Dana et al. 2009, Wacholder et al. 2010).
The aim of the study was to evaluate the antibody responses to HPV 16/18 vaccination and to clarify whether vaccination can reduce the rate of HPV infections among females and males.

The specific aims were:

1. To evaluate development of antibody responses following HPV 16/18 vaccination in adolescent girls aged 10-14 years and young women aged 15-25 years in time.

2. To compare HPV 16/18 antibody levels in serum and cervicovaginal secretions in young women aged 15-25 years.

3. To evaluate antibody responses following HPV 16/18 vaccination in adolescent boys and young men aged 10-18 years.

4. To compare the prevalence of secreted HPV 16/18 DNA in HPV 16/18 vaccinated and non-vaccinated young men.
4 MATERIALS AND METHODS

4.1 Study participants

The multicentric, randomized phase III study HPV 012 (Paper I) started in September 2004 and ended in July 2005. There were 17 study sites in Denmark, Estonia, Finland, Greece, Netherlands, and Russia. In Finland the study sites were in the cities of Seinäjoki and Tampere. Overall, study participants were recruited in schools (Finland), hospitals, children’s outpatient clinics, or among the general population by using recruitment letters or sessions, advertisement, leaflets, and articles in local newspapers.

An eligible participant was a healthy 10-25 year old female, who had been abstinent from sexual activity, or had used adequate contraception 30 days prior to vaccination and up to 2 months after completion of the vaccination series if of childbearing age, had a negative pregnancy test result, and had no more than six lifetime sexual partners (not applied in Finland). Individuals were excluded from the enrolment if they had used an investigational drug or vaccine within 30 days, immunoglobulins or blood products within 3 months, a chronic immune-modifying drug within 6 months, or planned to use any of these during the study period, were pregnant, planning to become pregnant, or breastfeeding, or had previously received an HPV vaccine. Informed consent was obtained from each participant or the participant’s parents or legally acceptable representative(s) before the onset of any study specific procedures. Participants below the legal age of consent were required themselves also to sign and date an informed assent.

The extension-study of HPV 012 (Paper II) continued from June 2006 to January 2009. All subjects from the HPV 012 study from Denmark, Estonia and Finland, who had received three doses of the HPV 16/18 vaccine and completed the primary study (at month 7), were invited to participate in the follow-up study. Subjects who missed the first visit of the extension study at month 18 were eligible to join the study at month 24. A new written informed consent from each participant was required before enrolment. From subjects below the legal age of consent, a written consent was required from a parent or legally acceptable representative. The participants from the extended HPV 012 study were included.
in the pooled data (Paper III) of four different phase III clinical trials (HPV 010, HPV 012, HPV 014, and HPV 028) with subjects aged 10-65 years (16-17 year olds in Finland). The participants from the extended 012-study were eligible and samples obtained at months 24 and 36 after the first vaccine dose were included in the pooled study.

The HPV 011-study (Paper IV) was a phase I/II randomized, observer-blind, parallel-group study that took place from April 2006 to January 2007 at seven study sites in Finland. The study participants were recruited by recruitment letters sent to the entire male birth cohort (parents or legal guardian in the case of minors) in the study site communities, by population census register, and by school recruitment sessions.

Boys aged 10-18 years were eligible to participate. Exclusion criteria at enrolment were usage of an investigational drug or vaccine within 30 days, usage of immune-modifying drugs within 6 months, and usage of immunoglobulins or blood -products within 3 months, or planning of using any of these during the study period. Previous HPV or HBV vaccination, previous HBV infection or known exposure to HBV within 6 weeks, or any suspected or confirmed immunosuppressive or immunodeficient condition all were also exclusion criteria at enrolment. Informed consent was obtained from each participant.

The HPV 040 -study is a Finnish phase III/IV community-based randomized trial, where the effectiveness of different HPV vaccination strategies (boys and girls vs. girls only) in reducing HPV prevalence in young females is studied. The participants were recruited in 11 municipalities where HPV 040 study had been carried out in 2007-2009 (birth cohorts 1992-1995 between ages 12 to 15) by recruitment letters (parents or legal guardian in the case of minors) by population census register and by recruitment sessions in schools (Paper V). Informed consent was obtained from each participant. The exclusion criteria were previous HPV or HBV vaccination, previous HBV infection or any suspected or confirmed immunosuppressive or immunodeficient condition.

All participants of the HPV 040 study and HPV 011 (birth cohorts 1992-1995) were offered a screening test for Chlamydia trachomatis at the age of 18.5 years. Of all male subjects anonymous first void urine (FVU) samples were included in paper V.
4.2 Study vaccine

The vaccine used in the studies was the HPV 16/18 vaccine (Cervarix™ GlaxoSmithKline Biologicals, Rixensart, Belgium) containing HPV 16 and HPV 18 L1-proteins self-assembled into VLPs and formulated with an AS04-adjuvant containing aluminium hydroxide and monophosphoryl lipid. Each dose of the vaccine contained 20 μg of HPV 16 and 18 L1 proteins adjuvanted with 550 μg of AS04 (500 μg of aluminium hydroxide and 50 μg of 3-O-desacyl-4´ monophosphoryl lipid A). Each vaccine dose was supplied in coded individual prefilled 0.5 ml syringes and administered in the deltoid muscle on a 0-, 1-, and 6-months schedule.

The control vaccine used in paper IV was hepatitis B vaccine (Engerix-B™) containing 10 μg of hepatitis B surface antigen and 250 μg of aluminium hydroxide. Each vaccine dose was supplied in prefilled 0.5 ml syringes and administered into the deltoid muscle on the same schedule as the HPV 16/18 vaccine.

4.3 Study design

The participants of the HPV 012 study (Paper I) were divided into two groups by age: 15-25 years (n = 458) and 10-14 years of age (n = 158). There were five parallel treatment groups: three groups of women aged 15-25 years who received one of three consecutive production lots (Lot 1, 2 and 3) of HPV 16/18 vaccine (Hi5-produced vaccine), and one group of women aged 15-25 years who received a differently manufactured HPV vaccine (Hi5/SF9-produced vaccine), and one group of adolescent girls aged 10-14 years who received the Hi5-produced vaccine (Lot 1). All subjects received three doses of the candidate vaccine according to a 0, 1, 6-month schedule. Blood samples were collected from each participant at the first visit before vaccination and at month 7 after the first vaccine (Figure 4).

The participants were scheduled to visit the study sites at months 18, 24, 36 and 48 after the first visit. Blood samples were collected at each visit and cervicovaginal samples (CVS) were collected in post-menarcheal subjects who volunteered for the procedure at months 24, 36 and 48 (not from minors in Finland) (Figure 4). CVS samples were collected by using ophthalmic sponges. The subject agreed to avoid
sexual intercourse and/or the use of intravaginal medications for 24-48 hours before the collection of CVS samples. To minimize blood contamination, collection was performed at least 2-3 days after last menstrual bleeding. The sponge was placed on the cervix for 30-60 seconds to absorb mucus. Two sponges were collected per subject and stored at ≤ -20°C, until used for antibody extraction (Papers II and III).

Figure 4. Study design of the HPV 012 study (Papers I, II, III)

The participants of the HPV 011 study (Paper IV) were divided into three groups by age: 10-12, 13-15, and 16-18 years. A randomization in a 2:1 ratio was used to administer either the HPV 16/18 vaccine or an HBV control vaccine on a 0, 1, 6-month schedule. Baseline blood samples were collected from each participant before the first vaccination and one month after the second and third vaccine doses to evaluate immunogenicity.

All participants of the HPV 040-study received either the bivalent HPV 16/18 vaccine (Cervarix™) or the hepatitis-B-vaccine (Engerix-B™). Starting from autumn 2010, when the first participant turned 18.5 years, an invitation/information letter was sent to each subject to participate in the Chlamydia trachomatis screening. Upon a web-page (www.rokotitus.net) request, a first void urine sampling kit was mailed to the participant. The samples were returned in prepaid envelopes to the FIMLAB laboratory, Tampere, Finland, for C. trachomatis
testing. The test results were delivered to the trial participants on the secure www.rokotitus.net web page, where personal identity was protected via pseudonymisation. Those testing *C. trachomatis* positive were invited to the community study sites for treatment including azithromycin prescriptions. Pseudonymised leftover samples and the extracted DNAs were labelled as being from HPV 16/18 vaccinated, HBV vaccinated, or unvaccinated individuals, and stored at -20°C for later use.

### 4.4 Laboratory analyses

HPV 16 and 18 antibodies were measured from blood samples, using a type-specific enzyme-linked immunosorbent assay (ELISA). Recombinant HPV 16 and 18 VLPs were used as coating antigens for antibody detection. Seropositivity was defined as a titre greater than, or equal to, the cut-off titre established at 8 ELISA units/ml (EU/ml) for HPV 16 and 7 EU/ml for HPV 18 (Harper et al. 2004) (Papers I, II, III and IV).

Antibody extraction from cervicovaginal secretion samples was performed at GSK Biologicals Laboratories, Belgium, and anti-HPV 16 and anti-HPV 18 IgG antibodies were measured and quantified according to the ELISA serum standardized protocol. The presence of blood in cervicovaginal samples was evaluated with a Hemastix® reagent strip test. Samples showing 200 erythrocytes/ml or more were excluded from statistical analyses to reduce potential bias due to blood contamination in mucosal IgG assessment (Castle et al. 2004, Schwarz et al. 2009) (Papers II and III).

The extracted DNAs from the FVU samples were analysed by a consensus PCR, using a modified general primer (MGP) followed by MALDI-TOF mass spectrometry on a sequenom platform, that allowed simultaneous separation and detection of short DNA sequences elongated with a single nucleotide. The modified primers were able to genotype 14 hr HPV types and the two most common lr HPV types; HPV 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 (Soderlund-Strand et al. 2008, Soderlund-Strand et al. 2009) (Paper V).
4.5 Statistical methods

Statistical analyses were performed using the SAS version 8.2 and ProcStatXact 5 in papers I and IV. The SAS version 9.1 and ProcStatXact version 7.0 were used in papers II and III. SPSS was used in analyses of HPV prevalences in paper V.

Noninferiority in the seroconversion rates was to be demonstrated, if the upper limit of the 95% CI (confidence interval) for the difference between the percentages of the subjects who seroconverted in each group was less than 10% one month after the third vaccine dose, and if the upper limit of the 95% CI for the GMT ratio between each group was less than 2 (Paper I). Asymptotic two-sided CIs for the GMT ratios were computed using an analysis of variance model of log10-transformed titres. Antibody titres below assay cut-off were given an arbitrary value of half of the cut-off value to calculate GMT (Papers I and IV).

In paper II, a pooled analysis was performed for the total vaccination cohort at each sampling time-point. Seropositivity rates and the proportion of women with CVS antibody titres greater than or equal to the limit of quantitation for the assay were calculated with exact 95% CI for HPV 16 and 18 antibodies at each time-point. GMTs of anti-HPV 16/18 antibodies in serum were calculated, as were the GMTs of anti-HPV 16/18 antibodies in CVS samples in women (Papers II and III).

For samples with detectable antibodies in both serum and CVS, the log10 IgG corrected values of the ratios were calculated between anti-HPV 16 or anti-HPV 18 IgG titres in CVS samples and total IgG in CVS. Similar log10 IgG corrected values of the ratios were calculated for serum samples and used together with the values for CVS to calculate Pearson correlation coefficient between the corrected serum and CVS sample values. In order to minimize variation in the antibody titres variation during the menstrual cycle, antibody titres (expressed in EU/mL) measured in CVS and in serum were divided by the amount of total IgG measured in µg/mL. This ratio (expressed in EU/µg) was used for the correlation (Papers II and III).

In paper IV, seropositivity rates and GMT with 95% CIs were calculated for antibodies to each HPV antigen. Immunogenicity analyses were performed on study subjects initially seronegative for each HPV antigen. The immune responses induced by the HPV 16/18 vaccine in boys were compared to those induced in adolescent and young women in paper I. To demonstrate noninferiority, the upper limit of 95% CI, for the difference between the percentage of participants who
seroconverted in each group one month after the third vaccine dose, was less than 10%, and the upper limit of 95% CI, for the GMT ratio between each group one month after the third dose, was below 2.

In paper V, a Chi-square test with 95% CIs was used for the evaluation of differences in the frequency of HPV positive individuals in HPV-vaccinated, HBV-vaccinated, and unvaccinated arms.

4.6 Ethical statement

For the HPV 012 study (Papers I, II and III) each centre’s Institutional Ethical Review Board had approved the study and the associated material (EudraCT-number 200400117326). The HPV 011 study (Paper IV) and all distributed material were approved by the ethical review committee of the Pirkanmaa Hospital District (EudraCT-number 200500594324). Ethical approvals for the HPV 040 vaccination study and the Chlamydia screening trials were obtained in 2007 from the Ethical Review Boards of the Pirkanmaa Hospital District, Tampere, Finland (EudraCT-number 200700173155), and the North Ostrobothnia Hospital District, Oulu Finland (310/2009), respectively (Paper V)
5 RESULTS

5.1 Antibody response following HPV 16/18 vaccination in adolescent and young women (Paper I)

The total vaccinated cohort included 616 subjects aged 10-25 years and the according-to-protocol (ATP) cohort included 553 subjects. At baseline, 10% of the TVC participants aged 15-25 years were seropositive for HPV 16, 9% for HPV 18, and 6% had both HPV 16 and HPV18 antibodies. Among the 10-14-years-old participants, 3% were seropositive for HPV 16 and 4% for HPV 18, but none had antibodies to both HPV 16 and 18. Immunogenicity analyses were based on the ATP cohort and were performed on initially seronegative subjects only. If seropositivity for one HPV type was detected at baseline, the subject was not eligible to corresponding antibody analyses, but was eligible to antibody analyses for other HPV types.

All subjects (100%) seroconverted to both antigen types one month after completion of the full vaccine regimen (at month 7). In terms of seroconversion rates, the immunogenicity in the age group 10-14 was noninferior to the 15-25 age group. The antibody geometric mean titres levels elicited by the vaccine were substantially higher for both HPV 16 and HPV 18 in the 10-14 age group than in the 15-25 age group (Figures 5 and 6).

![Figure 5](image URL)

**Figure 5.** GMTs for HPV 16 in girls (10-14 years) and young women (15-25 years) at month 7 (Formulation 1).
5.2 Development of antibody levels following HPV 16/18 vaccination in adolescent and young women in time (Papers II and III)

Of the total vaccinated cohort in the primary HPV 012 study, altogether 321 subjects participating in the extension study were eligible, from three countries: Denmark, Estonia and Finland. Out of these, 243 subjects entered the study, and 220 (51 from the 10-14 age group and 169 from the 15-25 age group at first vaccination) attended the last visit at month 48 after first vaccine dose. The ATP cohort for immunogenicity included 193 subjects, 50 from the 10-14 age group and 143 from the 15-25 age group. Mean age of the participants at the month 48 visit was 15.7 years for the former, and 24.2 years for the latter.

All seroconverted individuals remained seropositive for both antigens up to 48 months. The anti-HPV 16/18 antibodies declined somewhat after the month 7 peak in all age groups and plateaued at month 36.

In the 10-14 years age group, the GMTs for anti-HPV 16 and anti-HPV 18 at month 48 were 2862.2 (2129.3-3847.3) and 940.8 (714.8-1238.3), respectively. The corresponding GMTs for the 15-25 year old vaccinees were significantly lower, 1186.2 (1007.4-1396.8) for HPV 16, and 469.8 (394.7-559.2) for HPV 18. The GMTs were 2.4-2.9-fold higher for anti-HPV 16 antibodies and 2.0-2.5-fold higher.
for anti-HPV 18 antibodies in the 10-14 year old age group than in the 15-25 year old age group. Compared to another study in which the same serological methods were used, and in which the subjects cleared a natural HPV 16/18-infection and induced an immune response (Paavonen et al. 2007), the GMTs for anti-HPV 16 antibodies in this study were 96.0-fold among 10-14-year-old girls, and 39.8-fold among 15-25-year-old women. The corresponding GMTs for the anti-HPV 18 antibodies were 41.4-fold and 20.7-fold higher, respectively. Comparison of the plateau levels observed in subjects from another study, in which sustained efficacy of the HPV 16/18 vaccine was demonstrated (GlaxoSmithKline Vaccine HPV 007 Study Group 2009), showed that the GMTs in the HPV 012 study for anti-HPV 16 and -18 antibodies were 7.2-fold and 3.2-fold higher in the 10-14 year age group and 3.0- and 1.6-fold higher in the 15-25 year age group (Figure 7).

The pooled study (Paper II) combined results from four different studies HPV 010, HPV 012, HPV 014 and HPV 028, and analysed them as one dataset. In the pooled study, all vaccinated subjects seroconverted by month 7 and remained seropositive through month 36, for both antigens. Similarly, the GMTs for anti-HPV 16/18-antibodies in the serum of HPV 16/18 vaccinated subjects were substantially higher at all time-points than antibody levels reported for 15-25-year-old women who had cleared a natural HPV infection (Paavonen et al. 2007).

5.3 Comparison of antibody levels in serum and cervicovaginal secretions in young women following HPV 16/18 vaccination (Papers II and III)

Cervicovaginal secretion samples were collected from 665 HPV 16/18 vaccinated females aged 10-65 in four different phase III immunogenicity trials (HPV 010, HPV 014, HPV 012 and HPV 028). Of these, 350 were included in the pooled analysis. Subjects were excluded, if the CVS sample was contaminated with blood, the collected secretion sample volume was insufficient, the subject was lost to follow-up, or if sample results were unavailable. Control CVS samples were collected from a subset of 20 Al(OH)3-recipients from a placebo controlled study (HPV 028) (Paper II).

Positivity rates for anti-HPV 16/18-antibodies in CVS ranged between 71.1-87.0% for HPV 16 and 55.3-73.1% for HPV 18, from month 18 through month 36. At month 12, the positivity rates for anti-HPV 16/18-antibodies were 100%,
respectively, but only 12 subjects were analysed at that time-point. The placebo-vaccinated controls from the HPV 028 study had no detectable anti-HPV 16/18 antibodies in the CVS at month 12. At month 18, none of the subjects had detectable anti-HPV 16 antibodies, but 2/20 subjects had detectable anti-HPV 18-antibodies in the CVS (Paper II).

Strong correlation between anti-HPV 16/18 antibodies in serum and CVS was observed in women vaccinated with the HPV 16/18 vaccine, at each time-point up to 36 months post vaccination. Pearson correlation coefficients for the correlation between serum and CVS antibody titres were 0.84 - 0.92 for HPV 16 and 0.90 - 0.91 for HPV 18 (Paper II).

In the extended HPV 012 study, in which serum and CVS samples were collected up to four years, anti-HPV 16 and -18 antibodies were analysed in 69 and 66 CVS samples, respectively, from subjects in the 15-25 years age group (Paper III). Anti-HPV 16 antibodies were present in 84.1% (95% CI: 73.3, 91.8) of the CVS samples, and anti-HPV 18 antibodies in 69.7% (95% CI: 57.1, 80.4) of the samples. The correlation between antibody levels in serum and CVS was equally strong in the HPV 012 extension study, as compared to the pooled study. The correlation coefficients between levels in serum and CVS for HPV 16 antibodies were 0.93, 0.91 and 0.84 at months 24, 36 and 48, and 0.93, 0.91 and 0.90 for HPV 18 antibodies, respectively (Figure 8).

