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CERVICAL AND VAGINAL

HIGH-GRADE CANCER PRECURSORS

– AGE DEPENDENCE OF HUMAN PAPILLOMAVIRUS GENOTYPES

AND ALTERNATIVE MANAGEMENT STRATEGIES

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ACADEMIC DISSERTATION

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ABSTRACT

Nearly all humans acquire a human papillomavirus (HPV) infection during their lifetime. HPV is a necessary, but not sufficient, cause of cervical and vaginal cancer. The vast majority of HPV infections regress spontaneously, even the precancerous lesions (intraepithelial neoplasias) of the female genital tract that HPV causes. Secondary prevention of cervical cancer by organised screening has reduced rates by 80% in Finland and some other countries. Detected precancerous cervical lesions are treated with local excision or destruction, because the progressive or regressive nature of an individual lesion remains unknown. These procedures have a 90% initial cure rate but may predispose to late miscarriage or preterm birth in subsequent pregnancies. Prophylactic HPV vaccines targeting the two most common HPV types in cervical cancer (HPV 16 and 18) have been available for a little over a decade. A near eradication of HPV infections and precancerous lesions in adolescents has been demonstrated a decade after vaccination; however, the full effect of mass vaccination, especially on cancer rates, will only be seen decades later.

Characterising the prevaccination era HPV-type distribution can aid the assessment of the effect of vaccinations. Sensitivity of screening will suffer greatly when disease rates decrease after vaccinations. However, for decades there will be both unvaccinated and vaccinated women in screening, and HPV-type and age-specific information can aid in refining screening programs. HPV-type distribution also varies geographically; therefore, we assessed the current types causing morbidity in Finnish women. Our study of 1279 women referred to colposcopy for abnormal cytology found a distinct, age-related polarisation of HPV types; this revealed that HPV16/18 is much more common in young women (<30 years of age) than in women ≥45 years of age. Histological high-grade cervical disease was diagnosed in 503 women, and even in this group the type distribution remained polarised according to age. Two thirds of high-grade disease in young women were attributed to HPV16/18, whereas it was only found in one third of women ≥45. Other high-risk types and even HPV negativity were more common than HPV16/18 in high-grade disease in the older women.

We performed a meta-analysis on the outcomes of untreated CIN2, because treatment of cervical intraepithelial neoplasia (CIN) can lead to reproductive complications, and individual

previous studies have shown high spontaneous regression rates of moderate lesions (CIN grade 2, CIN2) especially in young women. Summary estimates from 36 studies showed the overall regression rate at two years to be 50% and the progression rate 18%. The two-year regression rate was 60% and the progression rate was 11% in a subgroup analysis of women <30 years of age (approximately 1000 women). Overall progression to invasive cancer was rare (0.5%, n=15/3160). In addition, we assessed the performance of a DNA methylation panel (S5 classifier) in predicting progression of untreated histological CIN2 in a prospective cohort study of 149 women (18-30 years of age). S5 was independently able to predict progression even when adjusted for age, initial cytology, cigarette smoking, and HPV16/18 status.

Vaginal intraepithelial neoplasia (VAIN) is more uncommon than CIN and presents mostly in older women. Contemporary treatment is mostly laser vaporisation, but recurrence occurs in up to a third, and repeated treatments can be scarring. HPV persistence is associated with recurrence. An immunomodulator imiquimod has been used in small studies with promising success rates. We recruited 30 women with histological high-grade VAIN into a three-arm, randomised trial comparing the efficacy of self-administered vaginal imiquimod, laser vaporisation, and expectant management. No progressions were observed during the four months of follow-up, and histological regression rates showed no significant differences between the study arms (80% in the imiquimod arm, 100% in the laser arm). HPV clearance, however, was significantly more common in the imiquimod arm (63%) than in the laser arm (11%) (p=0.05).

While we wait and hope for a widespread effect of the prophylactic HPV vaccines, it still remains important to refine who, when, and how to treat among women afflicted by HPV-related disease.

FINNISH SUMMARY

Lähes kaikki ihmiset saavat ihmisen papilloomavirusinfektion (human papillomavirus, HPV) jossain vaiheessa elämäänsä. HPV on välttämätön, mutta ei riittävä, kohdunkaula- ja emätinsyövän aiheuttaja. Valtaosa HPV-infektioista ja jopa sen aiheuttamista syövän esiastemuutoksista naisen synnytyselimissä paranee ilman hoitoa. Seulonta sekundääripreventiona on vähentänyt 80 % kohdunkaulasyöpätapauksista Suomessa ja joissain muissa maissa. Todetut kohdunkaulan esiastemuutokset hoidetaan paikallisella kirurgisella poistolla tai tuhoamisella, koska yksittäisen muutoksen paranemista tai etenemistä ei voida ennustaa. Muutoksen paikallisella poistolla on 90 % ensivaiheen onnistumisaste, mutta se voi altistaa myöhäiselle keskenmenolle tai ennenaikaiselle synnytykselle tulevissa raskauksissa. Profylaktisia HPV-rokotteita, jotka kattavat kohdunkaulasyövän kaksi yleisintä HPV-tyyppiä (HPV16 ja 18), on ollut saatavilla hieman yli vuosikymmenen ajan. Tutkimuksissa vuosikymmen lapsuudessa/nuoruudessa saatujen rokotusten jälkeen HPV-infektioiden ja esiastemuutosten on osoitettu lähes kokonaan hävinneen. Väestötason rokottamisen vaikutusta etenkin syövän esiintymiseen joudutaan silti odottamaan vielä vuosikymmeniä.

Ennen väestötasoista rokotekattavuutta on tärkeä tuntea tällä hetkellä sairastavuutta aiheuttavat HPV-tyypit, jotta rokotusten vaikutusta voidaan arvioida. Jatkossa seulonnan herkkyys tulee selvästi vähenemään, kun tautitapausten määrä pienenee. Vuosikymmenien ajan seulontaan tulee kuitenkin edelleen osallistumaan sekä rokottamattomia että rokotettuja naisia ja HPV-tyyppi- ja ikäkohtainen tieto voi auttaa parantamaan seulontaohjelmia. Lisäksi HPV-tyyppijakauma vaihtelee maantieteellisesti, joten arvioimme sairastavuutta aiheuttavia HPV-tyyppejä suomalaisissa naisissa. Totesimme 1279 kolposkopiaan solumuutoksen vuoksi lähetetyn naisen joukossa selvän ikään liittyvän jakauman HPV-tyypeissä. HPV16/18 oli paljon tavallisempi nuorilla naisilla (<30-vuotiaat) kuin ≥45-vuotiailla. Histologinen vaikea-asteinen muutos todettiin 503 naisella, ja tässäkin ryhmässä tyyppijakauma oli ikäryhmissä epätasainen. Kaksi kolmasosaa nuorten naisten vaikeista muutoksista liittyivät HPV16/18:aan ja vain kolmasosa yli 45-vuotiaiden. Vanhempien naisten vaikeissa esiastemuutoksissa muut korkean riskin virustyypit ja HPV-negatiivisuus olivat tavallisempia kuin HPV16/18.

Koska esiastemuutosten (cervical intraepithelial neoplasia, CIN) hoito voi johtaa raskauskomplikaatioihin ja yksittäiset aiemmat tutkimukset ovat osoittaneet keskivaikeiden esiastemuutosten (CIN2) spontaanin paranemistaipumuksen olevan suuri etenkin nuorilla naisilla, teimme meta-analyysin hoitamattomien CIN2-muutosten luonnollisesta kulusta. 36 tutkimuksesta saatu arvio näytti CIN2-muutoksen paranevan 50 % tapauksista kahdessa vuodessa kun taas 18 % muutoksista eteni. Vain alle 30-vuotiaita naisia sisältäneessä alaryhmäanalyysissä (noin 1000 naista) kahden vuoden kohdalla 60 % muutoksista parani kun vain 11 % eteni. Eteneminen syöväksi oli kaikkiaan harvinaista (0,5 %, n=15/3160). Lisäksi arvioimme DNA-metylaatioluokittelijan (S5 classifier) toimivuutta CIN2-muutoksen etenemistä ennakoivana tekijänä 149 nuoren (18-30-vuotiaan) naisen prospektiivisessa kohorttitutkimuksessa. S5 pystyi itsenäisesti ennustamaan muutoksen etenemistä iästä, lähtötilanteen solumuutoksen vaikeusasteesta, tupakoinnista ja HPV16/18-löydöksestä riippumatta.

Emättimen esiastemuutokset (vaginal intraepithelial neoplasia, VAIN) ovat harvinaisempia kuin kohdunkaulan muutokset ja esiintyvät pääasiassa vanhemmilla naisilla. Muutoksia hoidetaan nykyään pääasiassa laserilla, mutta tauti uusiutuu noin joka kolmannella ja etenkin uusintahoidot voivat olla arpeuttavia. HPV:n säilyminen on tunnettu muutosten uusiutumista ennakoiva tekijä. Immuunivasteen muuntelija imikimodia on käytetty aiemmin joissain pienissä tutkimuksissa lupaavin tuloksin. Rekrytoimme 30 naista, joilla oli todettu keskivaikea tai vaikea-asteinen VAIN-muutos, satunnaistettuun tutkimukseen, jossa verrattiin itseannostellun imikimodin, laserhoidon ja seurannan tehoa hoidossa. Yksikään muutoksista ei edennyt neljän kuukauden seurannassa ja paranemisaste oli yhtäläinen imikimodi- ja laserhoidolla (80 % ja 100 %). HPV:n häviäminen oli kuitenkin selvästi tavallisempaa imikimodiryhmässä (63 %) kuin laserryhmässä (11 %) (p=0.05).

Profylaktisten HPV-rokotusten laajaa vaikutusta odottaessa ja toivoessa on edelleen tärkeää tarkentaa keitä naisia, milloin ja miten hoidetaan HPV:n aiheuttamissa sairauksissa.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications and referred to in the text by their roman numerals (I-IV).

I Aro K, Nieminen P, Louvanto K, Jakobsson M, Virtanen S, Lehtinen M, Dillner J, Kalliala I. Age-specific HPV genotype distribution in high grade cervical disease in screened and unvaccinated women. Gynecol Oncol. 2019 Aug 154(2):354-359.

II Tainio K*, Athanasiou A, Tikkinen KAO, Aaltonen R, Cardenás Hernándes L, Glazer-Livson S, Jakobsson M, Joronen K, Kiviharju M, Louvanto K, Oksjoki S, Tähtinen R, Virtanen S, Nieminen P, Kyrgiou M, Kalliala I. Clinical course of untreated cervical intraepithelial neoplasia grade 2 under active surveillance: systematic review and meta-analysis. BMJ. 2018 Feb 27;360:k499.

III Louvanto K, Aro K**,** Nedjai B, Bützow R, Jakobsson M, Kalliala I, Dillner J, Nieminen P, Lorincz A. Methylation in predicting progression of untreated high-grade cervical intraepithelial neoplasia. Clin Infect Dis. 2019 Jul 25. doi: 10.1093/cid/ciz677

IV Tainio K*, Jakobsson M, Louvanto K, Kalliala I, Paavonen J, Nieminen P, Riska A. Randomised trial on treatment of vaginal intraepithelial neoplasia - Imiquimod, laser vaporisation and expectant management. Int J Cancer 2016 Nov 15;139(10):2353-2358.

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ABBREVIATIONS

AGC-FN	atypical glandular cells favor neoplasia
AGC-NOS	atypical glandular cells not otherwise specified
AIS	adenocarcinoma in situ
APC	antigen presenting cell
ASC-H	atypical squamous cells, cannot rule out HSIL
ASC-US	atypical squamous cells of undetermined significance
AUC	area under the receiver operating characteristic curve
C	cytosine
CI	confidence interval
CIN	cervical intraepithelial neoplasia
CIN2+	cervical intraepithelial neoplasia grade 2 or worse
CIN3+	cervical intraepithelial neoplasia grade 3 or worse
CpG site	cytosine followed by guanine in DNA
DNA	deoxyribonucleic acid
DS	dual staining (p16, Ki67)
dVIN	differentiated vulvar intraepithelial neoplasia
e.g.	exempli gratia
etc.	et cetera
G	guanine
HIV	human immunodeficiency virus
HPV	human papillomavirus
hrHPV	high-risk human papillomavirus
HSIL	high grade squamous intraepithelial lesion
IARC	International Agency for Research on Cancer
i.e.	id est
ISRCTN	International Standard Randomised Controlled Trial Number
ITT	intention-to-treat

Ki67	a cellular proliferation marker
LCR	long control region
LEEP	loop electrosurgical excision procedure
LLETZ	large loop excision of the transformation zone
LSIL	low grade squamous intraepithelial lesion
mITT	modified intention-to-treat
NILM	negative for intraepithelial lesion or malignancy
NSAID	nonsteroidal anti-inflammatory drug
OR	odds ratio
PCR	polymerase chain reaction
pRB	retinoblastoma protein
p16	a cellular protein reflecting the activity of the HPV E7 oncogene
p53	tumour protein 53
RCI	Reid colposcopic index
RCT	randomised controlled trial
ROC curve	receiver operating characteristic curve
RR	relative risk or risk ratio
SCJ	squamo-columnar junction
STM	specimen transport medium
TBS	the Bethesda system
TZ	transformation zone
uVIN	usual vulvar intraepithelial neoplasia
VAIN	vaginal intraepithelial neoplasia
VIN	vulvar intraepithelial neoplasia
VLP	virus-like particle
WHO	World Health Organisation
5-FU	5-fluorouracil

1 INTRODUCTION

Genital human papillomavirus (HPV) infections are extremely common, with nearly all humans having at least one infection during their lifetime (Bruni *et al.*, 2010). First HPV infections are usually acquired right after sexual debut (Winer *et al.*, 2003). The necessity of HPV in carcinogenesis in the uterine cervix is well established, but it alone is insufficient to cause cancer, because the majority of infections and even preinvasive lesions (intraepithelial neoplasias) resolve spontaneously (zur Hausen, 1977; Ho *et al.*, 1998; Walboomers *et al.*, 1999; Castle *et al.*, 2009). HPV is also recognised as a causative agent of neoplastic transformation in the vulva, vagina, anus, penis, and oropharynx (Forman *et al.*, 2012). It has been estimated that, on average, it takes decades from an incident HPV infection to development of cervical cancer. Currently there is no way to predict the outcome of an individual HPV infection despite some well-established risk factors of carcinogenesis.

Two major advances in HPV-related disease control have been made: cervical cancer screening and prophylactic vaccines. Organised nationwide screening programs based on cytology were started in developed countries such as Finland nearly 60 years ago and have led to an 80% reduction in cervical cancer incidence and mortality, because preinvasive lesions can be treated (Laara, Day and Hakama, 1987). Globally, cervical cancer is still the fourth most prevalent cancer in women (Ferlay *et al.*, 2018). High-risk HPV (hrHPV) testing has been established more recently as a more sensitive, albeit less specific, screening test expected to further reduce cancer rates in women attending screening (Koliopoulos *et al.*, 2017). Prophylactic HPV vaccines have been available for a little over a decade. The prevalence of HPV infection and preinvasive disease has been tremendously reduced in countries with high vaccine coverage of adolescents (Kavanagh *et al.*, 2017; Palmer *et al.*, 2019). Evidence also exists of herd immunity, especially with gender-neutral vaccination (Lehtinen, Söderlund-Strand, *et al.*, 2018a).

Unsolved issues remain despite these major advances. Local treatments of cervical preinvasive lesions are highly efficient in preventing cancer but can have important, long-term adverse effects, such as an increased risk of preterm birth or midtrimester miscarriage (Kyrgiou *et al.*, 2017). The adverse effects and natural history estimates of cervical intraepithelial neoplasias, especially in young women, have led to the adoption of expectant management strategies where lesions are actively surveilled in hope of spontaneous resolution. A predictive test for outcomes could revolutionise management algorithms by allowing allocation of patients with risk of progression to cancer to immediate treatment and saving those with low risk from treatment-related adverse effects. DNA methylation has shown promise in this area, because it has been shown to be able to predict which hrHPV infections lead to significant preinvasive disease (Lorincz *et al.*, 2016).

Treatment and detection of HPV-related disease at other sites than the cervix is more difficult. Vaginal disease is commonly revealed by cervical cancer screening, but treatment is complicated by anatomy and typical multifocal disease. Currently used treatments can also have serious long-term effects such as scarring, and recurrences are common in up to one third of patients, irrespective of treatment method (Gurumurthy and Cruickshank, 2012). Most current methods aim at excision or destruction of vaginal preinvasive lesions. A treatment targeting the causative agent, HPV, could potentially have better efficacy, because hrHPV persistence is a well-recognised risk factor for recurrence. Imiquimod, an immune response modulator, has been found promising in small, non-randomised studies (Buck and Guth, 2003; Haidopoulos *et al.*, 2005).

Great promise lies in the prophylactic vaccines in eradication of HPV if coverage on the population level is sufficiently high, but evidence of long-term effectiveness against cancer is still awaited. In the meantime, it is still important to better our understanding of the process of HPV-related carcinogenesis and optimise treatments. This thesis aims to answer some aspects of these issues.

2 REVIEW OF THE LITERATURE

2.1 HUMAN PAPILLOMAVIRUS INFECTION

Human papillomavirus (HPV) is an icosahedral, non-enveloped, double-stranded, 8000 base pair DNA virus belonging to the Papillomaviridae family. Papillomaviruses have been found to be both host-species-specific and tissue-specific, replicating in the basal layer of either cutaneous or mucosal surface epithelium. HPVs are divided based on DNA sequence analysis to five genera that are further divided into species. Over 200 HPV genotypes have been described, 40 of which are known to infect mucosal epithelium (Bzhalava, Eklund and Dillner, 2015). Thirteen of the mucosal HPVs are classified as group I or 2A carcinogens by the International Agency for Research on Cancer (IARC) and are commonly referred to as high-risk HPVs (hrHPV) (IARC, 2012). hrHPVs belong to various species of the alpha genera. Specific HPV genotypes (exempli gratia (e.g.) HPV16) have also been found to have different variant lineages and sublineages (Burk, Harari and Chen, 2013). A study on the geographical distribution of HPV16 lineages suggests that the ancestor of HPV16 was present in ancestral humans over 500 000 years ago (Pimenoff, de Oliveira and Bravo, 2017).

HPV is known to spread through direct epithelial contact and, most commonly, mucosal contact during sexual intercourse. A microtrauma is thought to be necessary for the virus to enter the basal cells of stratified epithelium, and the squamo-columnar junction (SCJ) in the female cervix is especially vulnerable (Schiller, Day and Kines, 2010; Doorbar *et al.*, 2012). Recent evidence exists that there are phenotypically distinct cells in the junctional area that are specifically targeted (Herfs *et al.*, 2012).

