FACULTY OF MEDICINE AND HEALTH SCIENCES

Evidence Regarding Primary and Secondary Prevention of Human Papillomavirus Related Gynaecological Cancers

Lan Xu

Student number: 00914081

Supervisors:
Dr. Marc Arbyn[1]
Prof. Dr. Steven Weyers[2]

[1]Unit of Cancer Epidemiology/ Belgian Cancer Centre, Sciensano, Brussels, Belgium
[2]Department of Obstetrics and Gynecology, Ghent University Hospital, Ghent, Belgium

A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Doctor in Health Science
Faculty of Medicine and Health Science
Academic year: 2019 - 2020

UNIVERSITEIT GENT

Members of the examination committee:

Prof. dr. Olivier Degomme (Chair)
International Centre for Reproductive Health, Ghent University, Belgium

Prof. dr. Philippe Tummers
Department of Gynecology, Ghent University Hospital, Ghent, Belgium

Prof. dr.Koen Van de Vijver
Department of Pathological Anatomy, Ghent University Hospital, Ghent, Belgium

Prof. dr.Wei Hong Zhang
International Centre for Reproductive Health, Ghent University, Belgium

Prof. dr. Davy Van den Broeck
International Centre for Reproductive Health, Ghent University, Belgium

Prof. dr.Gilbert Donders
General Obstetrics and Gynaecology, Regionaal Ziekenhuis Heilig Hart Tienen, Belgium

dr.Johan Van der Heyden
Lifestyle and Chronic Diseases, Sciensano, Brussels, Belgium

Table of Contents

Abbreviations

AGC	Atypical glandular cells
AIN	Anal intraepithelial neoplasia
AIS	Adenocarcinoma in situ
ASC-H	Atypical squamous cells of undetermined significance, in which a high-grade squamous intraepithelial lesion cannot be excluded
ASC-US	Atypical squamous cells of undetermined significance
ASIR	Age standardized incidence rate
ASMR	Age standardized mortality rate
CIN	Cervical intraepithelial neoplasia
CIR	Cumulative incidence rate
CMR	Cumulative mortality rate
Cobas	Cobas 4800 HPV test
cNPV	Complement of the negative predictive value
DNA	Deoxyribonucleic acid
DTA	Diagnostic test accuracy
dVIN	Differentiated VIN
ECDC	European Centre for Disease Prevention and Control
GP5+/6+P-EIA	GP5+/6+ PCR-based enzyme immunoassay
GP5+/6+-LMNX	GP5+/6+ PCR-LMNX genotyping
HBRT-H14	Hybribio's 14 High-risk HPV with 16/18 Genotyping Realtime PCR
HC2	Hybrid capture 2
HDI	Human development index
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
hr	High-risk
HSIL	High-grade squamous intraepithelial lesions
HPV16/18	HPV types 16 and 18 jointly

IARC	International Agency for Research on Cancer
IDT	Integrated DNA Technologies
INNO-LiPA	INNO-lipa HPV Genotyping Extra II assay
LBC	Liquid-based cytology
Linear Array	Linear Array HPV Genotyping Test
LOD	Lower level of detection
lr	Low-risk
LSIL	Low-grade squamous intraepithelial lesions
MALDI-TOF	Matrix-assisted laser desorption-ionization time-of-flight.
NILM	Negative for intraepithelial lesions and malignancies
ORF	Open-reading frame
Pap	Papanicolaou
PCR	Polymerase chain reaction
PICOS	Population, intervention, comparison,outcome and Study type
PPV	Positive predictive value
pRb	Retinoblastoma protein
PV	Papillomavirus
RCT	Randomized controlled trials
RIATOL qPCR	RIATOL qpcr HPV genotyping assay
RNA	Ribonucleic acid
RR	Relative risk
SCC	Squamous cell carcinoma
SIL	Squamous intraepithelial lesion
SIR	Standardized incidence rate
SMR	Standardized mortality rate
URR	Upstream regulatory region
VALGENT	Validation of HPV genotyping tests
VALHUDES	Validation of human papillomavirus assays and collection devices for HPV testing on self-samples and urine samples

VaIN	Vaginal intraepithelial neoplasia
VC	Viral concentration
VE	Vaccine efficacy
VLP	Virus like particle
VIN	Vulvar intraepithelial neoplasia
WHO	World health organization
YLD	Years lived with disability
YLL	Years of life lost
2vHPV	Bivalent HPV vaccine
4vHPV	Quadrivalent HPV vaccine
9vHPV	Nonavalent HPV vaccine

Glossary

Randomized controlled trials	A type of medical experiment that aims to reduce certain sources of bias when testing the effectiveness of new treatments; this is accomplished by randomly allocating subjects to two or more groups, treating them differently and then comparing them with respect to a measured response. The trial may be blinded, in which information which may influence the participants is withheld until after the experiment is complete.
Cost-effectiveness	The extent to which an intervention or prevention programme is effective in relation to its costs, i.e. euro/life years gained.
Co-testing	A screening strategy with two tests :microscopic examination of cervical cells and HPV testing on these cells.
Vaccine efficacy	Percentage reduction of disease in vaccinated group of people compared to an unvaccinated group, using the most favorable conditions.
Vaccine effectiveness	Real-world reduction of disease in population due to vaccine with evidence coming from observational studies.
Systematic review	A review of the evidence on a clearly formulated question that uses systematic and explicit methods to identify, select and critically appraise relevant primary research, and to extract and analyse data from the studies that are included in the review.
Meta-analyses	Meta-analysis is a quantitative, formal, epidemiological study design used to systematically assess the results of previous research to derive conclusions about that body of research. Typically, but not necessarily, the study is based on randomized, controlled clinical trials.
Cochrane collaboration	Cochrane is a not-for-profit organization and global network of health and social care practitioners, researchers, patient advocates and others, with a mission to promote evidence-informed decision making by producing high quality, relevant, accessible systematic reviews and other synthesized research evidence (www.cochrane.org).
Phases of clinical research	Phases are steps in which scientists conduct experiments with a health intervention in an attempt to obtain sufficient evidence for a process which would be useful as a medical treatment.
Phase II trials	Testing of drug on patients to assess efficacy and side effects with therapeutic dose on 100-300 patients with specific diseases.
Phase III trials	Testing of drug on patients to assess efficacy, effectiveness, and safety with therapeutic dose on 300-3000 patients with specific diseases.
PRISMA	PRISMA is an evidence-based minimum set of items for reporting in systematic reviews and meta-analyses. PRISMA focuses on the reporting of reviews evaluating randomized trials but can also be used as a basis for reporting systematic reviews of other types of research, particularly evaluations of interventions.
PICOS	A framework which is a mnemonic used in evidence-based medicine to frame and answer a clinical or health care related questions. It is also used to develop literature search strategies,

CHAPTER 1

1. General introduction and outline

1.1 Human papillomavirus

Human papillomaviruses (HPV) are small, non-enveloped, double-stranded viruses that belong to the papillomaviridae family and commonly infect mucosal or cutaneous epithelia in humans [1]. So far, over 200 HPV types have been identified and grouped in five genera indicated by a Greek letter (Alpha, Beta, Gamma, Mu, and Nu), this based on their nucleotide sequences and tropism to human epithelial tissues [2-4]. Although the papillomaviriadae family represents a highly diverse group of viruses, they share the same genome organization (**Figure 1.1**). The HPV viral genome is about 8000 base-pairs in length contains a non-coding upstream regulatory region (URR) on one strand and eight open-reading frames (ORFs) on the other strand, which are grouped into early and late genes. The early genes (E1, E2, E4, E5, E6 and E7) encode proteins that are necessary for viral cycle and cell transformation, whereas late genes (L1 and L2) encode two structural capsid proteins [5].

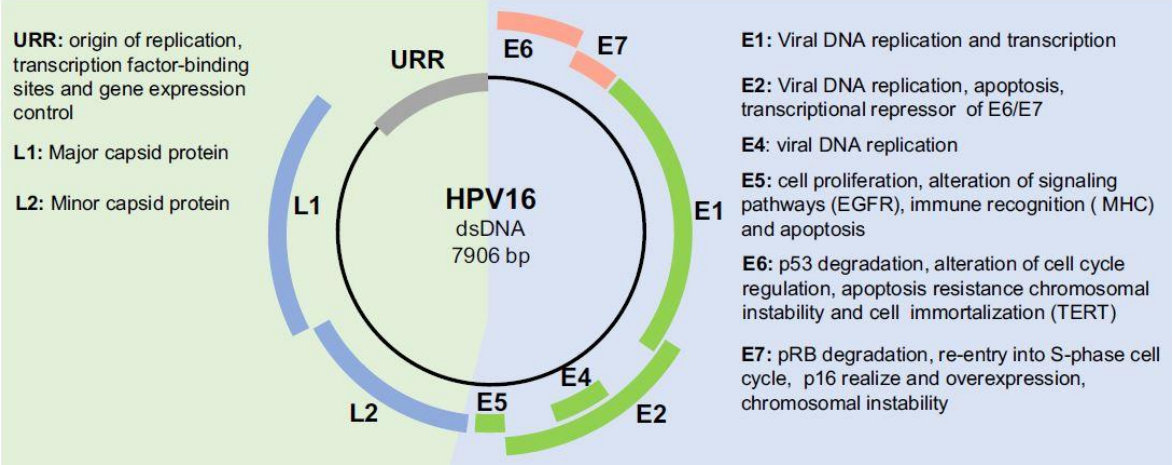


Figure 1.1 Genomic structure of HPV16, reproduced from de Sanjose,et al, 2018 [6]

Approximately 40 HPV types from Alpha genus are known to infect the genital mucosa [2]. According to the International Agency for Research on Cancer (IARC), HPVs are divided into high-risk (hr), probably/possibly carcinogenic types and low-risk (lr) based on their oncogenic potential [7]. 12 hrHPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52 and 58) are considered to be causally linked with cervical cancer and their immediate precursors. Several types (HPV26, 53, 66, 67, 69, 70 etc.) are considered to be 'possibly carcinogenic' and HPV68 is considered as 'probably carcinogenic'.HPV6 and HPV11 classified as lrHPV can cause benign diseases including genital warts and papillomas [8] .

HPV16 has the highest oncogenic potential and is the most frequently detected type in invasive cervical cancer, followed by HPV18 [9]. The HPV types 16 and 18 jointly (HPV16/18) cause more than 70% of all cervical cancers worldwide [10]. Moreover, HPV16 also causes a large proportion of all the other HPV-related, non-cervical cancers. The next most important five types (HPV31, 33, 45, 52 and 58) together with HPV16/18 are causally linked with approximately 90% of cervical cancers [11].

1.2 HPV infection and cancer development

HPV infects both men and women and is mainly transmitted through sexual contact [6, 12, 13]. The burden of cancers attributable to HPV infection is much higher in women due to high susceptibility to HPV infection in cervix uteri [14]. Although most women will at some time have been infected with HPV (an estimated lifetime risk of 80%) [15, 16], most (about 80%) of these infections clear spontaneously without clinical symptoms within 12 to 24 months [17, 18]. Only few persistent carriers of hrHPV types will progress to invasive cervical cancer, and some rarer cancers of non-keratinized mucosa and skin of the lower genital tract (vagina, vulvar and penis), the anus and the oropharynx [19].

The concept of HPV-mediated carcinogenesis consists of several phases, starting with HPV infection when the virus enters the basal layer of the epithelial cell through micro-abrasions, transition of productive (permissive) to transforming (non-permissive) HPV infection, development of precancerous lesions and finally progression to invasive cancer [20]. Cervical infections are the best model to understand the natural history of HPV infection and anogenital malignant [21] (**Figure 1.2**).

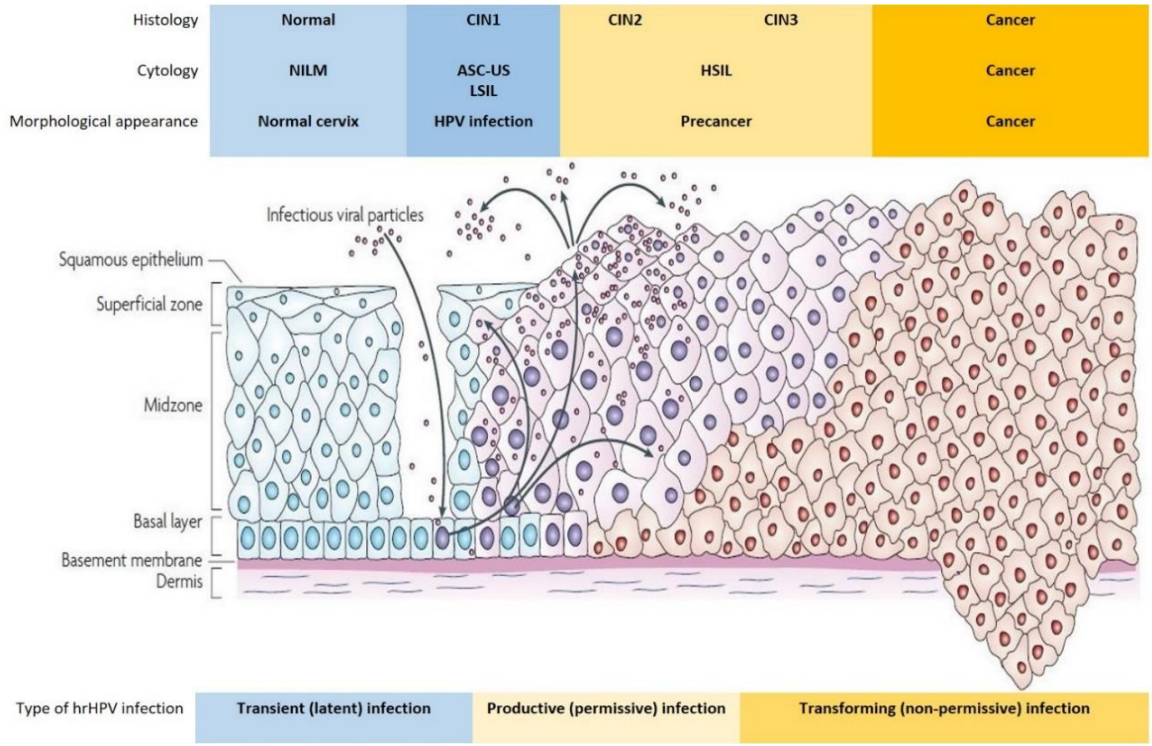


Figure 1.2 The schematic presentation of the HPV-mediated progression of cervical cancer and the histological and cytological terminology for potential morphological changes (adapted from Woordman et al., 2007 [5]). Histology system for squamouse intraepethlial lesion includes cervical intraepethelial neoplasia (CIN) grade 1 (CIN1), grade 2 (CIN2) and grade 3 (CIN3); The Bethesda System for abnormal cytological findings includes negative for intreapethelial lesion and malignancy (NILM), atypical squamous cells of undetermined significance (ASC-US), low-grade squamous intraepithelial lesions (LSIL), atypical squamous cells, cannot exclude a

high-grade lesion (ASC-H), atypical glandular cells (AGC), high-grade squamous intraepithelial lesions (HSIL), and Adenocarcinoma in situ (AIS).