In the same extension study, serum HPV 16 and 18 IgG antibody levels were evaluated at different time points and compared to the presence or absence of HPV 16/18 IgG antibodies in the CVS. Although all subjects remained seropositive for both anti-HPV 16 and 18 antibodies through month 48, the CVS anti-HPV 16/18 IgG antibodies were detectable only in about two thirds of subjects at month 48. Subjects without detectable CVS antibodies had significantly low serum antibody GMTs for anti-HPV 18 from the beginning, compared to subjects with detectable CVS antibodies (GMTs 1648.3 vs. 4011.4 at month 7 and 211.6 vs. 682.0 at month 48) and from month 24 onwards for anti-HPV 16 (GMTs 652.6 vs. 1479.4 at month 24 and 393.7 vs. 1260.8 at month 48). The difference in the HPV 16/18 serum antibody levels between subjects with or without detectable CVS antibodies increased with time.
Natural infection, subjects from study HPV 008 who were HPV 16 (a) or 18 (b) DNA negative and HPV 16 and 18 seropositive at baseline (i.e., who had cleared a natural infection) (Paavonen et al. 2007). Plateau phase. GMT at Months 45–50 (a) or at Months 75–76 (b) after the first vaccine dose from study HPV 007 in women aged 15–25 years (Total cohort; GMT=397.8 EL.U/ml (a) or GMT=279.4 EL.U/ml (b)). [10-14] (N= 49 for HPV 16 and N = 46 for HPV 18) [15-25] (N=123 for HPV 16 and HPV 18).
Figure 8. Correlation between serum and cervicovaginal secretion at Month 48 for HPV 16 (N = 58) (a) and HPV 18 (N = 46) (b) (standardised for total IgG) (TVC, 15-25 years) (Paper III).
5.4 Antibody responses following HPV 16/18 vaccination in males (Paper IV)

Of the altogether 270 male participants, a total of 181 from the HPV 16/18 group and 89 from the HBV group, were enrolled. The mean age was 14.4 years for the TVC. Altogether 97% of them received all three vaccine doses. At baseline, seropositivity was 3.5% for HPV 16, 7% for HPV 18 and 0.8% for both HPV 16 and HPV 18 antibodies. None of the 10-12 year old boys were baseline seropositive for either HPV 16 or HPV 18. All initially seronegative subjects who received the HPV 16/18 vaccine seroconverted for both the antigens by month 2, and all subjects seroconverted by month 7. During this time the antibody levels for HPV 16 antibodies increased 4-fold and for HPV 18 antibodies 2-fold.

Both the seroconversion rates and GMTs in boys aged 10-18 years were noninferior to those in for both seroconversion rates and GMTs compared to women aged 15-25 years (Paper I). Compared to women aged 15-25 the GMTs elicited by the HPV 16/18 vaccine were substantially higher at month 7 for both antigens in 10-18 year old boys. In boys aged 10-14 years HPV 16/18 antibody levels were higher than those reported for girls in the same age (Figure 9).

![Figure 9. GMTs for HPV 16 and HPV 18 antibodies in initially seronegative girls and boys and young women at month 7.](image-url)
5.5 Prevalence of HPV DNA between HPV 16/18 vaccinated and non-vaccinated young men (Paper V)

From the 1992-1993 male birth cohort altogether 395 (323 of 1283 vaccinated and 72 of 5193 unvaccinated) individuals donated a FVU sample for *C. trachomatis* screening at the age of 18-19 years. Pseudonymised leftover samples and the extracted DNAs were labelled as HPV 16/18 vaccinated, HBV vaccinated or unvaccinated, and analysed for the presence or absence of HPV DNA. In 6.9% of the samples from unvaccinated boys HPV DNA was detected. For vaccine types (16/18) and/or for HPV types (6/11/31/33/45/51), against which HPV 16/18 vaccine has been shown to induce cross-protection, the prevalence was approximately one third among the HPV 16/18 vaccinated boys as compared to the unvaccinated boys. Vaccine types HPV 16 or HPV 18 types were not detected among boys who had received the HPV vaccine (Table 3).

<table>
<thead>
<tr>
<th>HPV type</th>
<th>HPV 16/18 vaccinated n=323 Number of pos. (%)</th>
<th>Unvaccinated n=72 Number of pos. (%)</th>
<th>P for difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16/18</td>
<td>0 (0)</td>
<td>1 (1.4)</td>
<td>n.s.</td>
</tr>
<tr>
<td>6/11/31/33/45/51</td>
<td>8 (2.5)</td>
<td>4 (5.6)</td>
<td>n.s.</td>
</tr>
<tr>
<td>6/11/16/18/31/33/45/51</td>
<td>8 (2.5)</td>
<td>5 (6.9)</td>
<td>0.055</td>
</tr>
<tr>
<td>Other HPV</td>
<td>4 (1.2)</td>
<td>0 (0)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Any HPV</td>
<td>12 (3.7)</td>
<td>5 (6.9)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Table 3. Prevalence of human papillomavirus DNA detected in first void urine samples of 18 to 19 year old young men vaccinated with the HPV 16/18 vaccine as early adolescents between ages 12 to 15 years and in unvaccinated control men aged 18-19 years. Number of pos. = number of positive HPV DNA. n.s. = no significance.
The clinical development of the HPV 16/18 vaccine was initiated in 1999. The phase III studies, including the HPV 012 immunogenicity study, started in 2004 concomitantly with the large efficacy trial (HPV 008). Many similar HPV 16/18 vaccine immunogenicity trials were initiated at about the same time in different countries and in different age groups among women. The HPV 011 study was a phase I/II study and the first to study HPV 16/18 vaccine immunogenicity among males aged 10-18 years. Prevalences of secreted HPV DNA in urine among vaccinated and unvaccinated men have not been published before. HPV 16/18 vaccine efficacy trials in men are still missing.

The main purpose of the HPV 012 and 011 studies (Papers I, II, III and IV) was to evaluate vaccine immunogenicity in adolescent girls aged 10-14 years, boys aged 10-18 years, and young women aged 15-25 years. Regardless of age, all subjects seroconverted by month 7.

The seropositivity and high levels of vaccine-induced antibodies sustained remarkably unaltered in all female participants throughout 48 months. Soon after the third vaccine dose, the bivalent vaccine-induced antibody levels in female adolescents and young adults reached a plateau level, which was still substantially higher than the antibody levels induced by natural HPV infection (Paper III) (Paavonen et al. 2007, Schwarz et al. 2009). In the 15-25 years age-group, the serum HPV 16/18 antibody levels were somewhat higher than the corresponding antibody levels in HPV 16/18 vaccinated women of the same age in another study, where sustained protection against HPV 16/18 infection following the vaccination has been demonstrated for 8.4 years (Roteli-Martins et al. 2012). Interestingly, the antibody levels in our study (Paper III) were higher at month 48 than in a similar HPV 007 study in which the serological methods were the same. The studies applied somewhat different HPV 16/18 vaccine manufacturing modifications, but this may not have affected the immune response. The differences in GMTs are likely to be due to a population effect, as HPV 007 was conducted in Brazil,
Canada and the United States, and this study in Denmark, Estonia and Finland (GlaxoSmithKline Vaccine HPV 007 Study Group 2009).

In Paper III, the geometric mean titres were twice as high in females in the age-group of 10-14 years as in the age-group of 15-25 years. The humoral immune response to the HPV 16/18 vaccine was also stronger in boys in early adolescence, aged 10-14 years (Paper IV). This was expected, as the immune response is generally enhanced in younger populations compared to older age groups (Kumar et al. 2008), but the availability of intact B-cell clones in previously uninfected individuals might also have contributed. Although the immunogenicity analyses were performed on initially seronegative participants only, there might have been some subjects with HPV 16/18 seropositivity titres below the assay threshold, affecting the results in the older age group as compared to the younger age group.

Furthermore, the antibody levels elicited by the HPV 16/18 vaccine were high also in the 10-18 year old males (Paper IV). It is unclear why males tend to respond better to the bivalent vaccine than females. The difference could not be explained with baseline serostatus, as the analyses were done on initially seronegative individuals. On the contrary, the immunogenicity studies done with the quadrivalent vaccine show that men aged 16-26 years had lower antibody geometric mean titres to vaccine types than women of the same age (Hillman et al. 2012). Younger adolescent boys aged 10-15 years, however, responded with higher antibody levels than women aged 16-23 years. There are no studies on hormonal differences in males and females affecting HPV 16/18 vaccine responses that would explain the differences in the antibody levels. Hormonal contraceptive use during immunization with the quadrivalent vaccine does not affect the humoral responses, as measured in women at month 7 post vaccination (Giuliano et al. 2007a). Nor does smoking impair humoral antibody responses following HPV 16/18 vaccination (Simen-Kapeau et al. 2009a). Overall, the vaccine-induced immune responses at month 7 are comparable in males and females, and the differences in the antibody levels in this study are probably not relevant to the protective efficacy.

Long-term antibody levels induced by the bivalent vaccine have not yet been studied in men. The quadrivalent vaccine-induced immunologic responses in men have been comparable to those observed in women three years after immunization (Hillman et al. 2012). The antibody levels induced by the HPV 16/18 vaccine will
most likely remain high and result in long antibody persistence. Consequently, the vaccine would provide protection against HPV 16/18 infection for both genders and age groups. The long-term immunogenicity and efficacy of the bivalent vaccine in men needs to be studied further.

The serum antibody responses raised by the bivalent HPV vaccine appear to be generally higher than those raised by the quadrivalent HPV vaccine (Einstein et al. 2011). The antibody levels induced by the quadrivalent vaccine, especially those to HPV18, decline along with time to the level of antibodies elicited by natural infection. Still, the women vaccinated with the quadrivalent vaccine and whose antibody levels had declined to undetectable levels, remained protected against HPV infection and HPV-associated lesions up to four years (Joura et al. 2008). A booster dose of the quadrivalent vaccine five years after immunization induced a secondary response, after which the levels of antibodies to all vaccine HPV types were even higher than after the initial immunization doses (Olsson et al. 2009). This proves that the anamnestic memory exists despite decreased antibody levels.

Are the antibody levels induced by three doses of the bivalent vaccine unnecessarily high? Romanowski et al. reported results of high and stable antibody levels up to two years after two doses (months 0 and 6) of the HPV 16/18 vaccine (Romanowski et al. 2011). In a study by Safaeian et al., the HPV 16 antibody levels were at least 24 times higher and the HPV 18 antibody levels at least 14 times higher four years post vaccination among subjects who received two doses of the HPV 16/18 vaccine, and nine or five times higher, respectively, among subjects who received only one dose, than antibody levels induced by natural infection. Antibody levels following one-dose vaccination remained stable up to four years (Safaeian et al. 2013). These results suggest that even a single dose of the bivalent vaccine may induce long-term protection, but the immunological correlate of protection is not defined and further studies are needed. It is uncertain whether the quadrivalent vaccine would be effective after a single dose. The quadrivalent three-dose vaccine schedule may need an extra booster vaccine dose at some point, especially for HPV type 18, for the antibody levels to remain high, and for long-lasting protection.

The efficient antibody responses raised by the bivalent vaccine can be explained by the AS04-adjuvant in the vaccine preparation, which induces significantly high antibody responses for both HPV 16 and HPV 18 as compared to another bivalent
vaccine formulated with aluminium salt only (Giannini et al. 2006). This advantage is associated with the addition of monophosphoryl lipid A to aluminium salt. Monophosphoryl lipid A is a toll-like receptor 4 agonist that enhances humoral and cell-mediated response by rapidly triggering a local and transient cytokine response, which leads to an increased activation of antigen-presenting cells and results in an improved presentation of antigen to CD4+ T cells, and further antibody production and generation of memory B-cells. The elevated frequencies of memory B-cells have a positive impact on antibody persistence (Giannini et al. 2006). In this concept it is worth noting that the AS04 in the bivalent vaccine is different from the adjuvant AS03 used in the influenza H1N1 2009 Pandemrix™ vaccine and associated with increased narcolepsy incidence in Finland and some European countries. AS03 is composed of α-tocopherol, squalene and polysorbate 80, in an oil-in-water emulsion (Garcon et al. 2012).

The two HPV VLP vaccines, given with the normal three-dose schedule, give different immune responses, which probably influence the duration of protection. The four-year efficacy trials show that there are substantial differences in vaccine efficacies between women. This is especially true for high-grade lesions in baseline negative subjects as well as in the intention-to-treat group, irrespective of HPV in the lesion and in favour of the bivalent vaccine (Table 1) (Lehtinen et al. 2012, Munoz et al. 2010). In baseline negative individuals, the efficacy of the bivalent vaccine has been excellent against CIN3+ and AIS, irrespective of any HPV DNA in the lesion. This is probably associated with the different adjuvants in the vaccines but also with cross-protectivity. The proved cross-protective efficacy of the bivalent vaccine against the oncogenic non-vaccine HPV types 31, 33, 45 and 51 related lesions provides additional protection against cervical cancer and other HPV related diseases, and hence better the vaccine efficacy (Malagon et al. 2012). As the immune responses to HPV vaccination are also excellent in males, the vaccine is likely to protect also men against HPV-related diseases. Naturally this needs to be further evaluated.

The high vaccine-induced mucosal antibody levels correlated with serum HPV 16/18 antibodies and sustained up to 48 months (Paper III). Similar findings have been reported in other studies and also in women aged 26 to 55 years (Kemp et al. 2008, Schwarz et al. 2009). Comparing with the quadrivalent vaccine the bivalent vaccine induced CVS neutralizing antibodies levels higher for both HPV 16 and 18 corresponding with serum antibody levels (Einstein et al. 2011). The presence of
neutralizing antibodies at the cervical mucosa is important, as they prevent HPV from infecting the cervical basal cell layer at the transformation zone. Proof from principle studies does not yet exist, but neutralizing antibodies can be expected to transudate to all mucosal sites in the body, which is pivotal in preventing anogenital and oropharyngeal HPV infections.

The relationship of the vaccine-induced humoral antibodies with the mucosal antibody levels, as evaluated among women with regard to the presence or absence of cervicovaginal HPV 16 and HPV 18 IgG antibodies, revealed that about two thirds of the subjects had no detectable cervicovaginal HPV 16/18 antibodies (Paper III). Throughout the study, from month 24 to 48, subjects with detectable cervicovaginal antibodies had statistically significant, more than two to three times higher, serum geometric mean titres for HPV 16/18 antibodies than those devoid of cervicovaginal antibodies. Furthermore, a decline in serum antibody levels (rather than plateau) was evident in subjects without detectable cervicovaginal sample antibodies at month 48. These findings indicate that the vaccine-induced antibody levels at the lower genital tract mucosa depend on the vaccine-induced immune response reflected in the serum antibody level. In the subjects without detectable cervicovaginal antibodies, the geometric mean antibody titres for anti-HPV18 were lower from the very beginning, and for anti-HPV 16 from month 24 onwards. It is therefore possible that some individuals respond differently to the vaccine and might turn susceptible to HPV infection in future. Whether e.g. smoking or some other factors impaired the immune response, was not studied. According to a study by Nardelli-Haeflieger et al., hormonal contraceptive use by HPV 16 VLP-immunized women resulted in higher mucosal antibody levels than women who were ovulating (Nardelli-Haeflieger et al. 2003). The time of sampling with regard to the menstrual cycle was not reported in this study, but the levels of HPV-specific antibodies were normalized to total CVS antibody levels, to exclude possible menstrual cycle-dependent fluctuations.

The number of female subjects at month 48 follow-ups (Paper III) was lower than in the primary HPV 012 study, resulting in a limited number of subjects from whom CVS samples were collected. Cervicovaginal samples were not taken since the beginning of the study, although a comparison of the serostatus and the mucosal antibodies would have been of interest. The minimum level of protective antibody titre has not yet been established, but the minimum level of serum antibodies to be transudated to mucosa could probably be estimated in a larger
study. The HPV 16/18 IgG antibodies transudated to CVS in the 10-14 year old group were not studied due to the subjects’ young age but they would have been of interest later on in long-term follow-up. The proportion of baseline seropositive subjects that were excluded from the study was too small to be analysed for detection of secreted antibodies after natural infection. A correlation of neutralizing mucosal IgG antibodies with serum antibodies after natural HPV infection has been detected after HPV 16-infection, but not for other HPV types (Mbulawa et al. 2008). Whether the presence of mucosal antibodies reduces infectivity or transmission rates, has, however, not been studied.

Serum HPV 16/18 antibody levels that develop following natural genital HPV 16/18 infection are insufficient for protection against re-infection by HPV (Hildesheim et al. 2007) and are not cross-protective (Palmroth et al. 2010, Safaeian et al. 2010). The vaccine induced high HPV 16/18 antibody levels in the early adolescent males appeared to reduce the prevalence of HPV 16/18 DNA in 18-19 year old males, and probably also reduced the transmissibility of HPV 16 and 18 among them. The data on the utility of urine sampling for HPV DNA testing in women and men have been controversial due to sub-optimal sensitivity. The sensitivity of detecting HPV 16/18, or any high-risk HPV, by using a GP5+/6+ primer system in urine samples from women, was 75% and 84%, respectively, while the sensitivity was only 13% and 28% in male urine samples, respectively (Bissett et al. 2011). The modified general primer PCR system used in HPV analysis from urine samples in this study is more sensitive in detecting most of the high-risk HPV types than the classical GP5+/GP6+ primer system (Soderlund-Strand et al. 2009). Still, the number of subjects was too low to achieve significance in this study. A non-invasive test for detecting genital HPV DNA would be useful in screening and would increase compliance. Detecting urine HPV DNA in vaccinated and unvaccinated males and females needs further studies and larger sample sizes.

As HPV infection is usually acquired within three years of sexual debut, it is essential to provide preteens and young adolescents with the prophylactic HPV vaccine to decrease the burden of HPV-related diseases. Many countries have implemented HPV vaccination of girls in prevention of cervical cancer into vaccination programs. In countries like Australia with high vaccine coverage among females, herd immunity is achievable by female only vaccination (Donovan et al. 2011). However, in non-organized HPV vaccination programs, the coverage
rates remain quite low, at 30-40% (Rouzier & Giordanella 2010). The impact could be improved by extending vaccination to boys. If 80% coverage is reached among girls, the overall estimated impact is a 55% reduction of overall hrHPV prevalence and a 65% reduction of persistent hrHPV infection in females, and a 42% reduction in hrHPV prevalence in males, based on herd immunity (Vanska et al. 2013). It is not likely that 80% vaccine coverage will be reached. Current studies report an epidemic increase of HPV-associated diseases, such as anal and oropharyngeal cancers affecting both sexes (Bjorge et al. 1997, Mork et al. 2001, Nasman et al. 2009), which highlights the fact that men are not only vectors for transmitting HPV infections to females. Although HPV 16/18 vaccine efficacy trials are missing in males, the vaccine efficacies against anal and oropharyngeal HPV infections have been proved to be excellent in women (Herrero et al. 2013, Kreimer et al. 2011a). Consequently, HPV VLP vaccination of both genders would bring even better herd immunity, and would decrease the burden of HPV-related diseases in both females and males.
CONCLUSION

Both systemic and mucosal immune responses are important when a vaccine targeted mucosal infection is introduced. In this study it was shown that the HPV 16/18 vaccine generates excellent immune responses in both adolescent girls aged 10-14 years and young women aged 15-25 years, as well as in boys and young men aged 10-18 years. In women aged 15-25 years high serum antibody levels transudate to the mucosal surfaces where infection occurs, suggesting protection against vaccine HPV type infections which consequently reduces transmissibility. This can also be expected in men as the prevalence of HPV DNA in urine was reduced in HPV 16/18 vaccinated males compared to unvaccinated males, although larger sample sizes are needed to reach significance. Strong vaccine immune responses in age groups 10-25 years and in both genders due to the adjuvant system in the bivalent vaccine ensure a long-lasting duration of protection. Based on the demonstrated benefit of reduction in the rate of cervical precancerous lesions in vaccinated girls in large efficacy trials, the HPV vaccine implemented in vaccine programs in most developed countries focuses on reducing cervical cancer risk in women. As HPV is responsible for about 5% of all cancers worldwide in both genders, and the well tolerated bivalent vaccine provides excellent efficacy against high-grade cervical lesions and other HPV 16 and 18 related diseases, such as oropharyngeal and anal infections and lesions, the focus should be set on overall protection against HPV related diseases and reducing overall HPV burden. If the coverage of vaccination remains less than 80% the desired herd immunity might not be achieved. Consequently, the HPV 16/18 vaccine should be administered to both adolescent girls and adolescent boys prior sexual debut. By means of herd immunity this would be the only rational way to ensure a proper reduction in HPV transmission.