HPV relies on the host cells' cellular machinery to complete its life cycle, and its genome remains a low copy number extrachromosomal episome in the nucleus of

the basal cell in early stage infections (Stubenrauch and Laimins, 1999; Pyeon *et al.*, 2009). HPV does not kill the host cell (Doorbar *et al.*, 2012). The viral genome consists of a long control region (LCR) and early and late regions. Figure 1 shows a schematic representation of the HPV genome. These genes are expressed at different stages the host cell passes through in the proliferating and differentiating epithelium; the end result is assembled complete viral particles that are released from the surface of the epithelium (Fehrmann and Laimins, 2003). Early genes (for example E6, E7) are needed for viral replication and to promote host cell proliferation; late genes (L1, L2) code the viral capsid (Munger *et al.*, 1989). Mucosal HPV infections are mostly asymptomatic apart from those causing visible genital warts.

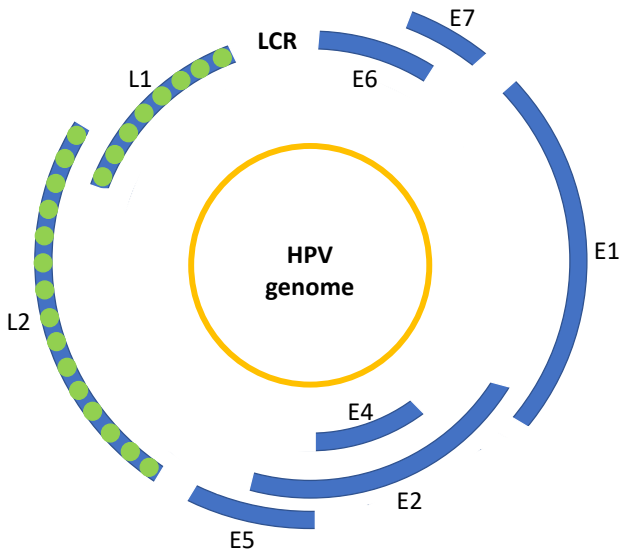

Figure 1 Schematic representation of the HPV genome. E6 inhibits tumour suppressor gene p53 and E7 retinoblastoma protein (pRB). L1 is the major capsid protein and L2 the minor capsid protein.

2.1.1 NATURAL HISTORY OF HPV INFECTION

Up to 90% of all HPV infections clear spontaneously within two years (Ho *et al.*, 1998; Moscicki *et al.*, 2006). Host immune response is generated when infected cells are shed from the surface of the epithelium and viral proteins are recognised by antigen presenting cells (APCs) and antibody producing B cells (Stanley, 2010a). APCs activate T cells, of which helper T cells enhance the antibody production of B cells, and cytotoxic T cells restrict the infection locally. A cell-mediated response to infection can be detected within weeks of infection, but a detectable antibody level occurs only after months (Stanley, 2006). HPV antibodies are thought to be genotype specific and, for an unknown reason, only approximately half of the infected individuals have a detectable humoral immune response (Mählck *et al.*, 1999; Carter *et al.*, 2000). Natural antibodies are likely to be protective against re-infection by the same genotype in approximately 50-70% of seropositive individuals (Lin *et al.*, 2013; Beachler *et al.*, 2016).

The precise pathways leading to persisting infections are poorly understood. The longevity of the infected basal stem cell has been implicated (Egawa, 2003; Doorbar, 2006). The infecting genotype clearly affects time to clearance, with HPV16 having the longest time to clearance (18-23 months), followed by 18, 31, 33, and 52 of the hrHPVs (Bulkmans *et al.*, 2007). Different lineages of the same HPV genotype also appear to have different oncogenic potential (Schiffman *et al.*, 2010). Low-risk HPV infections, however, usually clear within a few months (Stanley, 2010b). Infection with multiple HPV genotypes appears to have an increased risk of persistence; conversely, a co-infection with a low-risk genotype has been shown to promote clearance (Ho *et al.*, 1998; Trottier *et al.*, 2006; Sundström *et al.*, 2015).

Host cofactors associated with persistence and development of cervical neoplasia are mostly linked to a diminished immune response, such as immunosuppression (human immunodeficiency virus (HIV) infection, immunosuppressive medication), cigarette smoking, other sexually transmitted disease (Chlamydia trachomatis, Herpes simplex), and increasing age (Castellsague, Bosch and Munoz, 2002; Castle *et*

al., 2005; Castle *et al.*, 2011). Younger women, in contrast, have usually the highest exposure to HPV because of behavioural factors leading to more persistent infections and cervical intraepithelial neoplasias (Wellings *et al.*, 2006). Multiparity has also been implicated, but the mechanism is mostly thought to be indirect, and the increase was not as great in Finnish multiparous women as internationally reported, most likely because of lower incidence of other sexually transmitted infections (Hinkula *et al.*, 2004). Oral contraceptive use has been implicated as a risk factor for persistence and neoplasia development, although findings are not consistent (Ylitalo *et al.*, 1999; Giuliano *et al.*, 2004; Adhikari *et al.*, 2018). Persistence of an HPV infection is most likely multifactorial, including characteristics of both the virus and host.

A clinical persistent infection can be thought to be present when the same hrHPV(s) is repeatedly detected (Moscicki *et al.*, 2006). A clear sign of persistence can also be considered to be histopathological changes beyond signs of a productive HPV infection. Difficulty in defining persistence makes investigating the phenomenon also challenging. Differentiating between re-infection and a persistent infection is not always possible, and the onset of infection also cannot be reliably established, because it might have already persisted for any time period when first detected. The E6 and E7 viral genes become deregulated in persistent infections leading to cervical cancer precursors, and they may be integrated into the host genome, causing genetic instability and secondary somatic mutations leading to uncontrolled proliferation (Pett *et al.*, 2004; Isaacson Wechsler *et al.*, 2012). E6 and E7 are recognised as oncogenes inhibiting tumour suppressor genes (p53 and pRB) (de Sanjosé, Brotons and Pavón, 2018). The necessity of HPV infection in uterine cervical carcinogenesis has been well established, and this discovery was awarded the Nobel prize in 2008 (zur Hausen, 1977; Walboomers *et al.*, 1999).

There is increasing evidence of latent HPV infections presenting with HPV DNA presence in basal cells even when the virus and histological changes are undetectable with current standard diagnostic methods (Gravitt, 2011; Maglennon, McIntosh and

Doorbar, 2011). A proportion of infections currently labelled as cleared might thus actually be latent. A latent infection is thought to be controlled by the host immune response, especially tissue resident T cells, but reactivation may occur if immune response is diminished (Doorbar *et al.*, 2012; Gravitt, 2012). An example can be found, for example, in HIV-positive, sexually abstinent women in whom incident HPV detection progressively increases when CD4 cell counts decrease, i.e., immune response diminishes (Strickler *et al.*, 2005).

2.1.2 HPV EPIDEMIOLOGY

Anogenital HPV infections are extremely common: at least 80% of humans become infected at least once during their lifetime, and 10% of humans have a prevalent HPV infection at any given time (de Sanjosé *et al.*, 2007; WHO/ICO Information Centre on HPV and Cervical Cancer, 2007; Bruni *et al.*, 2010). HPV prevalence increases steeply after sexual debut, with about half being infected within three years (Winer *et al.*, 2003; Kjaer *et al.*, 2005). Risk factors for HPV acquisition are similar to those of HPV persistence and neoplasia development with the addition of number of lifetime sexual partners (Rositch *et al.*, 2012). Many young women who have acquired a genital HPV infection will acquire another one, and behavioural factors, including also the number of sexual partners the current partner has had, appear to lead to this clustering of infections (Burk *et al.*, 1996; Woodman *et al.*, 2001; Muñoz *et al.*, 2004; Vaccarella *et al.*, 2006; Trottier *et al.*, 2010).

Overall HPV prevalence is highest, at up to nearly 50%, in young women under the age of 25 and decreases thereafter until a second but smaller peak is sometimes seen in peri- and postmenopausal women (Castle *et al.*, 2005; Franceschi *et al.*, 2006; Schiffman *et al.*, 2010). The HPV point prevalence was found to be 33% in Finnish female first-year university students (Auvinen *et al.*, 2005). Age-specific prevalence curves, however, vary greatly geographically and according to income in populations (Franceschi *et al.*, 2006). The reason for the second prevalence peak remains under debate, because behavioural factors (such as new sexual partners) appear to be

insufficient to explain it (Bosch *et al.*, 2008). Reactivated latent infections after immune senescence have been proposed as a possible explanation. A second peak, however, is not seen universally in all studies (Schiffman, 1992; Franceschi *et al.*, 2006). A study from the USA reported hrHPV prevalence in a cervical cancer screening population to be 17.8% in 25-29-year-olds, but only 6.5% in women over 50 years of age, and 3.5% and 0.8%, were HPV16 positive in those groups, respectively (Monsonego *et al.*, 2015). Similar findings on age trends have been reported also from the UK and Finland (Sargent *et al.*, 2008; Leinonen *et al.*, 2013).

Globally, HPV point prevalence in general varies greatly from 20-30% in Africa and South America to 6-7% in Southeast Asia and Southern Europe (Clifford *et al.*, 2005; de Sanjosé *et al.*, 2007). A study in the 1990s found the hrHPV prevalence to be 7% in Finnish women of screening age (Syrjanen *et al.*, 1992). Genotype-specific prevalence shows distinct geographical patterns, but HPV16 is globally the most prevalent genotype, followed by HPV18 (Bruni *et al.*, 2010). Table 1 presents the estimated genotype-specific prevalence in women with normal cytology worldwide, in Europe, in a screening population in Sweden, and in an hrHPV test-positive screening population in Finland. In the Finnish study, 7.8% of the overall population tested hrHPV positive, but 30% of hrHPV test-positive women were found to be HPV negative in genotyping, which may have affected the overall prevalence results (Leinonen, 2013; Leinonen *et al.*, 2013). The reason for this is unclear, but genotyping was performed much later than the hrHPV test, which was directly analysed. When compared to overall European data, HPV16 and HPV18 are less prevalent, and HPV52 is more prevalent in Finland (de Sanjosé *et al.*, 2007; Bruni *et al.*, 2010). HPV52 was also found to be more prevalent than the European average in Denmark (Kjaer *et al.*, 2008).

Table 1. *Point prevalence of HPV genotypes in different geographical regions in women with normal cytology and in screening populations in Sweden and Finland.*

Genotype	Worldwide Bruni et al. 2010 n=215 568	Europe Bruni et al. 2010 n=129 646	Sweden Forslund et al. 2002 n=6123	Finland Leinonen thesis 2013 n=33 043
HPV16	3.2%	4.8%	2.1%	0.9%
HPV18	1.4%	0.9%	0.6%	0.4%
HPV31	0.8%	2.3%	1.1%	0.7%
HPV33	0.5%	0.6%	0.4%	0.3%
HPV52	0.9%	0.4%	0.3%	0.5%
HPV58	0.7%	0.4%	0.3%	0.4%

2.2 PROPHYLACTIC HPV VACCINES

Vaccines have been developed for the primary prevention of HPV infection. Prophylactic HPV vaccines contain virus-like particles (VLP) that mimic the viral capsid protein encoded in the L1 region of the viral genome of specific HPV genotypes (Schiller and Lowy, 2001). Three prophylactic vaccines have been or are commercially available. The bivalent vaccine targets HPV16 and 18, the quadrivalent vaccine targets the former two and HPV6 and 11 that commonly cause genital warts, and the 9-valent vaccine targets all the formerly mentioned and HPV31, 33, 45, 52, and 58 (FUTURE II Study Group, 2007; Paavonen *et al.*, 2007; Joura *et al.*, 2015). The quadrivalent vaccine has recently been replaced by the 9-valent vaccine.

Many developed countries and some developing countries have included the HPV vaccine in their national vaccination program. Most programs still include only vaccination of adolescent girls, such as the Finnish program that started in 2013, but some countries have moved to vaccinating gender neutrally, which results in better herd immunity (Lehtinen, Luostarinen, *et al.*, 2018; Lehtinen, Söderlund-Strand, *et al.*, 2018b). The National Institute for Health and Welfare recommended

commencing gender-neutral HPV vaccination in Finland in January 2019 (*The National Institute for Health and Welfare recommends including the HPV vaccine in the boys' vaccination programme - Press release - THL*, 2019). Current trends of fear of vaccine-related adverse events in the general public, among other factors, have affected uptake of the HPV vaccines (Ferrer *et al.*, 2014). Several studies, however, have shown no difference in long-term adverse events or adverse pregnancy outcomes in HPV-vaccinated and unvaccinated populations (Arnheim-Dahlstrom *et al.*, 2013; Lehtinen *et al.*, 2016; Arbyn *et al.*, 2018; Skufca *et al.*, 2018).

2.2.1 IMMUNOGENICITY

The magnitude of antibody response to the vaccines is vastly greater than that of a natural infection and has been demonstrated in all vaccinated subjects, in contrast to the lack of natural antibodies in many individuals after natural infection (FUTURE II Study Group, 2007; Paavonen *et al.*, 2007; Joura *et al.*, 2015). Vaccines are commonly administered as a two-dose regimen within 6 months. A three-dose regimen is recommended after adolescence or in immunocompromised individuals. Antibody levels are up to 100-fold higher after vaccination than after natural infection and remain elevated in the case of the bivalent vaccine for nearly a decade, but some waning has been shown for antibody levels with the quadrivalent vaccine (Villa *et al.*, 2006; Roteli-Martins *et al.*, 2012; Artemchuk *et al.*, 2018). Protection against infection and cervical neoplasias appears to remain high despite lowering serum antibody levels (Joura *et al.*, 2008; Einstein *et al.*, 2009). Immunogenicity of the vaccines has been demonstrated to be age-specific with a better response in children and adolescents under age 15 (Pedersen *et al.*, 2007; Perez *et al.*, 2008).

2.2.2 EFFICACY

The prophylactic vaccines have been shown to be highly efficacious against HPV infection and cervical intraepithelial neoplasia (CIN) in phase III trials. There is also evidence of cross-protection towards high-risk genotypes not targeted by the vaccines in the case of the bivalent and quadrivalent vaccine (Brown *et al.*, 2009;

Wheeler *et al.*, 2012; Woestenberg *et al.*, 2018). Table 2 (Villa *et al.*, 2006; Lehtinen *et al.*, 2012; Joura *et al.*, 2015) and 3 (Munoz *et al.*, 2010; Lehtinen *et al.*, 2012; Joura *et al.*, 2015; Huh *et al.*, 2017) show efficacy estimates of the prophylactic vaccines. The trials consisted of adolescents and young women (under 26 years of age) with follow-up up to approximately five years. The vaccines were found to be more efficacious in HPV-naïve women in comparison to individuals already harbouring HPV infection.

Table 2. *Vaccine efficacy against genotype-specific HPV infection in HPV-naïve subjects (%, 95% confidence interval (CI)).*

Outcome	Bivalent	Quadrivalent	9-valent*
HPV16	94.7 (91.8-96.7)	91.6 (73.3-98.4)	-
HPV18	92.3 (86.5-96.0)	91.6 (43.3-99.8)	-
HPV31	77.1 (67.2-84.4)	46.2 (15.4-66.4)	95.5 (90.7-97.9)
HPV33	43.1 (19.3-60.2)	28.7 (-45.1-65.8)	99.1 (95.2-100)
HPV45	79.0 (61.3-89.4)	7.8 (-67.0-49.3)	96.8 (92.1-98.9)
HPV52	18.9 (3.2-32.2)	18.4 (-20.6-45.0)	97.3 (95.3-98.7)
HPV58	-6.2 (-44.0-21.6)	5.5 (-54.3-42.2)	94.8 (91.0-97.1)

* 9-valent vaccine compared against the quadrivalent and found non-inferior in protection against HPV16/18

Table 3. *Vaccine efficacy against CIN grade 2 or worse (CIN2+) in HPV-naïve subjects and in intention-to-treat (ITT) populations (%, 95% CI)*

Outcome	Bivalent		Quadrivalent		9-valent*	
	naïve	ITT	naïve	ITT	naïve	mITT
CIN2+ (HPV16/18+)	99.0 (94.2-100.0)	60.7 (49.6-69.5)	100.0 (91.4-100.0)	53.0 (38.2-64.5)	-	-
CIN2+ (HPV16/18-)	64.9 (52.7-74.2)	33.1 (22.2-42.6)	42.7 (23.7-57.3)	19.3 (5.7-31.0)	97.4 (85.0-99.9)	71.4 (40.8-86.2)

* 9-valent vaccine compared against the quadrivalent and found non-inferior in protection against HPV16/18+ CIN2+; CIN2+ in the modified ITT (mITT) includes also high-grade vaginal and vulvar disease

The quadrivalent vaccine also shows high efficacy against genital warts: 97.1% (95% confidence interval (CI) 92.4-99.2%) in HPV-naïve women and 79.3% (95% CI 72.7-84.5%) in baseline HPV-positive women (FUTURE I/II Study Group, 2010). Efficacy of the quadrivalent vaccine against high-grade vaginal and vulvar intraepithelial neoplasia (VAIN/VIN2-3) irrespective of HPV genotype is also high: 77.1% (95% CI 47.1-91.5) in HPV-naïve women and 50.7% (95% CI 22.5-69.3) in baseline HPV-positive women (Munoz *et al.*, 2010).

A recent Cochrane review of prophylactic HPV vaccines includes 26 trials with over 70 000 participants with follow-up from 1.3 to 8 years (Arbyn *et al.*, 2018). The review concluded that there is high certainty evidence of vaccine protection against high-grade cervical lesions in young (15-26-year-old) women, and the effect is greatest against disease associated with HPV16/18 and in those who are hrHPV negative at time of vaccination. In older women there was moderate certainty evidence that vaccination reduces high-grade cervical disease in HPV16/18-negative women but not if they are unselected by hrHPV status. All of this emphasises the importance of vaccination in adolescence before exposure to HPV.

2.2.3 EFFECTIVENESS

The first national HPV vaccination programs started a little over a decade ago. Reports from the past five years from Scotland and Australia show significant real-life reductions in hrHPV infections in women vaccinated in adolescence (Kavanagh *et al.*, 2014, 2017; Tabrizi *et al.*, 2014; Cameron *et al.*, 2016; Machalek *et al.*, 2018; Garland *et al.*, 2018). A study from Scotland with over 8 000 participants showed a vaccine effectiveness of 89.1% (95% CI 85.1-92.3) against HPV16/18 in adolescent females vaccinated at 12-13 years of age with the bivalent vaccine (Kavanagh *et al.*, 2017). A cross-protective effect was seen with close to or over 80% effectiveness regarding HPV31/33/45 infections; the risk of infection by vaccine-related genotypes was also reduced in the unvaccinated population, implying herd immunity. Australian studies on the quadrivalent vaccine effectiveness showed similar reductions in HPV16/18 and evidence of herd immunity, but of less cross-protection (Tabrizi *et al.*, 2014; Garland *et al.*, 2018). Effectiveness data on the 9-valent vaccine is still awaited, as it has been available for a shorter period of time.