Cervical cancer develops mainly in the transformation zone in cervix uteri at the junction between the outer part (the ectocervix, a multilayer squamous epithelium) and the inner part (the endocervix, a single layer of glandular columnar epithelium) (**Figure 1.3**)

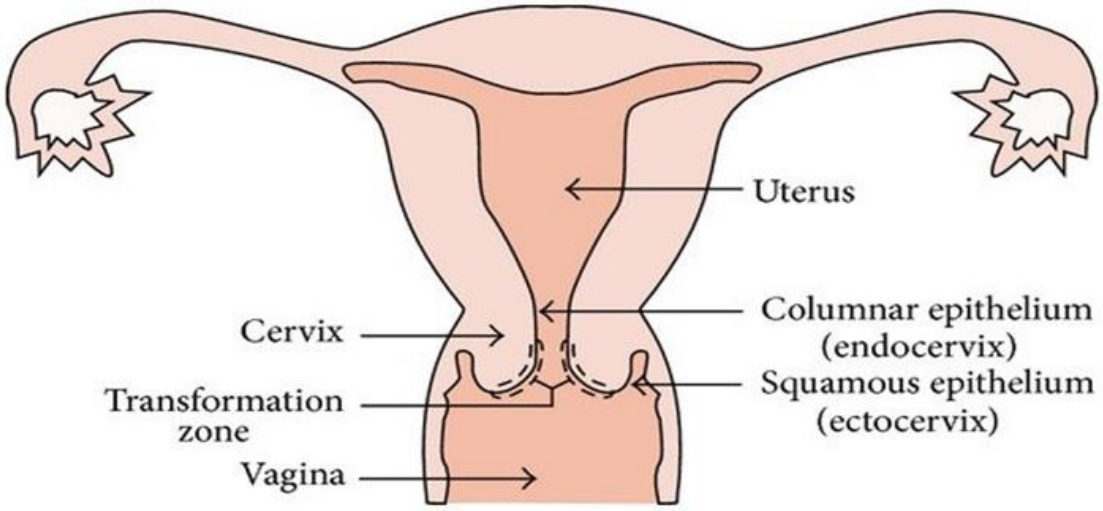


Figure 1.3 Anatomy of the uterus and cervix (adapted from Bengtsson et al. 2014[22]).

Infection with HPV is the main cause of cervical cancer, other risk factors may act together with HPV infections to increase the risk even more, including smoking, having a weakened immune system, becoming sexually active at a young age, having many sexual partners, chlamydia infection, long-term use of oral contraceptives, having multiple full-term pregnancies and people living in low economic status.

In this thesis, we used the histopathology-based cervical intraepithelial neoplasia (CIN) terminology [23] to describe the severity of cervical precancerous lesions, whereas the squamous intraepithelial lesion (SIL) terminology of The 2001 Bethesda System was used to describe cytological lesions [24]. A conversion table for different cytological and/or histological classification systems used to grade cervical (pre-)cancerous lesions is presented in supplementary information **Table 1.S1**.

Productive infection may give rise to productive CIN lesions. CIN1 or CIN2 are not considered as actual cervical precancer and frequently spontaneously regress within 1-2 years [20]. However, transforming infections may lead to CIN3 and cervical cancer. A transforming infection occurs when there is a deregulation in the expression of the viral E6 and E7 oncoproteins resulting in a deregulated cell cycle [25]. The E6 oncoprotein targets the p53 tumor suppressor protein, thereby interfering with p53-apoptosis and cell cycle control mechanisms [26, 27]. The E7 oncoprotein targets the retinoblastoma tumor suppressor protein (pRB) and leads to increased E2F activity with consequently uncontrolled cell proliferation. In

addition, complex formation of E6 and E7 with other cellular proteins does also contribute to the virus-mediated transformation process [28, 29]. Both oncoproteins can modulate the deoxyribonucleic acid (DNA) methylation machinery, thereby influencing cellular and viral gene expression [20].

CIN lesions generally regress, but the likelihood of progression to invasive cancers increases with the severity of the lesion and the duration of HPV infection. The progression from initial HPV infection to invasive cervical cancer usually takes 15-30 years [30, 31].

1.3 HPV prevalence

1.3.1 HPV infection in cervical site

For women with normal cytological results (NILM), the prevalence of 'active' HPV infection is estimated as 11.7% (95% CI: 11.6-11.7) globally. However, most HPV infections (70-90%) are asymptomatic and transient and will be cleared by host immune system within 1 to 2 years. HPV prevalence peaks in women less than 25 years (24%, 95%CI: 23.5-24.5) and then declines in older population. Sometimes, a modest second peak of HPV infection is observed in older women [32-34].

In women with normal cytology, HPV16 is the most frequent oncogenic type (3.2%), followed by HPV18 (1.4%), HPV52 (0.9%), HPV31 (0.8%), and HPV58 (0.7%). In women with abnormal cytological findings, HPV prevalence increases with lesions severity. HPV is detected in 52.5% (51.6-53.3%) of ASC-US lesions, 74.8% (74.3-75.3%) of LSIL lesions, and 88.9% (88.5-89.3%) of HSIL lesions. HPV16 is the most commonly detectable type in all grades of precancerous lesions, together with HPV18 accounts for approximately 71% of all cervical cancer cases [10, 35].

1.3.2 HPV infection in other anogenital sites

Less information is available on HPV prevalence in other anogenital sites (vulva, vaginal, anus and penis), but growing evidence is available suggesting that HPV infection is a risk factor for these cancers as well [7, 34].

The prevalence and type distribution of HPV in vulvar precancer and cancer have been summarized in systematic reviews and meta-analyses [36, 37]. HPV-related vulvar intraepithelial neoplasia (VIN) lesions are categorized as low-grade squamous intraepithelial lesions (LSIL, VIN1) and high-grade squamous intreapithelial lesions (HSIL, VIN2/3) [38, 39]. HPV prevalence is approximately 40% in vulvar cancer and 83% in vulvar HSIL [36]. HPV16 and 18 infection together account for 93% of HPV-related vulvar cancers [37].

It is generally accepted that HPV-related vaginal cancers and cervical cancers share similar risk factors. Therefore, vaginal cancer has often been treated similarly to cervical cancer [40]. Low-grade and high-grade vaginal intraepithelial neoplasia (VaIN) are classified as LSIL (VaIN1) and HSIL (VaIN2/3), respectively. The prevalence of HPV in vaginal cancer is about 78% [41]

and in VaIN2/3, it is estimated to be 91% [34]. HPV16 is the most common type found in vaginal cancer [12, 41].

Anal HPV infection can be detected in both women and men. HPV prevalence is high in women with history of cervical or vulvar cancer, in men having sex with men (MSM) and in some immunosuppressed populations, including those who are positive for human immunodeficiency virus (HIV) and patients with a history of organ transplantation [34]. HPV is detected in 92.7% of anal intraepithelial neoplasia (AIN) with a breakdown by grade of 91.5% in LSIL (AIN1) and 93.9% in HSIL (AIN2/3) [37]. HPV16 (73%) is the most frequently detected type in HPV-positive anal cancers and HPV18 is the second most common type and is found in about 5% of cancer cases [34].

About 50% of all penile cancers are associated with HPV infection [12]. Among HPV-related penile cancer patients, again, HPV16 is the most frequently detected type, followed by HPV18 and low-risk types HPV6 and HPV11 [42]. It is estimated from a cohort study that the median time to clearance of hrHPV types is 7.2 months and 12.2 months for HPV16 [43] .

1.3.3 Oral HPV infection

Detection of oral HPV infection is associated with some cancers of the oropharynx, oral cavity and larynx. The proportion of oropharyngeal cancers (mainly comprises the tonsils and base of the tongue sites) that are HPV-positive has increased over the last decades [44]. Globally, HPV16 is considerably the most prevalent type, accounting for 82% of all HPV positive cases, followed by HPV18 and a minority of other types [45]. For cancers of the oral cavity and larynx, the prevalence of HPV, which is much less frequent than in oropharynx cancer cases, is approximately 4% at both sites [13].

1.4 Burden of HPV-related anogenital cancer

In 2012, HPV has been identified as the cause of approximately 4.5% of new cancer cases (640,000) worldwide [41].

1.4.1 Burden of cervical cancer

Approximately 570,000 new cases of cervical cancer and 311,000 new death from the disease occurred in 2018 [46]. Cervical cancer ranks the fourth most common female cancer and the fourth leading cause of cancer death worldwide [47]. It is estimated that cervical cancer caused 7 million (95% CI, 6.5-7.4 million) disability-adjusted life-years (DALYs) in 2015, with 96% from years of life lost (YLLs) and 4% from years lived with disability (YLDs) [48].

An age-standardized incidence (ASIR) rate of 13.1 cases per 100,000 women was observed with considerable variations between countries (**Figure 1.4**). ASIR ranges from 2 to 75 per 100,000 women [46]. About 84% of all cervical cancers and 88% of all deaths caused by cervical cancer occurred in less developed countries (those with a human development index [HDI]<0.80). In the more developed regions, cervical cancer accounts for less than 1% of all

cancers in women [47]. China accounts for the highest number of new cases (106,000) whereas India accounts for the highest estimated number of deaths (60,000), respectively [46] (**Figure 1.5**).

Not surprisingly, HPV16 is the most prevalent type across all regions, detected in 60.5% (59.5 - 61.6) of cases of cervical cancer [34, 35]. Followed by HPV18, HPV45, HPV31, HPV33, HPV53 and HPV58 with variations in regions. Notably HPV16 is detected more often in squamous cell carcinoma (SCC) (61.7%) than in adenocarcinoma (50.0%) cases, while HPV18 and HPV45 are detected more frequently in adenocarcinoma (32.3% and 11.9%, respectively) than in SCC (8.3% and 5.4%, respectively) cases [35].

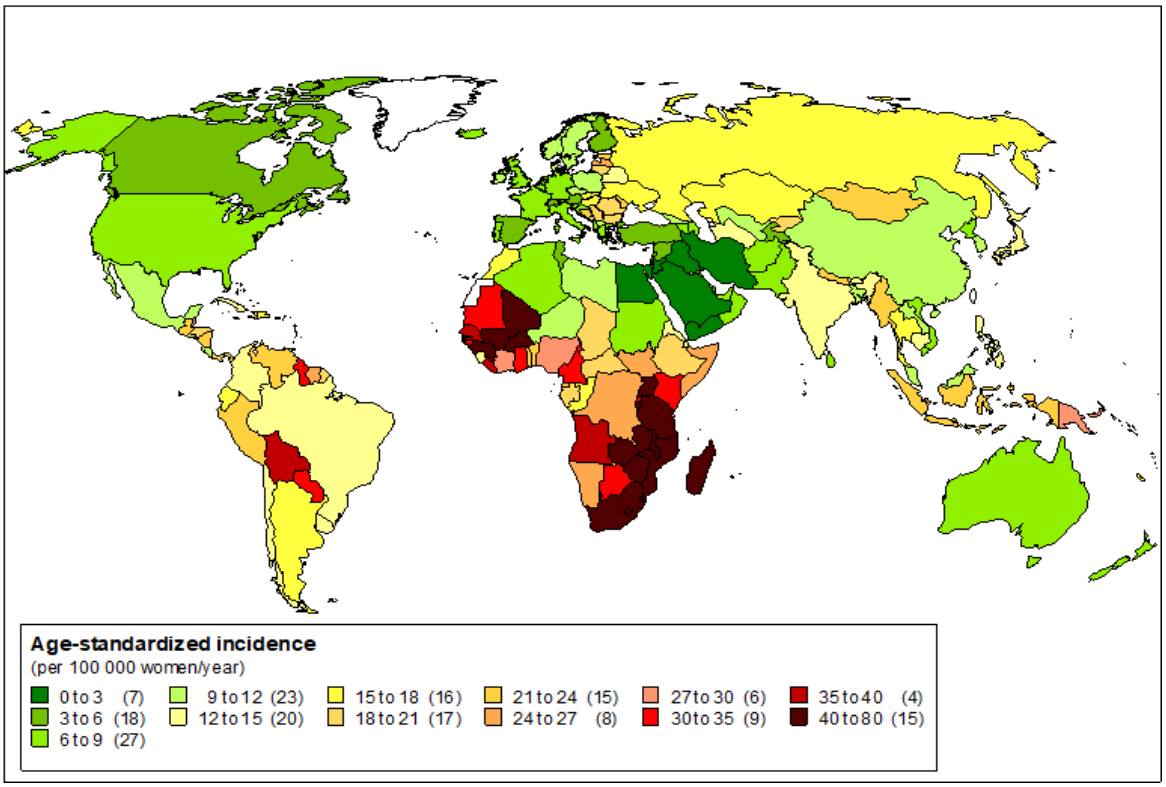


Figure 1.4 Geographic distribution of the world-age-standardised incidence rate (ASIR) of cervical cancer, by country, estimated for 2018 (per 100,000 women-years). Reproduced with permission from Arbyn et al. 2020 [46].