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References


Immunization of Early Adolescent Females with Human Papillomavirus Type 16 and 18 L1 Virus-Like Particle Vaccine Containing AS04 Adjuvant

Court Pedersen, M.D., Sc.D. a, Tiina Petaja, M.D. b, Gitte Strauss, M.D. c, Hans C. Rumke, M.D. d, Airi Poder, M.D. e, Jan Hendrik Richardus, M.D., Ph.D. f, Bart Spiessens, Ph.D. g, Dominique Descamps, M.D. g, Karin Hardt, Ph.D. g, Matti Lehtinen, M.D., Ph.D. b,*, and Gary Dubin, M.D. h, for the HPV Vaccine Adolescent Study Investigators Network

aOdense University Hospital, Odense, Denmark
bUniversity of Tampere, School of Public Health, Tampere, Finland
cBispebjerg hospital, Department of Dermato-venerology, Copenhagen, Denmark
dVaxinostics BV, University Vaccine Center, Rotterdam, The Netherlands
eTartu University Clinics, Dermatology, Tartu, Estonia
fMunicipal Public Health Service Rotterdam Area, Infectious Disease Control Rotterdam, Rotterdam, The Netherlands
gGlaxoSmithKline Biologicals, Rixensart, Belgium
hGlaxoSmithKline Biologicals, King of Prussia, Pennsylvania

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Abstract

Purpose: In female individuals 15–25-years of age, the AS04-containing human papillomavirus (HPV)–16/18 vaccine is highly immunogenic and provides up to 100% protection against HPV-16/18 persistent infection and associated cervical lesions up to 4.5 years. Optimal cervical cancer prevention will require prophylactic vaccination against oncogenic HPV 16 and 18 before the onset of sexual activity in early adolescent girls. To establish the feasibility of vaccination in girls 10–14 years of age, we compared the immunogenicity and safety in early adolescent female individuals to those 15–25 years in whom vaccine efficacy has been demonstrated.

Methods: We enrolled 773 female participants aged 10–14 years and 15–25 years to receive the HPV-16/18 L1 VLP AS04 vaccine, which was administered at months 0, 1, and 6. Serum samples were collected at months 0 and 7; antibodies to HPV 16 and 18 VLPs were measured by enzyme-linked immunosorbent assay. Vaccine safety was assessed at 7 or 30 days after each dose; serious adverse events were recorded during the entire study period.

Results: Both age groups achieved 100% seroconversion for HPV 16 and 18. Participants in the group aged 10–14 years were not only noninferior to those 15–25 years in terms of HPV 16 and 18 seroconversion rates but also had approximately twice as high geometric mean titers. The vaccine was generally safe and well tolerated.

Conclusions: These findings suggest that HPV vaccination during early adolescence is generally safe, well tolerated, and highly immunogenic. The observed higher antibody titers in the group 10–14 years of age are likely to result in longer antibody persistence. Overall, these data support the implementation of prophylactic HPV vaccination in this age group. © 2007 Society for Adolescent Medicine. All rights reserved.

Keywords: Human papillomavirus vaccine; Cervical cancer; Immunogenicity; AS04; Adolescent; Female
Cervical cancer is the second most common female cancer worldwide, with 493,000 new cases and 274,000 deaths reported in 2002 [1]. Persistent infection with genital human papillomavirus (HPV) types 16 and 18 is associated with highly increased risk for subsequent development of invasive cervical cancer (ICC) [2–4]. More than 99% of ICC cases are positive for at least one of the 15 oncogenic HPV types, which makes HPV infection a logical cause of cervical cancer [5]. In female individuals more than 15 years of age, HPV 16 and 18 are the most common HPV types found in ICC cases and cumulatively contribute to 70% of ICC worldwide [6].

Up to 20% of female adolescents will be infected with an oncogenic HPV type; among these types HPV 16 and 18 are commonly found [7–9]. In fact, 50% of the genital HPV types causing infections over a lifetime in a given woman are acquired during the first 3 years after sexual debut [10,11]. Almost all 40 HPV types that cause genital infections can be found in adolescent populations, and the persistence of genital oncogenic HPVs also among female adolescents is greater than that for nononcogenic HPVs [12].

Vaccination with prophylactic HPV virus–like particle (VLP) vaccines, comprising the L1 protein viral capsid, have demonstrated high levels of protection against HPV 16 and 18 incident infections, persistent infections and associated abnormal cytologic findings, and precancerous lesions [13–17]. Recent evidence of cross-protection against phylogenetically related HPV types using a bivalent HPV-16/18 L1 VLP AS04 vaccine indicates that prevention against incident infection may extend beyond HPV 16 and 18 [15]. The HPV-16/18 L1 VLP AS04 vaccine has also demonstrated a clinically acceptable safety profile up to 4.5 years in young women. These observations are further supported in other clinical trials using a vaccine also adjuvanted with AS04 [14,15,18,19].

Recently, the impact of vaccination with HPV 16 and 18 VLP vaccines has been estimated to substantially reduce low- and high-grade cervical abnormalities, such as atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial neoplasias (LSIL) abnormalities, high-grade squamous intraepithelial neoplasias (HSIL), and cancer. These estimates suggest that the vaccine may prevent 8–19% of ASCUS, 15–32% of LSIL, 41–57% of HSIL, and 65–77% of cancers. The total percentages of ASCUS, LSIL, HSIL, or cancer prevented by HPV-16/18 vaccination may increase with demonstration of cross-protection against cervical lesions [20].

Mathematical models evaluating the impact of the administration of a HPV-16/18 vaccine in 12-year-old girls, using efficacy estimates against HPV 16 and 18 associated ICC that ranges from 70% to 100%, predict that such a vaccine could contribute to a reduction in the lifetime risk of cancer of approximately 70% [21,22]. To reduce the burden of HPV 16/18 associated cervical disease, one important consideration in preventive strategies is to target early adolescent females before they engage in sexual activity.

The present phase III randomized study was performed to evaluate the immunogenicity and safety of the HPV-16/18 L1 VLP AS04 vaccine in early adolescent females 10–14 years of age as compared with those 15–25 years of age, an age group in which efficacy of the vaccine was previously shown in a clinical trial [14,15].

Methods

Study participants and ethics

The study took place from September 2004 to July 2005 in 17 centers in Denmark, Estonia, Finland, Greece, The Netherlands, and Russia. Study participants were recruited through hospitals, children outpatient clinics, schools, or in the general population. Recruitment tools included school recruitment sessions, recruitment letters, articles in local newspapers, leaflets, or advertisements. All distributed material had received prior approval by the Ethics Review Committees.

Prospective participants were enrolled in the study if they were female, aged 10–25 years, were abstinent from sexual activity, or were using adequate contraceptive precautions for 30 days before vaccination and up to 2 months after completion of the vaccination series if of childbearing potential, had negative pregnancy test results, and had no more than six lifetime sexual partners. Individuals were excluded from enrollment if they had used an investigational drug or vaccine within 30 days, chronic immunomodifying drugs within 6 months, immunoglobulins or blood products within 3 months or planned to use any of these during the study period, were pregnant or planning to become pregnant, breastfeeding, or had previously received HPV vaccine.

Each center’s Institutional Review Board approved the study and consent forms. Informed consent was obtained from each participant or participant’s parents/legally acceptable representative(s) before the performance of any study specific procedures. Participants below the legal age of consent were required to sign and date an informed assent. This study was registered with the European Clinical Trials Database.

Study design

This study included five parallel treatment groups in two age categories: 15–25 years (n = 458) and 10–14 years (n = 158). Participants aged 15–25 years were randomized to receive HPV-16/18 L1 VLP AS04 vaccine (one of three consistency lots; Groups 1, 2, and 3). Participants aged 10–14 years received HPV-16/18 L1 VLP AS04 vaccine from one of the three consistency lots (Lot 1, Group 4) (Figure 1). In the fifth treatment group, women 15–25 years of age (n = 154) were randomized to receive HPV vaccine...
prepared using a modified manufacturing process. These results are not included here.

A randomization blocking scheme (1:1:1:1:1) was used to ensure that treatments were assigned equally and randomly among the groups. The study vaccines were assigned treatment numbers from a randomization list generated at GlaxoSmithKline Biologicals (Rixensart, Belgium) using a standard SAS program (SAS Institute, Cary, NC). Participants were assigned a vaccine treatment number, and blinding was maintained to the individual treatment allocated. For all study participants, study personnel were blinded to the vaccine lot number.

**Study objectives**

One of the primary objectives of this study was to demonstrate lot-to-lot consistency of the vaccine in participants 15–25 years old (Groups 1, 2, and 3) in terms of immunogenicity. The secondary objectives were: (1) to demonstrate the noninferiority of the group aged 10–14 years compared with that in the group aged 15–25 years in terms of immunogenicity with the same vaccine lot; and (2) to compare immunogenicity results for all three lots combined: all groups aged 15–25 years together with immunogenicity results in those aged 15–25 years, in whom efficacy has been demonstrated [14]. In addition, vaccine safety was evaluated after each dose in all study participants. As a second primary endpoint, noninferiority in terms of immunogenicity between manufacturing processes, was also assessed. These results are not included here.

**Study vaccines**

Each dose of HPV-16/18 L1 VLP AS04 candidate vaccine (GlaxoSmithKline Biologicals, Rixensart, Belgium) contained 20 µg each of HPV 16 and 18 L1 proteins self-assembled as virus-like particles (VLP) and adjuvanted with AS04 (50 µg 3-O-desacyl-4′-monophosphoryl lipid A [MPL] and 500 µg aluminum hydroxide). The vaccine was produced using a Baculovirus Expression Vector System (BEVS) in which each type of VLP antigen was produced on a Hi-5 cell line derived from Trichoplusia ni. The vaccine was supplied in individual 0.5-ml prefilled syringes and administered into the deltoid muscle on a 0-, 1-, and 6-month schedule.

In the group 15–25 years of age, each participant received one of three manufacturing lots produced in a consecutive manner (consistency lots: Lots 1, 2, 3). Individuals in the group 10–14 years received the same vaccine lot (Lot 1) as one of the groups 15–25 years of age.
Serologic evaluation

At the initial and month 7 study visits, blood samples were collected from each participant to evaluate immunogenicity. All blood samples were evaluated for HPV 16 and HPV 18 antibodies using a type-specific enzyme-linked immunosorbent assay (ELISA) as reported elsewhere [15]. Seropositivity was defined as a titer greater than or equal to the assay threshold established at 8 ELISA U/ml (EU/ml) for HPV 16 and 7 EU/ml for HPV 18 [15].

Vaccine safety

On the day of vaccination, diary cards were given to participants to report solicited local and general symptoms during a 7-day follow-up period (days 0–6). Solicited local adverse events included pain, redness, and swelling at the injection site. Solicited general adverse events included fever, headache, fatigue, gastrointestinal symptoms (i.e., nausea, vomiting, diarrhea, abdominal pain), arthralgia, myalgia, rash, and urticaria. Grade 3 solicited adverse events were defined as pain that prevented normal activity, areas of redness or swelling greater than 50 mm, fever higher than 39.0°C (axillary temperature), urticaria distributed on at least four body areas, or events that prevented normal everyday activities. Urticaria or rash that appeared within 30 minutes of each vaccine dose was also documented by the investigator. Unsolicited signs and symptoms were reported within 30 days after each dose. Serious adverse events were reported throughout the study period. Serious adverse events were defined as any untoward medical occurrence that was life-threatening, required hospitalization, resulted in disability or incapacity, was an important medical event, resulted in death, or was a congenital anomaly/birth defect in the offspring of a study participant.

Statistical analysis

It was estimated that 360 evaluable participants aged 15–25 years (120 per group) were needed to achieve more than 92% power for consistency for the primary objective. This approach was used to rule out the null hypothesis that at least two of the three vaccine lots differed by more than twofold with respect to their geometric mean titers (GMTs).

Lot-to-lot consistency was demonstrated for all pairs of lots if, 1 month after the third dose, the two-sided 90% confidence intervals (CI) of the GMT ratio (i.e., ratio between GMTs) were within the clinical limit interval (0.5, 2).

To achieve the secondary objectives, 120 evaluable participants aged 10–14 years were needed. This approach provided 90% power to rule out that for a given HPV antigen, the difference between the percentage of individuals who seroconverted in the older age group when compared with the younger age group, was greater than 10%. Furthermore, it also provided 97% power to rule out that for a given HPV antigen, the GMT in the older group was more than twofold greater than in the younger group.

Noninferiority was demonstrated if, 1 month after the third dose, the upper limit of the 95% CI for the difference between the percentage of participants who seroconverted in each group was less than 10%, and if the upper limit of the 95% CI for the GMT ratio between each group was less than 2 (tests performed sequentially).

All sample size calculations were done using Pass 2000, assuming a standard deviation of 0.6 for the log_{10} transformed titers (based on another previously published study [14]) and assuming a baseline seroconversion rate of 95%.

The asymptotic two-sided confidence intervals for the ratio of GMTs were computed using an analysis of variance model on log_{10} transformed titers. Antibody titers below the cut-off of the assay were given an arbitrary value of half the cut-off value for the purpose of GMT calculation.

Safety analyses were based on the total vaccinated cohort. Incidence rates of solicited symptoms during the 7-day follow-up period and unsolicited symptoms during the 30-day follow-up period were tabulated with exact 95% CIs over all vaccine doses and for each treatment group. For the analysis of solicited symptoms, missing or nonevaluable measurements were not replaced and included only subjects with documented safety data (i.e., symptom sheet completed) per dose. The analysis of unsolicited adverse events and serious adverse events included all vaccinated participants. Participants who did not report an event were considered not to have experienced an event.

Statistical analyses were performed with SAS version 8.2 (SAS Institute, Cary, NC) and ProcStatXact 5 (Cytel Inc, Cambridge, MA).

Results

A total of 773 participants were enrolled within 3 months (September 5 to December 4, 2004). Study compliance was high (Figure 1), with almost all study participants (>95% in each group) receiving all three vaccine doses. The mean age was 20.2 years for the group 15–25 years and was 12.4 years for the group 10–14 years. Distribution of ethnicity was comparable among all groups (Table 1). In all, 21 participants withdrew from the study, including four for nonserious adverse events.

A computer programming error in the randomization web-based application used for treatment allocation to study participants was discovered during the trial. The extent of this web-based application error on the results was evaluated, and statistical analyses demonstrated that there was no statistically relevant effect on the validity of the immunogenicity results.

The trial profile for according-to-protocol (ATP) analyses and total vaccinated cohort analyses is described in Figure 1. Immunogenicity analyses were based on the ATP cohort and were performed on initially seronegative participants only. Participants seropositive for one HPV antigen at baseline were eliminated from the analysis for that anti-
Table 1
Demographic characteristics of participants receiving the human papillomavirus (HPV)–16/18 L1 VLP AS04 vaccine (total vaccinated cohort)

<table>
<thead>
<tr>
<th>Subjects 15–25-years</th>
<th>Subjects 10–14-years</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 458 (Lots 1–3)</td>
<td>N = 158 (Lot 1)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean age (years)</th>
<th>Country</th>
<th>Ethnic origin</th>
<th>Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.2</td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>12.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>The Netherlands</td>
<td>90 (19.7)</td>
<td>30 (19.0)</td>
<td></td>
</tr>
<tr>
<td>Russia</td>
<td>72 (15.7)</td>
<td>24 (15.2)</td>
<td>White/Caucasian</td>
</tr>
<tr>
<td>Greece</td>
<td>63 (13.8)</td>
<td>16 (10.1)</td>
<td></td>
</tr>
<tr>
<td>Estonia</td>
<td>59 (12.9)</td>
<td>21 (13.3)</td>
<td>Black</td>
</tr>
<tr>
<td>Denmark</td>
<td>116 (25.3)</td>
<td>43 (27.2)</td>
<td>Arabic/North African</td>
</tr>
<tr>
<td>Finland</td>
<td>58 (12.7)</td>
<td>24 (15.2)</td>
<td>East/South East Asia</td>
</tr>
<tr>
<td>Other</td>
<td>10 (2.2)</td>
<td>1 (0.6)</td>
<td></td>
</tr>
</tbody>
</table>

N = number of study participants; n (%) = number and percentage of participants.

Immunogenicity

Serostatus at study entry showed that 25% of the participants 15–25 years old were seropositive for HPV 16 and/or 18 antibodies 10% seropositive HPV 16 alone (n = 45), 9% HPV 18 alone (n = 40), and 6% for both HPV 16 and 18 (n = 27) Among participants 10–14 years old, 3% were positive for HPV 16 (n = 5), 4% for HPV 18 (n = 6), and none was infected with both HPV 16 and 18 types. Using the predefined statistical criteria, consistency was demonstrated among the three vaccine lots of vaccine. The GMTs (EU/ml) (95% CI) for HPV 16 were 7438.9 (6324.6–8749.6), 7150.3 (6038.1–8467.3), and 7297.2 (6136.8–8677.0) for Groups 1, 2, and 3, respectively. The upper limits of the 95% CIs for both seroconversion rates and GMT ratios were below the predefined limits for demonstrating noninferiority (Figure 2).

Safety

Compliance in returning symptom sheets was high (greater than 98% for all groups). Safety profiles were indistinguishable between the study groups; in addition, there was a similar frequency of reporting solicited local and general symptoms among groups. The most commonly reported solicited local symptom was pain at the injection site (Table 2). No urticaria or rash was reported by the investigator within 30 minutes of vaccine administration for any study participant.

The most frequently reported solicited general symptoms were fatigue, headache, and myalgia (Table 3). Most solicited adverse events were transient, lasting no longer than 2–3 days; more importantly, the incidence of adverse events did not increase with increasing number of doses. Grade 3 adverse events were reported infrequently.

In general, the frequency of unsolicited symptoms reported during the 30-day postvaccination period after each dose was similar between groups. Fewer symptoms were reported in the group aged 10–14 years (incidence of any grade unsolicited symptom: 16.5%; Grade 3: 2.1%) than in the group 15–25 years (pooled lots, incidence of any grade unsolicited symptom: 23.0%; Grade 3: 2.5%). The most frequently reported unsolicited adverse events were injection site reactions, headache, and influenza (reported respectively after 4.4%, 2.7%, and 1.4% of vaccine doses in the pooled group aged 15–25 years and after 1.5%, 1.3%, and 0.4% of vaccine doses in the group 10–14 years).

No participants withdrew from the study because of a serious adverse event. Four participants withdrew from the study because of nonserious adverse events. Three participants in the group 15–25 years of age reported three of the four nonserious adverse events that were considered by the investigator not to be related to study vaccination (fatigue, abortion threatened, and perioral rash). One participant in the group 10–14 years of age reported nausea, which was considered by the investigator to be causally related to vaccination.