Significant reductions of CIN after national vaccination program implementation of the HPV vaccine in Scotland, Australia, and five regions in the USA have been shown in a few studies, but long-term results are awaited, because vaccinated women are only starting cervical cancer screening (Crowe *et al.*, 2014; Pollock *et al.*, 2014; Hariri *et al.*, 2015; Cameron *et al.*, 2017; Palmer *et al.*, 2019). The most recent study from Scotland of over 100 000 young women showed a nearly 90% reduction of high-grade CIN (Palmer *et al.*, 2019). Two registry-based studies from the Nordic countries found vaccine effectiveness against CIN3 or worse (CIN3+) to be 66-90% a decade after vaccination (Lehtinen *et al.*, 2017; Kjaer *et al.*, 2018). First proof of protection against invasive cancer from a randomised setting has also been reported (Luostarinen *et al.*, 2018).

2.3 HPV-RELATED NEOPLASIAS OF THE FEMALE GENITAL TRACT

2.3.1 CERVICAL INTRAEPITHELIAL NEOPLASIA

2.3.1.1 Classification

The ectocervix is covered with stratified squamous epithelium and the endocervix with columnar (glandular) epithelium, although the location of the SCJ and surrounding transformation zone (TZ) differs according to age and hormonal status. Histopathological grading of preinvasive squamous disease of the uterine cervix, CIN, was first described by Richart in 1973 (Richart, 1973). He divided CIN into three grades based on the thickness of the abnormal cells in the squamous epithelium: CIN grade 1 (CIN1) remaining only in the basal layer, CIN grade 2 (CIN2) up to half of the thickness of the epithelium, and CIN grade 3 (CIN3) the full thickness of the epithelium. Invasive cervical cancer is characterised by the breach of the basal layer by the neoplastic cells and the possibility of metastatic disease.

This three-tier classification is known to suffer from great histopathological intra- and interobserver variability which, in conjunction with natural history estimates of different CIN grades, has led to a revised classification by the World Health Organisation (WHO) in 2014 (Ismail *et al.*, 1989; Stoler and Schiffman, 2001; WHO, 2003, 2014). CIN2 and 3 (dysplasia moderata, dysplasia gravis) are grouped together in the new histopathological classification as high-grade squamous intraepithelial lesion (HSIL), and CIN1 (dysplasia levis) and former HPV atypia/atypia condylomatosa et cetera (etc.) are replaced by low-grade squamous intraepithelial lesion (LSIL). LSIL is currently considered a sign of a productive HPV infection and not a true cancer precursor (Wright, 2006). Figure 2 shows a schematic representation of histological changes in cervical neoplastic disease and CIN classifications and possible relations to HPV infection.

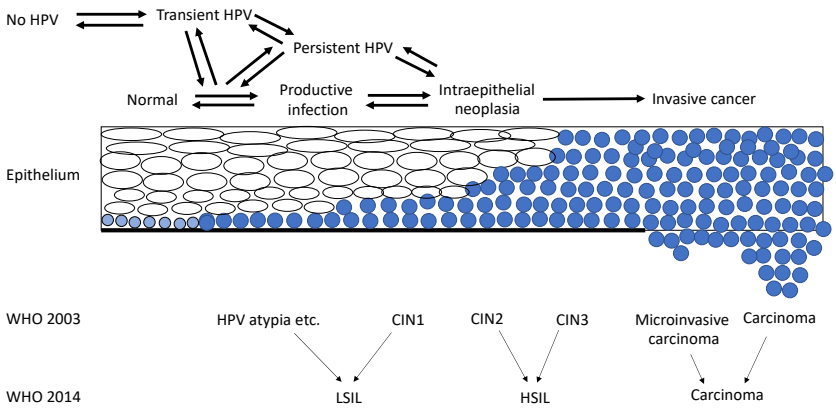

Figure 2 Schematic figure of histological changes in different CIN grades and carcinoma, different CIN gradings, and association with HPV infection

Classification of glandular lesions is more difficult and has been greatly revised throughout time. The new 2014 classification promotes the use of adenocarcinoma in situ (AIS) to describe a preinvasive glandular disease without any other precursors (WHO, 2014). AIS is recognised as a precursor to adenocarcinoma of the cervix (Sheets, 2002; Zaino, 2002).

2.3.1.2 CIN epidemiology and natural history

CIN is more common in young women, with a peak prevalence in the late 20s and early 30s that reflects the earlier peak of HPV infections after sexual debut (Finnish Cancer Registry, no date). Some HPV16 or 18 infections, however, appear to be more aggressive with even CIN3 developing in only a few years after infection (Winer *et al.*, 2005). Despite this, only a small proportion of HPV infections lead to CIN, and known risk factors are similar to those of persistent HPV infection. CIN in peri- and postmenopausal women is rare, at least in developed countries with efficient screening programs and adequate registries. However, cervical cancer incidence peaks at around 35-40 years of age (median age 45) in these countries, and remains

elevated in older women, when mortality also increases (Engholm *et al.*, no date; Finnish Cancer Registry, no date; Hallowell *et al.*, 2018).

As with HPV infections, spontaneous regression of CIN also occurs commonly. There is a higher tendency for spontaneous resolution when the CIN grade is lower. Table 4 provides a summary of some studies examining the natural history of different CIN grades. Even though estimates differ, possibly due to differences in study design and population, CIN1/LSIL regresses often spontaneously with progression to high-grade disease in approximately 10%.

Table 4. *Natural history estimates of different CIN grades*

Author (year)	CIN1/LSIL			CIN2			CIN3		
	Regr %	Persis %	Progr %	Regr %	Persis %	Progr %	Regr %	Persis %	Progr %
Östör (1993) [1]	57	32	11 (1)	43	35	22 (5)	32	56	12
Holowaty et al. (1999) [2]	44	-	11	33	-	16	-	-	-
Cox et al. (2003)	-	-	9	-	-	-	-	-	-
Elit et al. (2011)	-	5	4	-	-	-	-	-	-
Gurumurthy et al. (2014)	-	-	12	-	-	-	-	-	-
Nasiell et al.(1983)	-	-	-	54	16	30	-	-	-
Moscicki et al. (2010) [3]	-	-	-	63	17	12	-	-	-
Castle et al. (2009)	-	-	-	40	-	-	-	-	-
McCredie et al. (2008) [4]	-	-	-	-	-	-	-	-	17 (34)

[1] Progression to invasion in parentheses; [2] Rates within 2 years; [3] Progression and regression rates at 2 years, persistence at 3 years; [4] Progression to invasion at 5 years and at 20 years in parentheses

Estimates for CIN2 are varying, and many studies, especially in the last decade, have shown high regression rates in young women (Fuchs *et al.*, 2007; Moore *et al.*, 2007; Monteiro *et al.*, 2010; Moscicki *et al.*, 2010; McAllum *et al.*, 2011; Loopik *et al.*, 2016). However, a recent retrospective analysis of more than 2000 women with untreated

CIN2 (including CIN1/2 and CIN2/3) between the ages of 21 and 39 showed that only a fifth of them were able to return to routine screening after a median follow-up of 48 months (Silver *et al.*, 2018). Nearly half remained under colposcopic surveillance for low-grade lesions or persisting hrHPV. The study reported six cases (0.2%) of invasive cancer, half of which were characterised by failure to return for surveillance, and none occurred after negative cytology and hrHPV test.

Natural history estimates on CIN3 are historic, because those studies would now be considered mostly unethical. A review by Östör and a study from New Zealand show marked progression to invasive cancer, albeit regression or at least non-progression appears to happen also (Östör, 1993; McCredie *et al.*, 2008, 2010). As not all CIN3 lead to invasive cancer, characteristics of CIN3 cases have been evaluated, and greater lesion size was seen with advancing age, while HPV16-related CIN3 was diagnosed at a younger age (Schiffman and Rodríguez, 2008; Yang *et al.*, 2012; Wentzensen *et al.*, 2013). The natural history of glandular abnormalities is not well-described, because diagnostics are more difficult than in the case of squamous neoplasias (Krivak *et al.*, 2001; Ruba *et al.*, 2004). AIS is diagnosed simultaneously with high-grade squamous neoplasia in 50% of cases, and it has been estimated that the disease is multifocal in over 10% of cases (Östör *et al.*, 2000; Zaino, 2002).

2.3.1.3 HPV genotype distribution in CIN and cancer

HPV genotype distribution differs in different CIN grades. The proportion of HPV16/18-related CIN increases with increasing severity of findings. HPV16 was found in approximately 28% of CIN1, 40% of CIN2, and 58% of CIN3 in a meta-analysis of studies that included approximately 20 000 cases of CIN worldwide (Guan *et al.*, 2012). HPV18, in contrast, was found in approximately 10% of CIN irrespective of grade, with a steeper increase to 16% in invasive cancers. The proportion of HPV16 in cervical cancer in this study was estimated to be 63%. In studies on genotype distribution in cervical cancer, approximately 70% are associated with HPV16/18

(Wheeler *et al.*, 2009; de Sanjose *et al.*, 2010). A recent large population-based study from Sweden found 19% of invasive cervical cancers to be HPV negative, while only 3% of CIN3 were negative using genotyping for detection (Hortlund *et al.*, 2016; Lei *et al.*, 2018). Most likely most of the cancer cases were originally hrHPV positive, but with HPV negativity associated with advanced stage disease and older age at diagnosis, HPV might have become undetectable when the carcinogenic process proceeds. More rare HPV-negative cervical cancers also exist, primarily adenocarcinomas (McCluggage, 2016).

Other genotypes dominating in higher grade disease and cancer are HPV45, 52, 31, 33, and 58: approximately 4-11% of cases are attributed to these individual genotypes (Guan *et al.*, 2012). HPV16-related high-grade cervical changes have been found to be more common at a younger age in some studies (Porras *et al.*, 2009; Wheeler *et al.*, 2009; Castle *et al.*, 2010; Castle, Shaber, *et al.*, 2011). Also, up to 25% of histological LSIL is attributed to HPV16; conversely, some studies report 24-45% of LSIL to be associated with other than hrHPV genotypes (Cavalcanti *et al.*, 2000; Silveira *et al.*, 2015). In a Finnish study on cytological LSIL, over two-thirds were positive for hrHPV, and CIN2 or worse (CIN2+) was found in nearly 15% (Veijalainen *et al.*, 2015). HPV18 and 45 have been found to be more common in glandular disease in comparison to squamous disease (Clifford and Franceschi, 2008; Wheeler *et al.*, 2009; Castle, Shaber, *et al.*, 2011). Some HPV16 sublineages have been shown to be overrepresented in glandular disease, especially (Mirabello *et al.*, 2016).

2.3.1.4 CIN diagnostics

HPV infection and CIN are mostly asymptomatic; thus, the primary diagnostic approach for decades has been microscopy of exfoliated cervical and vaginal cells (cytology). Cervical cytology testing was first described by Georgios Papanicolau in the 1920s (Papanicolaou, 1928; Papanicolaou and Traut, 1941). The traditional method, in which three individually scraped samples are taken from the vaginal fornices, ectocervix, and endocervix and placed on a glass slide, is still called a

Pap(anicolau) smear. Liquid-based cytology, where exfoliated cells are collected with a brush or spatula and rinsed into a preservative solution, was developed in the 1990s in an attempt to reduce the false negative rate of conventional cytological samples (Lee *et al.*, 1997).

Cervical cancer is globally the fourth most common cancer in women, with an annual incidence of over half a million cases and over a quarter million annual deaths (Ferlay *et al.*, 2018). Cervical cancer is also an exceptional cancer, because precancerous lesions can be detected and treated with great cost effectiveness. These features make mass screening for cervical cancer justified according to WHO criteria (Wilson and Jungner, 1968). Organised cervical cancer screening with cytology has dramatically decreased cervical cancer incidence and mortality in many countries (Arbyn *et al.*, 2009). The nationwide organised screening program started in Finland in the 1960s has led to an 80% reduction in cervical cancer incidence and mortality (Laara, Day and Hakama, 1987; Nieminen, Kallio and Hakama, 1995; Hristova and Hakama, 1997; Anttila *et al.*, 1999). Hence, the majority of cervical cancer burden today remains in countries with low resources for screening and treatment. Finnish municipalities are obligated by legislation to organise cervical cancer mass screening for women between the ages of 30 and 60 at 5-year intervals.

Classification of cervical cytological findings has been revised throughout time, and the current recommendation is the Bethesda system (TBS) updated in 2001 (Table 5), which emphasises also the adequacy assessment of the sample (Solomon *et al.*, 2002). Cytological findings are considered an insufficient basis for diagnosing cervical disease; the preferred method is histology obtained by colposcopically directed biopsies. Cytology is used to screen for cervical abnormalities and, depending on local guidelines, different cut points are used for referral to further examinations. Possible symptoms of HPV infection and CIN include irregular or postcoital bleeding (Gulumser *et al.*, 2015). Women presenting with these symptoms should have cytological testing and be referred to colposcopy if a reason for abnormal bleeding

cannot be otherwise identified (Abu, Davies and Ireland, 2006; *Cytological abnormalities: Finnish Current Care Guidelines (online).*, 2019).

Table 5. *Overview of cervical cytology categories according to the Bethesda system (TBS 2001)*

<table>
<tr><th colspan="4">Normal findings</th></tr>
<tr><td colspan="2">NILM</td><td colspan="2">negative for intraepithelial lesion or malignancy</td></tr>
<tr><th colspan="4">Abnormal findings</th></tr>
<tr><td colspan="2">Squamous</td><td colspan="2">Glandular</td></tr>
<tr><td>ASC-US</td><td>atypical squamous cells of undetermined significance</td><td rowspan="2">AGC-NOS</td><td rowspan="2">atypical glandular cells not otherwise specified</td></tr>
<tr><td>LSIL</td><td>low grade squamous intraepithelial lesion</td></tr>
<tr><td>ASC-H</td><td>atypical squamous cells, cannot rule out HSIL</td><td rowspan="2">AGC-FN</td><td rowspan="2">atypical glandular cells favour neoplasia</td></tr>
<tr><td>HSIL</td><td>high grade squamous intraepithelial lesion</td></tr>
<tr><td>Squamous cell carcinoma</td><td>squamous cell carcinoma</td><td>Adenocarcinoma</td><td>adenocarcinoma</td></tr>
</table>

The accuracy of cytology, especially sensitivity, in finding high-grade cervical disease is highly varying. One meta-analysis found the sensitivity to be 30-87% with specificity of 86-100% (Nanda *et al.*, 2000). A study of over 60 000 women in Europe and North America found the sensitivity of cytology to be 53% and specificity 96%

(Cuzick *et al.*, 2006). Variability of estimated sensitivity can be attributed to the quality of the health care system, including sampling and interpretation of samples. In Finland sensitivity of conventional cytology in detecting CIN2+ has been reported to be up to 83% with a specificity of 94% when the cut point for cytology was set at LSIL or worse, and also the false-negative rate has been found to be low (Nieminen *et al.*, 2004; Lonnberg *et al.*, 2010). Sensitivity of cytology is especially poor in glandular abnormalities, and AIS has been found to have false-negative cytology in up to 50% (Nieminen, Kallio and Hakama, 1995; Ruba *et al.*, 2004; Sasieni, Castanon and Cuzick, 2009).

High-grade cervical disease is associated with persistent hrHPV infection; thus, hrHPV testing has been introduced for primary screening. Many large, randomised studies from different countries have shown hrHPV testing to have higher sensitivity, albeit less specificity, when compared to cytology in detecting high-grade cervical disease (Naucler *et al.*, 2007, 2009; Kitchener *et al.*, 2009; Ronco *et al.*, 2010; Castle, Stoler, *et al.*, 2011; Leinonen *et al.*, 2012; Rijkaart *et al.*, 2012). A recent Cochrane review of over 140 000 women in 40 studies reached the same conclusion (Koliopoulos *et al.*, 2017). A negative hrHPV test has a longer disease-free period even for cervical cancer when compared to negative cytology, making longer screening intervals possible and sensible, because transient infections would most likely subside in between screening rounds (Ronco *et al.*, 2014). The lower specificity of hrHPV testing, however, can lead to more referrals to colposcopy, because not all women harbouring an hrHPV infection present with any histological abnormalities or, moreover, abnormalities requiring treatment. It has been estimated that only approximately a third of women with a detectable single hrHPV infection present with cytological or histopathological abnormalities (Kovacic *et al.*, 2006). Most municipalities in Finland currently offer cytology-based screening, but there is an ongoing shift towards hrHPV screening with cytology triage (Veijalainen *et al.*, 2016, 2019).

Colposcopy is performed when cervical disease is suspected, usually based on cytological abnormalities. Urgency of colposcopy depends on clinical, cytological, and/or hrHPV test findings. Table 6 shows the timing of colposcopy according to cytology in the Finnish Current Care Guidelines (*Cytological abnormalities: Finnish Current Care Guidelines (online)*, 2019). A colposcope is a binocular microscope allowing magnification up to 40-fold (Anderson *et al.*, 1996). Topically applied acetic acid (3-5%) is used, causing coagulation of superficial intracellular proteins that results in whitening of the epithelium (acetowhitening) (Anderson *et al.*, 1996). Iodine can be used as an adjunct to acetic acid. This can be especially helpful for detection of vaginal lesions (Sopracordevole *et al.*, 2018).

Table 6. *Overview of recommendations for colposcopy timing by cytological finding according to Finnish Current Care Guidelines*

Reason for Colposcopy	Timing
Carcinoma	Immediately (within 1-7 days)
HSIL, ASC-H, AGC-FN	Within 1 month
LSIL	According to cytopathologist's recommendation[1]
ASC-US (repeated 2-3 times within 12-24 months or in over 30-year-olds concomitant hrHPV positivity)	Within 6 months
AGC-NOS	Within 2 months or according to cytopathologist's recommendation[2]
Abnormal endometrial cells	Within 1 month
[1] ≥30-year-olds within 6 months; <30-year-olds within 6 months if cytopathologist recommends colposcopy OR if a repeated smear within 6-12 months is abnormal after which colposcopy within 6 months (if repeat cytology is also ≤LSIL)	
[2] within 2 months if cytopathologist recommends colposcopy OR if a repeated smear within 4-6 months is abnormal	

Detected acetowhite or iodine-negative areas are further magnified and examined to see if changes suggestive of CIN are seen. In CIN the angioarchitecture subepithelially changes, resulting in mosaic-like surface structure, punctuation, and frank abnormal vessels. Acetowhitening, however, occurs also in metaplastic or regenerative epithelium, which makes colposcopic diagnosis challenging. Special attention should be paid to the most vulnerable area in the cervix, the TZ and SCJ. The location of the SCJ may vary and is not always visible, so the accuracy of colposcopy suffers. Scoring systems such as the Reid colposcopic index (RCI) and the Swede score have been developed to improve accuracy of colposcopic examination (Reid and Scalzi, 1985; Strander *et al.*, 2005).

The gold standard for diagnosis of CIN that should guide treatment decisions is colposcopically directed punch biopsies taken from the most abnormal areas seen in colposcopy (American College of Obstetricians and Gynecologists, 2008). Taking multiple biopsies has been shown to increase diagnostic accuracy, because colposcopy alone may lack in sensitivity (Massad and Collins, 2003; Gage *et al.*, 2006; Jeronimo and Schiffman, 2006). A meta-analysis of the accuracy of punch biopsies in diagnosing high grade cervical disease found the sensitivity in detecting CIN2 or worse (CIN2+) to be over 90% and the specificity to be approximately 25% (Underwood *et al.*, 2012). The authors point out, however, that the analysis included only women with positive punch biopsies, which may have resulted in bias, increasing sensitivity and lowering specificity.