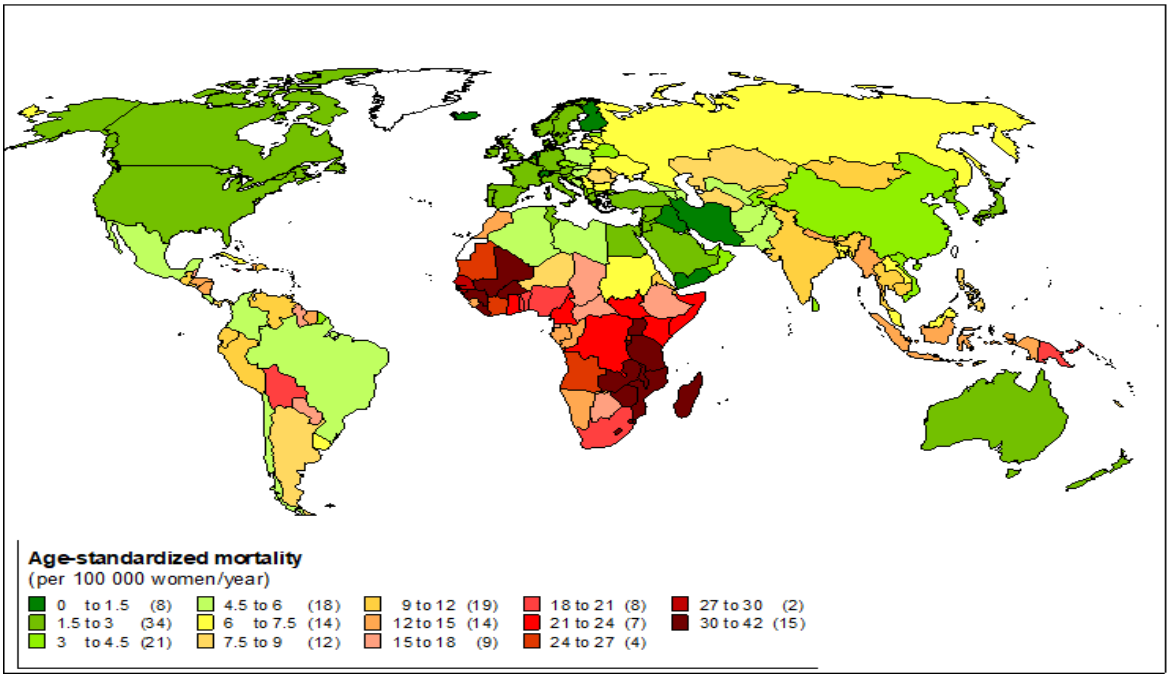


Figure 1.5 Geographic distribution of the world-age-standardised mortality rate of cervical cancer (ASMR) by country, estimated for 2018 (per 100,000 women-years). Reproduced with permission from Arbyn et al. 2020 [46].

1.4.2 Burden of other HPV-related anogenital cancers

Globally, an estimated 34,000 and 15,000 new vulva and vaginal cancer cases are diagnosed annually, with respectively 8,500 and 12,000 cases attributable to HPV [13]. There are about 40,000 new anal cancer cases occurring every year. About 35,000 of the new cases are considered to be caused by HPV infection, with 17,000 cases in men and 18,000 cases in women [13, 41]. The annual burden of penile cancer is estimated to be 26,000 with half of the cancer cases attributable to HPV (13,000 cases) [13].

The highest ASIR of HPV-attributable anogenital cancers other than cervix is observed in America and Australia, while most of the cases are accumulated in India (10,500), China (12,400) and Europe (14,600) [13] .

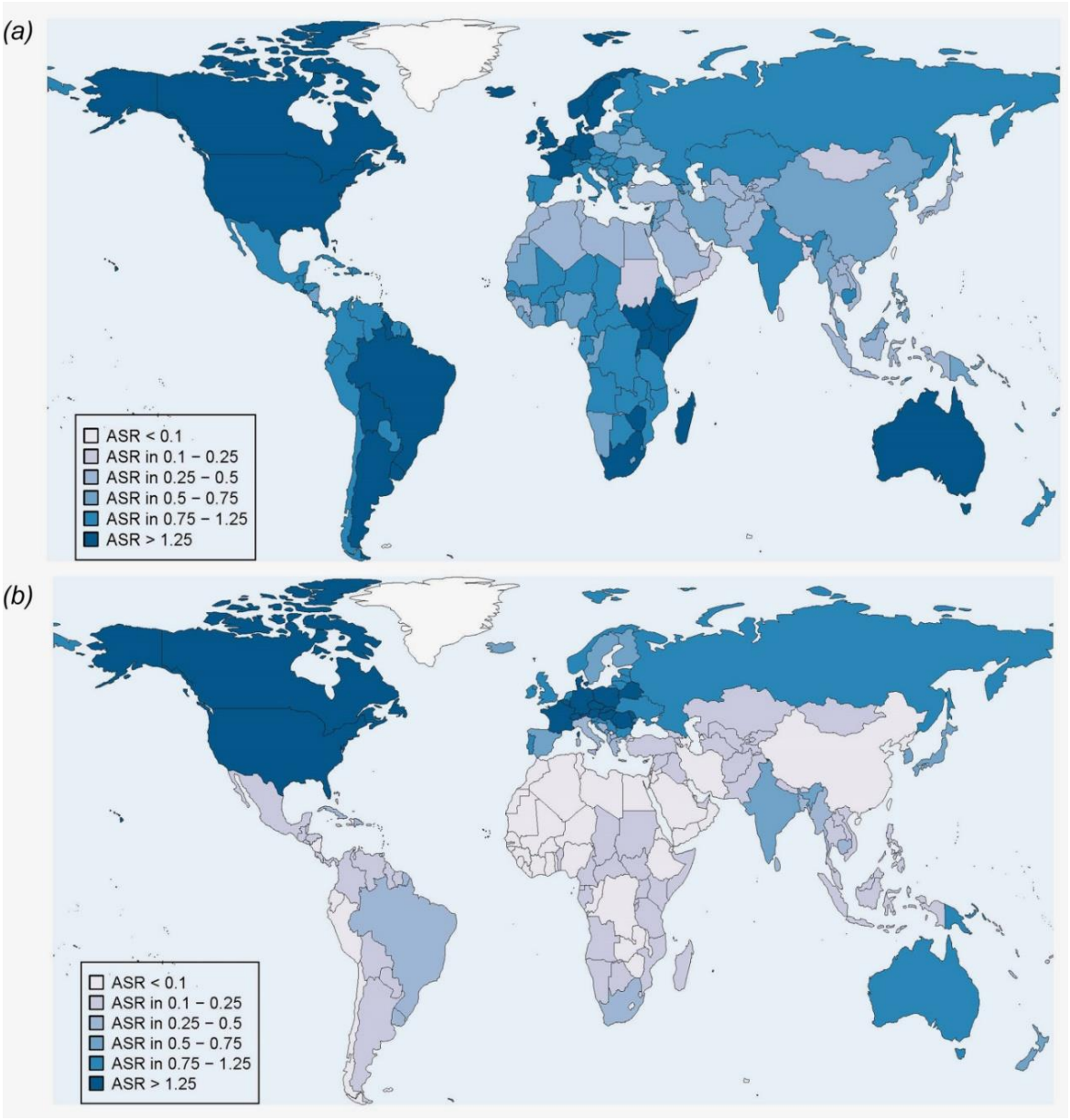


Figure 1.6 Age standardized (world) incidence rates (per 100,000) of cancer cases attributable to HPV in 2012, both sexes. Panel (a) Anogenital cancer cases (vulvar, vaginal, anal and penile). Panel (b) Head and neck cancer cases (oropharynx, oral cavity and larynx). Reproduced with permission from de Martel et al, 2017 [13].

1.4.3 Burden of head and neck cancer

Globally, approximately 456,000 head and neck cancers cases are diagnosed each year, among which 37,200 cases are attributable to HPV. To be more specific, 29,000 in the oropharynx, 4,400 in the oral cavity and 3,800 in the larynx. Around 30% of oropharyngeal cancers are caused by HPV which is higher than the prevalence of HPV in oral cavity and larynx cancers. Most of the HPV-related head and neck cancer cases are diagnosed in more

developed regions, with the highest age standardized incidence rates observed in Northern America and Europe [13].

1.5 Prevention strategies of HPV-related cancers

Cancer prevention strategies are usually existing at three levels: primary, secondary and tertiary prevention [49]. Primary prevention focuses on preventing the onset of disease or disorder, by reducing risk factors or providing protection in healthy individuals. Secondary prevention aims to detect and treat diseases in an early and asymptomatic stage in order to prevent progression (e.g. screening, early detection and effective treatment). Tertiary prevention aims to reduce the burden of disease for individuals with established clinical stage disease. All levels of cancer prevention strategies must be synthesized into a comprehensive framework in which both high- and low-resource settings should be taken into account for cost-effective and sustainable implementation in different countries [19].

Knowledge on the strong causal association of HPV infection and cervical cancer has led to the development of prophylactic HPV vaccines (primary prevention) and HPV assays to detect cervical precursors (secondary prevention).

1.5.1 Primary prevention: prophylactic HPV Vaccination

Prophylactic HPV vaccines aim to prevent initial HPV infection and subsequent lesions and are designed based on the concept that the major structural L1 gene of HPV automatically folds into non-infectious and non-oncogenic virus-like particles (VLPs) which mimics the viral capsid (shell) so that antibodies are induced [50]. Currently, three HPV vaccines are licensed: a bivalent (2vHPV) vaccine containing the L1 protein of HPV16 and HPV18 (Cervarix®, GSK), which are the two most carcinogenic HPV types and are estimated to cause approximately 70% of all cervical cancers worldwide [51]; a quadrivalent (4vHPV) vaccine (Gardasil®, Merck) containing the L1 protein HPV16 and HPV18, and in addition also two low-risk HPV types of HPV6 and HPV11 (therefore also protects against the majority of genital warts) [52, 53]; a nonavalent (9vHPV) vaccine (Gardasil-9®, Merck) targeting the same HPV types as the 4vHPV vaccine plus five other high-risk types of HPV31, HPV33, HPV45, HPV52 and HPV58 [54, 55]. For all three vaccines, the vaccination schedule depends on the age of the vaccine recipient. A 2-dose schedule (0, 6 months) is recommended for girls and boys 9 to 14 years old, and a 3-dose schedule (0,2,6 months) is recommended for persons starting the HPV vaccination series on or after the 15th anniversary, and for those known to be immunocompromised and/or HIV-infected.

Large randomized controlled trials (RCTs) have shown HPV vaccination to be effective and protect against vaccine-type related ano-genital precancer in young females and males [51] [52, 53] [54-58] . HPV VLP vaccines are generally safe with minor injection-site symptoms, no increased risk of serious adverse effects have been found [50].

So far, the documented duration of protection from infection and cervical lesions caused by HPV16/18 with the bivalent vaccine in a 3-dose schedule is 9.4 years. For 4vHPV vaccine, the duration of protection for vaccine-type HPV-related cervical, vulvar, vaginal lesions and genital diseases in a 3-dose schedule has been demonstrated for at least 10 years, while for the 9vHPV vaccine this is 5.6 years [59]. It is expected however that the duration of protection of all vaccines is much longer, possibly life-long.

1.5.2 Secondary prevention: screening

Screening is an important secondary prevention strategy with the aim to detect disease at an early stage in asymptomatic healthy population. The progression of CIN to cervical cancer is slow and CIN lesions can be detected relatively easy and can be treated effectively, thus making the secondary prevention of cervical cancer via screening possible [17, 31].

A wide range of suitable screening tests are available for cervical cancer. The traditional method is the Papanicolaou (Pap) smear cytology, involves microscopic examination of the exfoliated cells collected from the transformation zone of the cervix and staining them with Pap stain done by trained pathologists. The test was first introduced by George Papanicolaou in 1949 and was accepted as a screening tool in many countries since 1960s. Liquid-based cytology improves the quality of smears allowing faster reading of the slides and less inadequate smears. Well-organized screening programmes that use cytology-based screening for cervical cancer have reduced the incidence of cervical cancer substantially in several developed countries [60, 61]. However, despite its success, cytology-based screening has some limitations, including limited sensitivity and low reproducibility of the morphological reading of the test results [62]. Moreover, the management of women with equivocal or mildly abnormal cytological lesions can be quite challenging. As women with equivocal cytological diagnoses such as ASC-US or LSIL have a small but significantly increased risk of developing cervical cancer compared to women with normal smears and the challenge is to discriminate accurately between those having underlying or incipient cervical cancer precursors and those can be released safely back to routine screening programmes. The natural history of minor cytologic lesions is difficult to predict on the basis of cytomorphologic grounds which often regress spontaneously and do not require treatment. Referring all women with equivocal or mildly abnormal cytological lesions for further examination would mean an increase in over-diagnosis and overtreatment [63].

In order to overcome these limitations in cytology-based screening, HPV-based screening was introduced and is being implemented in more and more primary screening programmes worldwide. Evidence to support this paradigm shift is mainly coming from the four large-scale European RCTs, showed substantially lower cumulative incidence of cervical cancer and precancer after a negative HPV test than after a negative cytology result [64].