Eight serious adverse events occurred in eight participants: seven in the group 15–25 years old (myocarditis, pericarditis, gastric ulcer, acute sinusitis, heat stroke, insulin-dependent diabetes mellitus, and threatened abortion) and one in the group 10–14 years old (depression). None were fatal, and none were considered by the investigator to be related to study vaccination.
Discussion

Besides demonstration of consistency between different lots of the HPV-16/18 L1 VLP AS04 vaccine, this study showed that 100% seroconversion for both HPV 16 and 18 was achieved in all age groups. The immunogenicity of the vaccine was significantly higher when administered to early adolescents, with post-vaccination GMTs that were at least twofold higher than in the group aged 15–25 years for both HPV 16 and 18.

Previously published results in female individuals aged 15–25 years who were seronegative for HPV 16 and 18, and DNA negative for high-risk HPV types at study entry, show high vaccine efficacy for up to 4.5 years in the prevention of HPV 16/18 incident and persistent infections and associated cytohistological abnormalities [14,15]. When comparing the antibody responses reported in this published study to our current results, we observed that the GMTs in the group 10–14 years of age were substantially higher than the antibody levels obtained at 50–53 months postvaccination,
and where sustained vaccine efficacy has been observed [15]. In the group 15–25 years of age, GMT values are comparable between studies. These observations suggest that similar vaccine efficacy against HPV 16/18 related virological and clinical outcomes could be expected in the present study population.

In both age groups, the high level of antibody titers induced by the HPV-16/18 L1 VLP AS04 vaccine may in part be explained by the presence of the AS04 adjuvant in the vaccine formulation. Recently published clinical data show that the vaccine formulated with AS04 induced higher and sustained antibody levels against HPV 16 and HPV 18 and more robust memory B-cell responses when compared with the same HPV VLP vaccine formulated with conventional aluminum salt only [23].

Antibody responses in the group aged 10–14 years did not compromise the safety profile of the vaccine; on the contrary, there were actually fewer adverse reactions reported among individuals 10–14 years of age than in those 15–25 years. Assessments of safety show that the HPV-16/18 L1 VLP AS04 vaccine was well tolerated and similar safety profiles were observed in both age groups.

High prevalence of HPV 16/18 has been shown in adolescents, although a majority of HPV infections are frequently transient; there are a high number of infections that develop into abnormal cervical cytologic findings or precancerous cervical lesions [2,10]. Furthermore, adolescent girls may also have greater susceptibility to HPV infection and malignancy based on the anatomical features of the cervix, where there is an enlarged cervical transformation zone, the area in which almost all cancerous cervical lesions arise [24–26]. Thus, the administration of a prophylactic HPV vaccine before sexual debut should be considered when determining vaccine implementation.

Table 2
Incidence of solicited local symptoms reported during the 7-day follow-up period, overall per dose (total vaccinated cohort)

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Type</th>
<th>Subjects 15–25 years (N = 1341)</th>
<th>Subjects 10–14 years (N = 463)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%) (95% CI)</td>
<td>n (%) (95% CI)</td>
<td></td>
</tr>
<tr>
<td>Pain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>1147 (85.5) (83.5–87.4)</td>
<td>387 (83.6) (79.9–86.8)</td>
<td></td>
</tr>
<tr>
<td>Grade 3a</td>
<td>69 (5.1) (4.0–6.5)</td>
<td>17 (3.7) (2.2–5.8)</td>
<td></td>
</tr>
<tr>
<td>Redness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>530 (39.5) (36.9–42.2)</td>
<td>165 (35.6) (31.3–40.2)</td>
<td></td>
</tr>
<tr>
<td>&gt;50 mm</td>
<td>17 (1.3) (0.7–2.0)</td>
<td>4 (0.9) (0.2–2.2)</td>
<td></td>
</tr>
<tr>
<td>Swelling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>448 (33.4) (30.9–36.0)</td>
<td>155 (33.5) (29.2–38.0)</td>
<td></td>
</tr>
<tr>
<td>&gt;50 mm</td>
<td>22 (1.6) (1.0–2.5)</td>
<td>6 (1.3) (0.5–2.8)</td>
<td></td>
</tr>
</tbody>
</table>

N = number of documented doses (with safety diary cards returned); CI = exact confidence interval; n (%) = number/percentages of doses that were followed by at least one symptom.

a Pain that prevented normal activity.

Table 3
Incidence of solicited general symptoms reported during the 7-day follow-up period after administration of human papillomavirus (HPV)–16/18 L1 VLP AS04 vaccine, overall per dose (total vaccinated cohort)

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Type</th>
<th>Subjects 15–25 years (N = 1342)</th>
<th>Subjects 10–14 years (N = 463)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%) (95% CI)</td>
<td>n (%) (95% CI)</td>
<td></td>
</tr>
<tr>
<td>Arthralgia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>125 (9.3) (7.8–11.0)</td>
<td>45 (9.7) (7.2–12.8)</td>
<td></td>
</tr>
<tr>
<td>Grade 3a</td>
<td>4 (0.3) (0.1–0.8)</td>
<td>1 (0.2) (0.0–1.2)</td>
<td></td>
</tr>
<tr>
<td>Fatigue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>399 (29.7) (27.3–32.3)</td>
<td>137 (29.6) (25.5–34.0)</td>
<td></td>
</tr>
<tr>
<td>Grade 3a</td>
<td>11 (0.8) (0.4–1.5)</td>
<td>7 (1.5) (0.6–3.1)</td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>46 (3.4) (2.5–4.5)</td>
<td>18 (3.9) (2.3–6.1)</td>
<td></td>
</tr>
<tr>
<td>&gt;39.0°C</td>
<td>0 (0.0) (0.0–0.3)</td>
<td>2 (0.4) (0.1–1.6)</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>187 (13.9) (12.1–15.9)</td>
<td>55 (11.9) (9.1–15.2)</td>
<td></td>
</tr>
<tr>
<td>Grade 3a</td>
<td>5 (0.4) (0.1–0.9)</td>
<td>1 (0.2) (0.0–0.8)</td>
<td></td>
</tr>
<tr>
<td>Headache</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>402 (30.0) (27.5–32.5)</td>
<td>126 (27.2) (23.2–31.5)</td>
<td></td>
</tr>
<tr>
<td>Grade 3a</td>
<td>12 (0.9) (0.5–1.6)</td>
<td>5 (1.1) (0.4–2.5)</td>
<td></td>
</tr>
<tr>
<td>Myalgia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>403 (30.0) (27.6–32.6)</td>
<td>140 (30.2) (26.1–34.6)</td>
<td></td>
</tr>
<tr>
<td>Grade 3a</td>
<td>16 (1.2) (0.7–1.9)</td>
<td>5 (1.1) (0.4–2.5)</td>
<td></td>
</tr>
<tr>
<td>Rash</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>50 (3.7) (2.8–4.9)</td>
<td>24 (5.2) (3.3–7.6)</td>
<td></td>
</tr>
<tr>
<td>Grade 3a</td>
<td>1 (0.1) (0.0–0.4)</td>
<td>2 (0.4) (0.1–1.6)</td>
<td></td>
</tr>
<tr>
<td>Urticaria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>22 (1.6) (1.0–2.5)</td>
<td>5 (1.1) (0.4–2.5)</td>
<td></td>
</tr>
<tr>
<td>Grade 3b</td>
<td>0 (0.0) (0.0–0.3)</td>
<td>0 (0.0) (0.0–0.8)</td>
<td></td>
</tr>
</tbody>
</table>

N = number of documented doses (with safety diary cards returned); CI = exact confidence interval; n (%) = number/percentages of doses that were followed by at least one symptom.

a Symptom that prevented normal activity.
b Urticaria present over at least four body areas.
strategies that may provide potentially substantial public health benefits.

The present study demonstrates that the HPV-16/18 L1 VLP AS04 vaccine is generally safe and well tolerated and induces excellent immunogenicity in female individuals 10–25 years of age. The findings provide evidence that HPV vaccination during early adolescence is feasible and induces even higher HPV 16/18 antibody levels than in young adult women. Additional data and analyses are needed to determine long-term immunogenicity and safety of the HPV-16/18 L1 VLP AS04 vaccine when administered to an early adolescent population. However, all the presently available evidence is in support of administering the vaccine to early adolescent females.

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References

Correlation between levels of human papillomavirus (HPV)-16 and -18 antibodies in serum and cervicovaginal secretions in girls and women vaccinated with the HPV-16/18 AS04-adjuvanted vaccine

Tino F. Schwarz, 1,* Mariëlle Kocken, 2 Tiina Petäjä, 3 Mark H. Einstein, 4 Marek Spaczynski, 5 Jacqueline A. Louwers, 6 Court Pedersen, 7 Myron Levin, 8 Toufik Zahaf, 9 Sylviane Poncelet, 9 Karin Hardt, 9 Dominique Descamps 9 and Gary Dubin 10

Key words: human papillomavirus, HPV, prophylactic HPV vaccines, HPV-16/18 AS04-adjuvanted vaccine, cervicovaginal secretions

Abbreviations: CVS, cervicovaginal secretions; ELISA, enzyme-linked immunosorbent assay; GMT, geometric mean titer; HPV, human papillomavirus; IgG, immunoglobulin G; MPL, monophosphoryl lipid A; VLPs, virus-like particles

This pooled analysis of data from four Phase III clinical trials was undertaken to assess the correlation between levels of anti-human papillomavirus (HPV)-16/18 antibodies in serum and cervicovaginal secretions (CVS) in girls and women vaccinated with the HPV-16/18 AS04-adjuvanted vaccine. Serum and CVS samples were collected from a subset of women aged 10–65 years (n = 350) at pre-specified time-points from 7 to 36 months post-vaccination. Anti-HPV-16/18 antibody levels in serum and CVS were measured by enzyme-linked immunosorbent assay. Pearson correlation coefficients between serum and CVS antibody levels, standardized for total immunoglobulin G, were calculated at each time-point in women with detectable antibodies in both serum and CVS. All subjects had seroconverted at month 7 and remained seropositive through month 36 for both antigens. Geometric mean titers of anti-HPV-16/18 antibodies in serum were substantially higher at all time-points than those in a control group of women who had cleared a natural HPV infection in another trial. In women with detectable antibodies in both serum and CVS, good correlation was seen between HPV-16/18 antibody levels at all time-points (Pearson correlation coefficient: 0.84–0.92 for HPV-16 and 0.90–0.91 for HPV-18). The strong correlation between levels of HPV-16/18 antibodies in serum and CVS up to 36 months post-vaccination in girls and women vaccinated with the HPV-16/18 AS04-adjuvanted vaccine supports transudation of serum antibodies as the mechanism by which antibodies are introduced into CVS. These CVS antibodies may play a role in the protective efficacy of this vaccine.

Introduction

Persistent infection with an oncogenic human papillomavirus (HPV) type is the necessary cause for the development of cervical cancer, 1,2 the second most frequent cause of cancer death among women worldwide.3,4 Among the HPV types currently classified as ‘oncogenic’ or ‘high-risk,’ HPV-16 and HPV-18 are the most common, cumulatively accounting for just over 70% of all cases of invasive cervical cancer.5-7

Large-scale, randomized, controlled clinical trials have shown the HPV-16/18 AS04-adjuvanted vaccine (Cervarix 8) to be highly effective for the prevention of HPV-16 and HPV-18 infection and associated cervical lesions.8,9 This vaccine contains HPV-16 and HPV-18 virus-like particles (VLPs), produced in Trichoplusia ni insect cells by a recombinant baculovirus expression vector system, formulated with a proprietary immunostimulatory adjuvant system AS04, comprising monophosphoryl lipid A (MPL) and aluminum salt.10 MPL is a purified, detoxified derivative of the
lipopolysaccharide molecule of the bacterial wall of Salmonella minnesota and is a powerful immunoenhancer, known to act as a Toll-like receptor agonist.14,15

The mechanism by which prophylactic HPV vaccines induce protection has not been fully elucidated, but seems to involve both cellular immunity and neutralizing immunoglobulin G (IgG) antibodies across the cervical epithelium to prevent virus particles from infecting the cervical basal cell layer at the transformation zone where cervical cancers usually develop.16-19 Exudation of antibody-specific antibodies to be essential for reliable protection against HPV infection.21-29 Transudation of vaccine-specific serum neutralizing antibodies may prevent new infections or reinfection at another site in the cervical, vaginal or vulvar region, or prevent shedding of viral particles of an active infection.18

The presence of anti-HPV antibodies at the cervix has been reported in women with HPV-16 infection30 and after administration of the HPV-16/18 AS04-adjuvanted vaccine in four Phase III clinical trials [HPV-010 (NCT00423046),35 HPV-012 (NCT00196937),36 HPV-028 (NCT00456807),37 Overall age range of women in all studies at each time-point.]

As shown in Table 1, serum GMTs were consistently higher in women who had detectable anti-HPV-18 antibodies in CVS at all time-points. Strong correlation was observed between GMTs and geometric mean antibody titers (GMTs) of anti-HPV-16/18 antibodies in women who received the HPV-16/18 AS04-adjuvanted vaccine in four Phase III clinical trials [HPV-010 (NCT00423046),35 HPV-012 (NCT00196937),36 HPV-014 (NCT00169494/00337818),34 HPV-028, NCT00456807,37 of which 350 (52.6%) were included in this pooled analysis (Table 1). Reasons for exclusion from the analysis were contamination of samples with blood (i.e., samples above the predefined Hemastix® threshold) (71.4%), secretion volume not sufficient (10.5%), lost to follow-up (9.2%), lost sample (8.6%) and enzyme-linked immunosorbent assay (ELISA) serum result not available (0.3%). To serve as a negative control, CVS samples were also collected from a subset of 46 women who received Al(OH)3 in the placebo-controlled study (HPV-028),37 of which 20 were included in this analysis. The most common reason for exclusion of control samples was contamination of samples with blood.

Immunogenicity. All subjects had seroconverted by month 7 and remained seropositive through month 36 to both antigens (Table 1). Serum geometric mean titers (GMTs) of anti-HPV-16/18 antibodies in women who received the HPV-16/18 AS04-adjuvanted vaccine were substantially higher at all time-points than antibody levels in baseline serum samples in a control group of women who had evidence of having cleared a natural HPV infection in another trial.10 Positivity rates for anti-HPV-16/18 antibodies in CVS were 95.4% and 92.3%, respectively, at month 7 and ranged between 71.1–100% and 55.3–100%, respectively, from month 12 through month 36 (Table 1). In the placebo-controlled study (HPV-028), none of the subjects who received Al(OH)3 control had detectable anti-HPV-16 antibodies in CVS samples at month 12 or 18. Similarly, none of these subjects had detectable anti-HPV-18 antibodies in CVS samples at month 12, although 2/20 subjects (10%) had detectable anti-HPV-18 antibodies in CVS at month 18. Serum anti-HPV-18 GMTs in these two subjects at this time-point were 991 and 20.7 EU/ml, respectively.

As shown in Figure 1, serum GMTs were consistently higher in women who had detectable anti-HPV-16/18 antibodies in CVS at all time-points. Strong correlation was observed between GMTs and geometric mean antibody titers (GMTs) of anti-HPV-16/18 antibodies in women who received the HPV-16/18 AS04-adjuvanted vaccine in four Phase III clinical trials [HPV-010 (NCT00423046),35 HPV-012 (NCT00196937),36 HPV-014 (NCT00169494/00337818),34 HPV-028, NCT00456807,37 Overall age range of women in all studies at each time-point.]

Table 1. Number of women with evaluable serum and CVS samples and proportion with detectable anti-HPV-16/18 antibodies in serum and CVS at each time point (Total Vaccinated Cohort)

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Sampling time (month)</th>
<th>Studya</th>
<th>Age† (years)</th>
<th>No. with evaluable samples</th>
<th>Serum positivity rates % (95% CI)</th>
<th>CVS positivity rates % (95% CI)</th>
<th>Serum positivity rates % (95% CI)</th>
<th>CVS positivity rates % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-16/18 AS04-adjuvanted vaccine</td>
<td>7</td>
<td>HPV-010</td>
<td>18–45</td>
<td>65</td>
<td>100 (94.5, 100)</td>
<td>95.4 (87.1, 98.8)</td>
<td>100 (94.5, 100)</td>
<td>92.3 (83.0, 97.3)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>HPV-028</td>
<td>26–65</td>
<td>12</td>
<td>100 (73.5, 100)</td>
<td>100 (73.5, 100)</td>
<td>100 (73.5, 100)</td>
<td>100 (73.5, 100)</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>HPV-014/ HPV-028</td>
<td>15–65</td>
<td>152</td>
<td>100 (97.6, 100)</td>
<td>71.1 (63.2, 83.1)</td>
<td>100 (97.6, 100)</td>
<td>55.3 (47.0, 75.1)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>HPV-014/ HPV-012</td>
<td>10–55</td>
<td>216</td>
<td>100 (98.3, 100)</td>
<td>77.8 (71.6, 86.2)</td>
<td>100 (98.3, 100)</td>
<td>63.4 (56.6, 78.2)</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>HPV-012</td>
<td>10–25</td>
<td>108</td>
<td>100 (96.0, 100)</td>
<td>87.0 (79.2, 93.4)</td>
<td>100 (96.6, 100)</td>
<td>73.1 (63.8, 85.3)</td>
</tr>
<tr>
<td>Al(OH)3 control</td>
<td>12</td>
<td>HPV-028</td>
<td>26–65</td>
<td>10</td>
<td>0.0 (0.0, 30.8)</td>
<td>0.0 (0.0, 30.8)</td>
<td>0.0 (0.0, 30.8)</td>
<td>0.0 (0.0, 30.8)</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>HPV-028</td>
<td>26–65</td>
<td>20</td>
<td>0.0 (0.0, 16.8)</td>
<td>0.0 (0.0, 16.8)</td>
<td>10.0 (1.2, 31.7)</td>
<td>10.0 (1.2, 31.7)</td>
</tr>
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</table>

CVS, cervicovaginal secretion; 95% CI, 95% confidence interval *etrack/NCT study numbers: HPV-010, NCT00423046;35 HPV-012, NCT00169494/00337818;34 HPV-014, NCT00196937;36 HPV-028, NCT00456807;37 Overall age range of women in all studies at each time-point.
between anti-HPV-16/18 antibody levels in serum and CVS in women who received the HPV-16/18 AS04-adjuvanted vaccine at each time-point (Fig. 2). Pearson correlation coefficients for the correlation between serum and CVS antibody titers were 0.84–0.92 for HPV-16 and 0.90–0.91 for HPV-18.

**Discussion**

Cervical HPV infections are restricted to the intraepithelial layer of the mucosa and typically do not induce a vigorous systemic immune response. Only 50–60% of women infected with HPV develop serum antibodies after natural infection and these antibodies do not necessarily protect against subsequent infection by the same HPV type. Although the exact mechanism of action of prophylactic HPV vaccines has yet to be fully elucidated, vaccine-induced serum neutralizing IgG antibodies that transude to the site of infection appear to play a pivotal role in determining whether a subsequent HPV infection will be cleared or will persist, which may result in the development of cervical neoplasia.

This pooled analysis of data from four separate Phase III studies was undertaken to assess the correlation between anti-HPV-16/18 antibody levels in serum and CVS over time in girls and women vaccinated with the HPV-16/18 AS04-adjuvanted vaccine. All subjects were found to be seropositive to both antigens from month 7 through month 36, with anti-HPV-16/18 antibody GMTs at all time-points substantially higher than antibody levels reported in women who had evidence of having cleared a natural infection (i.e., who were seropositive, but HPV DNA negative, for the respective HPV type at baseline) in the Phase III PATRICIA study (NCT00122681/580299/008). In the absence of a serological correlate of protection, it is generally accepted that vaccination should induce higher serum antibody levels than those induced by natural infection.