2.3.1.5 CIN treatment

CIN is treated with local excisional and ablative surgical techniques. Nonsurgical methods have also been studied but are not currently used in clinical practice (de Vet *et al.*, 1991; Alvarez *et al.*, 2003; Grimm *et al.*, 2012; Rahangdale *et al.*, 2014).

Of the excisional techniques, cold knife conisation in an operating room under general anaesthesia was traditionally performed. Since the 1990s this has been replaced to a great extent with large loop excision of the transformation zone

(LLETZ), also known as loop electrosurgical excision procedure (LEEP) in an outpatient setting with local anaesthesia, but practices vary greatly by country (Prendiville, Cullimore and Norman, 1989; Petry *et al.*, 2008). Laser excision with carbon dioxide laser or needle excision may also be used. All of these excisional procedures aim at complete excision of the TZ, including the lesion. Excisional techniques provide a cone-shaped histological specimen that can be used to affirm the initial histological diagnosis and complete removal of the lesion.

Ablative techniques include laser vaporisation, cryotherapy, and radical diathermy that aim to destroy the lesion with margins. Ablative treatment should be restricted to patients with fully satisfactory colposcopy (fully visible transformation zone), no suspicion of invasion or glandular disease, and no discrepancy in cytological and histological diagnosis (Jordan *et al.*, 2009; Martin-Hirsch *et al.*, 2013). No local treatment method has been found to be superior with regard to treatment failure or associated morbidity (Martin-Hirsch *et al.*, 2013). All local treatments should be performed under colposcopic control (*Cytological abnormalities: Finnish Current Care Guidelines (online).*, 2019). Hysterectomy can be considered in cases of (repeated) local treatment failure (*Cytological abnormalities: Finnish Current Care Guidelines (online).*, 2019).

Finnish Current Care Guidelines discourage routine direct local treatment based on cytology and colposcopic appearance alone (see-and-treat). Exceptions to this are AGC-FN cytology, since the endocervical canal cannot be fully visualised; HSIL cytology when the colposcopic appearance is also that of high-grade disease; and HSIL and ASC-H cytology if the SCJ is not visible in colposcopy (select-and-treat) (*Cytological abnormalities: Finnish Current Care Guidelines (online).*, 2019).

The threshold to treat CIN has varied throughout time. Most treatment guidelines now suggest active surveillance for LSIL for up to two years, since it is considered a sign of a productive HPV infection with frequent spontaneous regression and low risk of progression (TOMBOLA Group, 2009; Massad *et al.*, 2013; *Cytological*

abnormalities: Finnish Current Care Guidelines (online)., 2019). A finding of CIN2+ is mostly considered the cut-off to proceed to treatment. Currently some treatment guidelines suggest active surveillance also for CIN2 in young women, because spontaneous regression rates are recognised to be higher (Massad *et al.*, 2013; *Cytological abnormalities: Finnish Current Care Guidelines (online).*, 2019). AIS should always be treated, and hysterectomy is recommended, but local excisional treatment can be considered in women who have not completed child bearing (*Cytological abnormalities: Finnish Current Care Guidelines (online).*, 2019).

Complications of local treatment include short-term and long-term ones. Short-term complications include pain, haemorrhage, discharge, and infection. Haemorrhage can occur during treatment or, secondarily, afterwards. Two studies have reported delayed bleeding after outpatient LLETZ in approximately 1-5% of patients, of whom over a fourth to a half required additional haemostatic procedures or hospital admission (Dunn, Killoran and Wolf, 2004; Mossa *et al.*, 2005). Self-reported moderate-to-severe bleeding or discharge occurs, however, in approximately 50% of women after LLETZ (Sharp *et al.*, 2009). Infection after LLETZ has been reported in 1-14% of cases, while it can be up to over a third after cold knife conisation (Kietpeerakool *et al.*, 2017).

Long-term complications include stenosis of the cervical canal and preterm birth or midtrimester miscarriage. Cervical stenosis prevents menstrual blood from exiting the uterus and can be thought to affect fertility by preventing sperm from entering the uterine cavity (Baldauf *et al.*, 1996). It also disturbs future cervical diagnostic procedures. Risk of cervical stenosis has been associated with increasing cone depth, but the risk is still quite low in general (Baldauf *et al.*, 1996; Mossa *et al.*, 2005). Local treatment of CIN increases the overall risk of preterm birth (<37 weeks of gestation) from 5.4% to 10.7% (relative risk (RR) 1.78, 95% CI 1.60-1.98) and the risk of extremely premature birth (<28-30 weeks of gestation) from 0.3% to 1.0% (RR 2.54, 95% CI 1.77-3.63) in a recent meta-analysis (Kyrgiou *et al.*, 2016). This risk has been found to be associated with treatment method, cone depth, and number of

treatments with cold knife conisation and repeated treatments causing the most risk and ablative techniques the least risk (Kyrgiou *et al.*, 2017). Women with previous CIN even without treatment were also found to have a slightly increased risk of premature birth when compared to women without a history of CIN (Kyrgiou *et al.*, 2016). Treatment of CIN has not been associated with reduced fertility (Kyrgiou *et al.*, 2015).

Women treated for CIN are known to have increased risk for CIN or cervical or vaginal cancer for up to or over twenty years after treatment (Soutter *et al.*, 1997; Kalliala *et al.*, 2005; Strander *et al.*, 2007; Rebolj *et al.*, 2012; Strander, Hällgren and Sparén, 2014). Local treatments, however, are highly effective with an initial success rate of over 90% (Martin-Hirsch *et al.*, 2013). Most recurrent cases occur within two years of treatment and a recurrence rate of 4-18% has been reported (Arbyn, Ronco, *et al.*, 2012). Risk factors for recurrent disease are positive excision margins (more so if the endocervical margin is positive versus the ectocervical), persistence of hrHPV, and older age at treatment (Flannelly *et al.*, 2001; Verguts *et al.*, 2006; Ghaem-Maghami *et al.*, 2007; Serati *et al.*, 2012; Strander, Hällgren and Sparén, 2014; Arbyn *et al.*, 2017). hrHPV positivity after treatment was found to be a more accurate predictor of recurrence risk than margin status in a recent meta-analysis (Arbyn *et al.*, 2017). The mechanism underlying late recurrence is not well understood, but disease or hrHPV hidden in endocervical crypts has been suggested, and reactivated latent infections seem a plausible explanation (Reich and Regauer, 2015).

Because of the residual or recurrent disease risk, guidelines recommend follow-up after treatment for CIN before returning women to routine screening. Finnish Current Care Guidelines advocate cytology and hrHPV testing six months after treatment. The guidelines recommend cytology and hrHPV testing two years after treatment, even in cases where both results were negative at six months (*Cytological abnormalities: Finnish Current Care Guidelines (online).*, 2019). A British study, however, concluded that return to routine screening was safe after treatment if

cytology was normal and the hrHPV test negative at six months (Kitchener *et al.*, 2008).

Long-term risk of recurrent disease after active surveillance of regressed low-grade abnormalities is not yet well established. An Australian retrospective study comprising a median of four years of follow-up found the recurrence risk of untreated, actively surveilled CIN1 or 2 in young women to be 12% and 17%, respectively (Wilkinson *et al.*, 2015).

2.3.2 VAGINAL INTRAEPITHELIAL NEOPLASIA

2.3.2.1 Classification

The vagina is covered with stratified squamous epithelium that can also be infected by HPV (Vuyst *et al.*, 2009). Vaginal intraepithelial neoplasia (VAIN) is classified in a similar fashion as CIN based on the thickness of abnormal cells in the epithelium. The previous three-tier classification (VAIN1, 2, 3) was replaced in 2014 with vaginal HSIL, including VAIN2 and 3, and vaginal LSIL, including VAIN1 and HPV atypia/atypia condylomatosa of the vaginal epithelium (WHO, 2003, 2014). HPV-related cancer of the vagina is mostly squamous cell carcinoma.

2.3.2.2 VAIN epidemiology and natural history

VAIN is, overall, much less common than CIN with an annual incidence rate of 0.2-0.3 per 100 000 women (Henson and Tarone, 1977; Sillman *et al.*, 1997). The low incidence of VAIN makes comprehensive research difficult. Predisposing factors to VAIN are immunosuppression and a history of HPV-related genital disease, with the possibility of concomitant disease (Sillman *et al.*, 1997; Gunderson *et al.*, 2013; Jentschke *et al.*, 2016). VAIN is more common in peri- or postmenopausal women in comparison to CIN (Gunderson *et al.*, 2013; Cong *et al.*, 2018).

VAIN is slow to progress, but high-grade VAIN has been estimated to have approximately a 10% risk of progressing to invasive vaginal cancer (Sillman *et al.*, 1997; Rome and England, 2000). Approximately 70% of vaginal carcinomas are HPV positive (Arbyn, de Sanjosé, *et al.*, 2012). Incidence of vaginal carcinoma has been increasing in Finland and other countries also for an unknown reason (Finnish Cancer Registry, no date). Spontaneous regression of VAIN also occurs, and low-grade VAIN can also be considered a sign of productive HPV infection that can be managed with active surveillance (Aho *et al.*, 1991; Massad, 2008; *Cytological abnormalities: Finnish Current Care Guidelines (online).*, 2019).

Genotype-specific data on VAIN are scarce, but HPV16 seems to predominate (Lamos *et al.*, 2016). An Italian study found the most common genotypes in high-grade VAIN to be HPV16 (23.3%), HPV18 (20.7%), and HPV31 (14.2%), reflecting also the genotype-distribution of high-grade CIN (Bogani *et al.*, 2017). A study in young women in the placebo arm of a vaccine trial found that over 50% of vaginal HSIL was associated with HPV16/18 and also nearly 20% of vaginal LSIL (Garland *et al.*, 2018). Approximately 50% of vaginal LSIL was associated with non-hrHPV genotypes.

2.3.2.3 VAIN diagnostics and treatment

VAIN is mostly asymptomatic. VAIN results often in abnormal cytology, and lesions can be visualised with colposcopy, although this is more challenging than in the cervix (Boonlikit and Noinual, 2010; Sopracordevole *et al.*, 2018). The vagina has a much larger surface area and has to be stretched in multiple directions for complete visualisation. Use of iodine can aid identification of abnormal areas. VAIN presents in a substantial proportion of cases after hysterectomy, especially if it has been performed for CIN or cervical cancer (Rome and England, 2000). Most VAIN occur in the upper third of the vagina, and multifocal lesions are common (Aho *et al.*, 1991; Boonlikit and Noinual, 2010). Diagnosis of VAIN should be based on colposcopically guided biopsies. Small studies have reported occult vaginal cancer associated with

high-grade VAIN in 12-28%, emphasising the need for adequate and even multiple biopsies of suspicious areas (Hoffman *et al.*, 1992; Indermaur *et al.*, 2005).

VAIN treatment is also challenging because of the anatomy and the possibility of multifocal disease (Gurumurthy and Cruickshank, 2012). Many different approaches in treatment have been used. Historically, surgery (partial or total vaginectomy) aiming at removal of the vaginal mucosa has been performed. Total vaginectomy causes significant morbidity and should be reserved for special circumstances (Gurumurthy and Cruickshank, 2012). Studies have concluded that upper vaginectomy has a position as treatment in unifocal disease of the vaginal vault, at least in patients in whom shortening of the vagina might not cause an issue and where invasion cannot be ruled out (Diakomanolis *et al.*, 2002; Indermaur *et al.*, 2005; Gurumurthy and Cruickshank, 2012). Upper vaginectomy can also be performed with loop excision (Fanning, Manahan and McLean, 1999). Smaller excisional procedures or laser excision can also be carried out (Julian, O'Connell and Gosewehr, 1992; Cheng *et al.*, 1999; Rome and England, 2000; Sopracordevole *et al.*, 2017; Bogani *et al.*, 2018).

Laser vaporisation or ablation is used often as first line of treatment, because it is more useful in multifocal disease and can be performed in an outpatient setting (Kim *et al.*, 2009; Gunderson *et al.*, 2013; Perrotta *et al.*, 2013; Wang *et al.*, 2014; Jentschke *et al.*, 2016). This, however, does not allow for additional histological diagnosis in contrast to excisional procedures and should be limited to cases where the lesion can be fully visualised. Internal radiation therapy (brachytherapy) has also been used. Small studies with different protocols have reported good success rates (Graham *et al.*, 2007; Blanchard *et al.*, 2011). It should not be considered as a first line treatment due to the adverse effects of radiation (Gurumurthy and Cruickshank, 2012).

A topically applied chemotherapeutic antimetabolite 5-fluorouracil (5-FU) has also been used. A recent meta-analysis of 14 moderate quality studies of 358 women

found a good success rate with some adverse effects (Tranoulis *et al.*, 2018). An earlier study, however, has reported chronic vaginal ulcerations requiring surgical treatment (Krebs and Helmkamp, 1991). Topically applied immunomodulator imiquimod has been used in small, nonrandomised studies with equal success rates to other therapeutic options (Buck and Guth, 2003; Haidopoulos *et al.*, 2005; Lin *et al.*, 2012; de Witte *et al.*, 2015). Both of these medical treatments are used in VAIN off label (Gurumurthy and Cruickshank, 2012).

Conclusive overall outcomes of different treatment methods are difficult to provide due to the small number of patients and varying follow-up in individual studies or case series. Success rates generally vary between 60-100% for every treatment method in one treatment round (Gurumurthy and Cruickshank, 2012). Equally, a major issue in VAIN treatment is the high residual disease and recurrence rate, leading to repeated treatments. Risk factors for recurrence are hrHPV persistence and multifocal disease, and it appears that younger patients with disease involving the vaginal vault have a higher recurrence risk (Dodge *et al.*, 2001; Frega *et al.*, 2007; Kim *et al.*, 2009). Additionally, all current treatment methods can have significant adverse effects, some of which are long term (especially vaginectomy, radiation therapy, 5-FU). Finnish Current Care Guidelines suggest follow-up with colposcopy six months after treatment (mostly laser vaporisation) with frequency of colposcopies determined by post-treatment hrHPV status and follow-up lasting at least three years in all circumstances before return to routine screening (*Cytological abnormalities: Finnish Current Care Guidelines (online).*, 2019).

2.3.3 VULVAR INTRAEPITHELIAL NEOPLASIA

The vulva has two main types of intraepithelial neoplasia: one type is related to HPV (usual vulvar intraepithelial neoplasia, uVIN) and the other (differentiated VIN, dVIN) to chronic dermatoses, including lichen sclerosus (del Pino, Rodriguez-Carunchio and Ordi, 2013; Halonen *et al.*, 2017). uVIN and associated squamous cell carcinoma occur at a younger age than dVIN and associated squamous cell carcinoma (de

Sanjosé *et al.*, 2013). Overall, approximately 90% of VIN is associated with HPV (especially HPV16, 31, and 18), but only 30% of vulvar squamous cell carcinoma is HPV positive (Arbyn, de Sanjosé, *et al.*, 2012; de Sanjosé *et al.*, 2013). The incidence of VIN has been reported to be increasing, although VIN is not as common as CIN (Judson *et al.*, 2006).

2.4 HPV-RELATED DISEASE OF OTHER ANATOMIC SITES

HPV-related disease is not restricted to the female genital tract, since hrHPVs can infect any mucosal epithelium. Overall, 600 000 of annual incident cancer cases (4.8% of global cancers) can be currently attributed to HPV (Arbyn, de Sanjosé, *et al.*, 2012; Forman *et al.*, 2012). HPV is recognised as a cause in approximately 70% of vaginal and anal cancers and in 40% of vulvar and penile cancers (Arbyn, de Sanjosé, *et al.*, 2012). Increasing numbers of cancers are recognised to have an association with HPV infection, such as oropharyngeal cancer, of which approximately 20-55% are attributed to HPV, depending on the exact anatomic site (Ndiaye *et al.*, 2014). Other cancers, such as colorectal, lung, urinary bladder, and prostate cancer, have been implicated to have an association with HPV, but evidence is still accumulating (IARC, 2012).

Cervical cancer and precursors, however, currently remain the disease with the strongest correlation to HPV infection and the possibility of secondary prevention by screening and treating premalignant disease. For the time being, other HPV-related disease control will be more dependent on primary prevention with vaccine immunity (Chaturvedi, 2010).

2.5 EPIGENETICS

Epigenetics means regulatory mechanisms of gene expression beyond the genetic sequence encoded in DNA. Epigenetic mechanisms can respond to external

environmental stimuli, leading to dynamic gene expression patterns (Lorincz, 2016). This kind of regulation of gene expression has been found to be important in foetal development, ageing, and death (Issa, 2000; Robertson, 2005). Epigenetic regulation can change in the course of a lifetime, while the DNA sequence remains generally the same, although sporadic mutations do occur. Epigenetic marks, however, can also be heritable and lead to genomic imprinting, i.e., epigenetically programmed gene expression patterns in offspring (Reik and Walter, 2001).

2.5.1 DNA METHYLATION

Methylation of DNA is the main mechanism through which gene expression is regulated epigenetically in mammals (Bird, 2002). Other more complex mechanisms involve intracellular protein complexes and post-translational methylation of histone proteins (Bird, 2002). DNA is most commonly methylated by the addition or removal of a methyl group (CH_3) to or from an aromatic ring of the nucleotide cytosine (C), followed directly by guanine (G) at so-called CpG sites (Bird, 2002). CpG islands comprising many CpG sites are mostly found close to promoter DNA regions responsible for gene transcription (Bird, 1986; Illingworth and Bird, 2009). Hypermethylation can lead to condensation of stretches of DNA, preventing its transcription, and hypomethylation can lead to increased transcription (Lorincz, 2016).

2.5.2 DNA METHYLATION, CARCINOGENESIS, AND CANCER

The role of methylation in carcinogenesis has been, and continues to be, extensively studied. In the past carcinogenesis was primarily seen as the clustering of unfortunate sporadic mutations in DNA, leading to the activation of oncogenes and deactivation of tumour suppressor genes, which could be further enhanced by an individual's genetic predisposing factors (Knudson, 1971). It is recognised now that, in fact, epigenetic mechanisms appear to be equally important in malignant

transformation and can lead to the same change in gene expression as actual mutations (Shen and Laird, 2013; Witte, Plass and Gerhauser, 2014).

The first findings in the 1980s were of mass hypomethylation of many CpG sites in malignant colorectal tumours in comparison to normal tissue (Feinberg and Tycko, 2004). A similar difference in methylation was seen between benign and premalignant colorectal tumours (Goelz *et al.*, 1985). This hypomethylation results in the activation of oncogenes and causes overall genomic instability (Lorincz, 2014). Hypermethylation of specific CpG islands, however, causes silencing of tumour suppressor genes and contributes to neoplastic transformation (Feinberg and Tycko, 2004). This kind of epigenetic change could be reversible, whereas actual silencing mutations in the genetic code currently are not (Lorincz, 2016).