In practice, the effectiveness of screening protocols depends not only on the clinical performance of the screening test, but also on the attendance rate, the adequacy of follow-up algorithms, and the availability and effectiveness of treatment [65]. HPV testing can also be done on a vaginal sample taken by women themselves. Previous meta-analyses on accuracy

of self-sampling has showed that polymerase-chain0reaction (PCR)-based HPV testing on self-samples was as sensitive and specific as on clinician taken cervical samples [66, 67]. By offering HPV self-sampling to women who do not participate in routine screening or who are reluctant for gynaecological examination, the attendance rates were increased [67, 68]. Few pathologies other than cervical cancer has such a wide range of means of prevention, moreover no high-quality screening programs are currently available to prevent HPV-related cancer other than cervical cancer in women [69] [70].

1.5.3 Tertiary prevention: treatment of cancers and precancer

As cervical cancer development passes over a long period taking a few years up to more than a decade precancerous lesions, early detection and treatment at this stage can prevent the majority of cervical cancers. Currently, invasive cervical cancer is treated by surgery and/or radiotherapy. Chemotherapy can complement the treatment regime in late stages [71, 72].

The treatment for vulva, vaginal and anal cancer is often involved 1 or a combination of treatments: surgery, radiation therapy, and/or chemotherapy. Treatment options and recommendations depend on several factors, including the type and stage of cancer, possible side effects, and the patient's preferences and overall health.

For the many women who have already been infected by HPV or developed HPV-related cancers, therapeutic HPV vaccines may be a solution. Currently, many candidate therapeutic HPV vaccines are under development or being evaluated in clinical trials, although no breakthrough has been reached yet.

1.6 Objective and overview of methodology

The main objective of this thesis is to synthesize evidence regarding primary and secondary prevention of cervical cancer and other HPV-related cancers, to conduct systematic reviews to update and extend current evidence in the field with new technologies and strategies. This thesis also aims to conduct primary research studies eligible to be included in meta-analyses to guide current clinical practices related to prevention of HPV-related gynaecological cancers.

1.6.1 Systematic review and Meta-analysis

Systematic reviews and meta-analyses are essential source of information for evidence-based medicine [73], which is a process of integrating the best evidence for the development of evidence-informed guidelines and policy [74]

Cochrane Collaboration (www.cochrane.org) is an international, independent, not-for-profit organization that promotes and supports systematic reviews evaluating health-care interventions and diagnostics tests accuracy and facilitates evidence-based clinical decision-making. Cochrane also develops methodologies for summarizing and reporting evidence, currently publishes five main types of systematic reviews and meta-analyses: (1) reviews of the

effects of interventions; (2) reviews of diagnostic test accuracy (DTA); (3) reviews of prognosis; (4) overview of reviews and (5) reviews of methodology.

In this thesis, we mainly focus on the intervention reviews and DTA reviews and the methodology developed by the Cochrane Collaboration for systematic reviews and meta-analyses were followed [74, 75]. A systematic review starts with a comprehensive literature search based on a structured search strategy targeting a research question for pre-defined PICOS, which stands for population, intervention, comparison, and outcomes according to the Cochrane Collaboration. Meta-analysis, which is a subset of systematic reviews, is a statistical procedure used to synthesize quantitative findings from all selected studies to provide more precise estimates of a treatment effect or the accuracy of a diagnostic test, or other outcomes, than any individual studies included in the pooled analysis [76]. The variation across studies (heterogeneity) must be considered, and possible sources of heterogeneity should be discussed [75]. Moreover, the PRISMA (Preferred Reporting Items for Systematic reviews and Meta-analyses) statement for reporting of meta-analyses and other recommendations developed by Cochrane Collaboration for systematic review were also followed [77].

1.6.2 Validation of HPV assays

As mentioned in section 1.5.2, currently HPV-based screening is being incorporated in more and more screening programmes worldwide. As a result of that, the number of commercially available HPV assays has increased substantially in the past decade. Nowadays, over 254 HPV assays and at least 425 test variants are available on the global market [78], however, the clinical performance of these assays differ significantly and only few have been clinically validated.

Only clinically validated HPV assays can be used in primary cervical cancer screening [79]. The Hybrid Capture 2 assay (HC2; Qiagen, Hilden, Germany) [64, 80-82] and GP5+/6+ PCR-based enzyme immunoassay (GP5+/6+-EIA; Diassay, Rijwijk, the Netherlands) [83-85] are HPV DNA assays that had been clinically validated for primary screening based on longitudinal evidence obtained from large RCTs. Therefore, HC2 and GP5+/6+-EIA are accepted as the standard comparator tests in evaluations of alternative HPV tests [86]. Based on this, a team of HPV experts in virology, molecular biology, statistics, and epidemiology has developed guidelines for hrHPV test requirements for primary cervical cancer screening and validation guidelines for candidate HPV assays [86]. These guidelines, also known as Meijer guideline, have been world-widely adopted and have generated lists of approved new HPV tests accepted for use in cervical cancer screening [86]. To be more specific, Meijer guideline states that the clinical sensitivity of a candidate HPV assay for detection of CIN2+ should not be lower than 90%, and the clinical specificity for CIN2+ not lower than 98%, compared the GP5+/6+ -EIA or the HC2 assay. Before a new HPV assay can be used for screening purposes, non-inferiority compared to the GP5+/6+-EIA or the HC2 should be shown.

Currently, 11 HPV assays have been proven to fulfil these criteria and are therefore considered as clinically validated for primary cervical screening [79]. A list of the validated assays together with their characteristics and genotyping capacity is summarized in **Table 1.1**

Table 1.1 Characteristics of hrHPV tests validated using Meijer guideline.

	HrHPV assay	Nucleic acid targeted	Type of amplification	Genes Targeted	Separate genotyping	Internal control for human genes
Standard comparator tests						
*	HC2	DNA	Signal	Several (undefined)	No	No
*	GP5+/6+ -EIA	DNA	Target	*L1*	No	No
Fully validated assays						
1	Abbott RealTime High Risk HPV test	DNA	Target	*L1*	16,18 & 12 other hr types	*β-globin*
2	Onclarity HPV assay	DNA	Target	*E6/E7*	16,18,31,45,51,52;33-58; 56-59-66; 35-39-68	*β-globin*
3	Cobas 4800 HPV test	DNA	Target	*L1*	16,18 & 12 other hr types (see HC2+ 66)	*β-globin*
4	HPV-Risk assay	DNA	Target	*E7*	16, 18& 13 other hr types (see HC2+66&67)	*β-globin*
5	PapilloCheck HPV-screening test	DNA	Target	*E1*	16,18,31,33,35,39,45; 51, 52, 53, 56, 58, 59, 66, 68; 70, 73, 82; 6, 11, 40, 42, 43, 44.	*ADAT1*
&	APTIMA HPV assay	RNA	Target	*E6/E7*	No	*No*
Partially validated assays						
6	GP5+/6+-LMNX	DNA	Target	*L1*	Separate typing of 16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68; Phr types:26,53, 73, 82.	Human DNA fragment located on chromosome 14
7	Riatol qPCR HPV genotyping assay	DNA	Target	*E6/E7*	Separate typing of 16,18,31,33, 35, 39, 45, 51,52,56,58,59,66,68; phr53 and lrHPV6,11.	*β-globin*
8	Cervista HPV HR Test	DNA	Signal	*L1/E6/E7*	14 hr types. Separate typing 16,&18.	human *histone 2*
9	MALDI-TOF	DNA	Target	*L1*	Separate typing of hr HPV types 16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68.	*β-globin*

hrHPV, high-risk human papillomavirus; *ADAT1* gene, adenosine deaminase tRNA specific 1; phr, potentially high-risk; qPCR, quantitative PCR; MALDI-TOF, matrix-assisted laser desorption-ionization time-of-flight.
***** HC2 and GP5+/6+ -EIA are clinically validated in randomised efficacy trials and therefore used as standard comparator tests to validate other HPV assays.
&APTIMA HPV assay targeting E6/E7 mRNA, however, Meijer guideline was set up for HPV DNA assay. Adapted from Arbyn et al., 2015[79].

The Meijer guideline has been beneficial for translation of HPV testing into clinical practice. However, one of the difficulties for validation of assays is obtaining samples from representative populations. The VALGENT (Validation of HPV Genotyping Tests) framework facilitates the comparison and validation of HPV tests by providing a set of samples derived from women attending routine screening, enriched with cytological abnormal samples. To allow comparison with other HPV assays, each VALGENT panel includes a clinically validated comparator assay [87]. VALGENT started in 2012 as a joint effort between HPV molecular biologists and clinical epidemiologists. As from VALGENT-2, standardized operational procedures in sample collation and processing to internationally approved recommendations for HPV test validation were applied.

In VALGENT-1, samples were provided by the Algemeen Medisch Laboratorium (AML) laboratory in Belgium with mixed opportunistic and organised screening. However, since VALGENT-2 sample collection was always nested within an organised screening programme, using well-annotated specimens, freshly collected or archived from existing cervical cytology biobanks, linked to comprehensive screening and pathology registries which is undoubtedly considered as a strength of the VALGENT framework. By selecting continuous samples from women attending screening enriched with a controlled selection of abnormal samples, both statistical power and representativeness can be assured which are crucial for clinical validation of HPV assays for primary screening. Moreover, by linkage with outcomes from subsequent screening rounds, final disease status may be obtained allowing for longitudinal assessment.

1.7 Outline of the thesis

1.7.1 Part I: Primary prevention of HPV-related diseases: prophylactic HPV vaccination

The first part of this thesis focuses on the evidence on efficacy and safety of prophylactic HPV VLP vaccines derived from RCTs.

Several phase II and phase III trials have been conducted and many reviews have tried to summarise the results [88-99]. However, none of them combined information on all the available endpoints. Vaccine efficacy is different when evaluating for different exposure groups (participants with no hrHPV infection, with no HPV infection of HPV types included in the vaccines, or regardless of HPV infection status at enrolment) and different age groups (young or mid-adult participants). Information on adverse effects associated with the administration of HPV vaccines are of particular importance for health professionals and general population. Clinical trials were primarily designed to demonstrate efficacy against cervical precursors.

In **chapter 2**, we evaluate the harms and protection of prophylactic HPV vaccines against cervical precancer and HPV16/18 infection in adolescent girls and women.

Contents in Chapter 2 have been published as part of a full Cochrane review on 7 May 2018 [100] and a summary of important finding of this review was published afterwards in *Expert Review of Vaccines [101]* in which safety and primary outcomes on clinical efficacy of 2vHPV

and 4vHPV vaccines against HPV infection and associated cervical precursors were included. Other reviews have assessed protection against ano-genital infection and lesions in males [58] and genital warts in female [102]. However, the efficacy of HPV vaccines against vulvar and vaginal cancers and their precursors have not been previously assessed.

In **chapter 3**, the published phase II and phase III literature on the efficacy of HPV prophylactic vaccines against vulvar and vaginal cancer and their precursors were systematically evaluated.

1.7.2 Part II: Secondary prevention of cervical cancer: clinical validation of HPV genotyping assay for primary screening

Few pathologies other than cervical cancer has high-quality screening programs available at the level of secondary prevention [69, 70]. The second part of this thesis focuses on cervical cancer screening strategies in HPV-based scenarios, more specifically, validation of HPV tests and cut-off optimisation assuring HPV genotyping assays fulfil requirement to be accepted for use in primary cervical cancer screening.

Only clinically validated HPV tests should be used in HPV-based primary screening setting. Although a substantial number of HPV assays are available on the market, few of them have been clinically validated for use. One of the difficulties is finding appropriate test specimen representative for primary screening [87]. The VALGENT framework facilitates the comparison and validation of HPV genotyping assays for clinically relevant outcomes using sample-populations appropriate for primary cervical cancer screening. The VALGENT framework contains several iterative sample panels collated in countries with organized cervical cancer screening programs. Following the international validation guidelines, each VALGENT panel includes a standard comparator assay [86].

The Roche Linear Array HPV genotyping test (Linear Array; Roche Molecular Diagnostics, Branchburg, NJ, USA) is a frequently used HPV genotyping tests, which enables consensus and type-specific detection of 37 HPV types. The Linear Array has not been validated previously to the international validation guideline.

In **chapter 4**, using the VALGENT-3 sample collation, the clinical performance of the Linear Array was evaluated in comparison to the standard comparator assay (HC2) and verified whether the Linear Array fulfils the requirements for use in screening. Additionally, we compared the analytical performance of the Linear Array for partial genotyping with that of another clinical validated hrHPV DNA assay, Abbott RealTime High Risk HPV test.

The INNO-LiPA HPV Genotyping *Extra* II assay (INNO-LiPA; Fujirebio Europe, Ghent, Belgium) was launched by the company in 2015. It is an assay has full genotyping capacity targeting 32 HPV types.

In **chapter 5**, INNO-LiPA was evaluated in VALGENT-3 and verified whether hrHPV testing with INNO-LiPA fulfils the minimal requirements for use in primary screening. In addition, type-specific concordance was compared between the INNO-LiPA and Linear Array.

Another test being evaluated within the VALGENT-3 framework is the RIATOL qPCR HPV genotyping assay is a laboratory developed test (RIATOL qPCR, Antwerp, Belgium) which has been routinely used in AML for more than 12 years. Besides a qualitative result for 14 hrHPV genotypes, the assay also quantifies viral concentration (VC) of each targeted HPV genotype.

In **chapter 6**, we assessed the clinical performance of RIATOL qPCR VALGENT-3 and to identify the optimal clinical VC cut-offs to assure that the test fulfils the required accuracy performance criteria for primary cervical cancer screening.

Hybribio's 14 High-risk HPV with 16/18 Genotyping Realtime PCR (HBRT-H14) is approved by the China Food and Drug Administration and widely used in China. However, the clinical accuracy of HBRT-H14 based on international validation criteria for cervical cancer screening has not been assessed.

In **chapter 7**, we evaluated the clinical accuracy of the HBRT-H14 relative to HC2. Additionally, the clinical performance of HBRT-H14 was optimized by comparing to the previously clinically validated HPV genotyping assay: Linear Array and Cobas 4800 HPV test (Cobas; Roche Molecular Systems, Alameda, CA, USA).