Positivity rates for anti-HPV-16 and anti-HPV-18 antibodies in CVS ranged between 71.1–100% and 55.3–100%, respectively, over the 36 months of follow-up. Women who had detectable anti-HPV-16/18 antibodies in CVS were consistently found to have higher antibody levels in serum. Indeed, a strong correlation was observed between antibody levels in serum and CVS at all time-points, supporting transudation of vaccine-induced antibodies into CVS. Cervical cancers usually develop at the metaplastic zone between the squamous and columnar epithelium in the cervix known as the transformation zone. Results of the placebo-controlled trial (HPV-028) show the detection of antibodies in CVS to be vaccine-dependent. While anti-HPV-16/18 antibodies were detected in CVS samples of women who received the HPV-16/18
As women are exposed to HPV infections when they become sexually active and remain at risk throughout their entire lives, vaccination must induce long-term protection. The high correlation between HPV-16 and HPV-18 IgG antibody levels in serum and CVS in this pooled analysis persisted through 36 months after the first vaccination. All women remained seropositive for anti-HPV-16/18 antibodies at this time, with CVS positivity rates at all time-points, anti-HPV-16/18 antibodies were only detected at month 18 in CVS samples in two subjects who received Al(OH)₃. The serum anti-HPV-18 antibody GMTs seen in these subjects (991 and 20.7 EU/ml, respectively) and the fact that these subjects had no detectable anti-HPV-18 antibodies in serum or CVS samples at Month 12 suggest that this was most likely due to natural infection.

**Figure 2.** Correlation of ratio between CVS and serum samples by time for (A) HPV-16 and (B) HPV-18. Titers were measured using an ELISA assay for detection of anti-HPV-16 and 18 antibodies for both CVS and serum samples. The scatter plots show the ratio (specific IgG/total IgG) transformed to linear log₁₀ values and plotted.
of 87.0% and 73.1%, respectively. Results of HPV-014 confirm that the correlation between serum and CVS anti-HPV-16/18 levels in women vaccinated with the HPV-16/18 AS04-adjuvanted vaccine is independent of age, with high correlation seen in all age groups in this study including post-menopausal women, who would be expected to produce significantly less CVS due to decreased estrogen production.34 Correlation coefficients were 0.90 in women aged 15–25 years, 0.73 in women aged 26–45 years and 0.88 in women aged 46–55 years for HPV-16 and 0.91, 0.82 and 0.93 in the three age groups, respectively, for HPV-18.34 The HPV-16/18 AS04-adjuvanted vaccine has also been shown to induce higher anti-HPV-16/18 serum antibody levels and CVS positivity rates than another prophylactic HPV vaccine containing HPV-16 and HPV-18 VLPs formulated with amorphous aluminum hydroxyphosphate sulfate salt.35

Positivity rates in this pooled analysis were determined by direct ELISA, which measures levels of both neutralizing and non-neutralizing antibodies. A pseudovirus-based neutralization assay (PBNA) has been developed to directly measure levels of HPV type-specific neutralizing antibodies believed to be relevant for protection.44 However, use of the PBNA assay to measure anti-HPV-16/18 neutralizing antibody levels in CVS samples is associated with methodological challenges that may potentially reduce the sensitivity of this assay and variability is higher than with the ELISA assay.50 High correlation has been demonstrated between the PBNA and the direct ELISA for the measurement of antibody responses in both serum and CVS samples.33 This correlation has been shown to persist for up to 6.4 years after vaccination and to be independent of the age of the vaccine recipient, suggesting that the direct ELISA is a good surrogate for neutralizing activity and well-suited for evaluating antibody responses induced by HPV vaccines.

Antibody levels in CVS have been shown to vary during the menstrual cycle, most likely due to hormonal fluctuation.31,47 Total and HPV-specific IgG show similar fluctuations, and decrease around the time of ovulation. To address this fluctuation in CVS antibody levels, total IgG titers were also determined in both serum and CVS samples to allow for standardization of immunogenicity results prior to evaluation of the correlation, by dividing anti-HPV-16 and anti-HPV-18 IgG titers by the corresponding total IgG titer. Oral contraceptive use has also been reported to influence cervical IgG levels.31,47 However, this does not appear to be an important modifier of the degree of correlation observed between anti-HPV-16/18 antibody titers in serum and CVS samples.33

The main limitations of the analysis described in this paper are the fact that this is a pooled analysis of data from different studies, the variation in duration of follow-up between studies and the low number of samples in some groups (e.g., month 12 data and the negative control group in Study HPV-028). Almost half of all CVS samples collected were not suitable for analysis, mainly due to blood contamination (i.e., samples above the Hemastix® threshold for inclusion), which was anticipated as the collection method involved application of the sponge for at least 30 seconds to the cervix. In addition, anti-HPV-16 and anti-HPV-18 IgA or secretory IgA levels in CVS were not investigated. IgG is the principal immunoglobulin present in the female genital tract.48,49 While systemic immunization is known to induce poor levels of mucosal IgA in animal models,31 IgA antibodies have been reported in serum and CVS in women with persistent HPV-16 infection.40,50 However, levels of serum IgA antibodies to HPV-16 VLPs were much lower than levels of serum IgG antibodies, making IgA antibodies more difficult to detect.

In summary, the main public health goal of prophylactic HPV vaccines is to reduce the incidence of cervical cancer and precancer by preventing the establishment of persistent cervical HPV infections. The strong correlation between levels of HPV-16/18 antibodies in serum and CVS up to 36 months after vaccination in girls and women vaccinated with the HPV-16/18 AS04-adjuvanted vaccine supports transudation of serum antibodies into CVS. High levels of CVS antibodies are likely to play an important role in the protective efficacy of this vaccine by preventing virus particles from infecting the cervical basal cell layer at the transformation zone.

Subjects and Methods

Study design. Data on antibody levels in CVS in girls and women vaccinated with the HPV-16/18 AS04-adjuvanted vaccine were pooled from four Phase III studies: HPV-010 (NCT00423046), HPV-012 (NCT00169494/00337818), HPV-014 (NCT00196937)34 and HPV-028 (NCT00456807)37 (Table 1). In Study HPV-010, a total of 1,106 women were stratified by age (18–26, 27–35 and 36–45 years) and randomized (1:1 ratio in each age stratum) to receive HPV-16/18 AS04-adjuvanted vaccine or another prophylactic HPV vaccine (Gardasil®, which contains HPV-6, 11, 16 and 18 VLPs formulated with amorphous aluminum hydroxyphosphate sulfate salt), according to their respective three-dose schedules (0, 1, 6 or 0, 2, 6 months).35 In this paper, only results for the HPV-16/18 AS04-adjuvanted vaccine are presented. Study HPV-012 compared the immunogenicity and safety of the HPV-16/18 AS04-adjuvanted vaccine in a total of 158 girls aged 10–14 years and 458 women aged 15–25 years.36 Study HPV-014 was an open-label, age-stratified study undertaken to assess the immunogenicity and safety of the HPV-16/18 AS04-adjuvanted vaccine in 666 women aged 15–25, 26–45 and 46–55 years.34 Study HPV-028 was an ancillary study performed to undertake supplementary immunologic testing in 100 women participating in an ongoing Phase III efficacy trial (HPV-015 [NCT00294047]).37 In this study, women aged 26 years and over were randomized in a double-blind manner to receive either the HPV-16/18 AS04-adjuvanted vaccine or placebo (aluminum hydroxide [Al(OH)3]). In all studies, girls/women randomized to receive the HPV-16/18 AS04-adjuvanted vaccine received three doses administered intramuscularly according to a 0, 1, 6 month schedule.

Serum and CVS samples were collected in a subset of participants at selected study sites at pre-specified time-points ranging from 7 to 36 months after the first vaccine dose. In Study HPV-028, women who received Al(OH)3 also had CVS samples collected to serve as a negative control. CVS samples were only taken from post-menarcheal girls/women who agreed to the sampling.
All studies were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines and all study protocols and amendments, informed consent/assent forms and recruitment materials were approved by the Institutional Review Board or equivalent at all participating centers. Written informed consent (or informed assent with written consent from a parent or legal representative if below the legal age of consent) was obtained from all participants.

**Collection of CVS samples.** CVS samples were collected using ophthalmic sponges (Merocel® Eye Spear or Sponge Points [Medtronic; Jacksonville, Florida USA]). Women volunteering for this procedure were requested to avoid sexual intercourse and/or the use of intravaginal medications (including intravaginal contraceptives) for 24–48 hours before visits at which CVS samples were to be taken. To minimize blood contamination, collections were performed at least 2–3 days after cessation of menstrual flow. The sponge was placed in contact with the cervix for 30–60 seconds to absorb mucus. In HPV-010, HPV-012 and HPV-014, two sponges (one primary and one backup sample) were collected per woman and stored at -20°C or -70°C until antibody extraction. In HPV-028, only one sample was collected from each woman at each time-point. If cytology was to be performed at the same visit, CVS samples were collected first to avoid blood contamination and subsequent detection of serum antibodies in CVS.

**Antibody extraction.** Antibody extraction from CVS samples was performed as previously described in references 33, 34, 45 and 51. To reduce any potential bias in mucosal IgG assessment that may be introduced by blood contamination during sample collection, the presence of blood in CVS-extracted samples was determined by measuring the concentration of erythrocytes using the Hemastix® test (Siemens Medical Solutions Diagnostics Europe Ltd.; Dublin, Ireland). During the test validation phase, some changes were introduced to the previously described methods. For instance, an aliquot of 10 μl of extracted sample was used for the test instead of 5 μl which enabled more accurate measurement of the erythrocyte concentration. The use of just 5 μl could result in underestimation of the true erythrocyte concentration. Consequently, the cut-off for blood contamination was increased from 80 to 200 erythrocytes/μl. For HPV-014 (months 18 and 24) and for HPV-012 (month 24), the Hemastix® test was performed using 5 μl and samples showing 80 erythrocytes/μl or more were excluded from statistical analysis. For HPV-010 (month 7), HPV-012 (month 36) and HPV-028 (months 12 and 18), the Hemastix® test was performed using 10 μl and only samples showing 200 erythrocytes/μl or more were excluded from statistical analysis.

**Antibody testing.** In all studies, anti-HPV-16 and anti-HPV-18 IgG antibodies in serum and CVS-extracted samples were measured by HPV VLP enzyme-linked immunosorbent assay (ELISA) as previously described in references 8, 34 and 46. CVS-extracted samples were serial diluted, starting from a dilution of 1/10. The final antibody titer for each CVS-extracted sample was calculated by multiplying the ELISA titer by the dilution factor used during the antibody extraction step. In serum samples, seropositivity was defined as an antibody titer greater than or equal to 8 EU/ml for HPV-16 and 7 EU/ml for HPV-18. For CVS-extracted samples, positivity was defined as an antibody titer greater than or equal to the assay limit of quantitation (i.e., ≥0.35 EU/ml for HPV-16 and ≥0.58 EU/ml for HPV-18).

To address possible fluctuations in CVS antibody levels during the menstrual cycle,31,47 a method was developed to standardize HPV-specific antibodies. Total IgG concentration was also measured using a human total IgG sandwich ELISA developed and validated by GlaxoSmithKline Biologicals, and the ratio of anti-HPV-16 or anti-HPV-18 IgG titer divided by the total IgG concentration was calculated for each individual serum and CVS sample.

**Statistical analysis.** A pooled analysis was performed for the total vaccinated cohort (i.e., all women who received at least one dose of HPV-16/18 AS04-adjuvanted vaccine, and who had CVS and serum samples available) at each sampling time-point. For each antibody at each time-point, seropositivity rates and the proportion of women with CVS antibody titers greater than or equal to the limit of quantitation for the assay were calculated with exact 95% confidence intervals (CI). Geometric mean titers (GMTs) of anti-HPV-16/18 antibodies in serum were calculated overall and in women with and without detectable anti-HPV-16/18 antibodies in CVS.

In women with detectable antibodies in both serum and CVS, the log_{10} IgG corrected values of the ratios calculated between anti-HPV-16 or anti-HPV-18 IgG titers in CVS samples and total IgG in CVS, and the log_{10} IgG corrected values of the ratios calculated between anti-HPV-16 or anti-HPV-18 IgG titers in serum samples and total IgG in serum were used to calculate the Pearson correlation coefficient between paired corrected serum and CVS samples for each subject. All statistical analyses were performed using SAS version 9.1 and Proc StatXact version 7.0.

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Cervarix is a registered trademark of the GlaxoSmithKline group of companies. Gardasil is a registered trademark of Merck & Co., Inc.

References

Long-term persistence of systemic and mucosal immune response to HPV-16/18 AS04-adjuvanted vaccine in preteen/adolescent girls and young women

Tiina Petäjä1,2, Court Pedersen3, Airi Poder4, Gitte Strauss5, Gregory Catteau6, Florence Thomas6, Matti Lehtinen1 and Dominique Descamps6

1 University of Tampere, School of Public Health, Tampere, Finland
2 Central Hospital of Seinäjoki, Obstetrics & Gynecology, Seinäjoki, Finland
3 Department of Infectious Diseases Q, Odense University Hospital, Odense, Denmark
4 Tartu University Clinics, Dermatology, Tartu, Estonia
5 Bispebjerg Hospital, Department of Dermato-Venereology, Copenhagen, Denmark
6 GlaxoSmithKline Biologicals, Wavre, Belgium

Vaccination against oncogenic human papillomavirus (HPV) types is one key intervention for cervical cancer prevention. This follow-up study assessed the persistence of the systemic and mucosal immune responses together with the safety profile of the HPV-16/18 AS04-adjuvanted vaccine administered to young women aged 10–25 years. Serum and cervicovaginal secretion (CVS) samples were collected at prespecified time-points during the 48-month follow-up period. Anti-HPV-16/18 antibody levels in serum and CVS were measured by enzyme-linked immunosorbent assay (ELISA). At Month 48, all subjects remained seropositive for serum anti-HPV-16 and -18 antibodies. As previously observed, anti-HPV-16 and -18 antibodies levels (ELISA Units/mL) were higher in subjects vaccinated at the age of 10–14 years (2862.2 and 940.8) compared to subjects vaccinated at the age of 15–25 years (1186.2 and 469.8). Moreover, anti-HPV-16 and -18 antibodies in CVS were still detectable for subjects aged 15–25 years (84.1% and 69.7%, respectively). There was a strong correlation between serum and CVS anti-HPV-16 and -18 antibodies levels (correlation coefficients = 0.84 and 0.90 at Month 48, respectively) supporting the hypothesis of transudation or exudation of serum immunoglobulin G antibodies through the cervical epithelium. The HPV-16/18 AS04-adjuvanted vaccine had a clinically acceptable safety profile. In conclusion, this follow-up study shows that the HPV-16/18 AS04-adjuvanted vaccine administered to preteen/adolescents girls and young women induces long-term systemic and mucosal immune response and has a clinically acceptable safety profile up to 4 years after the first vaccine dose.

Cervical cancer is the second most common cancer among women worldwide, with nearly 500,000 new cases and approximately 270,000 deaths each year.1,2 Human papillomavirus (HPV) infection with oncogenic types is well recognized as the necessary cause of virtually all cervical cancers, and HPV DNA has been found in 99.7% of all cases.2–4 HPV types 16 and 18 are the most common oncogenic HPV types, responsible for about 70% of all cervical cancers.5,6

Key words: cervical cancer, human papillomavirus, HPV-16/18 adjuvanted vaccine, long-term immune response, cervicovaginal secretion

Abbreviations: AE: adverse event; ATP: according-to-protocol; CI: confidence interval; CVS: cervicovaginal secretion; ELISA: enzyme-linked immunosorbent assay; EL.U: ELISA Units; GCP: good clinical practice; GMT: geometric mean titer; GSK: GlaxoSmithKline; HPV: human papillomavirus; IgG: immunoglobulin G; NOCD: new onset of chronic disease; R: correlation coefficient; SAE: serious adverse event; TVC: total vaccinated cohort; VLP: virus-like-particle

Conflict of interest: C. Pedersen has conducted vaccine trials and clinical research studies with SanofiPasteur, Wyeth, GlaxoSmithKline, Merck Sharp & Dohme, Tibotec, Roche, ScheringPlough, Pfizer, and BristolMyersSquibb. He received financial support from GSK Biologics through his institution to conduct our study as well as travel grants and honoraria for courses and conference. M. Lehtinen obtained grants through his employers the National Institute for Health and Welfare and University of Tampere from GSK Biologics and Merck&Co., Inc. G. Strauss received financial support from GSK Biologics through her institution to complete our study and declared no personal conflict of interest. T. Petäjä and A. Poder declared they have no conflict of interest. G. Catteau, F. Thomas and D. Descamps are GlaxoSmithKline Biologics’ employees. F. Thomas and D. Descamps own GSK Biologics stock options.

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Correspondence to: Tiina Petäjä, University of Tampere, School of Public Health, Sorinkatu 1, 33100 Tampere, Finland, Tel: +358 503951191, Fax: +358 335518057, E-mail: tiina.petaja@uta.fi

In their lifetime, up to 80% of women will acquire an HPV infection.\(^7\) The majority of these natural infections resolve within two years,\(^8\) but the induced antibody levels are low and many women do not seroconvert at all.\(^10,11\) Moreover, the prevalence and the persistence of infection with oncogenic HPV types appear to be higher compared to nononcogenic HPV types.\(^7,9,12\) Immunity conferred by natural infection may not reliably protect against (re)-infection, hence, vaccination against oncogenic HPV types is important for the overall strategy of cervical cancer prevention.

To this end, GlaxoSmithKline (GSK) Biologics has developed a L1 protein virus-like-particle (VLP) vaccine (Cervarix\(^\text{®}\)) against oncogenic types HPV-16 and HPV-18 formulated with the AS04 adjuvant system.\(^13\) In previous trials, the HPV-16/18 AS04-adjuvanted vaccine (HPV-16/18 vaccine) was shown to have a clinically acceptable safety profile,\(^14\) to be immunogenic\(^15,16\) and to prevent incident and persistent HPV-16/18 infection and associated cervical neoplasia.\(^16–20\) In addition, the HPV-16/18 vaccine has shown cross-protection against some nonvaccine oncogenic HPV types, including HPV -31, -33, and -45.\(^16,17,19,21\) Long-term efficacy and immunogenicity, along with a clinically acceptable safety profile, were demonstrated up to 7.3 years after vaccination of women aged 15–25 years with this vaccine.\(^18,19,22\) In women aged 15–55 years, the HPV-16/18 vaccine induced a robust immune response, in serum and cervico-vaginal secretions (CVS), which has been shown to persist for at least 24 months.\(^23\)

The mechanism by which the HPV-16/18 vaccine induces protection is not completely understood. The HPV-16/18 vaccine prevents HPV infection of basal cells in the cervical epithelium, likely through the induction of high and sustained titers of neutralizing immunoglobulin G (IgG) antibodies. The level of serum antibodies induced by HPV-vaccination is 10–100 times higher than that following natural infection.\(^16,18,19,24,25\) Most vaccine-induced genital tract antibodies are reported to derive from the circulation by transudation or exudation across the cervical epithelium to the cervical mucus, where they bind to the HPV’s outer shell (capsid) and prevent infection of host cells.\(^26–28\) These antibodies may, however, also be actively transported or locally produced in the cervical mucosa by a mucosal immunization.\(^29\)

Vaccination against oncogenic HPV types before sexual debut is important since 50% of HPV infections in women are acquired during the first three years of their sexually active lives\(^30,31\) and the incidence of HPV infections remains high up to ten years after sexual debut.\(^7,32,33\) Moreover, the highest rates of HPV infections have consistently been found in women younger than 25 years of age. The duration of vaccine-induced protection is therefore critical as women are at risk of infection with an oncogenic HPV type throughout their sexually active life.\(^8,34,35\)

The initial phase of our study (Pedersen et al.,\(^15\) Month 7) provided evidence that the HPV-16/18 vaccine elicited higher anti-HPV-16/18 antibody levels in preteen/adolescent girls (aged 10–14 years) as compared to young adult women (aged 15–25 years) and had a clinically acceptable safety profile.