Studies of methylation patterns, methylomics, are currently widely incorporated into the study of cancer genomics in a variety of malignant diseases (Witte, Plass and Gerhauser, 2014). Currently, methylation can be seen to show promise in cancer risk evaluation, early detection, prognosis, and therapeutic response. For example, aberrant methylation patterns in non-small cell lung cancer patients could be identified in sputum three years prior to clinical diagnosis (Palmisano *et al.*, 2000). Another study of several types of solid malignant tumours and haematopoetic malignancies has shown hypermethylation of promoter region DNA to be present already in precancerous or normal tissue (Sproul *et al.*, 2012). Further research is still ongoing and needed before clinical use for the majority of possible methylation applications.

2.5.3 DNA METHYLATION IN CIN AND CERVICAL CANCER

DNA methylation has also been a focus of research interest in cervical neoplasias, because none of the current diagnostic methods or known risk factors can explain why neoplastic transformation happens in some HPV infected individuals and not in others. Both viral and host genome methylation have been investigated.

Widespread hypomethylation was seen initially in the HPV16 genome and overall with correlation to neoplasia severity (Badal *et al.*, 2003; de Capoa *et al.*, 2003). Specific HPV16 CpG sites of interest were identified later on, and hypermethylation was seen with an increasing severity of lesions (Mirabello *et al.*, 2012, 2013; Lorincz *et al.*, 2013). Furthermore, CpG targets in other hrHPV genotypes and also a plethora of host genes able to differentiate between CIN grades and cancer have been identified (Wentzensen *et al.*, 2012; Kalantari *et al.*, 2014; Louvanto *et al.*, 2015; Clarke *et al.*, 2017). FAM19A4, a host gene, has also been been shown to be more often positive in methylation testing in high-grade cervical disease when the hrHPV infection has persisted longer (De Strooper *et al.*, 2014). In a British nested case control study within a screening trial, DNA methylation in women without cytological abnormalities showed aberrant patterns in those who were subsequently diagnosed with CIN2+ (Teschendorff *et al.*, 2012).

Based on these results, viral and/or host DNA methylation have been seen to show promise in development of a biomarker test for the accurate detection and prediction of high-grade cervical disease (Cuschieri *et al.*, 2018). A triage test for hrHPV screening-positive women is also called for because of the low specificity of hrHPV testing (Cuzick *et al.*, 2012). Currently, cytology is commonly used as a triage test, but this suffers from subjectivity of interpretation and modest sensitivity even if combined with immunostaining (p16, Ki67) (Cuzick *et al.*, 2012; Cuschieri *et al.*, 2018). HPV genotyping (HPV16/18) has also been proposed to solve this issue, but it appears to perform similarly to cytology (Castle, Stoler, *et al.*, 2011). A recent study from the United States, however, shows dual staining (DS) with p16 and Ki67 to outperform cytology in triage of hrHPV-positive women and extended follow-up without colposcopy to be safe in DS and HPV16/18 genotyping-negative women (Wentzensen *et al.*, 2019). A major advantage of a methylation-based test would be the objectivity of interpretation in contrast to methods relying on microscopy of cytological specimens (Cuzick *et al.*, 2012; Cuschieri *et al.*, 2018). Viral and host CpG-site methylation have been studied in various combinations and generally have been

found to have a sensitivity of a little under 90% and specificity of 50-70% in detecting CIN2/3+, which to date is not performing better than its proposed counterparts (Lorincz, 2016).

A DNA methylation test, QIAsure Methylation Test (Qiagen, Venlo, Netherlands), is commercially available for triage of hrHPV-positive women. It tests for hypermethylation of the host genes FAM19A4 and hsa-mir124-2 (De Strooper *et al.*, 2016) and has been shown to have a sensitivity of 67% in detecting CIN3 and 100% of cervical cancer also in self-sampling (De Strooper *et al.*, 2016; Luttmer *et al.*, 2016). The combination of HPV16/18 genotyping results was shown to increase sensitivity further, albeit with a commensurate loss of specificity.

A DNA methylation classifier (S5) has been developed comprising the late regions of HPV16, 18, 31, 33, and the promoter region of a human tumour suppressor gene EPB41L3 (Brentnall *et al.*, 2014). It has been shown to perform well as a triage test for hrHPV screening-positive women and outperformed HPV16/18 genotyping in detection of CIN3+ with sensitivities of 0.84 (95% CI 0.62-0.94) and 0.58 (95% CI 0.36-0.77), respectively (p=0.04) (Lorincz *et al.*, 2016). The corresponding specificities were 0.63 (95% CI 0.58-0.68) and 0.69 (95 % CI 0.64-0.74), respectively (p=0.07). In another study it also outperformed abnormal cytology in combination with HPV16/18 genotyping in detecting prevalent CIN2/3 and did not fail to detect any of the prevalent or incident cancer cases within the follow-up period (Cook *et al.*, 2018).

3 AIMS OF THE STUDY

The goal of this thesis was to characterise the HPV genotypes causing gynaecological morbidity in Finnish women prior to prophylactic vaccinations, and to assess novel approaches in evaluation and treatment of cervical and vaginal intraepithelial neoplasia.

The aims of the individual studies were:

1. To assess the age-specific HPV genotype distribution in Finnish women with cytological abnormalities that cause clinical morbidity

2. To evaluate the clinical course of untreated CIN2 and adherence to active surveillance protocols

3. To assess the performance of a DNA methylation classifier in predicting clinical outcomes of untreated CIN2

4. To evaluate the efficacy of self-administered vaginal imiquimod in treatment of VAIN

4 SUBJECTS AND METHODS

4.1 SUBJECTS (STUDY I, III, IV)

Three studies (I, III, IV) were conducted in separate patient cohorts recruited at the Colposcopy Centre of the Department of Obstetrics and Gynecology in Helsinki University Hospital. Table 7 describes the characteristics of the prospective studies (I, III, IV). The studies have been granted approval to be carried out by the Hospital District of Helsinki and Uusimaa and the recruited women gave written informed consent in accordance with the Declaration of Helsinki. All studies were registered in the International Standard Randomised Controlled Trial Number (ISRCTN) registry.

The first cohort (study I) recruited a total of 1302 patients 18 years of age or older who had been referred to colposcopy for abnormal cytology (Table 8). Recruitment started in January 2014 and ended in May 2016. The study was carried out in collaboration with Karolinska Institute, Stockholm, Sweden. The study protocol was approved by Helsinki University Hospital's Ethical Committee (130/13/03/03/2013).

The second prospective study (study III) was performed in a cohort study that recruited women 18 to 30 years of age with histologically confirmed CIN2. Recruitment started in September 2013 and finished in December 2018. The first 149 patients with at least two 6-monthly follow-up visits completed were included in the current study, and the study was carried out in collaboration with the Center for Cancer Prevention, Wolfson Institute of Preventive Medicine, Queen Mary University of London, UK, and the Karolinska Instute, Stockholm, Sweden. The study protocol was approved by Helsinki University Hospital's Ethical Committee (131/13/03/03/2013).

The third prospective study (study IV) was a randomised, controlled trial (RCT) that recruited patients 18 years of age or older with histologically confirmed VAIN2-3 or

VAIN1 that had persisted for two years. The study recruited 30 patients between December 2012 and May 2015. The study protocol was approved by Helsinki University Hospital's Ethical Committee (385/13/03/03/2012) and the Finnish Medicines Agency Fimea (EudraCT 2012-005377-31).

Table 7. *Characteristics of the prospective studies (I, III, IV)*

	Study I	Study III	Study IV
Design	Prospective cohort	Prospective cohort	Randomised controlled trial (RCT)
N of participants	1279	149	30
Inclusion criteria	18 years of age or above Referred to colposcopy for abnormal cytology	18-30 years of age Histological CIN2 TZ type 1-2 Lesion size ≤3/4 of the TZ ≥2 follow-up visits	18 years of age or above Histological VAIN2-3 or VAIN1 persisting ≥2 years[1]
Exclusion criteria	No baseline HPV genotyping result	Previous treatment for CIN/VAIN/VIN or corresponding cancers Pregnancy or lactation Known HIV positivity Immunosuppressive medication No common language	Concomitant diagnosis of CIN2+ Lack of reliable contraception premenopausally Pregnancy or lactation Known HIV positivity Vaginal cancer No common language
Protocol[2]	Colposcopy (examination, treatment, and follow-up) according to Finnish current care guidelines Endocervical brush sample for HPV genotyping at recruitment	6-monthly colposcopy up to 24 months Endocervical brush sample for HPV genotyping and methylation analyses at recruitment LLETZ on progression (CIN3+), persistence of CIN1-2 at end of study, on patient request or moving out of the region	Randomisation (imiquimod, laser, active surveillance) Colposcopy at 4, 8, and 16 weeks hrHPV testing at recruitment and 16 weeks Laser vaporisation or surgery at end of study for persistence or progression (VAIN2-3+)
Main outcome measure	Age-specific HPV genotype distribution by cervical histology	Accordance of S5 DNA methylation to progression to CIN3+	Rate of histological regression to ≤VAIN1
Study registration	ISRCTN10933736	ISRCTN91953024	ISRCTN45751386

[1] No patients with persistent VAIN1 were eventually recruited; [2] Colposcopy in all studies included punch biopsies (or LLETZ in study I) and/or conventional cytology by discretion of the colposcopist, and routine biopsies at the end of the study in studies III and IV

Table 8. *Characteristics of 1279 women referred to colposcopy for abnormal cytology (study I).*

	All N=1279		<30 N=339		30-44.9 N=614		≥45 N=326	
Age median (range)	35.1 (19.2-83.7)		26.1 (19.2-29.9)		35.2 (30.0-44.9)		51.4 (45.0-83.7)	
Referral cytology	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**
Repeat ASC-US	10.6	135	14.5	49	6.0	37	15.0	49
LSIL	39.3	502	26.6	90	44.6	274	42.3	138
ASC-H	24.9	318	31.6	107	23.6	145	20.3	66
HSIL	19.8	253	25.1	85	21.2	130	11.7	38
AGC-NOS	3.4	43	1.5	5	2.6	16	6.8	22
AGC-FN	2.2	28	0.9	3	2.0	12	4.0	13
Cervical histology								
NILM	30.0	383	20.9	71	24.6	151	49.4	161
LSIL	29.3	375	31.3	106	30.3	186	25.5	83
CIN2	18.6	238	28.0	95	17.9	110	10.1	33
CIN3	17.7	226	15.9	54	22.8	140	9.8	32
AIS	1.5	19	1.5	5	1.8	11	0.9	3
Cervical cancer	1.6	20	0.9	3	1.3	8	2.8	9
No sample	1.4	18	1.5	5	1.3	8	1.5	5
LLETZ	37.2	476	24.2	82	43.8	269	38.3	125

4.2 METHODS (STUDY I, III, IV)

4.2.1 COLPOSCOPY AND LOCAL TREATMENT

Colposcopies were performed with 5% acetic acid with or without Lugol's iodine solution, depending on the colposcopist's preference. A senior colposcopist with national accreditation or at least 100 colposcopies performed annually was always present at colposcopy. Cytology and punch biopsies were taken at the discretion of the colposcopist, and routine punch biopsies were taken at the final visit of studies III and IV. An extra endocervical brush sample was obtained for HPV genotyping in

studies I and III, and endocervical cells obtained with the sample were also used for methylation analyses in study III. An hrHPV test (endocervical or from the vaginal vault in cases with previous hysterectomy) was taken in study IV at the recruitment and exit visits. Colposcopists were unaware of the HPV genotyping and S5 DNA methylation results.

LLETZ (study I and III) was performed under local anaesthesia and colposcopic guidance at the outpatient clinic. Laser vaporisation of VAIN (study IV) was also performed under local anaesthesia and colposcopic guidance at the outpatient clinic with carbon dioxide laser 6-12W continuous beam to a depth of 2 mm with 2 mm margins.

The study I participants were examined, treated, and followed-up according to Finnish Current Care Guidelines. Figure 3 presents a flow chart of study visits in study III. Decisions to treat at 24 months for persistence in cases of CIN1/LSIL were made on an individual basis in study III, taking into account the cytological finding and colposcopic appearance. Figure 4 presents a flow chart of study IV.

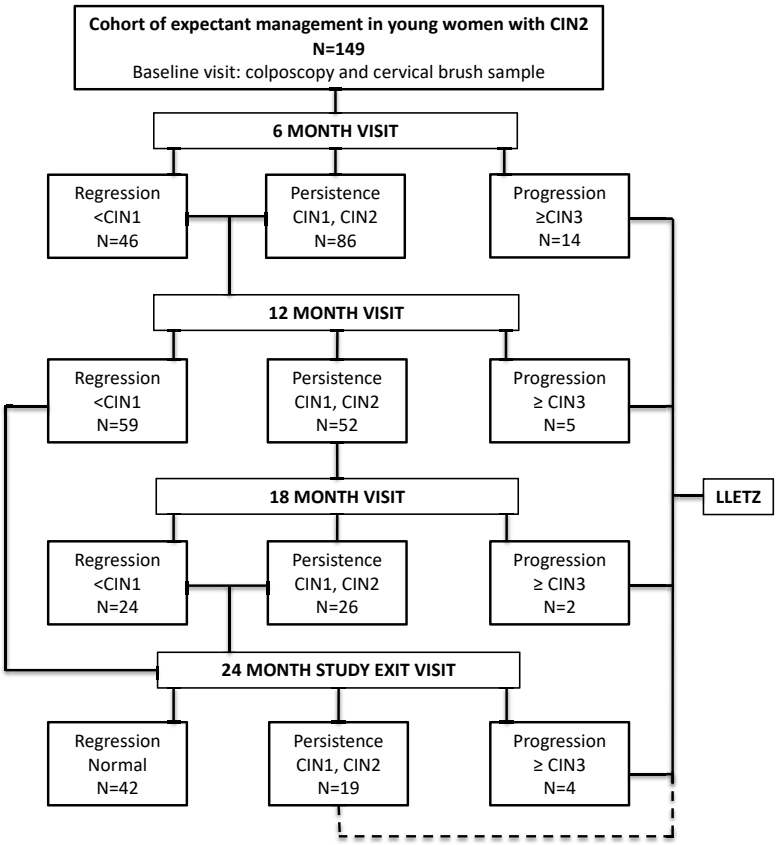

Figure 3 Flow chart on the cohort study on expectant management of CIN2 in young women (study III). Eighty-six women have completed the study (either progression treated or finished 24 months of follow-up). Follow-up is still ongoing for 63 women. Among the 19 women with persistence at 24 months, seven LLETZ were performed with CIN2 histology found in the cone, and the remaining 12 are followed-up according to guidelines. Redrawn from Louvanto et al. Clin Infect Dis. 2019 Jul 25. doi: 10.1093/cid/ciz677

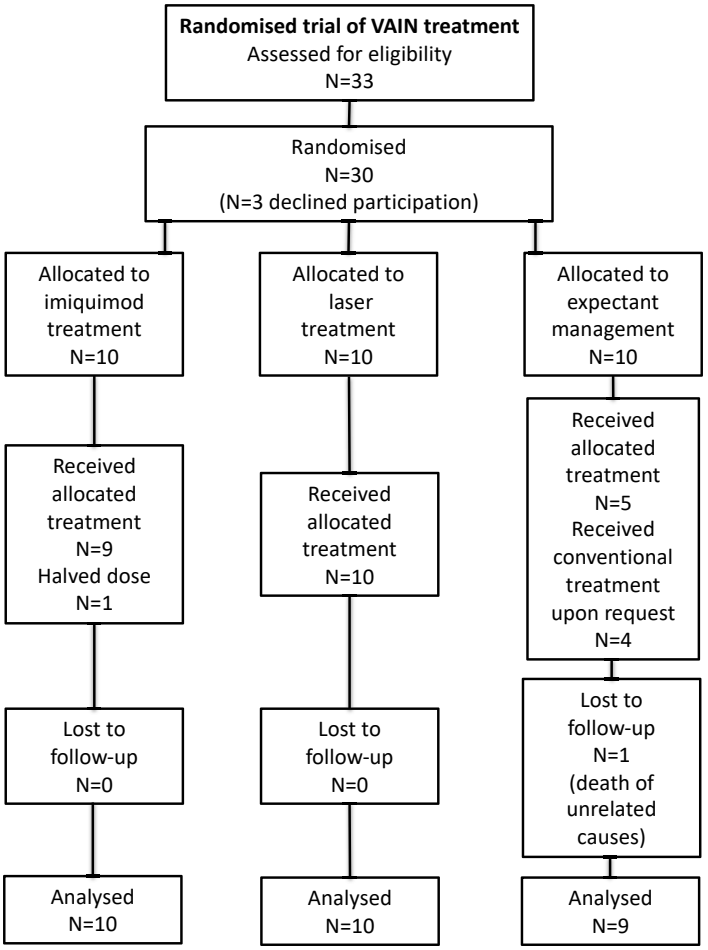

Figure 4 Flow chart on the progress of a randomised trial on VAIN treatment (study IV). Redrawn from Tainio et al. Int J Cancer 2016 Nov 15;139(10):2353-2358.

4.2.2 RANDOMISATION AND TREATMENT (STUDY IV)

Study IV's three treatment groups were vaginal self-administered imiquimod, laser vaporisation, and active surveillance. The patients were randomised 1:1:1 by computer-assisted, permuted-block randomisation with random block sizes of four to six, and the investigators did not participate in the process. Allocation concealment was achieved by sequentially numbered, sealed opaque envelopes. The study visits for all arms were at 4, 8, and 16 weeks after the enrolment visit.

Patients allocated to active surveillance had no other intervention than punch biopsies at the baseline and exit visit (16 weeks) and also at the 8-week visit if new lesions were suspected. Laser vaporisation was performed in the laser group at the recruitment visit, as previously described.

12.5 mg of imiquimod was manufactured into vaginal suppositories (inactive binding materials: macrogol 400 1.35 g and macrogol 6000 0.9 g) by the Pharmacy of Helsinki University Hospital. Patients self-administered the total of 14 suppositories in the evening before bedtime over a period of eight weeks. The dose was 12.5 mg weekly for the first two weeks, and 12.5 mg doses twice a week 3-4 days apart in the remaining six weeks.

The patients received all suppositories from the investigators with written and oral instructions for use and storage (room temperature) along with a diary for recording use and any adverse effects (separate fields for application dates, flu-like symptoms, fever, local irritation, lower abdominal pain and other symptoms). They were instructed to halve the suppositories longitudinally and continue the treatment with half a dose (6.25 mg) if adverse effects were not tolerable after medication (paracetamol and/or non-steroidal anti-inflammatory drug (NSAID)).

4.2.3 COLLECTION OF CLINICAL DATA

Data on referral reasons, patient background (chronic illnesses, current medications, cigarette smoking, parity, contraception, hormone replacement therapy, previous cytological and histological gynaecological findings and treatments, history of sexually transmitted diseases), and clinical findings (RCI, colposcopic diagnosis) were retrieved from the structured electronic colposcopy database. Age at recruitment was recorded.