1.7.3 Part III: secondary prevention of cervical cancer: triage of women with atypical cervical cytology

Although HPV-based screening is increasingly being incorporated into cervical cancer screening protocols, cytological examination of a Pap smear is still the main form of screening methods used worldwide. The management of an abnormal cytological lesion depends on the severity of the lesion and its underlying future risk of high-grade CIN and cancer. General consensus exists to refer all women with HSIL directly to colposcopy [103, 104]. For women with ASC-US, triage with hrHPV testing is recommended in many cervical cancer screening protocols worldwide [105-107]. However, divergent recommendations are seen in literature regarding the management of women with LSIL and ASC-H.

Due to the high prevalence of HPV in women with LSIL, triage with hrHPV testing is less informative in the management of LSIL- positive women. To avoid immediate referral of all patients to colposcopy, alternative triage tools are needed to identify the minority of women with LSIL with underlying or incipient high-grade lesions. Partial genotyping for HPV16 and HPV18 (HPV16/18 genotyping) has been proposed as a candidate triage marker. A previous systematic review and meta-analysis [108], published in 2017, indicated that although

HPV16/18 genotyping has reduced sensitivity, it may be useful as an additional triage tool in LSIL hrHPV positive women in a two-step triage scenario.

In **chapter 8**, current evidence on the usefulness of HPV16/18 genotyping as a triage marker for the management of women with LSIL is updated using new accuracy data obtained from the international VALidation of HPV GENotyping Tests (VALGENT) framework.

Women with ASC-H are usually immediately referred for colposcopy according to American and European guidelines [109-111]. However, triage may reduce the burden of the diagnostic workup and prevent overtreatment and adverse effects associated with the excision of lesions [112, 113].

In **chapter 9**, a systematic review and meta-analysis was performed in order to assess the accuracy of hrHPV testing and other molecular markers for triaging women with a cytological result of ASC-H to predict the presence or development of cervical precancer.

Finally, in **chapter 10** a general discussion was provided in which we discussed how the results and findings in this thesis may contribute to further improvement of primary and secondary prevention of HPV-related diseases strategies. Propositions for future research were also discussed in the final chapter.

Supplementary Information

Table 1.S1 Conversion table for different cytological and/or histological classification systems used to grade cervical (pre-)cancerous lesions of the uterine cervix. Adapted from the 2nd edition of the European Guidelines for Quality Assurance in Cervical Cancer Screening [110, 114].

<table>
<tr><td>Papanicolaou</td><td>WHO</td><td>CIN (Richart, 1973)[23]</td><td>TBS 1991 (Luff, 1992)[115]</td><td>TBS 2001 (Solomon, 2002)[24]</td><td>LAST (Darragh, 2012) [116]</td></tr>
<tr><td>Cytology</td><td>Histology</td><td>Histology</td><td>Cytology</td><td>Cytology</td><td>Histology</td></tr>
<tr><td>I</td><td colspan="3">Normal</td><td>NILM</td><td rowspan="3"></td></tr>
<tr><td rowspan="3">II</td><td colspan="2" rowspan="3">Atypia</td><td>Infection, reactive repair</td><td rowspan="2"></td></tr>
<tr><td rowspan="2">ASCUS</td></tr>
<tr><td>ASC-US</td><td></td></tr>
<tr><td></td><td colspan="2">Atypical glandular cells</td><td>AGUS</td><td>AGC</td><td></td></tr>
<tr><td rowspan="4">III</td><td rowspan="2">Mild dysplasia</td><td>Condyloma</td><td rowspan="2">LSIL</td><td rowspan="2">LSIL</td><td rowspan="2">LSIL

(including p16-CIN2, p16-unclear CIN)</td></tr>
<tr><td>CIN I</td></tr>
<tr><td rowspan="2">Moderate dysplasia</td><td rowspan="2">CIN II</td><td rowspan="3">HSIL</td><td rowspan="3">HSIL</td><td rowspan="3">HSIL (including p16+ CIN2, p16+ unclear CIN)</td></tr>
<tr><td rowspan="2">IV</td><td>Severe dysplasia</td><td rowspan="2">CIN III</td></tr>
<tr><td>CIS</td></tr>
<tr><td></td><td>AIS</td><td>CGIN</td><td>AGUS</td><td>AIS</td><td></td></tr>
<tr><td>V</td><td colspan="4">Invasive carcinoma (micro-invasive, stages I-IV) (squamous cell carcinoma, adeno-carcinoma, adenosquamous carcinoma)</td><td>SISCCA</td></tr>
</table>

AGC: atypical glandular cells; AGUS: atypical glandular cells of undetermined significance; ASC-H: atypical squamous cells cannot exclude HSIL; AIS: adenocarcinoma in situ; ASC-US: atypical squamous cells of undetermined significance; CGIN: cervical glandular intraepithelial neoplasia; CIN: cervical intraepithelial neoplasia; CIS: carcinoma in situ; HSIL: high -grade squamous intraepithelial lesion; LAST: Lower Anogenital Squamous Terminology Standardization; LSIL: low-grade squamous intraepithelial lesion; NILM: Negative for epithelial abnormality; SSSCCA: superficially invasive squamous cell carcinoma ;TBS: the Bethesda System; WHO: World Health Organisation

PART I

PRIMARY PREVENTION OF HPV-RELATED DISEASES: PROPHYLACTIC HPV VACCINATION

CHAPTER 2

2. Efficacy and safety of prophylactic HPV vaccines. A Cochrane review of randomized trials

Adapted from:

Arbyn M*, **Xu L***, Simoens C, Martin-Hirsch PPL. Prophylactic vaccination against human papillomaviruses to prevent cervical cancer and its precursors. Cochrane Database of Systematic Reviews 2018, Issue 5. Art. No.: CD009069. DOI:10.1002/14651858.CD009069.pub3.
*Joint first author

Arbyn M, **Xu L**. Efficacy and safety of prophylactic HPV vaccines. A Cochrane review of randomized trials. Expert Review of Vaccines 2018; 17(12): 1085-1091.

Abstract

Introduction: Recently, the evidence on efficacy and safety of prophylactic HPV vaccines derived from randomised trials was published in the Cochrane database of Systematic reviews. A summary of this Cochrane review is presented below.

Areas covered: Only trials involving mono-, bi- (2v) and quadri-valent (4v) HPV vaccines were included. Trials evaluating the nona-valent (9vHPV) vaccine were excluded since women in the control group received the 4vHPV vaccine. Main outcomes were histologically confirmed cervical precancer lesions distinguishing those associated with vaccine HPV types and any cervical precancer. Exposure groups were women aged: 15-26 or 24-45 years being initially negative for high-risk HPV (hrHPV) or negative for the vaccine types and women unselected by HPV status.

Expert commentary: All evaluated vaccines offered excellent protection against cervical intra-epithelial neoplasia of grade 2 or 3 (CIN2+ or CIN3+) and adenocarcinoma-in-situ (AIS) associated with HPV16/18 infection in young women who were not initially infected with hrHPV or HPV16/18. Vaccine efficacy was lower when all women regardless of HPV DNA status at enrolment were included. In young women, HPV vaccination protected also against any cervical precancer but the magnitude of protection was lower than against HPV16/18 associated cervical precancer. Vaccine efficacy was lower in mid-women (aged 24-45 years). No protection against cervical precancer was found in mid-adult unselected by HPV DNA status at enrolment. Trials were not empowered to address protection against cervical cancer. Occurrence of severe adverse events or adverse pregnancy outcomes was not significantly higher in recipients of HPV vaccines than in women included in the control arms.

2.1 Background

The Cochrane Collaboration is an international, independent not-for-profit organization that evaluates interventions for disease prevention, treatment & rehabilitation by producing systematic reviews of primary research using established methods for summarising and reporting evidence. On May 7, 2018, our review on efficacy and safety of HPV vaccines was published in the Cochrane Library[100]. That day the Cochrane Collaboration set up a press conference which received wide international coverage[117].

Cervical cancer is etiologically linked with persistent high-risk HPV infection. A dozen of HPV types is considered as carcinogenic [1]. The world-wide annual incidence is estimated at about 530,000 cases and approximately 70 percent of them are caused by HPV types 16 or 18[10, 118]. Prophylactic HPV vaccines contain virus-like-particles [VLPs] consisting of the major L1 protein of the capsid. Administration by intramuscular injection triggers production of antibodies that are believed to prevent new type-specific infections and subsequent development of cervical intra-epithelial neoplasia (CIN)[119]. CIN of grade 2 (CIN2), and in particular, CIN3 and adenocarcinoma in situ (AIS), are considered as precursor lesions which may develop into invasive squamous or adenocarcinoma of the uterine cervix [1, 23, 120, 121].

In this paper, we highlighted the main findings of the recently published Cochrane review that addressed two main questions: 1) does the prophylactic HPV vaccination protect against cervical HPV infection and cervical (pre-)cancer? and 2) what are the harms associated with being vaccinated? [100].

2.2 Methods

We included results from randomised trials published in peer-reviewed journals comparing effects in women who received one to three doses of prophylactic HPV vaccines with those who received the control product. We also included non-peer-reviewed sources (clinicaltrials.gov and www.gsk-clinicalstudyregister.com) reporting on severe adverse effects. The control product was the adjuvant without HPV VLPs or a vaccine protecting against other infectious agents. Standard Cochrane methodology was applied to retrieve references, extract data and compute pooled relative risks [1]. The current paper is restricted to the following main outcomes: a) CIN2+, CIN3+ and adenocarcinoma in-situ (AIS) related to the HPV types included in the vaccine; b) any CIN2+, CIN3+ and AIS irrespective of HPV types. We separated results for three exposure groups, according to the presence of absence of HPV DNA at enrolment: a) high-risk HPV-negative; b) negative for the HPV vaccine types; c) regardless of HPV status. Randomised trials addressing vaccine efficacy typically enrolled young women (15-26 years) or mid-adult women (24-45 years).

2.3 Results

2.3.1 Included trials

We included 26 trials that enrolled almost 74,000 women, followed over a time span of six months in smaller studies to three to six years in most of the larger phase-3 studies, with longest follow-up reaching 8-9 years in two extended phase-2 trials [122, 123]. One trial evaluated a prototype monovalent HPV16 vaccine[123, 124], 18 trials evaluated the bivalent (2vHPV) vaccine containing antigens of HPV16/18 (GSK, Rixensart, Belgium) [51, 125-146] and seven trials evaluated the quadrivalent (4vHPV) vaccine [52, 53, 147-152] containing antigens of HPV6/11/16/18 (MSD, Whitehouse Station, NJ, USA). Trials assessing the nonavalent (9vHPV) vaccine (containing the antigens of HPV6/11/16/18/31/33/45/ 52/58) were not included in the Cochrane review, since the efficacy was not evaluated against a non-HPV vaccine control group. Ten of the 26 included trials measured efficacy against HPV16/18 infection and associated lesions. None was large enough to document protection against cervical cancer. Most studies enrolled young women and only three recruited women of 24 years or older.

2.3.2 Efficacy in women who were hrHPV-negative or HPV16/18-negative at baseline

Three or at least one dose of HPV vaccine confer excellent protection against 6-month persistent HPV16/18 infection and against CIN2+, CIN3+ and AIS associated with these types in young women who were hrHPV-negative or HPV16/18-negative at enrolment (RR≤ 0.10, **Table 2.1, Figure 2.1 & 2.2**). Good protection against CIN2+ associated with HPV16/18 was observed also in mid-adult HPV16/18-negative women who received three doses (RR=0.16,

95% CI 0.04 to 0.74) but protection was moderate for the group that received at least one dose (RR=0.30, 95% CI 0.11-0.81).

When the protection against any high-grade lesions irrespective of HPV types is considered, we observe a lower but still significant protection. However, the efficacy against any CIN2+ and any CIN3+ was better with the 2vHPV vaccine. Protection against any high-grade lesions was not documented for mid-adult women.

2.3.3 Efficacy in all women regardless of baseline HPV DNA status

In young women, regardless whether HPV was present or not, HPV vaccines protect against 6-month persisting HPV16/18 infection and against CIN2+ (**Figure 2.3**), CIN3+ and AIS associated with HPV16/18 (RR between 0.36 and 0.55). The efficacy against any CIN3+ differed by vaccine valency: RR 0.55 (95% CI 0.43-0.71) and 0.81 (95% CI 0.69-0.96) for the 2vHPV and 4vHPV vaccine, respectively (**Figure 2.4**).

Vaccination of mid-adult women reduced 6-month persistent HPV16/18 infection. However, risks of CIN2+ associated with HPV16/18 or any CIN2+ were not different in vaccinated and control arms (unity included in 95% CI around RR).

Table 2.1 Summary of the vaccine efficacy estimates, by age group, outcome and HPV DNA status at enrolment, for women who received at least one dose or three doses of 2vHPV or 4vHPV vaccine.