Here, we present extended follow-up data from that study where the persistence of the serum and mucosal immune response and the safety profile of the HPV-16/18 vaccine have been evaluated up to 4 years after vaccination of preteen/adolescent girls and young adult women.

**Material and Methods**

**Study objectives**

The primary objective of this follow-up study was to evaluate the long-term immunogenicity of the HPV-16/18 vaccine in young women (vaccinated at the age of 10–25 years with three vaccine doses) who completed a visit 48 months after the first vaccine injection (Month 48).

The secondary objectives were: (i) to compare the immune responses to the HPV-16/18 vaccine in sera from subjects enrolled in our study with responses measured in sera from adults of previous studies in which efficacy has been shown;\(^16,19,22\) (ii) to evaluate anti-HPV-16 and -18 antibodies responses in CVS samples and to compare the antibody levels in CVS with antibody levels in sera from subjects vaccinated pre- and postmenarche; and (iii) to evaluate the safety of the HPV-16/18 vaccine during long-term follow-up.

**Study design**

The primary study took place from September 2004 to July 2005 in 17 centers in Denmark, Estonia, Finland, Greece, The Netherlands, and Russia (Fig. 1). The primary study included healthy female subjects divided in two age groups: the 10–14 years age group (subjects aged 10–14 years at the time of first vaccine injection) and the 15–25 years age group (subjects aged 15–25 years at the time of first vaccine injection). Participants of the 15–25 years age group were randomized (1:1:1) to receive one of three consecutive production lots of the industrial scale HPV-16/18 vaccine. A randomization blocking scheme was used to ensure that treatments were assigned equally and randomly. All participants of the 10–14 years age group received vaccine from the same production lot. Another group of women aged 15–25 years received an HPV-16/18 vaccine prepared using a modified manufacturing process and were not included in the publication.\(^15\)

The extension study was conducted in Denmark, Estonia and Finland from June 2006 to January 2009 as a phase III, open, multicentric, follow-up study (NCT00337818) designed to evaluate the safety and immunogenicity of the HPV-16/18 vaccine up to Month 48 in subjects vaccinated at the age of 10–25 years (Fig. 1).

**Study population and ethics**

Participants were enrolled in the primary study if: (i) they were abstinent from sexual activity, or were using adequate contraceptive precautions for 30 days before vaccination and
up to 2 months after completion of the vaccination series; (ii) they had negative pregnancy test results (if they were of childbearing potential); and (iii) they had no more than six lifetime sexual partners. Individuals were excluded from the extension study at the time of study entry if they had used an investigational product, a nonregistered product or chronic immune-modifying drugs. Exclusion criteria included also the administration of immunoglobulins or blood products within 3 months prior to a blood sampling.

To be eligible for the extension study, the subjects had to have participated in the primary study in Denmark, Estonia or Finland, to have received three doses of the HPV-16/18 vaccine (at Months 0, 1 and 6) and to have completed the Month 7 visit. 15 Subjects who missed assessment at Month 18 (i.e., first visit of the extension study) were eligible to join the study at Month 24.

All participants had to sign the written informed consent before enrollment. For subjects below the legal age of consent, written informed consent had to be obtained from a parent or legally acceptable representative and, in addition, the subject had to sign and personally date a written informed assent.

The study protocol, any amendments, the informed consent and other information that required preapproval were reviewed and approved by a national, regional, or investigational centre Ethics Review Committee or Institutional Review Board.

Our study was conducted in accordance with good clinical practice (GCP) and all applicable regulatory requirements, including, where applicable, the Declaration of Helsinki. All distributed material had received prior approval by the Ethics Review Committees.

**Study vaccines**

The HPV-16/18 vaccine (GSK Biologicals, Rixensart, Belgium) contains HPV-16 and -18 L1 proteins self-assembled as VLP and is formulated with AS04, an adjuvant system known to enhance the vaccine’s immunogenicity. Each dose of the HPV-16/18 vaccine contains 20 μg of each HPV-16 and -18 L1 proteins adjuvanted with 550 μg of AS04 (500 μg aluminium hydroxide and 50 μg 3-O-desacyl-4′-monophosphoryl lipid A). The HPV-16/18 vaccine was supplied in individual 0.5-mL prefilled syringes and administered into the deltoid muscle on a 0-, 1- and 6-month schedule (Fig. 1). 15

**Serologic and CVS evaluations**

In the follow-up study, blood samples were collected at Months 18, 24, 36 and 48 for measurement of anti-HPV-16 and -18 antibody titers in serum (Fig. 1). All blood samples were evaluated for anti-HPV-16 and -18 antibodies using type-specific enzyme-linked immunosorbent assay (ELISA) at GSK Biologicals Laboratories, Rixensart, Belgium.

Anti-HPV-16 and -18 antibodies were also measured in CVS samples collected at Months 24, 36 and 48 in postmenarcheal subjects who volunteered for the procedure (Fig. 1). CVS samples were collected using ophthalmic sponges (Merocel® Eye Spear or Sponge Points [Medtronic Inc; Jacksonville, Florida, USA]). The sponge was placed in contact with the cervix for 30–60 seconds to absorb mucus. Antibody extraction from CVS samples was performed at GSK Biologicals Laboratories, Rixensart, Belgium and anti-HPV-16 and -18 IgG antibodies were detected and quantified according to ELISA serum standardized protocols as previously published. 16,23,37

To avoid any bias in results, the presence of blood in CVS samples was evaluated using the Hemastix® (Bayer Healthcare LLC) reagent strip test. After extraction of antibodies from CVS by two washing steps, a fixed volume of extracted sample was dispensed onto the strip test end. After one minute, the color of the test pad was matched to the color chart on the bottle label. Results were expressed as 0, 10, 25, 80, or 200 erythrocytes per μL.

**Vaccine safety**

The occurrence of serious adverse events (SAEs), medically significant adverse events (AEs), new onset of chronic

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1. **Figure 1.** Primary and extended follow-up study design: Vaccine administration, serologic and cervicovaginal secretion evaluation. M: Month.
diseases (NOCDs) and pregnancies was recorded throughout the entire study period. AEs, withdrawal due to AE(s), pregnancies and their outcomes were described in detail. SAEs were further evaluated for their clinical relevance and relationship to vaccination.

SAEs were defined as any untoward medical occurrence that was life-threatening, required hospitalization, resulted in disability or incapacity, was an important medical event, resulted in death, or was a congenital anomaly/birth defect in the offspring of a study participant. Medically significant AEs were defined as adverse events prompting emergency room or physician visits, which were not related to common diseases or routine visits for physical examination or vaccination or AEs not related to common diseases. NOCDs included autoimmune conditions, allergies and asthma.

**Statistical methods**

The primary immunogenicity analysis was performed on the according-to-protocol (ATP) immunogenicity cohort which included all evaluable subjects, i.e., subjects who were included in the primary study ATP immunogenicity analyses, meeting all eligibility criteria, complying with the procedures and intervals defined in the protocol with no elimination criteria during the study and for whom serology results were available for a particular blood sampling time point (at Month 18, 24, 36 or 48) of the extension phase. A supplementary analysis was performed on the Total Vaccinated cohort (TVC) which included all vaccinated subjects who received three doses of HPV-16/18 vaccine in the primary study and for whom data were available.

Seropositivity rates (with 95% confidence interval [CI]) were calculated for anti-HPV-16 and -18 in both groups. Seropositivity was defined as a titer greater than or equal to the assay threshold established at 8 ELISA Units/mL (ELU/mL) for anti-HPV-16 and 7 ELU/mL for anti-HPV-18.\(^{15,19}\) The range and distribution of antibody concentrations in serum were tabulated by geometric mean titers (GMTs) and their 95% CI for anti-HPV-16 and -18 at each time-point.

In the absence of an accepted serological correlate of protection, descriptive comparisons were performed between anti-HPV-16 and -18 GMTs in our study and anti-HPV-16 and -18 GMTs in women aged 15–25 years who cleared a natural infection and mounted an immune response in the Phase III PATRICIA trial.\(^{16}\) The immunogenicity results were also compared to the anti-HPV-16 and -18 GMTs from the plateau phase of the HPV-007 efficacy study at Months 45–50.\(^{19,22}\)

For the subjects who volunteered to provide CVS samples, correlation between serum and CVS antibody concentrations was calculated by Pearson coefficient. To minimize the antibody titer variation during the menstrual cycle, antibody titers (expressed in ELU/mL) measured in CVS and in serum were divided by the amount of total IgG measured in μg/mL.\(^{28}\) This ratio (expressed in ELU/μg) was used for the correlation. CVS samples with Hemastix® value >200 erythrocytes per μL were excluded from analyses.

Safety analyses were performed on the TVC. The percentages of subjects reporting at least one SAE, NOCD or other medically significant AE throughout the study (between Month 0 and Month 48) were tabulated with their exact 95% CI per treatment group.

The analyses were performed using Statistical Analysis System 9.1 and Proc StatXact 7.0.

**Results**

**Study population**

Of the 616 subjects who received at least one dose of HPV-16/18 vaccine prepared using standard manufacturing process in the primary study, 321 were from the three countries that accepted to participate in the extension study. Of these, 243 subjects entered the extension study, 220 subjects (51 aged 10–14 years and 169 aged 15–25 years at the first vaccination) attended the visit at Month 48 and 193 subjects (50 from the 10–14 years age group and 143 from the 15–25 years age group) were included in the ATP cohort for immunogenicity (Fig. 2).

At Month 48, the mean ages of participants (TVC cohort) were 15.7 years for the group of subjects aged 10–14 years at the time of first vaccine injection and 24.2 years for the group of subjects aged 15–25 years at the same time. Almost all subjects were White/Caucasian (98.2%). When compared to the demographic characteristics of the primary study, subjects in the two age groups participating in the extension study were of similar age and heritage (data not shown).

**Immunogenicity**

Most subjects of the 10–14 and 15–25 years age groups were seronegative for anti-HPV-16 antibodies (100% and 87.2%, respectively) and for anti-HPV-18 antibodies (95.8% and 87.2%, respectively) prior to vaccination. All initially seronegative subjects had seroconverted after vaccination and remained seropositive for both antibodies up to 4 years after the first vaccine dose (Table 1).

Immunological kinetic profiles showed that anti-HPV-16/18 antibodies peaked at Month 7, then gradually declined in all age groups tending towards a plateau (Fig. 3). At Month 48, GMTs (ELU/mL) (95% CI) for anti-HPV-16 in initially seronegative subjects were 2862.2 (2129.3–3847.3) in the 10–14 years age group and 1186.2 (1007.4–1396.8) in the 15–25 years age group (Fig. 3a). Anti-HPV-18 antibody GMTs were respectively 940.8 (714.8–1238.3) and 469.8 (394.7–559.2) for the same age groups and time-point (Fig. 3b). At all time-points postvaccination, GMTs were higher in subjects of the 10–14 years age group compared to subjects of the 15–25 years age group for anti-HPV-16 antibodies (between 2.4- and 2.9-fold) and anti-HPV-18 antibodies (between 2.0- and 2.5-fold).

In initially seronegative subjects of the 10–14 and 15–25 years age groups, anti-HPV-16 antibody GMTs at Month 48
were, respectively, 96.0- and 39.8-fold higher than anti-HPV-16 antibody levels achieved in subjects who cleared a natural infection and mounted an immune response in the Phase III PATRICIA trial (Fig. 3a).\textsuperscript{16} Anti-HPV-18 antibody GMTs were respectively 41.4- and 20.7-fold higher than levels after a natural infection for the same age groups (Fig. 3b). Compared to the plateau level observed in subjects from another study in which sustained efficacy of the HPV-16/18 vaccine has been demonstrated,\textsuperscript{19,22} anti-HPV-16 antibody titers were respectively 7.2- and 3.0-fold higher in the 10–14 years and the 15–25 years age groups (Fig. 3a). Anti-HPV-18 antibody titers were ~3.2- and 1.6-fold higher than the plateau level for the same age groups (Fig. 3b).

At Month 48, anti-HPV-16 and -18 antibody testing in CVS was performed on 69 and 66 samples (with HemaStix\textsuperscript{®} < 200 erythrocytes/μL) from subjects of the 15–25 years age group. Anti-HPV-16 and -18 antibodies were detected in CVS from 84.1\% (95% CI: 73.3, 91.8) and 69.7\% (95% CI: 57.1, 80.4) of subjects. Similar data were observed in CVS samples tested at Month 24 and Month 36.

Serum anti-HPV-16 and -18 IgG antibody response was evaluated at different time-points in subjects with and without detectable anti-HPV-16/18 IgG antibodies in their CVS at Month 48 (Table 2). All subjects remained seropositive for anti-HPV-16 and -18 antibodies at Month 48. However, subjects with detectable cervicovaginal anti-HPV-16 IgG antibodies displayed higher anti-HPV-16 serum GMTs than subjects without detectable antibodies in CVS. The difference in anti-HPV-16 GMTs between subjects with and without detectable cervicovaginal antibodies increased with time (from 1.7 fold at Month 7 to 3.2 fold at Month 48). A comparable trend was observed for anti-HPV-18 IgG antibodies.

A strong and direct correlation was observed between antibody levels in serum and CVS from the subjects of the 15–25 years age group throughout the study. The correlation coefficients (R) between antibody levels in serum and CVS for anti-HPV-16 antibodies were 0.93, 0.91 and 0.84 at Month 24, 36 and 48, respectively. For anti-HPV-18 antibodies the correlation coefficients were 0.93, 0.91 and 0.90 for the same time-points (Fig. 4).

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Figure 2. Flow of participants through the primary and extension study and definition of analyses cohorts. *Greece, Russia and The Netherlands declined participation in the extension study. TVC: total vaccinated cohort; ATP: according-to-protocol; N: number of subjects; M7: Month 7; M48: Month 48; S+: seropositive.
Throughout the study (Month 0 to Month 48), a total of 27 SAEs were reported by 23 subjects. Four subjects (2.5% CI: 0.7, 6.4) reported 4 SAEs in the 10–14 years age group and 19 subjects (4.1% CI: 2.5, 6.4) reported 23 SAEs in the 15–25 years age group. None of these events was considered to be possibly related to the study vaccination by the investigator.

Twenty-two subjects (13.9% CI: 8.9, 20.3) reported 24 medically significant AEs in the 10–14 years age group and 92 subjects (20.1% CI: 16.5, 24.1) reported 144 medically significant AEs in the 15–25 years age group. The most frequently reported medically significant AEs were depression (11), cystitis (9), asthma (6) and acne (5).

Three subjects (1.9% CI: 0.4, 5.4) in the 10–14 years age group and 19 subjects (4.1% CI: 2.5, 6.4) in the 15–25 years age group reported respectively 3 and 20 NOCDs (based on GSK assessment). The most frequently reported NOCDs were asthma (5) and hypothyroidism (3). None of these events were considered as possibly related to study vaccination by the investigator.

Of 45 pregnancies reported (one in the 10–14 years age group and 44 in the 15–25 years age group), there were 35 normal births, one stillbirth, one therapeutic abortion and one missed abortion in the same subject, four elective terminations and three premature infants.

### Discussion

This long-term follow-up study was designed to evaluate the immunogenicity and safety of the HPV-16/18 vaccine in healthy female subjects (aged 10 to 25 years at the first vaccine injection) up to four years after administration of the first vaccine dose. The vaccine had a clinically acceptable safety profile and high serum and mucosal anti-HPV-16 and -18 antibody levels were observed up to Month 48. All initially seronegative subjects seroconverted and remained seropositive for both anti-HPV-16 and -18 antibodies throughout the study, regardless of their age at vaccination. The immunological kinetic profiles, showing peak titers gradually declining and tending towards a plateau in all age groups, were similar to those observed in other HPV-16/18 vaccine clinical efficacy trials.16,19,23 In line with previous studies, anti-HPV-16 and -18 serum antibody titers at Month 48 were still substantially higher than titers elicited by a...
natural infection, and they were above antibody levels associated with sustained protection observed in previous clinical trials. This high and sustained immune response induced by the vaccine may in part be explained by the presence of the AS04 adjuvant system in the vaccine formulation.

Results from the primary study showed that the vaccine induced higher antibody titers when administered to young adolescents aged 10–14 years as compared to young women aged 15–25 years. The results of Month 7 were not unexpected since immune response to vaccination is known to

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**Figure 3.** Geometric mean titers of anti-HPV-16 (a) and -18 (b) antibodies in initially seronegative subjects aged 10–14 years and 15–25 years at the time of first vaccination (ATP immunogenicity cohort Month 48). 10–14 years: subjects aged 10–14 years at the time of first vaccine dose; 15–25 years: subjects aged 15–25 years at the time of first vaccine dose; GMT: geometric mean titer; Error bars: 95% CI: 95% confidence interval; Seropositivity rates shown above bars. Results of Month 18 were not presented since only eight subjects attended the visit. Natural infection: GMTs of subjects from Study HPV-008 who were HPV-16 (a) or -18 (b) DNA negative and seropositive at baseline (i.e., who had cleared a natural infection; GMT: 29.8 EL.U/mL (a) or GMT: 22.7 EL.U/mL (b)) Plateau phase: GMTs of subjects from Study HPV-007 in women aged 15–25 years at Months 45–50 after the first vaccine dose (Total cohort; GMT: 397.8 EL.U/mL (a) or GMT: 297.3 EL.U/mL (b)).
be enhanced in younger populations compared to advancing age groups. Of importance, the same trend was observed throughout the four years of follow-up in the extension study, which therefore brings new findings of major significance with regards to the expected long-lasting protection.

Preclinical studies have demonstrated the role of species-specific antibodies, directed against HPV L1 capsid protein, in protection against HPV infection and subsequent lesion development in three distinct animal model systems (canine oral papillomavirus, cottontail rabbit papillomavirus and bovine papillomavirus). One possible mechanism of protection at the mucosal surface is the transudation/exudation of serum IgG antibodies into CVS. In humans, high levels of mucosal antibodies could prevent virus particles from infecting the cervical basal cell layer at the transformation zone, which is the metaplastic zone between the squamous and columnar epithelium in the cervix where cervical cancers usually develop. Serum IgG antibodies are thought to transudate or exudate at this site across the cervical epithelium. Indeed, even if serum antibody levels decreased with time in both groups, the drop-off was slightly higher in subjects without detectable cervicovaginal antibodies than subjects with no detectable cervicovaginal anti-HPV-16 and -18 IgG antibodies. The strong correlation between serum and CVS antibodies persisted until Month 48, suggesting that transudation/exudation of serum IgG antibodies to the cervical epithelium is long-standing. Results of three other studies confirm that women who had detectable anti-HPV-16/18 antibodies in CVS reported consistently higher antibody levels in serum, regardless of age.

In our study, mucosal immune response to the HPV-16/18 vaccine was evaluated in CVS samples provided by subjects on a voluntary basis. The correlation between CVS and serum titration for antibodies against HPV-16 and HPV-18 was assessed and analysed. At Month 48, 84.1% and 69.7% of subjects vaccinated at the age of 15 to 25 years still had detectable antibody titers in their CVS samples for respectively anti-HPV-16 and anti-HPV-18. The strong correlation between serum and CVS antibodies persisted until Month 48, suggesting that transudation/exudation of serum IgG antibodies to the cervical epithelium is long-standing. Results of three other studies confirm that women who had detectable anti-HPV-16/18 antibodies in CVS reported consistently higher antibody levels in serum, regardless of age.