Data on cytological and histological samples and diagnoses taken at visits were retrieved from the hospital's electronic patient records. Cytology was reported

according to the Bethesda system. A shift was made from the WHO 2003 histopathological classification to the WHO 2014 classification during the study periods. The institution's pathologists, however, reported HSIL at different anatomic sites, also according to the WHO 2003 classification: cervical HSIL as either CIN2 or CIN3 and vaginal HSIL as VAIN2 or VAIN3. Reporting of low-grade lesions shifted from HPV atypia, atypia condylomatosa, etc., and CIN1 or VAIN1 to LSIL. Studies III and IV's data were collected according to the WHO 2003 classification, and study I grouped low-grade lesions as LSIL. During the study period hrHPV testing in routine clinical practice was performed with Hybrid Capture 2 (Digene Corporation, Gaithersburg, MD, USA; Qiagen, Venlo, Netherlands) until April 2014 and thereafter with Aptima (Hologic, Marlborough, MA, USA). Study IV analysed the hrHPV test results.

The worst histological diagnosis was included in the analysis in study I if LLETZ was performed based on punch biopsy results from the recruitment visit within 1-2 months. The baseline histological diagnosis of CIN2 was re-reviewed by an expert pathologist in study III. The worst histological diagnosis for a time-point from punch biopsies or LLETZ cone was also recorded.

The patients reported adverse effects and use of imiquimod treatment in a separate written form (study IV).

4.2.4 LABORATORY ANALYSES

4.2.4.1 Sample handling and HPV genotyping (study I, III)

The cells collected with the endocervical brushes in specimen transport medium (STM, Qiagen GMBH, Hilden, Germany) were stored immediately at -20° C and later divided into three aliquots without adding any medium and stored at -80° C.

One aliquot was sent frozen to the Karolinska Institute for HPV genotyping. DNA was extracted from the samples, and a modified GP5+/6+ primer set was used for

polymerase chain reaction (PCR) , as described previously (Söderlund-Strand, Carlson and Dillner, 2009). Genotyping was performed with the Bioplex 200 Luminex system (Bio-Rad, California).

4.2.4.2 DNA methylation analyses (study III)

One aliquot of the endocervical cells stored in STM was sent frozen to the Center for Cancer Prevention, Wolfson Institute of Preventive Medicine, Queen Mary University of London, for DNA methylation analyses. DNA was extracted with the QIAamp DNA Mini Kit (Qiagen Inc, Hilden, Germany). Two hundred nanograms of DNA were used in the bisulfite conversion reactions using the EZ DNA methylation kit (Zymo research, Irvine, USA) to convert unmethylated cytosines to uracil. Converted DNA (equivalent of 1600 cells per sample) were amplified by methylation-independent PCR primers, and the amplicons were tested by pyrosequencing for DNA methylation of EPB41L3 and the late regions of HPV16, HPV18, HPV31, and HPV33.

4.2.5 OUTCOME PARAMETERS

hrHPV genotypes were grouped for analyses in study I as HPV16/18+, other hrHPV+ (HPV31/33/35/39/45/51/52/56/58/59/66/68+), hrHPV not directly targeted by prophylactic HPV vaccines (non-vaccine hrHPV+: HPV35/39/51/56/59/66/68+), other HPV than high-risk (only low-risk HPV: HPV6/11/30/40/42/43/53/61/67/69/70/73/74/81/83/86/87/89/90/91+), and HPV negative. Other hrHPV and non-vaccine hrHPV were considered positive only if HPV16/18 were not present, and only low-risk HPV was positive only if hrHPVs were not present. The individual HPV groups were positive if any or multiples of the included types in the individual groups were present.

Cervical histological findings were grouped in study I as less than HSIL (<HSIL), including NILM and LSIL; HSIL or worse (HSIL+), including CIN2, CIN3, AIS, squamous cell carcinoma, and adenocarcinoma of the cervix; CIN3+, including CIN3 and squamous cell carcinoma; and adenocarcinoma in situ or worse (AIS+), including AIS

and adenocarcinoma. Women were grouped into three categories based on age: <30 years of age, 30-44.9 years of age, and ≥45 years of age.

Study III outcomes were divided as regression, persistence, and progression based on histological diagnosis. CIN2 regression was defined as <CIN1 according to the WHO 2003 classification. CIN1 and CIN2 were considered as persistence and ≥CIN3 as progression. Cases with high grade cytology (ASC-H, HSIL) at follow-up time points but <CIN1 histology were classified as persistence. For some analyses, women with persistence were grouped together with regression or progression, respectively. HPV genotyping results were recorded as a binary, with women positive in genotyping either for HPV16 or 18 or both classified as positive (HPV16/18+) and as negative if neither was found (HPV16/18-), and similarly for HPV16/18/31/33+ and HPV16/18/31/33-. The S5 methylation classifier was defined with percentages of the methylation measured in biomarker variables as 30.9(EPB41L3) + 13.7(HPV16L1) + 4.3(HPV16L2) + 8.4(HPV18L2) + 22.4(HPV31L1) + 20.3(HPV33L2). Cut-offs for S5 were set at the previously validated cut point of S5 = 0.8 or at the upper tertile of S5 defined as any value within the upper 1/3 of methylation values identified for each methylation biomarker in the specific outcome category.

Study IV considered regression as partial if VAIN2-3 regressed to VAIN1. Complete regression was defined as <VAIN1 according to the WHO 2003 classification. Persistence was defined as VAIN2-3 at the end of the study. Progression for VAIN2 was defined as ≥VAIN3 and as vaginal carcinoma for VAIN3. hrHPV clearance was defined as a positive hrHPV test in the baseline visit and a negative test at the exit (16 week) follow-up visit; persistence was defined as a positive hrHPV test at both time points.

4.2.6 STATISTICAL ANALYSES

Study I estimated risk ratios (RR) of being HPV genotype group positive between different age groups according to histological findings using binomial logistic

regression; the youngest age group (women <30 years of age) was set as the referent group.

Study III compared differences in baseline characteristics in the different clinical outcome groups (regression, persistence, progression) with Mann-Whitney or Fisher's exact test or nonparametric test for trend, as applicable. The associations of mean methylation level or the upper tertile level of different methylation markers and various clinical outcome comparisons were evaluated with unconditional logistic regression odds ratios (OR) and 95% confidence intervals. Multivariable models of logistic regression were used to evaluate confounding factors in methylation and outcome comparisons. Receiver operator characteristic (ROC) analysis by comparing area under the curve (AUC) was used to test the performance of the methylation marker and screening protocols. Missing baseline DNA methylation status (n=8) of HPV16 were imputed with zero for HPV16-negative samples (n=5) and by the median for HPV16 positive samples for HPV16-positive women (n=3). Missing values for EPB41L3 (n=8) were imputed by the median independent of their HPV genotyping result. Eight women without HPV genotyping results were imputed as HPV negative.

Study IV compared the baseline characteristics and findings between the three arms. The cytological and histological findings and hrHPV status were compared at 16 weeks. Additional comparisons between two individual arms were performed (imiquimod vs. laser, imiquimod vs. expectant management, and laser vs. expectant management). Frequency tables were analysed using the Chi 2 test or Fisher's exact test for categorical variables, and means of continuous variables were analysed using nonparametric (Kruskal-Wallis) tests for two and multiple independent samples, respectively. The results were analysed according to intention-to-treat analyses.

In studies I and III statistical analyses were done in STATA version 15 (STATA Corp., College Station, TX) and in study IV STATA version 13 was used (STATA Corp., College Station, TX).

4.3 ELIGIBILITY CRITERIA (STUDY II)

Study II was a systematic review and meta-analysis carried out in national and international collaboration (study registration PROSPERO2014: CRD42014014406). Original studies were included that reported on outcomes of histologically diagnosed CIN2 not treated at diagnosis. Other inclusion criteria were expectant management for three or more months and a follow-up diagnosis with either histology and/or cytology. A histological diagnosis was always preferred to a cytological one. Studies on pregnant women, HIV-positive women, studies in which fewer than 10 patients completed follow-up, studies without a defined follow-up period or merging CIN2 with CIN1 or CIN3, and studies published in other languages than English were excluded.

4.4 METHODS (STUDY II)

4.4.1 LITERATURE SEARCH, DATA EXTRACTION, AND RISK OF BIAS APPRAISAL

Three databases (Medline, Embase, and the Cumulative Index to Nursing and Allied Health Literature (CINAHL)) were searched for publications between 1 January 1973 and 20 August 2016. Reference lists of included studies were also hand searched. Abstracts were screened independently in duplicate, and full texts of eligible studies from screened abstracts were screened similarly. Figure 5 presents a flow chart of the screening process.

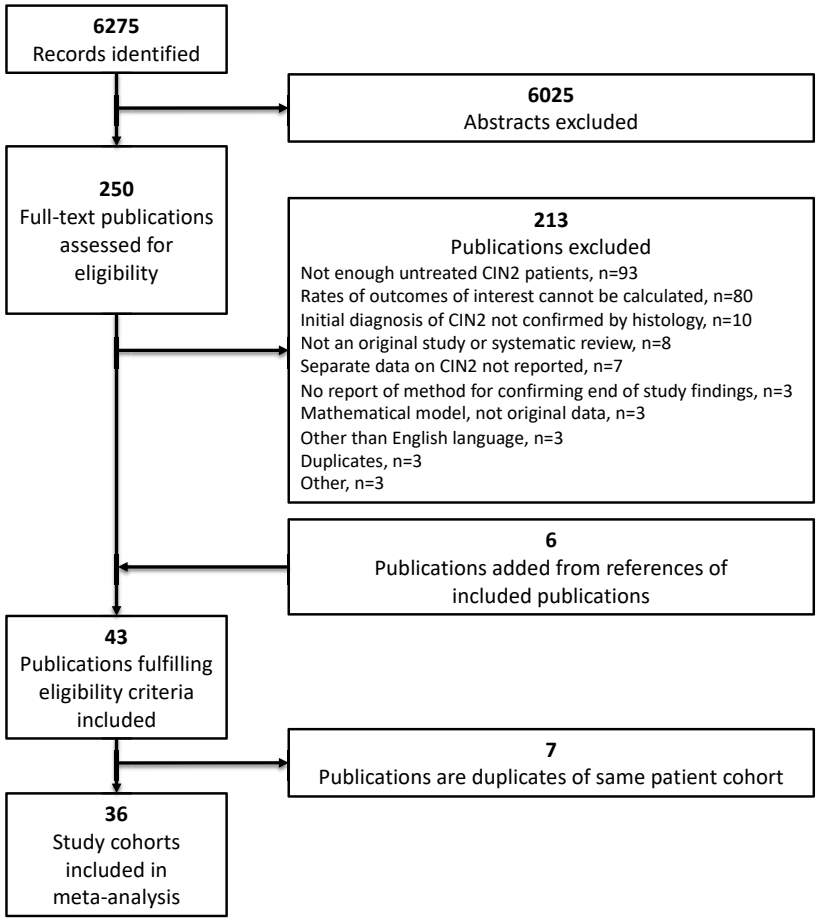

Figure 5 Flow chart of the publication evaluation process in a systematic review and meta-analysis of the clinical course of untreated CIN2 (study II). Redrawn from Tainio et al. BMJ. 2018 Feb 27;360:k499.

Data extraction and risk of bias assessment were performed independently in duplicate by two investigators. Progression, persistence and regression rates from each study were extracted, as well as the name of the first author, year of publication, the design and setting, the total number of participants, the number of participants with the outcomes of interest at different time points, geographical region, and the number of baseline hrHPV- or HPV16/18-positive women, or both.

Risk of bias was assessed using a modified version of the Cochrane Collaboration's risk of bias tool by evaluating each study according to five criteria (Table 9).

Table 9. *Risk of bias assessment tool and definitions*

Domain	Low risk of bias	High risk of bias
Assessment of exposure	Secure record (e.g. hospital records)	Structured interview, self-written report
Confirmation of initial (CIN2) diagnosis	Histological confirmation	Confirmation by cytology, colposcopy and/or HPV testing
Assessment of outcome	Outcome confirmed with multiple methods including histology	Outcome confirmed only with cytology, colposcopy or HPV testing
Loss to follow-up	Loss to follow-up <10%	Loss to follow-up >20% or not adequately reported
Representativeness of cohort	All eligible (CIN2) cases in a predefined time period and population included	Not fulfilling low risk criteria, predefined age range is not considered high risk of bias

For each risk of bias criterion, studies were judged to have either a high or a low risk of bias. Studies were classified at high risk of bias overall if at least one criterion was at high risk of bias.

4.4.2 OUTCOME PARAMETERS

Study II used the definition of progression, persistence, and regression given by the authors of the original publications in each study. Recognising that there would be heterogeneity in definitions between studies, regression and persistence definitions were classified into two groups: "strict" or "lenient" (Table 10). For studies reporting more than one outcome definition (strict and lenient), the strict definition was used in the main analyses.

Table 10. *Cytological and histological criteria for strict and lenient definitions of regression and persistence*

	Strict regression	Lenient regression	Strict persistence	Lenient persistence
Cytology	Normal	ASC-US, LSIL	ASC-US, LSIL, ASC-H, HSIL	ASC-H, HSIL
Histology	Normal	Normal, CIN1	CIN1, CIN2	CIN2

Progression was defined as histological CIN3 or worse (CIN3+) in 29 studies and as a worsening cytological finding during follow-up in the remaining seven. Regression, persistence, progression, and default rates were defined as the ratio of the number of women with an outcome divided by the number of women attending follow-up at that time point. The studies were grouped to the closest time point (3, 6, 12, 24, 36, and 60 months of active surveillance) based either on the exact, the median, or mean follow-up time. Loss to follow-up was defined as the actual number of women lost to follow-up in prospective studies and as the number participants with missing follow-up data in retrospective studies.

4.4.3 STATISTICAL ANALYSES

Pooled proportions for each outcome were meta-analysed separately at the 3, 6, 12, 24, 36, and 60-month time-points using the metaprop command in STATA. The exact binomial score test-based confidence intervals with the Freeman-Tukey double arcsine method were used to stabilise the variances for individual studies, where many of the proportions were close to the margins of the interval (0 or 100%). Heterogeneity between studies was assessed using the I^2 metric of inconsistency. A single predefined set of sensitivity analyses was performed to explore heterogeneity sources. Additional subgroup analyses were performed to further explore the heterogeneity sources and differences in summary estimates, including according to

the age range (studies with only ≤30-year-olds and >30-year-olds, respectively) and according to baseline hrHPV or HPV16/18 status.

Analyses were performed in STATA version 13 (STATA Corp., College Station, TX).

5 RESULTS

5.1 AGE-SPECIFIC HPV GENOTYPE DISTRIBUTION

Study I recruited 1302 women referred for cytological abnormalities; valid HPV genotyping results were available for 1279. The most prevalent genotype in the 1279 women was HPV16 (28.3%), but the prevalence declined steeply with increasing age between age groups, as did prevalence of HPV16/18. Conversely, HPV negativity increased between age strata (Table 11).

Table 11. *HPV genotype and genotype-group distribution in 1279 women referred to colposcopy for abnormal cytology (single genotypes irrespective of multiple infections)*

	All n=1279		<30 n=339		30-44.9 n=614		≥45 n=326	
	%	n	%	n	%	n	%	n
HPV type								
HPV negative	17.3	221	9.7	33	13.7	84	31.9	104
HPV16	28.3	362	38.6	131	30.9	190	12.6	41
HPV18	5.8	74	7.1	24	5.7	35	4.6	15
HPV31	9.9	126	11.8	40	11.1	68	5.5	18
HPV33	3.9	50	5.6	19	3.6	22	2.8	9
HPV45	4.5	57	4.4	15	5.4	33	2.8	9
HPV52	7.7	98	8.9	30	7.7	47	6.4	21
HPV groups								
HPV16/18	33.2	425	44.0	149	35.8	220	17.2	56
Other hrHPV	38.1	487	36.0	122	40.2	247	36.2	118
Non-vaccine hrHPV	15.2	195	13.9	47	15.0	92	17.2	56
Only low-risk HPV	11.4	146	10.3	35	10.3	63	14.7	48

Other hrHPV (HPV31/33/35/39/45/51/52/56/58/59/66/68), non-vaccine hrHPV (HPV35/39/51/56/59/66/68), only low-risk HPV (HPV6/11/30/40/42/43/53/61/67/69/70/73/74/81/83/86/87/89/90/91)

Altogether, 503 cases of histological HSIL+ were identified (Table 12, Figure 5), and 56.7% were associated with HPV16/18. There was a pronounced decrease of HPV16/18-associated HSIL+ with increasing age: 64.3% in women <30 years of age, 58.4% (RR 0.91, 95% confidence interval 0.78-1.06) in women 30-44.9 years, and 35.1% (RR 0.55, 95% CI 0.39-0.75) in women ≥45 years. However, other hrHPVs

(HPV31/33/35/39/45/51/52/56/58/59/66/68) were associated with 31.9% of HSIL+ in women <30 years of age, 36.8% in women 30-44.9 (RR 1.16, 95% CI 0.88-1.52), and 54.6% in women ≥45 (RR 1.71, 95% CI 1.26-2.33). A similar increase was seen with nonvaccine-related genotypes (HPV35/39/51/56/59/66/68). The proportion of HPV-negative HSIL+ cases increased with increasing age with 6.5% of women ≥45, with HSIL+ being HPV negative (RR 5.10, 95 % CI 1.01-25.68).

Table 12. *Age-specific HPV group distribution and risk ratios (RR) of HSIL+ with women <30 as the referent group*

	HPV16/18 n=425		Other hrHPV n=487		Non-vaccine hrHPV n=195		Only low-risk HPV n=146		HPV neg n=221	
	n (%)	RR (95% CI)	n (%)	RR (95% CI)	n (%)	RR (95% CI)	n (%)	RR (95% CI)	n (%)	RR (95% CI)
HSIL+ <30 N=157	101 (64.3)	Ref	50 (31.9)	Ref	8 (5.1)	Ref	4 (2.6)	Ref	2 (1.3)	Ref
HSIL+ 30-44.9 N=269	157 (58.4)	0.91 (0.78-1.06)	99 (36.8)	1.16 (0.88-1.52)	15 (5.6)	1.09 (0.47-2.52)	3 (1.1)	0.44 (0.10-1.93)	10 (3.7)	2.9 (0.65-13.15)
HSIL+ ≥45 N=77	27 (35.1)	0.55 (0.39-0.75)	42 (54.6)	1.71 (1.26-2.33)	12 (15.6)	3.06 (1.30-7.17)	3 (3.9)	1.53 (0.35-6.66)	5 (6.5)	5.10 (1.01-25.68)
HSIL+ Total N=503	285 (56.7)		191 (38.0)		35 (7.0)		10 (2.0)		17 (3.4)	

Other hrHPV (HPV31/33/35/39/45/51/52/56/58/59/66/68), non-vaccine hrHPV (HPV35/39/51/56/59/66/68), only low-risk HPV (HPV6/11/30/40/42/43/53/61/67/69/70/73/74/81/83/86/87/89/90/91)

When separating high-grade squamous disease into CIN2 and CIN3+ similar age-group specific patterns of HPV group distribution were seen, although CIN3+ was overall more commonly associated with HPV16/18 than CIN2 (64.2% and 47.5%, respectively) (Figure 5). There were only 25 cases of AIS+, but all cases in women <30 years of age were associated with HPV16/18, while only a third of the cases in women ≥45.