		A	B	C
	Outcome	hr HPV DNA- ≥ 1 dose	HPV16/18 DNA- 3 doses	Regardless of HPV ≥ 1 dose
	Age group 15-26			
	High-grade intraepithelial neoplasia associated with HPV16/18			
1	CIN2+	0.01 (0.00 to 0.05)^{b2q1} [Fig1] ⊕⊕⊕⊕	0.07 (0.03 to 0.15)^{b4q2} [Fig2a] ⊕⊕⊕⊕	0.46 (0.37 to 0.57)^{b1q2} [F3a] ⊕⊕⊕⊕
2	CIN3+	0.01 (0.00 to 0.10)^{b1q1} ⊕⊕⊕⊕	0.07 (0.02 to 0.29)^{b1q2} ⊕⊕⊕⊕	0.55 (0.45 to 0.67)^{b1q1} ⊕⊕⊕⊕
3	AIS+	0.10 (0.01 to 0.82)^{b1q1} ⊕⊕⊕	0.12 (0.02 to 0.70)^{b1q2} ⊕⊕⊕	0.36 (0.17 to 0.78)^{b1q1} ⊕⊕⊕
	Any high-grade intraepithelial neoplasia irrespective of HPV types			
4	CIN2+	0.33 (0.25 to 0.43)^{b4} ⊕⊕⊕⊕ 0.57 (0.44 to 0.76)^{q1} ⊕⊕⊕	0.40 (0.25 to 0.64)^{b2q1} ⊕⊕⊕⊕	0.70 (0.58 to 0.85)^{b2q1} [F4a] ⊕⊕⊕⊕
5	CIN3+	0.08 (0.03 to 0.23)^{b2} ⊕⊕⊕⊕ 0.54 (0.36 to 0.82)^{q1} ⊕⊕⊕	-	0.55 (0.43 to 0.71)^{b2} [Fig4] ⊕⊕⊕⊕ 0.81 (0.69 to 0.96)^{q1} [Fig4] ⊕⊕⊕
6	AIS+	0.10 (0.01 to 0.76)^{b1q1} ⊕⊕⊕	-	0.32 (0.15 to 0.67)^{b1q1} ⊕⊕⊕⊕
	Persistent HPV16/18 infection			
7	6M persisting	0.07 (0.05 to 0.90)^{b1} ⊕⊕⊕	0.06 (0.05 to 0.08)^{b4} ⊕⊕⊕⊕	0.44 (0.38 to 0.51)^{b2} ⊕⊕⊕
	Age group 24-45			
	High-grade intraepithelial neoplasia associated with HPV16/18			
8	CIN2+	-	0.16 (0.04 to 0.74)^{b1q1} [Fig2b] ⊕⊕⊕	0.74 (0.52 to 1.05)^{b1q1} [Fig3b] ⊕⊕⊕
9	CIN3+	-	-	-
10	AIS+	-	-	-
	Any high-grade intraepithelial neoplasia irrespective of HPV types			
11	CIN2+	-	-	1.04 (0.83 to 1.30)^{b1q1} [F4b] ⊕⊕
12	CIN3+	-	-	-
13	AIS+	-	-	-
	Persistent HPV16/18 infection			
14	6M persisting	-	0.11 (0.06 to 0.20)^{b1q1} ⊕⊕⊕⊕	0.57 (0.47 to 0.69)^{b1q1} ⊕⊕⊕⊕

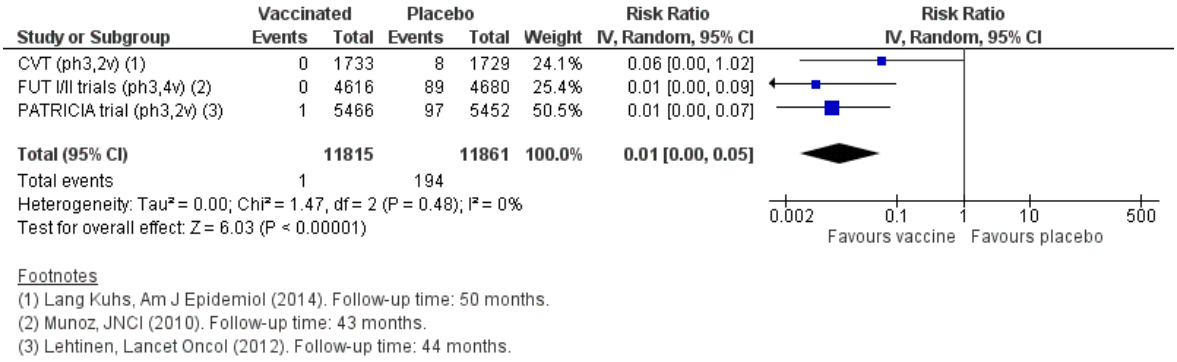
Level of protection (RR)
Excellent: RR ≤0.10, 1 excluded from CI
Good: RR>0.10 & ≤ 0.20, 1 excluded from CI
Moderate: RR>0.20 & ≤ 0.80, 1 excluded from CI
Weak: RR>0.80 & <1, 1 excluded from CI
No protection: 1 included in CI
Adverse protection: RR>1 and 1 excluded from CI

Quality of evidence*
High ⊕⊕⊕⊕
Moderate ⊕⊕⊕
Low ⊕⊕
Very low ⊕

How to read Table 2.1 We distinguish seven main end points arranged by rows: CIN2+, CIN3+ and AIS associated with HPV16/18 (rows 1-3); any CIN2+, CIN3+ and AIS associated with HPV16/18 (rows 4-6); persistent HPV16/18 infection (row 7). The first seven rows concern young women aged 15-26 year. The next seven rows (8-14) concern mid-adult women aged 14-45 years. We further distinguish 3 exposure groups arranged by columns: women being at baseline hrHPV-negative at baseline, having received at least one dose (col A); women being at baseline negative for HPV16.18 at baseline, having received all 3 doses; women regardless of HPV status at base line, having received at least one dose.

The index in superscript after the 95% CI corresponds with the number of trials where the 2vHPV (b) or 4vHPV (q) vaccines were assessed (for instance b1q2: meta-analysis of 3 trials, one with the 2vHPV and two with the 4vHPV vaccine).

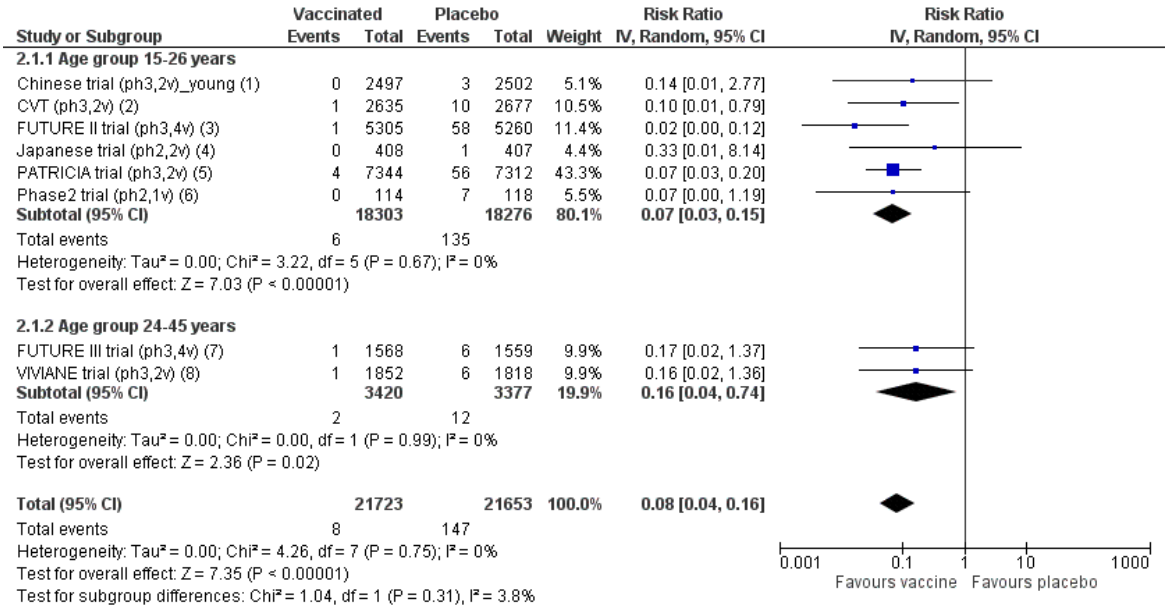
* The quality of evidence, assessed according to GRADE guidelines[153] is based on the quality of studies[154], number of studies, consistency and precision of estimates. ⊕ ⊕ ⊕ ⊕ means high quality, ⊕ means very low quality. Adapted from Arbyn et al, 2018 [100]



Footnotes
(1) Lang Kuhs, Am J Epidemiol (2014). Follow-up time: 50 months.
(2) Munoz, JNCI (2010). Follow-up time: 43 months.
(3) Lehtinen, Lancet Oncol (2012). Follow-up time: 44 months.

Figure 2.1 Relative risk to develop CIN2+ associated with HPV16/18 infection in women vaccinated with at least one dose of 2vHPV or 4vHPV vaccine vs control women, restricted to women who were hrHPV negative at enrolment.

Note: the phase of the trial (2 or 3) and the valency of the vaccine is indicated between brackets after the identification of each trial. CVT: Costa Rica Vaccination Trail; FUT I/II: FUTURE I & II trials. Reproduced from Arbyn et al, 2018 [100].



Footnotes
(1) Zhu, Int J Cancer (2014). Follow-up: 15 months.
(2) Hildesheim, Vaccine (2014). Follow-up time: 53.8 months.
(3) The FUTURE II study group, New Eng J Med (2007). Follow-up time: 36 months.
(4) Konno, Int J Gynecol Cancer (2010). Follow-up time: 24 months.
(5) Paavonen, Lancet (2009). Follow-up time: 35 months.
(6) Rowhani-Rahbar, Vaccine (2009). Follow-up time: 102 months.
(7) Castellsague, Br J Cancer (2011). Follow-up time: 45 months.
(8) Wheeler, Lancet Infect Dis (2016). Follow-up time: 71 months. Age range is larger and contains data for women of 46 years and older

Figure 2.2 Relative risk to develop CIN2+ associated with HPV16/18 infection in women vaccinated with 3 doses of 2vHPV or 4vHPV vaccine vs control women, restricted to women who were HPV16/18 negative at enrolment, stratified by age-group. Reproduced from Arbyn et al, 2018 [100].

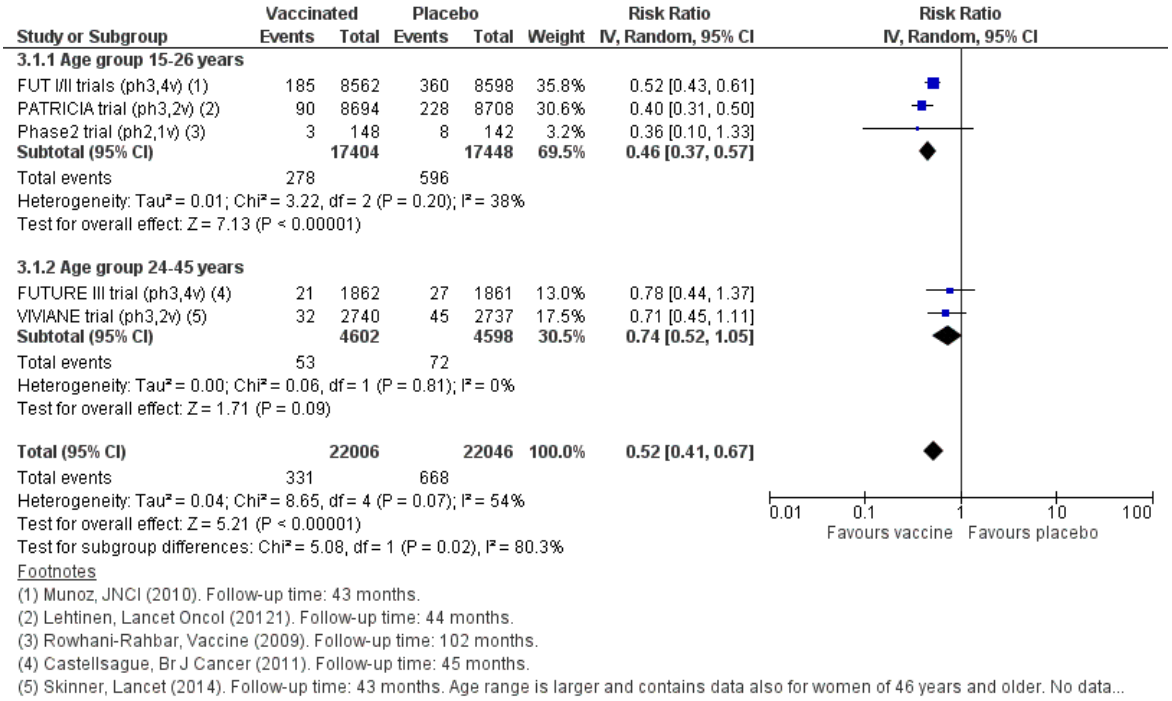


Figure 2.3 Relative risk to develop CIN2+ associated with HPV16/18 infection in women vaccinated with at least one dose of 2vHPV or 4vHPV vaccine vs control women, including all women regardless of HPV DNA status at enrolment, stratified by age-group. Reproduced from Arbyn et al, 2018 [100].

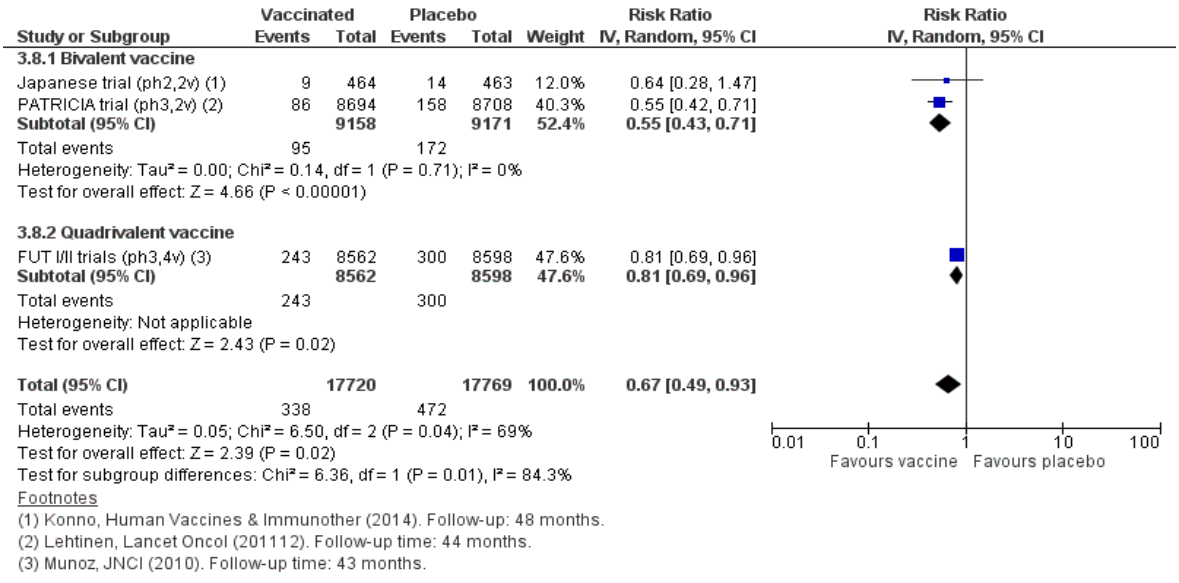


Figure 2.4 Relative risk to develop any CIN3+ irrespective of infection with HPV types in young women vaccinated with at least one dose of 2vHPV or 4vHPV vaccine vs control women, including all women regardless of HPV DNA status at enrolment, stratified by vaccine brand. Reproduced from Arbyn et al, 2018 [100].