The immune response was further evaluated regarding the presence or absence of detectable cervicovaginal anti-HPV-16 and -18 IgG antibodies 48 months after the first vaccine injection. Throughout the study, subjects with detectable CVS antibodies had higher serum GMTs for anti-HPV-16/18 IgG antibodies than subjects with no detectable cervicovaginal anti-HPV-16/18 antibodies. This suggests that the antibody transudation from serum to CVS depends on the antibody level in the serum. Indeed, even if serum antibody levels decreased with time in both groups, the drop-off was slightly higher in subjects without detectable CVS antibodies at Month 48. On the other hand, in the group without detectable CVS antibodies, lower GMTs were observed from the beginning for anti-HPV-16 and from Month 24 onwards for anti-HPV-16. In the absence of an accepted serological correlate of protection, it is unclear whether this group may respond differently to the vaccine and might turn susceptible to infections caused by different HPV types at different times in the future.

Table 2. Comparison of serum antibody response in HPV-16/18 vaccinated young women with and without detectable cervicovaginal anti-HPV-16/18 IgG antibodies at Month 48 (TVC, 15–25 years age group)

<table>
<thead>
<tr>
<th>Anti-HPV-16/18 IgG</th>
<th>Month</th>
<th>Seropositivity rate (95% CI)</th>
<th>GMT (EL.U/mL) (95% CI)</th>
<th>N</th>
<th>Seropositivity rate (95% CI)</th>
<th>GMT (EL.U/mL) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;LOQ</td>
<td></td>
<td>27.3 (6.0, 61.0)</td>
<td>5.4 (3.8, 7.7)</td>
<td>19</td>
<td>31.6 (12.6, 56.6)</td>
<td>7.5 (4.0, 14.1)</td>
</tr>
<tr>
<td>M7</td>
<td>11</td>
<td>100 (71.5, 100)</td>
<td>4173.5 (2782.2, 6260.7)</td>
<td>20</td>
<td>100 (83.2, 100)</td>
<td>1648.3 (1204.8, 2255.1)</td>
</tr>
<tr>
<td>M24</td>
<td>11</td>
<td>100 (71.5, 100)</td>
<td>652.6 (381.7, 1115.7)</td>
<td>18</td>
<td>100 (81.5, 100)</td>
<td>228.4 (174.4, 299.0)</td>
</tr>
<tr>
<td>M36</td>
<td>9</td>
<td>100 (66.4, 100)</td>
<td>547.1 (308.1, 971.6)</td>
<td>18</td>
<td>100 (81.5, 100)</td>
<td>290.2 (200.7, 419.5)</td>
</tr>
<tr>
<td>M48</td>
<td>11</td>
<td>100 (71.5, 100)</td>
<td>393.7 (245.3, 631.7)</td>
<td>20</td>
<td>100 (83.2, 100)</td>
<td>211.6 (147.7, 303.3)</td>
</tr>
<tr>
<td>≥LOQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRE</td>
<td>58</td>
<td>17.2 (8.6, 29.4)</td>
<td>6.3 (4.7, 8.5)</td>
<td>45</td>
<td>17.8 (8.0, 32.1)</td>
<td>6.5 (4.1, 10.2)</td>
</tr>
<tr>
<td>M7</td>
<td>58</td>
<td>100 (93.8, 100)</td>
<td>7148.6 (5560.9, 9189.6)</td>
<td>46</td>
<td>100 (92.3, 100)</td>
<td>4011.4 (3180.5, 5059.4)</td>
</tr>
<tr>
<td>M24</td>
<td>54</td>
<td>100 (93.4, 100)</td>
<td>1479.4 (1212.7, 1804.7)</td>
<td>44</td>
<td>100 (92.0, 100)</td>
<td>843.3 (641.5, 1108.6)</td>
</tr>
<tr>
<td>M36</td>
<td>55</td>
<td>100 (93.5, 100)</td>
<td>1400.2 (1143.0, 1715.2)</td>
<td>43</td>
<td>100 (91.8, 100)</td>
<td>816.2 (632.3, 1053.7)</td>
</tr>
<tr>
<td>M48</td>
<td>58</td>
<td>100 (93.8, 100)</td>
<td>1260.8 (1037.2, 1532.7)</td>
<td>46</td>
<td>100 (92.3, 100)</td>
<td>682.0 (533.2, 872.4)</td>
</tr>
</tbody>
</table>

Abbreviations: GMT, geometric mean titer; LOQ, limit of quantification, percent of individuals below (<) or above (≥) (8 and 7 EL.U/mL for anti-HPV-16 and -18 IgG antibodies, respectively); N, number of subjects with pre-vaccination results available; PRE, Pre-vaccination; M7, Month 7; M24, Month 24; M36, Month 36; M48, Month 48; 95% CI, 95% confidence interval.
The safety analysis revealed a clinically acceptable profile. The frequency of SAEs, medically significant AEs and NOCDs were comparable to those reported in previous analyses.\textsuperscript{15}

The main limitations of our study were the number of subjects in the 48-month follow-up study which was lower compared to the primary study, the limited number of subjects from whom CVS samples were assessable and the fact that CVS samples were not collected since the beginning of the study. The proportion and the demographic characteristics of subjects in the initially 10–14 years and 15–25 years age groups were, however, conserved in the follow-up study.

In conclusion, the long-term immunogenicity in serum and CVS of the HPV-16/18 vaccine for both HPV-16 and HPV-18 was demonstrated. The vaccine had a clinically acceptable safety profile when administered to healthy female subjects aged 10 to 25 years. Moreover, the previously observed higher anti-HPV-16/18 antibody levels in early adolescents as compared to young adults persisted up to 4 years after the first vaccine dose. The strong correlation between levels of anti-HPV-16/18 antibodies in serum and CVS up to Month 48 supports long-term transudation or exudation of serum IgG antibodies to the cervical epithelium. These data support the administration of HPV-16/18 vaccine in preteen/adolescent girls and in young women.

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Figure 4. Correlation between antibody levels in serum and CVS samples at Month 24, Month 36 and Month 48 for anti-HPV-16 (a) and -18 (b) antibodies. The scatter plots show the ratio (specific IgG/total IgG) transformed to linear $\log_{10}$ values (TVC, 15–25 years age group). M24: Month 24; M36: Month 36; M48: Month 48; $R$: correlation coefficient.
We thank Claire Verbelen, Mélanie Muylaert and Denis Sohy for providing editorial assistance and article coordination on behalf of GlaxoSmithKline Biologics.

Cervarix® is a registered trademark of the GlaxoSmithKline group of companies. Merocel® Eye Spear is a registered trademark of Medtronic Inc. Hemastix® is a registered trademark of Bayer Healthcare LLC.

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The human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine (Cervarix™) has been shown to be well-tolerated and immunogenic in females aged 10 to 55 years, and up to 100% effective for the prevention of HPV-16/18 infection and associated precancerous cervical lesions in females aged 15 to 25 years. This study is the first to evaluate the immunogenicity and safety of the vaccine in males.

Methods: Healthy males aged 10 to 18 years were randomized (2:1 ratio) to receive HPV-16/18 AS04-adjuvanted vaccine (n = 181) or hepatitis B virus (HBV) control vaccine (n = 89) at 0, 1, and 6 months, and were followed for 7 months.

Results: All initially seronegative subjects in the HPV-16/18 group seroconverted for HPV-16 and 18 (ELISA) at month 2. At month 7, all subjects were seropositive, and the HPV-16 and -18 antibody levels were, respectively, four- and twofold higher than at month 2. The anti-HPV-16 and -18 antibody responses for males aged 10 to 18 years were higher than those reported for females aged 15 to 25 years and 10 to 14 years, respectively, in a previous study. The reactogenicity profiles of the HPV-16/18 AS04 and HBV vaccines were similar, except that pain and swelling at the injection site were more common in the HPV-16/18 group. However, vaccine-related symptoms did not affect compliance with the three-dose course, which was equally high (97%) in both groups.

Conclusions: The HPV-16/18 AS04-adjuvanted vaccine is immunogenic and well tolerated in boys aged 10 to 18 years. However, further data on the potential public health benefits of vaccination of boys are required before any recommendations can be made.

Keywords: Human papillomavirus; Cervical cancer; Vaccine; Immunogenicity; Male; Adolescent
and 90% of anal cancers [8]. HPV infections are rapidly acquired after sexual debut, and up to 20% of adolescent women ≤18 years of age will be infected with an oncogenic HPV type [9–11]. The prevalence of HPV infection in adolescent men is not accurately known, but in a study conducted in men aged 18 to 40 years recruited from the general U.S. population, HPV prevalence in anogenital sites and semen was 51% [12]. If we assume that 50% of the genital HPV types causing infections in the life time of a given woman are acquired during the first 3 years following sexual debut [13], the occurrence of infections in men is probably comparable in the first years of sexually active life. By the ages of 14, 15, and 18 years, respectively, 30%, 50%, and 70% of Finnish adolescent men have had their sexual debut [14], and by analogy up to 35% of them may have acquired HPV infection [15].

Prophylactic HPV L1 protein virus-like particle (VLP) vaccines have demonstrated high levels of protection against HPV-16 and 18 cervical infections and associated precancerous cervical lesions [16,17]. The HPV-16/18 AS04-adjuvanted vaccine (Cervarix™) has been extensively evaluated in women, and is currently licensed for use in females in over 50 countries. (Cervarix™, Engerix-B™ and Boostrix™ are trade marks of the GlaxoSmithKline group of companies.) This vaccine has been administered to approximately 16,000 girls and women aged 10 years and above in Phase II/III clinical trials, and has been shown to be generally well tolerated [18]. The vaccine has also been shown to be immunogenic and efficacious for protection against HPV-16/18 infection and associated cervical lesions in women aged 15 to 25 years for up to 6.4 years after vaccination [17,19,20]. Data from clinical studies show that protection conferred by the HPV-16/18 AS04-adjuvanted vaccine may extend beyond HPV-16 and -18 in females [17,19].

For preventive strategies it is important to consider at what age to target adolescents, and whether vaccination of both adolescent women and men is beneficial. The HPV-16/18 AS04-adjuvanted vaccine is currently only licensed for use in women, although another HPV vaccine has been licensed for use in boys aged 9 to 15 years in some countries. A mathematical model evaluating the impact of HPV vaccination in 12-year-old women in Finland, assuming that the vaccine has 100% efficacy against HPV-16-associated invasive cervical cancer and 70% coverage, predicts that vaccination could contribute to a reduction in the lifetime risk of HPV-16-associated cervical cancer of up to 70% [21]. Using the same model, it is reported that the concomitant vaccination of male adolescents would result in the prevention of up to an additional 20% of cases of cervical cancer [21]. However, a cost-effectiveness analysis found no real added benefit for male vaccination if very high vaccine coverage is achieved in women [22].

The present study was performed to evaluate the immunogenicity and safety of the HPV-16/18 AS04-adjuvanted vaccine in boys aged 10 to 18 years. An additional objective of the study was to compare the immune response of boys to that of adolescent and young adult women in a separate trial [23], in whom protective efficacy of this vaccine has previously been demonstrated [17,19].

Methods

Study participants and ethics

The study (580299/011/NCT00309166) took place from April 2006 to January 2007 at seven study sites in Finland. Study participants were recruited by population-based recruitment letters sent to the entire target male birth cohort (parents or legal guardian in the case of minors) in the study site communities by the population census register and by school recruitment sessions. All distributed material had received prior approval by the Ethical Review Committee of the Pirkanmaa Hospital District (PSHP).

Boys aged 10 to 18 years were eligible to participate in this study. Individuals were excluded from enrollment if they had used an investigational drug or vaccine within 30 days, chronic immune-modifying drugs within 6 months, immunoglobulins or blood products within 3 months, or planned to use any of these during the study period, had previously received an HPV vaccine, or had previously been vaccinated against hepatitis B virus (HBV), had a known clinical history of HBV infection, or known exposure to HBV within the previous 6 weeks, or had any confirmed or suspected immunosuppressive or immunodeficient condition including HIV infection.

Informed consent was obtained from each participant or their parents/legal guardian prior to the performance of any study procedures. Participants below the legal age of consent (15 years) were also required to sign and date an informed assent. These participants were informed about the trial to an extent compatible with their understanding. The PSHP Ethical Review Committee approved the study and consent forms, and this study is registered with the European Clinical Trials Database.

Study design

This was a phase I/II, observer-blind, parallel-group, randomized study. The 270 participants were age-stratified according to three age groups: 10 to 12 years (n = 70), 13 to 15 years (n = 104), and 16 to 18 years (n = 96). Participants were randomized (2:1 ratio) to receive either the HPV-16/18 AS04-adjuvanted vaccine or a HBV control vaccine. A randomization blocking scheme was used to ensure that balance between treatments (2:1) and approximately equal distribution across the three age strata was maintained. The study vaccines were assigned treatment numbers from a randomization list generated at GlaxoSmithKline Biologicals (Rixensart, Belgium) using a standard SAS program (SAS Institute, Cary, NC). Participants were assigned a vaccine treatment number; blinding was maintained to the individual treatment allocated. All study personnel were blinded to the
vaccines used, except the study nurse administering the vaccines.

**Study vaccines**

Each dose of HPV-16/18 L1 VLP AS04-adjuvanted candidate vaccine (Cervarix™) contained 20 μg each of HPV-16 and -18 L1 proteins self-assembled as VLP and adjuvanted with AS04 (50 μg 3-O-desacyl-4′-monophosphoryl lipid A [MPL] and 500 μg aluminum hydroxide). The vaccine was produced using a Baculovirus Expression Vector System in which each type of VLP antigen was produced on a Hi-5 cell line derived from Trichoplusia ni. Each dose of the hepatitis B virus control vaccine (Engerix-B™) contained 10 μg hepatitis B surface (HBs) antigen and 250 μg aluminum hydroxide. The vaccines were supplied in identifiable 0.5-mL prefilled syringes and administered into the deltoid muscle on a 0-, 1-, and 6-month schedule.

**Serological evaluation**

Blood samples were collected from each participant before the first vaccination and 1 month after the second and third doses (months 2 and 7, respectively) to evaluate immunogenicity. All blood samples were evaluated for HPV-16 and HPV-18 antibodies using a type-specific enzyme-linked immunosorbent assay (ELISA) as reported elsewhere [19]. Seropositivity was defined as a titer greater than or equal to the assay threshold established at eight ELISA Units/mL (EU/mL) for HPV-16 and 7 EU/mL for HPV-18 [19].

The HBV vaccine used in this study is marketed in Finland with an upper age limit of 15 years. Hence, as this formulation was considered investigational for subjects aged 16 to 18 years, levels of anti-HBs antibodies at month 7 were evaluated by ELISA in boys aged 16 to 18 years who received the HBV vaccine, to ensure that sufficient protection against HBV was provided. If a boy was not seroprotected (anti-HBs antibody concentration of at least 10 mIU/mL) he was offered an additional dose of HBV vaccine using the formulation licensed for use in Finland according to the study protocol.

**Vaccine safety**

Participants used diary cards to report solicited local and general symptoms during a 7-day follow-up period after each vaccine dose (days 0–6). Solicited local adverse events included pain, redness, and swelling at the injection site. Solicited general adverse events included fever, headache, fatigue, gastrointestinal symptoms (i.e., nausea, vomiting, diarrhea, abdominal pain), arthralgia, myalgia, rash, and urticaria. Grade 3 solicited adverse events were defined as pain that prevented normal activity, redness or swelling larger than 50 mm, fever higher than 39.0°C (axillary temperature), urticaria distributed on at least four body areas, or events that prevented normal, everyday activities. Urticaria or rash that appeared within 30 minutes of each vaccine dose was also documented by the investigator.

Unsolicited signs and symptoms were reported within 30 days after each dose. Serious adverse events (SAEs), new onset chronic diseases (NOCDs) (e.g., diabetes mellitus, autoimmune diseases, asthma, allergies, etc.), and other medically significant conditions (MSCs) were reported throughout the study period. An SAE was defined as any untoward medical occurrence that resulted in death, was life-threatening, required hospitalization, resulted in disability or incapacity, was an important medical event or was a congenital anomaly/birth defect in the offspring of a study subject. MSCs were defined as nonserious adverse events prompting either emergency room or physician visits not related to either common diseases or routine visits for physical examination or vaccination, or SAEs not related to common diseases. Common diseases included upper respiratory infections, sinusitis, pharyngitis, gastroenteritis, urinary tract infections, and injury.

**Statistical analysis**

We estimated that 144 evaluable boys in the HPV-16/18 AS04-adjuvanted vaccine group were needed to demonstrate with at least 98% power, that the seroconversion rates at month 7 were not less than 90% for a given HPV antigen. All sample size calculations were done with Pass 2005, using a one proportion power analysis, one-sided test, type I error of 2.5% with the Bonferroni adjustment of beta. Immunogenicity analyses were based on the according-to-protocol (ATP) cohort and were performed on participants initially seronegative for the considered antigen (i.e., participants seropositive for HPV-16 antigen at baseline were eliminated from the HPV-16 analysis, but were still evaluable for HPV-18 analysis provided they were HPV-18 seronegative, and vice versa). Seropositivity rates and geometric mean titers (GMT) with 95% confidence intervals (95% CI) were calculated for antibodies to each HPV antigen. Asymptotic two-sided confidence intervals for the ratio of GMTs were computed using an analysis of variance model on log_{10} transformed titers. Antibody titers below the cutoff of the assay were given an arbitrary value of half the cutoff value for the purpose of GMT calculations.

The immune responses induced by the HPV-16/18 AS04-adjuvanted vaccine in boys in this study were compared to those induced in adolescent and young adult women in a separate study [23]. The separate historic comparator study was a randomized, parallel-group trial conducted at six study sites in Denmark, Estonia, and Finland, and included women aged 10 to 25 years stratified into two age groups (10–14 years and 15–25 years). The HPV-16/18 AS04-adjuvanted vaccine schedule was the same as that used in this study (0, 1, and 6 months), and anti-HPV-16/18 antibody levels were measured at months 0, 7, 18, and 24. Noninferiority of the immune responses induced by the HPV-16/18 AS04-adjuvanted vaccine in boys in this study compared to those
induced in women aged 15 to 25 years in the historic comparator study was demonstrated if the upper limit of the 95% confidence interval (CI) for the difference between the percentage of participants who seroconverted in each group 1 month after the third dose (month 7) was below 10% and if the upper limit of the 95% CI for the GMT ratio between each group 1 month after the third dose (month 7) was below 2 (tests performed sequentially).

Safety analyses were based on the total vaccinated cohort. Incidence rates of solicited symptoms during the 7-day follow-up period and unsolicited symptoms during the 30-day follow-up period were tabulated with exact 95% CI over all vaccine doses and for each treatment group. For the analysis of solicited symptoms, missing or nonevaluable measurements were not replaced, and included only boys with documented safety data (i.e., symptom sheet completed) per dose. All vaccinated boys were included in the analysis of unsolicited adverse events, SAEs, NOCDs, and MSCs. Participants who did not report an event were considered to have not experienced an event.

Statistical analyses were performed with SAS version 8.2 (SAS Institute) and ProcStatXact 5 (Cytel Inc., Cambridge, MA).

**Results**

A total of 270 participants (HPV-16/18 group, n = 181; HBV group, n = 89) were enrolled and vaccinated over a period of approximately 2 months (April 5 to June 10, 2006). Study compliance was excellent (Figure 1), and 97% of boys in both groups (HPV-16/18 and HBV) received all three vaccine doses. The demographic profile of the two groups was similar. In the total vaccinated cohort the mean age of boys was 14.4 years. As this was a study in one country only, the distribution of ethnicity was homogeneous in both groups (Table 1).