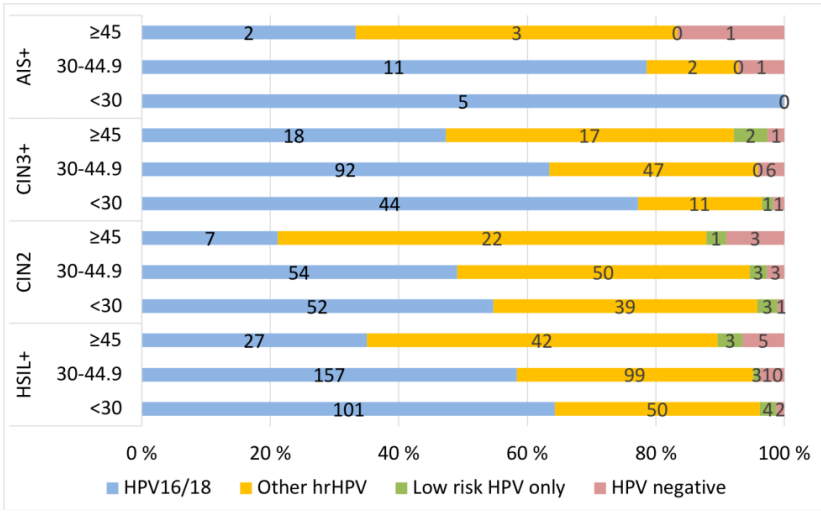

Figure 6 Age-group specific HPV genotype group distribution in different histological categories. Redrawn from Aro et al. Gynecol Oncol. 2019 Aug 154(2):354-359.

5.2 UNTREATED CIN2

Thirty-six studies with 3160 women were included in the meta-analysis of the clinical course of untreated CIN2 (study II). Seven control arms of randomised controlled trials, 16 prospective cohort studies, and 13 retrospective studies were included. The mean follow-up in the studies was 16 months (range 3-72 months). Half of the studies were considered as low-risk of bias. The most common reasons for a high-risk of bias assessment were rate of loss to follow-up (14 studies) and vagueness of the description of the follow-up method (five studies).

The regression rate at 24 months was 50% and the progression rate was 18% in the main analysis. The regression rate at 24 months was 60% and progression rate only 11% in a subgroup analysis of women <30 years of age (approximately 1000 women for all the outcomes) (Table 13). The rates were 44% and 23%, respectively, in women ≥30 years of age.

Table 13. *Pooled rates for outcomes at 24 months in women with untreated CIN2*

		24 months		
		Regression	Persistence	Progression
Main analysis	N of studies N obs/N att	11 819/1470	8 334/1257	9 282/1445
	Summary % (95% CI; I^2)	50 (43-57; 77)	32 (23-42; 82)	18 (11-27; 90)
<30-year-olds	N of studies N obs/N att	4 638/1069	2 226/938	3 163/1033
	Summary % (95% CI; I^2)	60 (57-63; 0)	23 (20-26; 97)	11 (5-19; 67)
≥30-year-olds	N of studies N obs/N att	7 181/401	6 108/319	6 119/412
	Summary % (95% CI; I^2)	44 (36-52; 61)	35 (23-49; 83)	23 (12-37; 89)
Low risk of bias	N of studies N obs/N att	5 653/1176	3 275/1049	3 181/1049
	Summary % (95% CI; I^2)	45 (33-58; 88)	35 (21-51; 89)	20 (12-30; 76)

N of studies: number of studies included in analysis; N obs: number of outcomes observed; N att: number of women attending follow-up time-point

The progression rates increased with the length of follow-up. The progression rate at six months was 13% (4 studies, 42/278 women, 95% CI 8-20%; I^2 42%) and 24% at 36 months (three studies, 105/370 women, 95% CI 12-39%; I^2=87%) in the main analysis. The vast majority of progressions were to CIN3. Among the 3160 women included, 15 cases of AIS were reported (0.5%) and 15 invasive cervical cancers were additionally reported (0.5%). Thirteen of these were stage IA1, and two were of more advanced stage.

Very few studies reported on outcomes according to baseline hrHPV (three studies) or HPV16/18 (two studies) status. hrHPV- and HPV16/18-negative women had a low risk of progression at 24 months at 3% (1/23 women, 95% CI 0%-24%; I^2=0%) and 5% (1/62 women, 95% CI 0%-28%; I^2=76%), respectively. 25% of hrHPV-positive women and 21% of HPV16/18-positive women progressed at 24 months (38/161 women 95% CI 14%-38%; I^2=51%, and 7/56 women, 95% CI 8%-37%; I^2=58%, respectively). Overall, most women regressed within 24 months irrespective of baseline hrHPV or HPV16/18 status.

Loss to follow-up summary estimates varied highly according to the study design. Loss to follow-up rates were consistently around 10% in prospective cohort studies most likely reflecting a real-life clinical situation.

5.2.1 S5 IN OUTCOME PREDICTION OF UNTREATED CIN2

In the prospective cohort study of 149 young women (study III) with untreated, histologically confirmed CIN2, outcome rates were in line with the findings of the meta-analysis (study II), although follow-up is still ongoing for 63 of the women (42%) (Figure 3). Eighty-eight of the women regressed to <CIN1 (59%), 25 progressed to ≥CIN3 (17%), and 36 persisted as CIN1/2 (24%). The women's mean age was 26.0 years, 52% (67/128) were current cigarette smokers, and 82% (116/141) were positive for any hrHPV genotype on the baseline visit. Overall, the most common HPV genotypes were HPV16 (43%, 61/141), HPV31 (13%, 19/141), HPV18 (8%, 11/141), and HPV33 (6%, 8/141). The baseline characteristics of the women did not differ statistically between the outcome categories (regression, persistence, progression) except for any hrHPV genotype positivity between the regression and persistence categories.

In a multivariable model, the odds ratios (OR) of the S5 classifier in different outcomes showed the S5 classifier to be an independent predictor of outcomes when adjusted for HPV16/18/31/33-status, initial cytology, cigarette smoking, and age among the regression versus progression group (crude OR 1.17, 95% CI 1.06-1.30; adjusted OR 1.12, 95% CI 1.00-1.27).

When comparing odds ratios for S5 results (high-tertile and >0.8 cut-offs) between different outcome categories, the highest OR of 4.84 was reached in the comparison of regression versus progression with the high-tertile cut-off (Table 14). The corresponding area under the curve (AUC) from the >0.8 S5 classifier cut-off was 0.718. Nearly all outcome comparisons reached statistical significance.

Table 14. *S5 classifier high-tertile and 0.8 cut-off odds ratios (OR) and area under the receiver operating characteristic curve (AUC) with 95% confidence intervals (95% CI) in different outcome comparisons of untreated CIN2. Significant results in bold. AUC for S5 at the >0.8 cut-off.*

Clinical outcome comparison	High-tertile S5 OR (95% CI)	0.8 cut-off S5 OR (95% CI)	0.8 cut-off S5 AUC (95% CI)
Regression vs. persistence	**2.61 (1.03-6.61)**	1.04 (0.95-1.14)	0.567 (0.46-0.68)
Regression vs. progression	**4.84 (1.35-17.41)**	**1.17 (1.06-1.30)**	**0.718 (0.61-0.83)**
Persistence vs. progression	2.86 (0.88-9.33)	**1.15 (1.01-1.30)**	**0.676 (0.54-0.82)**
Regression/persistence vs. progression	**4.48 (1.27-15.77)**	**1.16 (1.06-1.28)**	**0.706 (0.60-0.81)**
Regression vs. persistence/progression	**2.68 (1.27-5.64)**	**1.10 (1.02-1.19)**	**0.630 (0.54-0.72)**

When comparing ORs for progression and persistence, with regression as the referent group, high-tertile S5 positivity was found to be a significant prognostic variable (OR 3.39, 95% CI 1.35-8.50), whereas HPV16/18 genotyping positivity was not (Table 15). Additional analyses associated HPV16/18/31/33 positivity with persistence (OR 3.50, 95% CI 1.44-8.52) and also to progression to a slightly lesser extent (OR 3.17, 95% CI 1.15-8.68).

Table 15. *Odds ratios (OR) for outcomes with regression as the referent group with S5 high-tertile positivity and HPV16/18 genotyping positivity*

Outcome	S5 high-tertile OR (95% CI)	HPV16/18 positivity OR (95% CI)
Regression	1.00 Ref	1.00 Ref
Persistence	1.33 (0.58-3.07)	1.99 (0.91-4.35)
Progression	3.39 (1.35-8.50)	2.38 (0.96-5.91)

The performance of the S5 classifier alone and in combination with other possible predictive markers was tested in different outcome comparisons. The highest AUC was 0.735 (95% CI 0.621-0.849) when comparing regression with progression and S5 >0.8 and cytology ≥HSIL was regarded as positive. Combining HPV16/18 genotyping positivity provided no additional advantage (Figure 7). Addition of HPV16/18/31/33 genotyping also did not provide additional advantage with the exception of comparison of regression vs. persistence/progression (AUC of 0.666, 95% CI 0.580-0.752).

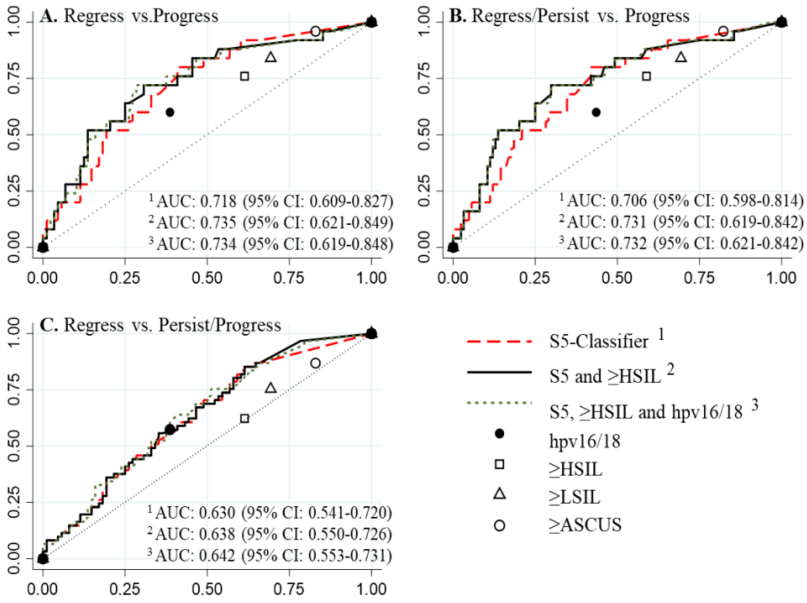

Figure 7 Receiver-operating characteristic (ROC) curves for the performance of S5-classifier alone and in combination with other tests in different clinical outcome categories. The points 0.00 and 1.00 are for the ROC start and end points for the single tests. Redrawn from Louvanto et al. Clin Infect Dis. 2019 Jul 25. doi: 10.1093/cid/ciz677

5.3 TREATMENT OF VAIN

Study IV included 30 women (median age 54, range 31-82) with histologically confirmed vaginal HSIL. Half of the women had been previously treated for one or multiple HPV-related genital diseases: 37% (11/30) for VAIN, 23% (7/30) for CIN, 13% (4/30) for cervical cancer (over five years ago) and 10% (3/30) for VIN. Four patients

were diagnosed with concomitant CIN1 and one with VIN3. Eleven women (37%) had previously had a hysterectomy, and seven (23%) were current cigarette smokers.

At baseline 25 women had VAIN2 (83%), and five had VAIN3 (17%). Four of the women with VAIN3 were in the imiquimod arm, while none were in the laser arm. Multifocal VAIN was found in 63% (19/30) of the women. At baseline 77% (20/26) were found to be hrHPV positive. No significant differences existed between the study arms by any of the baseline characteristics or findings.

None of the lesions progressed during the 16 weeks of follow-up. No differences were seen in the histological regression rates between the arms (Table 17). hrHPV clearance at the end of the study was significantly higher in the imiquimod arm at 63% when compared to 11% of the laser arm (p=0.05). One patient in the expectant management arm died during the study period due to unrelated causes (lung cancer), and four were treated conventionally (laser vaporisation) on request. Three of the five untreated patients had complete histological regression at the end of the study.

None of the women discontinued the imiquimod treatment, but one halved the dose. All women, however, reported adverse effects from the treatment. Flu-like symptoms (including a rapidly subsiding fever up to 39° Celsius within 12 hours of imiquimod application for four women) were reported by 9/10 women, local irritation of the vagina and vulva by 6/10, and lower abdominal pain by 3/10. None needed immediate evaluation for the symptoms, which were alleviated with paracetamol or NSAID medication. Vulvar irritation was seen in two women at intermediate study visits and were treated with local estrogen cream. One woman had a vulvar ulceration at the intermediate study visits, but this had healed by the end of the study.

Table 16. *Histological and hrHPV end of study results from the randomised trial of VAIN treatment*

		Imiquimod (N=10)		Laser (N=10)		Expectant management (N=9)		p-value [1]
Histology		**N**	**%**	**N**	**%**	**N**	**%**	
Regression of disease (any)		8	80	10	100	6	67	0.474
	Complete regression [2]	7	70	9	90	4	44	0.582
	Partial regression [3]	1	10	1	10	2	22	0.721
Persistent disease		2	20	0	0	3	33	0.474
hrHPV status [4]		**n/N**	**%**	**n/N**	**%**	**n/N**	**%**	
	Cleared HPV	5/8	63	1/9	11	1/6	17	0.05
	Persistent HPV	3/8	38	6/9	67	3/6	50	0.437

[1] Analyses between imiquimod and laser arms (none of the analyses between two other individual arms reached statistical significance); [2] defined as <VAIN1; [3] defined as VAIN1; [4] n=number cleared or persisted; N=number of total tested both at baseline and at 16 weeks

6 DISCUSSION

6.1 HPV IN CERVICAL AND VAGINAL PRECANCEROUS DISEASE

HPV infection is recognised as a necessary factor in the carcinogenic processes of the cervix and vagina (zur Hausen, 1977; Walboomers *et al.*, 1999; Arbyn, de Sanjosé, *et al.*, 2012). Study I found that, overall, 83% (1058/1279) of women with abnormal cytology were positive for any HPV genotype at colposcopy when referred according to guidelines aiming to find all relevant disease while omitting common transient infections. Over two thirds (>900) of the women in study I were positive for hrHPV genotypes, including a third for HPV16/18. Low-risk genotypes were only found in approximately 10%. The distribution of high- and low-risk genotypes in study I followed well-recognised patterns, since the proportion of hrHPV-related disease increases with the severity of findings (Guan *et al.*, 2012).

Over 90% of women diagnosed with histological HSIL+ were positive for hrHPV genotypes, including over half for HPV16/18 (study I). Among young women with CIN2 (study III), 82% were positive for any hrHPV genotype and approximately half for HPV16/18 within one to two months of the histological CIN2 diagnosis. An American study made a similar finding of approximately half of CIN2 in young women being attributed to HPV16/18 (Moscicki *et al.*, 2010). Study IV found that 77% of women with high-grade VAIN were hrHPV positive within 1-2 months of the histological diagnosis, which is in line with previous reports (Gunderson *et al.*, 2013; Rhodes, Chenevert and Munsell, 2014; Jentschke *et al.*, 2016).

HPV16 was the most commonly observed genotype (28.3%) in study I, as expected (Bruni *et al.*, 2010; Leinonen *et al.*, 2013). HPV52 has distinct geographical prevalence patterns, with it being among the most common genotypes in Denmark, Eastern Europe, Africa and Asia and, according to a previous study, also in Finland (Kjaer *et al.*, 2008; Bruni *et al.*, 2010; Leinonen *et al.*, 2013). Our study confirms the finding

regarding Finland. However, HPV52 is more uncommon in the rest of Europe (Bruni *et al.*, 2010).

Samples tested for HPV (genotyping or hrHPV testing) from the prospective studies were obtained one to six months after the initial cytological diagnosis (study I) or within one to two months of histological diagnosis (studies III and IV). This could have allowed time for HPV clearance, because a small proportion of high-grade disease was found to be HPV negative via either genotyping or hrHPV testing (Ho *et al.*, 1998). The women in study III and IV also had punch biopsies taken prior to recruitment that can promote disease regression (Trimble *et al.*, 2005; Mark *et al.*, 2019). Genotyping detects more HPV genotypes and is more sensitive than commercial hrHPV tests, which have higher thresholds for positive results aimed at finding only clinically relevant disease (Meijer *et al.*, 2009).

6.1.1 EFFECT OF AGE ON HPV GENOTYPE DISTRIBUTION

HPV16/18 is found in approximately 70% of cervical cancers globally (de Sanjose *et al.*, 2010; Guan *et al.*, 2012). Age-specific HPV genotype distribution has been mostly described in women with normal cytology, in screening populations, and in women with cervical cancer (Franceschi *et al.*, 2006; de Sanjose *et al.*, 2013). Data on age-specific HPV genotype distribution in histological HSIL, however, are relatively sparse. Our study found HPV genotype distribution in highly screened women with abnormal cytology warranting colposcopy to be distinctly polarised by age. All hrHPV genotypes were more uncommon with advancing age, but the decrease was most pronounced for HPV16. Conversely, the proportion of women found HPV negative in genotyping increased with advancing age.

A similar age-related pattern remained even when assessing only the women with histological high-grade cervical disease. The pattern remained when separating high-grade squamous histologies (CIN2 and CIN3+) and glandular abnormalities (AIS+). In all high-grade disease categories, however, the proportion of disease attributed to

other high-risk genotypes than HPV16/18 was greater with advancing age, and it is notable that the median age of the women in the ≥45 years of age group in study I was only 51. The proportion of HPV genotyping negative high-grade disease also increased markedly in the oldest age-group.

Our finding is consistent with a few reports on age-specific genotype distribution in primarily cervical cancer when invasive cancers related to HPV16/18 were diagnosed at a younger age (Wheeler *et al.*, 2009; Carozzi *et al.*, 2010; Brotherton *et al.*, 2017). An American study including CIN3 and AIS showed no age-specific pattern in those histological entities but did see one in invasive cervical cancer in samples retrieved from 1980 to 2000 (Wheeler *et al.*, 2009). The study also noted that the overall proportion of disease attributed to HPV16 had declined over the past decades, while other hrHPVs — excluding HPV18 — had become more common. Our more recent study could have affected the differing results regarding CIN3 and AIS. The observed time trend in genotype distribution in the American study might also be linked to participation in cervical cancer screening as the authors themselves noted. Women in Finland are, in general, highly screened, because the organised nationwide screening program started in the 1960s, and 90% of women currently have a smear within every five years either within the organised program or opportunistically (Working group set by National Institute for Health and Welfare (THL), 2011).