2.3.4 Adverse events

Short-term local adverse events were noted more frequently among receivers of HPV vaccine compared to those who received the control product (RRs 1.18 to 1.73). The risks of overall systemic events and serious adverse events were similar in the HPV vaccine arms as in the control arms (RR 1.02, 95% CI 0.98-1.07 and 0.98, 95% CI 0.92 to 1.05, respectively). Pooled estimates derived from data published in peer-reviewed journals matched well with those from publically accessible registries. The mortality ratio (deaths occurring in the vaccine versus in the control arm) was 1.29 (95% CI 0.85-1.98). The level of certainty about a relation between death and vaccination was judged as low. This judgement was motivated by the broad confidence interval and the heterogeneity by age group (no excess in risk in vaccinated young women [RR=0.98, 95 % CI 0.59] vs higher risk in vaccinated mid-adult women [RR=2.36, 1.10-5.03]). No pattern in the cause or timing of deaths has been established among mid-adult women.

2.3.5 Pregnancy outcomes

In vaccinated women who became pregnant around the period of vaccination, we did not find significantly increased risks of miscarriage (RR 0.88, 95% CI 0.68-1.14), termination of pregnancy (RR 0.90, 95% CI 0.80-1.02), stillbirths (RR 1.12, 95% CI 0.68-1.83) or congenital abnormalities (RR 1.22 95% CI 0.88-1.69).

2.4 Expert commentary

The randomised trials demonstrated excellent protection against cervical cancer precursors associated with the HPV vaccine types among young women who were not infected at baseline with these types or with hrHPV types in general (see **Figure 2.1**). These findings provide the evidence to consider girls or young women before onset of sexual activity as first target of routine vaccination programmes. However, less studies reported outcomes in this high-risk HPV DNA negative population. More trials documented outcomes in women who were at baseline negative for the HPV types included in the vaccine (see **Figure 2.2**) that are the target for the typical per-protocol outcome analyses.

Protection was lower when young women, who were already exposed to HPV, were also included. The findings in this larger group (often called the intention-to-treat population), reflect the expected effectiveness for catch-vaccination in young women aged 15-26 years. No differences in efficacy against HPV16/18 associated lesions between vaccine brands were found.

Since other hrHPV types also cause cervical precancer, protection induced by a HPV16/18 vaccine against any CIN2+, CIN3+ or AIS is as expected lower than against HPV16/18 associated lesions. The potential impact of immunisation with bi- or multi-valent vaccines has been estimated using weighted pooled prevalence of HPV types in cervical cancer and its precursors and the derived population risks attributed to HPV types [33, 155, 156]. How HPV vaccination, with this or that vaccine, will change the burden of cervical (pre-) cancer, will be

the target of careful surveillance consisting in linking HPV vaccination data with screening and pathology/cancer registries [157, 158].

Our Cochrane review has revealed remarkable differences in protection against any CIN2+ and CIN3+ between the 2vHPV and 4vHPV vaccines in both the restricted hrHPV-negative group and in the larger group regardless of initial HPV status. Moreover, the review demonstrated for the first time that the evaluated HPV vaccines significantly reduce the risk of AIS associated with HPV16/18 and any AIS. Another first finding was that less than three doses protected against precancer associated with HPV16/18 infection in young women. For all end point and exposure groups, for which data were available, vaccine efficacy was lower in older women.

The Cochrane review could not provide the evidence that HPV vaccines protect against invasive cervical cancer. A WHO expert committee had advised that high-grade CIN and AIS or worse was a sufficient endpoint for HPV vaccination trials, given the knowledge of the natural history of HPV infection and CIN derived from cervical cancer screening studies, and given the excessive resources and long duration of studies required to prove vaccine efficacy for this outcome[159]. Neither occurrence of rare adverse events nor certain adverse pregnancy outcomes associated with HPV vaccination could be excluded with a high level of certainty. Therefore, health authorities must organise pharmacovigilance activities and long-term surveillance joining vaccine and morbidity registries to complete observations from the trials. Reviews performed on observational good-quality follow-up data on HPV vaccines did not raise safety concerns up to now [160-162]. Pooling of effectiveness and safety from population-wide linkage studies should be priority for future systematic reviews.

2.5 Key points

- 2vHPV and 4vHPV vaccines induce excellent protection against persistent HPV16/18 infection and associated precursor lesions in females who are not infected with high-risk HPV. Girls and young women should therefore be the first target of HPV vaccination campaigns.

- The efficacy of HPV vaccines is lower when also women already infected with HPV are included.

- Vaccine efficacy decrease by age. No protection against any CIN2+/AIS was found in the group of older women (aged 24 or older) unselected by HPV DNA status at enrolment.

- Similar rates of serious adverse events were observed in the experimental and control arms of randomised trials.

- Careful population-wide surveillance of HPV vaccine effectiveness (targeting also incidence of HPV-related cancers) and safety (including also rare conditions such as neurologic and auto-immune syndromes) should be set up by linking vaccination, cervical cancer screening and morbidity registries.

2.6 Five-year view

In collaboration with colleagues of the Cochrane editor's office and other systematic reviewers we aim to update and complete the Cochrane review and cover: a) safety and efficacy of the nona-valent HPV vaccine as well as other newly developed HPV vaccines; b) assessment of protection of HPV vaccines against genital warts and HPV infection in other anatomical locations and associated (pre-)cancer lesions (vaginal, vulvar, penile, anal, oropharyngeal); c) complete gaps in information by exploring data from the grey literature and requesting data from principle investigators. Moreover, we want to stimulate international collaboration in order concentrate resources and avoid inefficient multiplication of efforts.

Funding

M. Arbyn and L. Xu were supported also by the COHEAHR Network (grant No. 603019), funded by the 7th Framework Programme of DG Research and Innovation, European Commission (Brussels, Belgium). M. Arbyn was also supported by the Belgian Cancer Centre and the Cochrane Gynaecological Cancer Review Group.

The authors are very grateful for the support from Cochrane's editorial office and the Cochrane Gynaecological Cancer group.

Declaration of interest

Details on potential conflicts of interest of MA can be found in the original Cochrane review[100]. LX: no conflicts of interest.

CHAPTER 3

3. Prophylactic vaccination against human papillomaviruses to prevent vulvar and vaginal cancer and its precursors

Adapted from:

Xu L, Selk A, Garland SM, Fabrizio B, Kyrgiou M, Weyers S, Arbyn M. Prophylactic vaccination against human papillomaviruses to prevent vulvar and vaginal cancer and their precursors. Expert Review of Vaccines 2019; 18(11): 1157-1166.

Abstract

Introduction: Safety and efficacy of prophylactic HPV vaccines against HPV infection and associated cervical cancers and precursors is well documented in the literature, however, their efficacy against vulvar and vaginal endpoints has not been previously assessed.

Areas covered: Published results of trials involving licensed HPV vaccines were included. Main efficacy outcomes were histologically confirmed high-grade vulvar and vaginal precancer distinguishing those associated with vaccine HPV types and any vulvar and vaginal precancerous lesions. Exposure groups included women aged 15–26 or 24–45 years being initially negative for high-risk HPV (hrHPV), negative for the HPV vaccine types, and women unselected by HPV status.

Expert opinion: Our results show that the HPV vaccines are equally highly efficacious against vuval/vaginal disease as previously noted for cervical disease. The vaccines demonstrated excellent protection against high-grade vulvar and vaginal lesions caused by vaccine-related HPV types among young women who were not initially infected with hrHPV types or types included in the vaccines (vaccine efficacies more than 90%). No protection against high-grade vulvar and vaginal lesions associated with HPV16/18 was observed for mid-adult women. Trials were not powered to address protection against invasive cancers.

Keywords: HPV vaccines; vulvar cancer; vaginal cancer; systematic review; meta-analysis; randomized clinical trials

3.1 Introduction

Persistent infection with oncogenic (high risk[hr]) HPV is a prerequisite for premalignant and malignant lesions of the cervix, a proportion of vagina, vulva, anus, penis, and oropharynx, whilst the low risk HPV6 and 11 cause the majority of genital warts[163, 164]. The hrHPV types 16 and 18 collectively cause approximately 70% of all the cervical cancers worldwide[10], 40% vulvar cancers and 65% vaginal cancers[163, 164]. Vaccination with the prophylactic 2vHPV and 4vHPV vaccines has resulted in a significant reduction of the prevalence of HPV vaccine-related genotypes and their associated lesions [165-167].

Vulvar cancer is rare condition and accounts for about 4% of all gynaecological malignancies[168]. It is estimated that there are 30,000 new cases diagnosed annually worldwide[163]. The incidence of vulvar cancer and lesions (VIN) has increased in recent years, particularly amongst younger women [169, 170]. This increase is believed to be due to a rise in HPV infection and associated disease [171, 172]. HPV prevalence is approximately 40% in vulvar cancer and 83% in vulvar high grade squamous intraepithelial lesion (HSIL) [36]. For HPV-related vulvar cancers, HPV16 and 18 infections together account for 93% of them [37].

Most vulvar cancers (more than 90%) are squamous cell carcinomas (SCC) [173] and develop from two distinct pathways with their own precursor lesions: 1) basaloid or warty carcinomas

and 2) keratinizing carcinomas. In general, basaloid or warty carcinomas are the most common in young women, whereas keratinizing carcinomas are more common in post-menopausal women. Basaloid or warty vulvar carcinomas are related to HPV infection in 86% of cases and are preceded by vulvar HSIL [36]. They have similar risk profiles as cervical cancer. In contrast, keratinising vulvar carcinomas represent the majority of the vulvar cancers (more than 60%) [34], are seen in older women and not associated with HPV(rarely contain HPV ~2% of cases) [36].They tend to arise in a background of lichen sclerosus or chronic inflammatory dermatoses, or both, and their immediate cancer precursor lesion is known as *differentiated VIN* (dVIN). The terminology for vulvar precancers has changed several times in the past decades. HPV-related VIN can be categorized as low-grade squamous intraepithelial lesions (LSIL, VIN 1) and high-grade squamous intraepithelial lesions (HSIL, VIN2/3) [38, 39].A historical overview of successive terminologies is summarized in **Table 3.1**.

Table 3.1 Historic terminologies for vulvar precancerous

Year of publication	ISSVD 1986 (Wilkinson 1986[174])	ISSVD 2004 (Sideri 2005[175])	LAST 2012 (Darragh 2013[39])	ISSVD 2015 (Bornstein 2016[38])
Terminology categories	VIN1	Flat condyloma	LSIL	LSIL
	VIN2 VIN3	VIN, usual type (basaloid and warty)	HSIL	HSIL
	Differentiated VIN	Differentiated VIN		Differentiated VIN

Source: table adapted from Bornstein 2016[38].
ISSVD: The International Society for the Study of Vulvovaginal Disease
LAST: The Lower Anogenital Squamous Terminology
LSIL: Low grade squamous intra-epithelial lesions
HSIL: High grade squamous intra-epithelial lesions

Compared to vulvar cancer, vaginal cancer is a more rarecancer with an estimated 15,000 new cases annually worldwide, representing about 2% of all gynaecological malignancies[163, 168]. It is generally accepted that HPV-related vaginal cancers and cervical cancers share similar risk factors and HPV infection. Low-grade and high-grade vaginal intraepithelial neoplasia (VaIN) are classified as LSIL (VaIN1) and HSIL (VaIN2/3), respectively. Women with a history of cervical intraepithelial neoplasia are at higher risk of developing VaIN and vaginal cancer and vice versa[176].The average prevalence of HPV is about 70%[12] and 91% in vaginal cancer and in VaIN2/3, respectively[34]. HPV16 is the most common type found in vaginal cancer and precancer [12].

To date, three prophylactic vaccines have been licensed based on efficacy, immunogenicity and safety evidence in RCTs: a 2vHPV vaccine containing the L1 protein of HPV types 16 and 18 (Cervarix ®, produced by GlaxoSmithKline (GSK), Rixensart, Belgium); a 4vHPV vaccine , containing the L1 protein of HPV6, HPV11, HPV16 and HPV18 (Gardasil®, manufactured by Merck, Sharpe & Dome (Merck), Whitehouse Station, NJ, USA) and a nonavalent (9vHPV) vaccine containing the L1 protein of 4vHPV with the addition of the next 5 commonest causing

cervical types HPV31, HPV33, HPV45, HPV52 and HPV58 (Gardasil®9, produced by the same company).

A Cochrane review on the safety and clinical efficacy of 2vHPV and 4vHPV vaccines against HPV infection and associated cervical pre-cancer lesions has been published recently [101] [100]. Other reviews have assessed protection against anogenital infection and lesions in males [58] and genital warts in females [102]. However, the efficacy of HPV vaccines against vulvar and vaginal cancers and their precursors has not been previously assessed. In this systematic review we aim to systematically appraise the published phase II and phase III literature on the efficacy of HPV prophylactic vaccines against vulvar and vaginal cancer and their precursors. More specifically we aim to explore efficacy in women previously not exposed to HPV infection (HPV DNA negative and seronegative for the vaccine HPV types) and in all women irrespective of the HPV DNA and serology status at enrolment.