The trial profile for ATP analyses and total vaccinated cohort analyses were almost identical (Figure 1).

### Immunogenicity

At study entry, 11.2% of the participants were seropositive for HPV-16 and/or -18 antibodies (3.5% seropositive for HPV-16 alone [n = 9], 7% for HPV-18 alone [n = 18] and 0.8% for both HPV-16 and HPV-18 [n = 2]). In the

---

**Table 1**

Demographic characteristics of participants receiving the HPV-16/18 AS04 or HBV vaccines (total vaccinated cohort)

<table>
<thead>
<tr>
<th></th>
<th>HPV-16/18 N = 181</th>
<th>HBV N = 89</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD) age (years)</td>
<td>14.4 (2.14)</td>
<td>14.4 (2.02)</td>
</tr>
<tr>
<td>Ethnic origin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White/Caucasian</td>
<td>177 (97.8)</td>
<td>88 (98.9)</td>
</tr>
<tr>
<td>Black</td>
<td>1 (0.6)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Arabic/North African</td>
<td>1 (0.6)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Central/South Asian</td>
<td>1 (0.6)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Other</td>
<td>1 (0.6)</td>
<td>1 (1.1)</td>
</tr>
</tbody>
</table>

N = number of study participants.  
\( n(\%) \) = number and percentage of participants.
At month 2, 100% of initially seronegative boys who received the HPV-16/18 AS04-adjuvanted vaccine had seroconverted for HPV-16 and HPV-18 (ELISA) and all boys were seropositive 1 month after completion of the full vaccination course at month 7. At month 7, antibody levels for HPV-16 and HPV-18 were, respectively, fourfold and twofold higher, compared to month 2 (Figure 2).

The immune response in boys aged 10 to 18 years in this study was shown to be noninferior for both seroconversion rates and GMTs to that seen in women aged 15 to 25 years in the historic comparator study [23], an age range in which a high degree of protection against HPV-16/18 infection and associated cervical lesions has been shown [17, 24]. With regard to seroconversion rates, 100% of boys and women seroconverted for anti-HPV-16 and anti-HPV-18 antibodies at month 7. With regard to antibody levels, the HPV-16/18 AS04-adjuvanted vaccine elicited substantially higher GMTs at month 7 for both antigens in boys aged 10 to 18 years (HPV-16: 22639.7 [19825.5–25853.4] and HPV-18: 8416.1 [7215.0–9817.1]) compared with women aged 15 to 25 years (HPV-16: 7292.9 [6623.7–8029.7] and HPV-18: 3318.8 [3023.1–3643.5]) [23] (Figure 3). The antibody levels at month 7 in the subset of boys aged 10 to 14 years in this study (HPV-16: 27891.6 [23975.6–32447.2] and HPV-18: 10593.7 [8875.8–12644.0]) were also higher than those reported in girls of the same age in the historic comparator study (HPV-16: 17272.5 [15117.9–19734.1] and HPV-18: 6863.8 [5976.3–7883.0]) [23] (Figure 3). Antibody levels at month 7 in the subset of boys aged 10 to 14 years in this study were also higher than those reported in the subset of boys aged 15 to 18 years (HPV-16: 18606.3 [15095.7–22933.5] and HPV-18: 6805.5 [5346.8–8662.0]).

Safety

Pain and swelling at the injection site were more frequent in the HPV-16/18 vaccine group than in the control HBV vaccine group (Table 2). However, higher levels of solicited local symptoms did not affect compliance with vaccination, as evidenced by 97% of boys in both vaccine groups completing the three-dose vaccination course.
The most frequently reported solicited general symptoms were headache, fatigue, and myalgia; myalgia was more frequent in the HPV-16/18 vaccine group than in the control vaccine group (Table 3). Most solicited adverse events were transient (lasting not longer than 2–3 days) and the incidence of adverse events did not increase with subsequent doses. Grade 3 adverse events were reported infrequently. Very low to low frequencies of urticaria (0.8% in both the HPV-16/18 and control HBV vaccine groups) or rash (3.6% and 1.9% in the two groups, respectively) were reported by the investigator within 30 minutes of vaccine administration for any study participant.

In general, the frequency of unsolicited symptoms reported during the 30-day postvaccination period following each dose was similar between groups: 15.7% and 15.6% in the HPV-16/18 and control HBV vaccine groups, respectively.

Two SAEs occurred in 2 participants receiving the HPV-16/18 AS04-adjuvanted vaccine (Crohn’s disease and epilepsy). The boy diagnosed with Crohn’s disease had symptoms that may have been related to the disease prior to the first dose of vaccine, and the boy with epilepsy had a family history of this condition. Neither of the SAEs were fatal, and both events were considered by the investigator to be unrelated to study vaccination. No participants withdrew from the study because of a SAE. One participant in the HPV-16/18 vaccine group withdrew from the study because of a nonserious adverse event (panic reaction after the first vaccine dose), which was not considered related to the study vaccine. Three NOCDs were reported: two in the HPV-16/18 group (Crohn’s disease and atopic dermatitis) and one in the control HBV vaccine group (asthma). The percentage of MSCs reported did not differ between groups (12.2% in the HPV-16/18 group and 11.2% in the control vaccine group).

**Discussion**

This is the first study in which Cervarix™ has been administered to boys. This HPV-16/18 AS04-adjuvanted vaccine was highly immunogenic, inducing seroconversion for both antigens in all boys and very high antibody titers for both HPV-16 and HPV-18. The immune response was shown to be noninferior in terms of seroconversion rates between boys aged 10 to 18 years in this study compared with women aged 15 to 25 years from a separate study.[23], an age range for which efficacy for the prevention of HPV-16/18 infection and associated precancerous cervical lesions has been demonstrated.[17,19] The HPV-16/18 AS04-adjuvanted vaccine elicited substantially higher antibody levels for

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Incidence of solicited local symptoms reported during the 7-day follow-up period following administration of HPV-16/18 AS04 or HBV vaccines, overall per dose (total vaccinated cohort)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptom</td>
<td>Type</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
</tr>
<tr>
<td>Pain</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>Grade 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Redness</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>&gt;50 mm</td>
</tr>
<tr>
<td>Swelling</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>&gt;50 mm</td>
</tr>
</tbody>
</table>

N = number of documented doses (with safety diary cards returned); CI = exact confidence interval; n (%) = number/percentage of doses that were followed by at least one symptom.

<sup>a</sup> Pain that prevented normal activity.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Incidence of solicited general symptoms reported during the 7-day follow-up period following administration of HPV-16/18 AS04 or HBV vaccines, overall per dose (total vaccinated cohort)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptom</td>
<td>Type</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>Grade 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fatigue</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>Grade 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fever</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>&gt;39.0°C</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>Grade 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Headache</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>Grade 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Myalgia</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>Grade 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rash</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>Grade 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Urticaria</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>Grade 3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

N = number of documented doses (with safety diary cards returned); CI = exact confidence interval; n (%) = number/percentage of doses that were followed by at least one symptom.

<sup>a</sup> Symptom that prevented normal activity.

<sup>b</sup> Urticaria distributed on at least four body areas.
both antigens in boys aged 10 to 18 years or 10 to 14 years, respectively, when compared with women aged 15 to 25 years or girls aged 10 to 14 years, respectively, from a previous study [23]; postvaccination antibody levels for both HPV-16 and -18 antibodies were observed to be up to threefold higher in boys than in women. This observation has also been described for another HPV vaccine [25]. As already described in women [23], the HPV-16/18 AS04-adjuvanted vaccine elicited higher antibody levels for both antigens in the group of young boys aged 10 to 14 years compared with the group of boys aged 15 to 18 years.

The HPV-16/18 AS04-adjuvanted vaccine was generally well tolerated, with a similar safety profile to the HBV vaccine used as a control, with the exception of increased reporting of some solicited symptoms (pain/swelling and myalgia). These differences in reactogenicity did not, however, impact on acceptance of the vaccine, with almost all boys completing the full three-dose vaccination course. The reactogenicity profile of the HPV-16/18 AS04-adjuvanted vaccine in boys in this study was similar to that reported previously in women [18,23,24]. Furthermore, the reactogenicity profile was also similar to that reported for a combined diphtheria, tetanus, and acellular pertussis vaccine (Boostrix™) in adolescents aged 10 to 18 years [26], in whom localized pain was reported for 75% to 90% of subjects, and localized swelling was reported for 21% to 35% of subjects (compared to 72.3% and 10.7%, respectively, for the HPV-16/18 AS04-adjuvanted vaccine in this study). In a previous direct comparison of the HPV-16/18 AS04-adjuvanted vaccine and placebo containing aluminum hydroxide in women aged 15 to 25 years of age [24], greater local reaction rates were observed in the vaccine group, but general symptom rates were equivalent to placebo, and neither local nor general vaccine related symptoms affected overall subject compliance.

Because HPV infections can be rapidly acquired after sexual debut, an important target population for the HPV-16/18 AS04-adjuvanted vaccine is preteens and young adolescents to provide protection prior to onset of sexual activity and exposure to oncogenic HPVs. The vaccine formulated with AS04 has been shown to induce higher and more sustained antibody levels against both HPV-16 and HPV-18 and more robust memory B-cell responses when compared to the same HPV-16/18 antigens formulated with conventional aluminum salts only [27]. Results of this study indicate that the HPV-16/18 AS04-adjuvanted vaccine is generally safe and well tolerated, and induces excellent immunogenicity in boys aged 10 to 18 years. However, the public health value of vaccination of boys against oncogenic HPV types has yet to be determined. The burden of HPV-associated diseases such as anal, penile, and oropharyngeal cancers in men is much less than that of cervical cancer in women [8], and potential public health benefits of male vaccination on this basis alone would be expected to be limited. However, HPV infection is common in men [12] and is readily transmitted [28], influencing disease rates in both men and women.

The impact of vaccination of both male and female adolescents on the reduction of female disease (cases of cervical cancer) has been explored in a number of mathematical modeling studies. Results of these studies vary, with some suggesting that vaccination of adolescent men may confer additional benefit over vaccination of women alone [19,21,29,30], whereas others show no real added benefit for male vaccination when very high vaccine coverage is achieved in women [22]. As such models are dependent on underlying assumptions such as the epidemiology of HPV infection in the region or country of interest, and vaccine coverage, it is not surprising that estimates of the benefits of male vaccination vary. Further data are clearly required before recommendations can be made regarding the use of HPV vaccines in males.

References

with human papillomavirus types 16 and 18 in young women; an interim analysis of a phase III double-blind, randomised controlled trial. Lancet 2007;369:2161–70.


Prevalence of human papillomavirus (HPV) DNA in men vaccinated with the HPV16/18 vaccine as early adolescents

Tiina Petäjä¹, Anna Söderlund-Strand², Tuomas Lehtinen¹*, Tiina Eriksson¹, Joakim Dillner³, Matti Lehtinen¹

¹Medical School and School of Health Sciences, University of Tampere, Finland;
²Department of Medical Microbiology, University of Lund, Malmö, Sweden;
³Department of Medical Epidemiology, Karolinska Institute, Stockholm, Sweden

*Corresponding author. Mailing address: University of Tampere, 33014 Tampere, Finland; Tel: +358465749019; Fax: +358206106251; E-mail: tuomas.lehtinen@uta.fi
Abstract

Both the quadrivalent and the bivalent human papillomavirus (HPV) vaccines are highly immunogenic, safe and efficacious in females. The former has yielded similar results in immunogenicity, safety and efficacy, and the latter has been shown to be immunogenic and safe in males. We explored HPV PCR prevalences overall, and for the vaccine-covered (16/18), and partially-covered types (6/11/31/33/45/51) HPV types, in former HPV16/18 vaccinated and unvaccinated, 1992-1994 born, male participants of a community randomized trial (2007-9) on the effectiveness of HPV vaccination strategies. Residents of the trial communities were offered *Chlamydia trachomatis* screening at the age of 18.5 years. Three-hundred and twenty three vaccinated and 72 unvaccinated males provided first void urine (FVU) samples for Chlamydia testing. DNA extracted from the pseudonymized FVU samples was genotyped for HPV DNA using PCR followed by Luminex assay and MALDI-TOF mass spectrometry.

The overall HPV prevalence in the 395 samples was low (4.3 %), partly due to the relatively low sensitivity (70%) of the FVU testing in detecting HPV DNA. No samples from vaccinated individuals tested positive for vaccine type (16/18) HPV DNA. There were no statistically significant HPV prevalence differences between the vaccinated and unvaccinated individuals sampled, but the difference in complete/partially vaccine covered HPV 6/11/16/18/31/33/45/51 prevalence: 8/323 (2.5%) vs. 5/72 (6.9%) was borderline significant (P= 0.055) suggesting efficacy against HPVPCR positivity for the bivalent vaccine in males. Main limitations of our pilot study were small sample size and low HPV DNA prevalence in the adolescent males. Larger randomized efficacy studies in populations with HPV infection peak are warranted.

**Key words:** antibody, avidity, genital infection, HPV, prevalence
**Background**

Human papillomavirus (HPV) is the major cause of anogenital cancers both in females and males. High-risk (hr) HPV type infections are highly common in males with prevalences ranging from 5 to 35% or more (1-4). Clustering of infections is also common as more than 20% of adult males are positive for multiple HPV types (3, 4). A recently rapid increase in the number of HPV infections and HPV related cancers in men has been noted (5).

There are now two HPV vaccines in use: (bivalent HPV16/18 Cervarix™ and quadrivalent HPV6/11/16/18 Gardasi™, both of which are immunogenic and safe also in males (6, 7). The bivalent vaccine has been reported to be not less immunogenic in males 10 to 18 years of age than in similarly aged females (6). The quadrivalent vaccine has yielded similar results in men aged 16 to 26 (7). The efficacy of the quadrivalent vaccine against persistent vaccine-type HPV infections is over 90% against vaccine HPV types in baseline HPV negative males (8).

In this pilot study we assessed the overall and vaccine-covered, cross-protected HPV types 6/11/31/33/45 included (9, 10), HPV PCR prevalence in HPV 16/18 vaccinated and unvaccinated young adult males approximately four years post vaccination.
Material and Methods

Participants

The study participants were enrolled in 11 municipalities participating in 2007-2009 a community randomized trial on the effectiveness of different (boys and girls vs. girls only) HPV vaccination strategies in reducing HPV prevalence in young females (11). In the 11 municipalities, where early adolescent boys (birth cohorts 1992-1995 between ages 12 to 15 years, together with similarly aged girls) had received either the bivalent HPV16/18 (Cervarix™) vaccine (90%) or hepatitis B-virus (HBV, Engerix™vaccine (10%), screening for Chlamydia trachomatis was offered to both young male and female residents of the trial communities, when exiting the trial or at the age of 18.5 years starting from Autumn 2010. Following an invitation / information letter, first void urine (FVU) sampling kits for C. trachomatis testing were mailed upon a web-page (www.rokotititus.net) request. The samples were returned in prepaid envelopes to the FIMLAB (Tampere, Finland) laboratory for C. trachomatis testing. Test results were delivered to the trial participants on the secure (personal identity protected via pseudonymization) www.rokotiitus.net web-page. Those testing C. trachomatis positive were invited to the community study sites for treatment including azithromycin prescriptions.

From the 1992-1993 male birth cohorts altogether 395 (323 of 1283 vaccinated and 72 of 5193 unvaccinated) donated a C. trachomatis screening (FVU) sample. Pseudonymized left-over samples and the extracted DNAs were labelled as being of an HPV16/18 vaccinated, an HBV vaccinated or an unvaccinated person and stored at -20°C for later use.
Ethical clearance to the separate HPV vaccination and the Chlamydia screening trials were obtained in 2007 from the Ethical Review Boards of the Pirkanmaa Hospital District, Tampere, Finland (Eudra-CT–number 2007-001731-55) and the North Ostrobothnia Hospital District, Oulu Finland (310/2009), respectively.

**Laboratory analyses**

The extracted DNA from the FVU samples were used for HPV genotyping using a cost-effective, high-throughput HPV-genotyping method that performs genotyping of HPV 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. The HPV DNA analyses were performed by a consensus PCR using the MGP primer system (12) followed by MALDI-TOF mass spectrometry on the SEQUENOM platform that allowed simultaneous separation and detection of short DNA sequences elongated with a single nucleotide (13). This semi-automated method has been found proficient using the 2010 WHO HPV labnet proficiency panel.

**Statistical analysis**

HPV, hrHPV, vaccine covered and vaccine-type HPV DNA prevalences were compared with the Chi-square test using SPSS software.
**Results and Conclusions**

The overall prevalence of HPV DNA in the 395 samples analysed was 4.3 % (17 samples tested positive with both methods used). Twelve samples (3.7%) of the 323 HPV 16/18 vaccinated individuals and 5 (6.9%) samples of the 72 unvaccinated individuals tested positive for any HPV type (Table 1). No samples from the vaccinated individuals tested positive for HPV16/18, and 8 (2.5 %) tested positive for HPV types 6/11/31/33/45/51 reported to be partially covered by the bivalent vaccine (9, 10). Corresponding findings in the unvaccinated group were 1 (1.4%) and 4 (5.6%). None of the differences were statistically significant, but the difference for completely/partially covered HPV types was borderline significant (Table 1), suggesting protective efficacy for the bivalent vaccine in males.

The overall HPV prevalence in the samples was low. This may have been partly due to the young age (18 to 19 years) of the individuals sampled (1-4, 11) and partly due to the relatively low sensitivity of the FVU sample testing in detecting HPV DNA (70 %) (14, 15), especially when compared to direct anogenital sampling (16). A difference in sexual behaviour in those vaccinated and/or sampled when compared to the entire adolescent population may also have contributed to the low prevalence (17, 18).

The main study limitations were limited sample size and the low sample HPV prevalence. Also the participant selection was not randomized, possibly resulting in bias as the
behavioural differences between HPV vaccinated and unvaccinated, and those sampled compared to the population could not be ruled out (18). Our findings warrant further studies with a larger sample size in populations with peak HPV incidence.

Conflicts of interest
JD and ML have obtained grants from Merck&Co. Inc. and GSK-Biologicals (ML) for HPV vaccination studies through their employers.

Acknowledgements
Dr. Saara Kares at the FIMLAB laboratories is gratefully acknowledged for DNA extraction.
References


Table 1. Prevalence of human papillomavirus (HPV) DNA detected in first void urine samples of 18 to 19 year old young men vaccinated with the bivalent HPV16/18 vaccine as early adolescents between ages 12 to 15 years and in unvaccinated, similarly-aged control men.

<table>
<thead>
<tr>
<th>HPV type</th>
<th>HPV16/18 vaccinated</th>
<th>Unvaccinated</th>
<th>P for difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N / number of pos. (%)</td>
<td>N / number of pos. (%)</td>
<td></td>
</tr>
<tr>
<td>16/18</td>
<td>323 / 0 (0)</td>
<td>72 / 1 (1.4)</td>
<td>n.s.</td>
</tr>
<tr>
<td>6/11/31/33/45/51</td>
<td>323 / 8 (2.5)</td>
<td>72 / 4 (5.6)</td>
<td>n.s.</td>
</tr>
<tr>
<td>6/11/16/18/31/33/45/51</td>
<td>323 / 8 (2.5)</td>
<td>72 / 5 (6.9)</td>
<td>0.055</td>
</tr>
<tr>
<td>Other HPV</td>
<td>323 / 4 (1.2)</td>
<td>72 / 0 (0)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Any HPV</td>
<td>323 / 12 (3.7)</td>
<td>72 / 5 (6.9)</td>
<td>n.s.</td>
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