The core reason for this polarisation of genotypes in high-grade cervical disease remains unclear based on these and previous data. In addition to differing screening attendance rates, it may be caused by a longer sojourn period before high-grade disease with genotypes other than HPV16/18 appear (Wheeler *et al.*, 2009). Another explanation might be latent HPV infections re-activating with menopausal immune senescence (Castle *et al.*, 2005). Of note, the prevalence of HPV18 in study I showed only a small reduction with advancing age (7.1% in women <30 and 4.6% in women ≥45). It has been suggested that HPV18-related disease is not as easily detected in cytology-based screening as is HPV16-related disease (Khan *et al.*, 2005).

We found the rate of HPV-negative, high-grade cervical disease to be low, as expected (3.4%, 17/503); however, the rate increased with advancing age up to 6.5% in women over 45 years of age. A recent large study on invasive cervical cancers from Sweden found only 80% to be HPV positive in genotyping (Lei *et al.*, 2018). Their study associated HPV negativity with older age at diagnosis. These findings raise questions on the accuracy of hrHPV-based screening in older women. Sweden is currently cotesting (hrHPV test and cytology) approximately 41-year-old women who have not previously been screened with an hrHPV test (*Cervixcancerprevention: Nationellt vårdprogram och konsekvenser av införande av Socialstyrelsens rekommendationer gällande screening juni 2015*, no date).

Finding an even more efficient screening and triage algorithm is highly important despite the great success of cytology-based cervical cancer screening programs. Screening based on hrHPV testing has increased the sensitivity of screening when compared to cytology (Koliopoulos *et al.*, 2017). Nevertheless, a problem remains with the decreased specificity, setting great demands on the triage test. Cytology triage is currently implemented in Finland (Veijalainen *et al.*, 2016). A recent study from the United States showed p16/Ki67 dual staining to be able to reduce colposcopy referral rates by approximately 30% (Wentzensen *et al.*, 2019). Using HPV16/18 genotyping to decide on the urgency of colposcopy in hrHPV-positive women has also been suggested, but studies assessing the method were not analysed in separate age strata (Castle, Stoler, *et al.*, 2011; Stoler *et al.*, 2011). In light of our data, high-grade cervical disease related to HPV16/18 was more uncommon than disease related to other high-risk genotypes in women ≥45 years of age, raising questions about the appropriateness of HPV16/18 genotyping as a triage test, at least in highly screened populations.

Taking into account the recent findings of genotype distribution and HPV negativity in the cervical cancer of older women, these data should not be overlooked when applying any adjunctive screening technologies. Prophylactic vaccinations will also greatly reduce the sensitivity of screening, but for decades there will still be

unvaccinated women also attending screening programs. The decision made in Sweden to cotest women in their 40s who have not been previously screened with a hrHPV test seems valid based on our findings. Cotesting with dual staining could possibly further improve sensitivity, and methylation can possibly replace this in the future, if sufficient evidence is accumulated.

The effect of prophylactic HPV vaccinations in a real-life setting is currently being seen in women in their 20s in countries that adopted the vaccination into national programs early on (Kavanagh *et al.*, 2017; Garland *et al.*, 2018; Palmer *et al.*, 2019). A steep decline is evident in at least HPV16/18 prevalence and related precancerous lesions. This phenomenon will most likely be seen in all regions with sufficient vaccine coverage. Decade-long efficacy in cancer prevention is very likely based on current findings but will only be seen much later. Moreover, it is not yet known how long lasting cross-protective efficacy against other hrHPV genotypes will be for the bivalent or quadrivalent vaccines (Artemchuk *et al.*, 2018). The polarisation of genotype distribution by age observed in study I does not inform us on when the women in the oldest age group have acquired the hrHPV genotypes, other than HPV16/18 that were more common in HSIL+ cases. Therefore, it cannot be reliably deduced what the effect of prophylactic vaccination in adolescence will mean for women in their 40s or 50s despite a likely near-eradication of HPV16/18. Concurrently, at least in developed countries, exposure patterns to HPV can be expected to change, because monogamous, life-long relationships are not as common as in the past (Vaccarella *et al.*, 2006; Bosch *et al.*, 2008).

6.2 OUTCOMES OF UNTREATED CIN2 WITH REGARD TO AGE

Our meta-analysis of the clinical course of untreated, histologically confirmed CIN2 (study II) was able to show an age-specific pattern of more frequent regression and less frequent progression in younger women (<30 years of age). This finding confirms the adequacy of some treatment guidelines already suggesting active surveillance as an alternative for immediate treatment for CIN2 in young women (Massad *et al.*, 2013). Study I found that the burden of high-grade preinvasive cervical disease (CIN3

and AIS) was in women 30-44.9 years of age (151/245 cases), but the burden of CIN2 was in women <30 (95/238 cases), highlighting the importance of the issue of CIN2 management.

Of most concern in CIN2 active surveillance protocols is the risk of progression to invasive cervical cancer. The studies included in the meta-analysis showed progression to invasive disease to be mostly associated with older age and longer follow-up. The majority of invasive disease (n=11/15) was reported from a single Japanese study in which none of the cases of invasive disease were diagnosed in women under the age of 30 (Hosaka *et al.*, 2013). Age at diagnosis could not be determined for the remaining four cases from the original publications. The majority of cases of AIS diagnosed during the active surveillance of untreated CIN2 (n=14/15), however, were found in young women under age 25 (Loopik *et al.*, 2016; Munro *et al.*, 2016). Loss to follow-up is another concern of active surveillance, but it is reassuring that the 10% loss seen in the prospective studies of meta-analysis most likely best reflects a real-life situation.

When comparing the reported natural history of CIN3 to the finding of our meta-analysis, a 0.5% progression to invasion rate for CIN2, the natural histories of these two CIN grades appear to be very different (McCredie *et al.*, 2008). Progression to invasion for CIN3 was reported to be 17% at 5 years and 34% at 20 years in the New Zealand study (McCredie *et al.*, 2008). Our finding raises questions on the appropriateness of combining CIN2 and CIN3 as histological HSIL in the updated WHO classification (WHO, 2014). It is widely accepted that CIN3 should be treated, and the new histological HSIL can hinder a more personalised approach in CIN2's management. However, the long-term risk of recurrent disease in women with regressed CIN2 initially managed with active surveillance is not currently confirmed.

The findings of the meta-analysis are important, especially for very young women, as they are the ones most likely to plan future pregnancies and have the greatest likelihood of spontaneous disease regression and the least risk of progression. The

combination of this and the negative impact of local CIN treatments have been shown to have on future pregnancies (Kyrgiou *et al.*, 2016, 2017) justify consideration of active surveillance, at least in selected young women. Shared decision making with the patient and appropriate information on the risks of both active surveillance and treatment are of key importance.

Active surveillance may increase costs and demands on health care services, since more visits and testing most likely are needed than if a woman is treated and re-seen for a test of cure. CIN2 cases persisting beyond two years most likely should be treated, as progression was seen to increase with time, according to our results. A firm recommendation for an active surveillance protocol is difficult to provide, because follow-up protocols of studies included in the meta-analysis varied highly. The most common follow-up in prospective low-risk of bias studies, however, was colposcopy every three to four months with cytology and routine punch biopsies or punch biopsies if progression was suspected.

6.3 S5 CLASSIFIER IN OUTCOME PREDICTION OF CIN2

When considering an individual woman with CIN2, active surveillance instead of immediate treatment always bears the risk of disease progression despite adhering to a follow-up protocol. A predictive biomarker for outcomes could aid clinical decision making in the future and change the outline of active surveillance protocols of CIN2, because cases with risk of progression could be treated immediately and cases with low risk could be managed expectantly. Our study (study III) is, to our knowledge, the first to show DNA methylation to be able to independently predict the risk of progression of untreated, high-grade cervical disease in a longitudinal study.

Other biomarkers have also been tested as progression markers for untreated CIN2. p16 has been found to be inconsistent in two studies (Guedes *et al.*, 2007; Omori *et al.*, 2007). A previous study has also shown baseline HPV16/18 positivity to perform relatively poorly in outcome prediction of CIN2 in young women (Moscicki *et al.*,

2010). However, persisting hrHPV (the same genotype found in consecutive samples) was more closely associated with persistence or progression in that study. Our meta-analysis (study II), although based on a small number women, shows that only 25% of baseline HPV16/18-positive and 21% of baseline hrHPV-positive women with CIN2 experienced progression at two years. Our study III shows that baseline HPV16/18/31/33 positivity was associated with persistence of CIN2 and even progression, although it did not predict it quite as well as the S5 classifier.

DNA methylation of many different candidate genes of both HPV and the host have previously been shown to be able to differentiate between different CIN grades and invasive cancer (Mirabello *et al.*, 2012, 2013; Wentzensen *et al.*, 2012; Kalantari *et al.*, 2014; Louvanto *et al.*, 2015; Clarke *et al.*, 2017). Methylation of other candidate genes and also the S5 classifier have previously been shown to be able to predict high-grade cervical disease in hrHPV-positive women (Brentnall *et al.*, 2014; De Strooper *et al.*, 2014; Lorincz *et al.*, 2016). Our study demonstrates the ability of the S5 classifier also to differentiate between progressive and regressive high-grade cervical disease. The S5 classifier, in contrast to DNA methylation tests of host genes, has not shown improved sensitivity when combined with HPV16/18 genotyping (De Strooper *et al.*, 2014; Lorincz *et al.*, 2016; Cook *et al.*, 2018). HPV16/18 genotyping did not add any additional advantage in our current study, either.

CIN histological grading suffers from interobserver variability (Ismail *et al.*, 1989; Stoler and Schiffman, 2001), so having a well-performing predictive biomarker for preinvasive cervical disease overall could even make further histological grading beyond “CIN” unnecessary. Based on our meta-analysis (study II) and a previous study from New Zealand, the natural histories of CIN3 and CIN2 appear to be very different (McCredie *et al.*, 2008). Still, even the majority of untreated CIN3 lesions do not progress to invasive cancer, opening a possibility of expectant management in cases of CIN3 if outcomes could be reliably predicted. A predictive test of CIN outcomes could also save costs due to the likely need for fewer follow-up visits.

6.4 IMIQUIMOD IN TREATMENT OF VAIN

Treatment of VAIN is burdensome for both patients and caregivers. Recurrences are common, and repeated surgical and laser treatments can especially be mutilating through vaginal scarring (Perrotta *et al.*, 2013; Wang *et al.*, 2014; Jentschke *et al.*, 2016; Kim, Lee and Lee, 2018). A treatment targeting the cause (hrHPV) of VAIN instead of the outcome (mucosal lesions) could be beneficial. Imiquimod activates the local immune response by cytokine release and dendritic cell activation, resulting in activation of both innate and acquired immunity (Schon and Schon, 2007). Imiquimod is topically used to treat external genital warts. The imiquimod dosage and delivery system used in our study was the same as used in an Austrian study exploring the use of imiquimod in high-grade CIN treatment, in which they observed a 73% regression rate compared to 39% with placebo (Grimm *et al.*, 2012). Imiquimod has also shown very promising results in VIN treatment (van Seters *et al.*, 2008). These facts make it also an attractive option for treatment of VAIN.

Study IV's study population was similar to those of previous VAIN treatment studies (Gunderson *et al.*, 2013; Rhodes, Chenevert and Munsell, 2014). Our study showed equal short-term (16-week) efficacy of vaginal imiquimod to conventional laser vaporisation in histological regression rates. However, the hrHPV clearance rate in the imiquimod group was higher, possibly leading to a promise of lower recurrence rates, since hrHPV persistence has been shown to be a risk factor (Frega *et al.*, 2007; Hee Seung Kim *et al.*, 2009; Wang *et al.*, 2014; Jentschke *et al.*, 2016). Of note, three out of five women in the expectant management group had complete histological regression at the end of the study period, while the remaining two had persisting VAIN2. The most plausible explanation for the regression appears to be the punch biopsies taken for the initial diagnosis.

Imiquimod treatment had short-term adverse effects in all women using it. None of the women discontinued treatment despite the adverse effects. Many women in study IV had recurrent VAIN, making them highly motivated to try a new treatment. All current treatments of VAIN also have adverse effects. A self-administered medical

treatment might be found more attractive by some in comparison to surgical or laser treatment. It is also possible that multifocal disease could be better treated with vaginal suppositories. A new treatment option could well be welcomed by both patients and caregivers.

6.5 STRENGTHS AND WEAKNESSES

The results in study I should be generalisable, because the women came from an unselected population referred to the single referral colposcopy centre in the Helsinki metropolitan area that serves a population base of approximately one million people. Only a small number of genotyped samples in study I were found to be invalid or were not taken in (n=23/1302), and HPV genotyping was performed at an international reference laboratory; thus, missing data should not have a major effect on our results. Study participants were referred to colposcopy according to Finnish Current Care Guidelines, so the observed hrHPV distribution should reflect the genotypes causing clinical morbidity, omitting most transient infections.

A weakness of study I is that the results cannot be used to assess distribution of hrHPV in the whole population. Study participants were asked whether they have been vaccinated for HPV, but this could not be confirmed elsewhere, which might introduce recall bias. The data on vaccination status were not necessarily recorded in patient records. Most likely the number of vaccinated women in the study is very low, since they could not have been vaccinated as a part of the national program that started only in 2013. Another shortcoming of the study is the low number of invasive cancer cases and individual hrHPV infections, excluding HPV16. Most invasive cervical cancer cases were diagnosed in women ≥45 (9/20 cases), which is in line with the mean age of cervical cancer diagnosis (45 years) in Finland (Finnish Cancer Registry, no date).

The regression rates in study II were high even at the most conservative estimates and despite the great observed heterogeneity in the summary estimates. The

observed interstudy heterogeneity that was not reduced in sensitivity and subgroup analyses is most likely related to the inherent difficulty in the histological classification of CIN (Ismail *et al.*, 1989; Stoler and Schiffman, 2001). The meta-analysis could not take the lesions' clinical features into account (type of transformation zone, lesion size), which most likely would also affect outcomes and should be considered in clinical practice. A strength of the study is the comprehensive literature search and duplicate evaluation performed at all stages. The meta-analysis is, to our knowledge, the first to be performed on histological CIN2 natural history. A most often cited previous review on CIN natural history included cytological diagnoses of CIN. It included neither a weighted meta-analysis nor took into account the length of follow-up or the age of the women (Östör, 1993).

The strengths of study III include the study's novelty, the unique study population, the rigorous follow-up scheme the women adhered to, and the re-assessment of the initial histological CIN2 diagnosis. Only a small proportion of endocervical cell samples were missing (n=8/149). Overall, the loss to follow-up rate in the study is extremely low (data not included in the current publication). The S5 classifier was rigorously assessed against different possible progression markers to minimise bias according to the REMARK guidelines (McShane *et al.*, 2006). A weakness of our current study is that the women who have not completed the 24 months of follow-up (63/149, 42%) and are now classified as regressed or persisting might switch outcome categories over time, e.g., a case classified as persistence might regress or progress eventually. The study was also restricted to young women and the uniform histological diagnosis of CIN2, making the generalisability of the results to women of all ages and other CIN grades uncertain.

In study IV a weakness of the proof of principle pilot study is the small sample size (n=30) and the short length of follow-up (4 months). The study design was decided upon because previous data on imiquimod treatment of VAIN were scarce, and power calculations could not be performed. The randomised study design can be considered a strength as previous studies using imiquimod have been non-

randomised introducing most likely selection bias. Moreover, the largest previous study on imiquimod treatment of VAIN, which included 56 young women (median age 20), used primarily only colposcopy for diagnosis and follow-up without histological confirmation (Buck and Guth, 2003). A second published study on imiquimod treatment of high-grade VAIN had only seven subjects but a mean follow-up of 18 months (range 5-31 months) (Haidopoulos *et al.*, 2005). hrHPV clearance was not assessed in either of the previous studies.

The women in study IV adhered well to the study protocol with no losses to follow-up except one death due to unrelated causes. The women were also highly compliant, though 4/10 women in the expectant management arm requested laser vaporisation during the study period. The women were also rigorously examined with colposcopy, cytology, histology, and hrHPV testing. However, hrHPV test results at baseline and/or the end of study were missing from 7/30 patients, and hrHPV clearance could not be assessed. The hrHPV test used was switched during the study period from Hybrid Capture 2 to Aptima, which might have somewhat impacted the results, since some women might have initially been tested with Hybrid Capture 2 and followed-up with Aptima. The imiquimod arm had 4/10 women with VAIN3 and the laser arm none, which might have biased the results.

6.6 FUTURE IMPLICATIONS

The reason for the age-specific polarisation of HPV genotypes would be of clinical interest. This information could aid estimating the long-term efficacy of the prophylactic HPV vaccines and help design future screening policies. It is possible that the polarisation is mostly due to screening, since it appears that HPV16-related disease is most easily detected. Latent infections as such and in combination with immune senescence offer another plausible explanation. We did not analyse data on previous abnormal cytology, previous CIN treatments, or sexual habits and history in study I.

A randomised trial comparing active surveillance with local treatment is called for despite the reassuring finding of our meta-analysis on outcomes of untreated CIN2. Long term follow-up results of actively surveilled and regressed CIN2 cases is also of great interest, because the recurrence rate remains unknown. It is important to bear in mind, despite the lack of data, that recurrences after local treatment do also occur both in the short and long term.

DNA methylation presents an interesting option for biomarker development for CIN outcomes. Overall, methylation research is still in its early stages, and refinement of genes of interest and combinations of them is called for. The S5 classifier comprising both a host gene and several hrHPV genotype genes seems to be currently performing better than methylation tests solely testing host genes. If study III's results can be replicated, that could change the outline of active surveillance protocols for CIN2 and perhaps even CIN on a broader scope. Further refinement of the DNA methylation assays is also a priority (Lorincz, 2016). Changes in methylation during follow-up of high-grade CIN could also be of interest.

Imiquimod presents an interesting option in treatment of HPV-related genital disease, which has historically been mostly surgical or destructive. The results from our randomised proof of principle pilot study on VAIN treatment are very preliminary and need to be validated in a larger group of patients with longer follow-up. If imiquimod would prove to be non-inferior to laser vaporisation, the current treatment of choice, it would provide a welcome alternative to repeated treatments in cases of recurrence. The optimal dose of imiquimod and treatment length need to be further explored, as well as the possibility of repeated imiquimod treatments.

7 CONCLUSIONS

The following conclusions can be drawn from this thesis

1. Current and future screening strategies should take into account the uneven distribution of HPV16/18-related high-grade cervical disease according to age in highly screened women.

2. Active surveillance instead of immediate treatment of CIN2 can be justified in selected young women who are willing to adhere to monitoring and whose personal preference, after adequate information on risks, is active surveillance.

3. The S5 DNA methylation classifier was able to differentiate between regressive and progressive CIN2. DNA methylation as a predictive biomarker for outcomes of cervical preinvasive disease should be further investigated and validated.

4. Self-administered imiquimod treatment may have potential as an efficacious option for laser vaporisation in high-grade VAIN, provided further studies show positive long-term results.

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10 ORIGINAL PUBLICATIONS