3.2 Methods

3.2.1 Search strategy and selection criteria

We systematically searched and reviewed the literature and report it in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guideline [177]. In this review, we restricted to peer reviewed RCTs with no language restriction. To be eligible, a study had to investigate the efficacy of vaccination (with a licensed vaccine) against HPV-associated high-grade vulvar and vaginal lesions that enrolled female participants, without any age restriction and distinguishing females' exposure to HPV infection or serology status at enrolment.

The electronic bibliographic databases searched were the Cochrane Central Register of Controlled Trials (CENTRAL), MEDLINE and Embase. The search strategy for MEDLINE is included in the Supplementary material. We searched databases from 2002 (year of publication of the first phase II trial) until March 2019. We downloaded all titles and abstracts retrieved by electronic searching to a bibliographic database stored in Reference Manager (Thompson Reuters, Toronto, Canada)

We added any references we obtain by hand searching and removed duplicates. Two reviewer authors (LX and AS) independently verified inclusion and exclusion of eligible studies and any disagreements were discussed. MA was consulted in case of no consensus was reached between LX and AS.

The primary outcomes are histologically confirmed high-grade VIN lesions (VIN2, VIN3) and high-grade vaginal lesions (VaIN2, VaIN3), or high-grade squamous intraepithelial lesions (HSIL) in either location and invasive vulvar or vaginal cancer. The secondary outcomes include incident and persistent vulvar or vaginal infection with vaccine HPV types (HPV6, HPV11, HPV16 and HPV18, separately and jointly) and with hrHPV types other than HPV16/18. Safety of HPV vaccines was not assessed since already evaluated in the recent Cochrane review [18].

3.2.2 Data extraction and quality assessment

Data extraction was done by two review authors (LX, AS). Differences were resolved between review authors by discussion or by appeal to a third review author (MA) if necessary. We extracted detailed information on study design, inclusion and exclusion criteria, characteristics of included participants with initial HPV status, number of vaccines doses addressed and primary and secondary outcomes.

The Cochrane risk of bias tool was used to assess the following study design items: random sequence generation, allocation concealment, blinding of outcome assessment, incomplete outcome data, selective reporting, other biases [74, 154]. Studies were categorized as being at high, low or unclear risk of bias. The Grading of Recommendations, Assessment, Development and Evaluation (GRADE) approach was used to assess the overall quality of the evidence [178, 179]. In the current review only randomized clinical trials were included, therefore the rating for each outcome started as high-quality evidence and was downgraded for the following considerations [179]: (1) Risk of bias due to flawed study design; (2) Inconsistency (both quantitative or qualitative); (3) Imprecision (relating to the width of the 95% confidence interval and number of participants in the analysis); (4) Indirectness and (5) Publication bias.

3.2.3 Statistical analysis

In this systematic review and meta-analysis, we computed relative risks (RRs) and their 95% confidence intervals (CIs) from the data extracted from the original studies and visualized in forest plots. The random-effects models with inverse variance weighting were applied [29]. The heterogeneity was assessed by the Q- and I-square statistics [30]. Statistical analyses were performed using Review Manager version 5.3 (The Cochrane Collaboration). We reported vaccine protection as vaccine efficacy (VE) which is defined as (1-relative risk)×100%, where the relative risk (RR) is the ratio of proportions with events among subjects who received the HPV vaccine vs control subjects. Vaccine protection was also expressed at risk difference (risk in 10,000 non-vaccinated and risk in 10,000 vaccinated population).

3.3 Results

3.3.1 Characteristics of included trials

In total, 698 references were identified from the search in Medline and Embase. The last search was done on March 23, 2019. After removing duplicates and reading of the titles, a total of 71 potentially relevant English publications remained for review of the abstract and full manuscript. Finally, 8 manuscripts were eligible for inclusion and was enriched [52, 54, 55, 94, 151, 171, 180-185] with 5 manuscripts [54, 94, 152, 180, 186] included through previous search for the Cochrane review on the safety and efficacy of prophylactic HPV vaccines against HPV infection and associated cervical cancer and its precursors [100]. In total, 13 manuscripts describing characteristics and results of six randomized trials were selected for this review. Multiple reports of the same study were available, therefore, the unit of interest in this meta-analysis is each trial rather than each report. The selection process of the literature is summarized in the

PRISMA flow diagram in **Figure 3.1**. The list of excluded studies and reasons for exclusion can be provided on request.

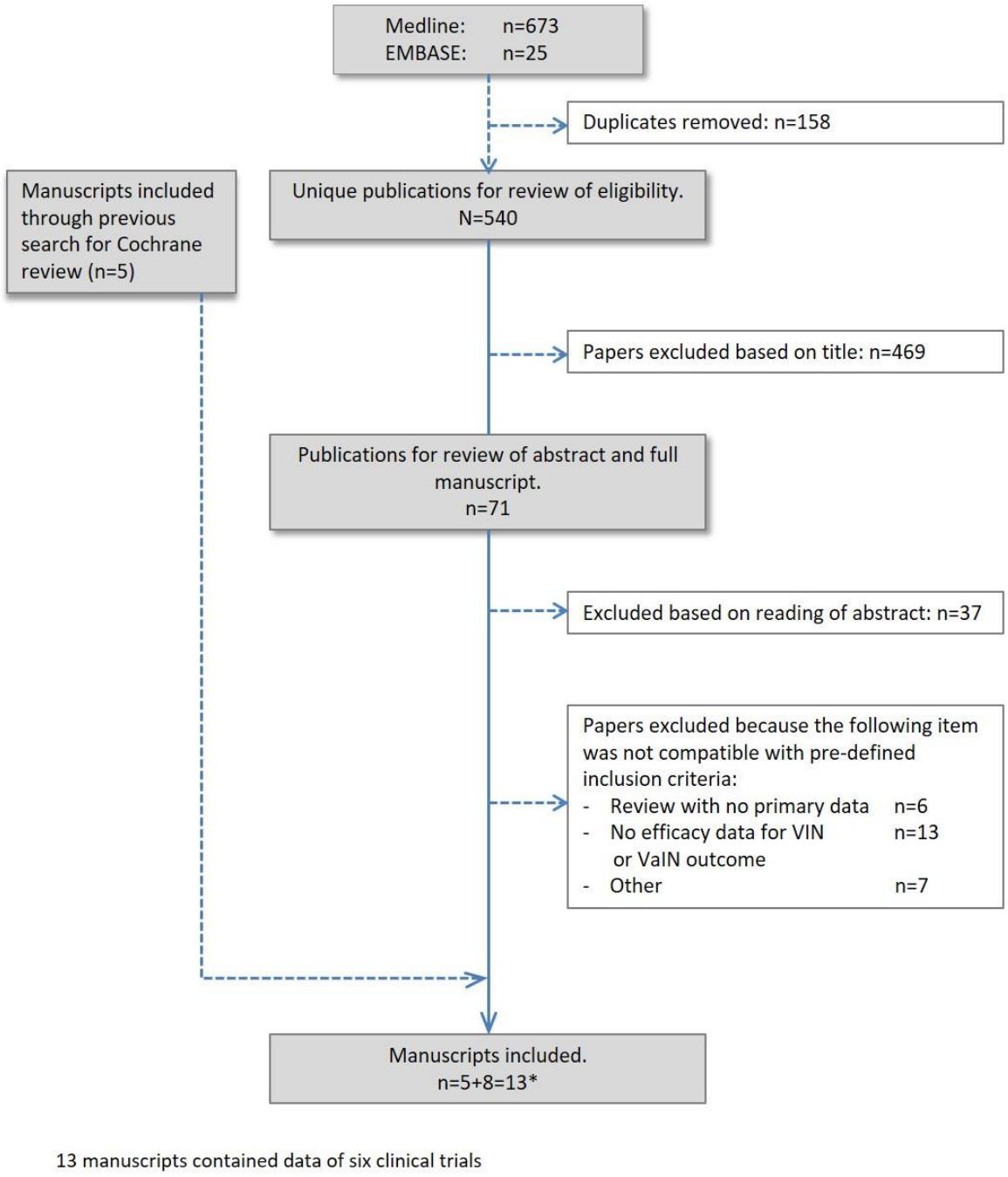


Figure 3.1 PRISMA flow chart of study selection. *13 manuscripts include 6 randomized trials.

Six distinct randomized trials were included in our review. The studies comprised data of a total of 37,768 study participants (**Table 3.1**). Two pooled studies were also included in this review because results were only reported in pooled analyses for certain endpoints [94, 151, 180, 183,

184, 187]. The main study characteristics and relevant references are presented in **Table 3.2**. One study evaluated the effects of the 2vHPV vaccine [171], eleven reports of four trials evaluated the 4vHPV vaccine, whereas one trial compared the 9vHPV with the 4vHPV vaccine [54, 55]. Participants who received the 9vHPV vaccine constituted the experimental group and participants who received the 4vHPV vaccine were considered as the comparison group. Only one trial was a phase II evaluation[184] and the five other trials were phase III evaluations. Five studies included young women (15-26 years) and only one study included participants of mid-adult women (24-45 years).

We judged the risk of bias of all of the six included trials to be low according to the six criteria incorporated in Cochrane's tool for assessing risk of bias in randomized trials [100].

Due to the reporting in the trials of composite endpoints that combined moderate and severe vulvar and vaginal lesions, in this analysis the composite endpoint was accepted to be used for incidence of VIN2-3 or VaIN2-3 (VIN2+/VaIN2+). The summary of VE estimates are presented in **Table 3.3**. The results are summarized by age group, endpoint, and HPV DNA/serology status at enrolment for women who received at least one dose or three doses of vaccine. HPV vaccine protection estimates is expressed as risk difference are presented in **Table 3.4**.

Table 3.2 Characteristics of included studies

Study (Trial ID)	Reference (Author,year)	N randomized	Age group (years)	Vaccine/ Comparator	Follow-up duration (month)
qHPV phase 2 trial (NCT00365716)	Joura, 2007[184]	1,158	16-23	qHPV vs aluminium-containing placebo	36
FUTURE I trial (NCT00092521)	Garland, 2007[188]	5,455	16-24	qHPV vs aluminium-containing placebo	58.8
FUTURE II trial (NTC00092534)	Joura, 2007[184]	12,167	15-26	qHPV vs aluminium-containing placebo	58.8
Pooled qHPV trials (NCT00092521+ NTC00092534+ NCT00365716)	Olsson, 2009[187]	18,172	16-24	qHPV vs aluminium-containing placebo	40
	Joura, 2007[184]				36
	Kjaer, 2009				42
FUTURE I/II trials (NCT00092521+ NTC00092534)	Future II study group, 2007[180]	17,622	15-26	qHPV vs aluminium-containing placebo	58.8
	Munoz, 2010[151]				43
	Joura, 2012[183]				
FUTURE III (NCT00090220)	Munoz, 2009 [186]	3,819	24-45	qHPV vs aluminium-containing placebo	48
	Castellsague, 2011[152]				48
Costa Rica Trial (NCT00128661)	Lang Kuhs, 2014[171]	1,044*	18-25	bHPV vs Hepatitis A licensed Havrix vaccine	48
9vHPV trial (NCT00543543)	Joura, 2015[54]	14,215	16-26	9vHPV vs qHPV	42
	Huh et al 2017[55]				60
Total	37,768				

*Analysis restricted to women who had vulvar specimen collected and had HPV results available. Intention-to-treat population only.

Table 3.3 Summary of the vaccine efficacy estimates, by age group, outcome, and HPV DNA status at enrollment, for women who received at least one dose or three doses of HPV vaccines.

		A	B	C	D	E
		Enrolment status & number of doses administered				
	Outcome	hr HPV DNA- ≥1 dose	HPV16/18 DNA- ≥1 dose	HPV16/18 DNA- 3 doses	Regardless of HPV ≥1 dose	Prior HPV infection ≥1 dose
	Age group 15-26					
	High-grade vulval or vaginal lesions due to HPV16/18 or HPV16/18/6/11					
1	VIN2+/VaIN2+	95% (62 to 99%) ⊕⊕⊕[2]	95% (73 to 99%) ⊕⊕⊕⊕	94% (53 to 99%) ⊕⊕⊕⊕	71% (40 to 86%) ⊕⊕⊕⊕	79% (-327 to 99%) ⊕⊕[1,2]
2	VIN2+	-	-	96% (38 to 100%) ⊕⊕⊕[2]		
3	VaIN2+	-	-	95% (19 to 100%) ⊕⊕⊕[2]		
	High-grade vulval or vaginal lesions irrespective of HPV or due to whatever HPV type					
4	VIN2+/VaIN2+	77% (48 to 90%) ⊕⊕⊕[2]	-	-	48% (8 to 71%) ⊕⊕⊕[1]	
5	VIN2+	-	-	-	52% (12 to 73%) ⊕⊕[1,2]	
6	VaIN2+	-	-	-	43% (-16 to 72%) ⊕⊕[1,2]	
	HPV16/18 infection					
7	Incident vulvar infection	-	-	-	54% (5 to 78%) ⊕⊕[1,2]	
	Age group 24-45					
	High-grade vulval or vaginal lesions due to HPV16/18 or HPV16/18/6/11					
8	VIN2+/VaIN2+	-	-	-	-399% (-10297 to 76%) ⊕⊕1,2	
9	VIN2+	-	-	-	-	-
10	VaIN2+	-	-	-	-	-
	High-grade vulval or vaginal lesions irrespective of HPV or due to whatever HPV type					
11	VIN2+/VaIN2+	-	Not estimatable	Not estimatable	-	-
12	VIN2+	-	-	-	-	-
13	VaIN2+	-	-	-	-	-
	HPV16/18 infection					
14	Incident vulvar infection	-	-	-	-	-

Level of protection
excellent, ≥90%, 0% excluded from CI
good, ≥80% & <90%, 0% excluded from CI
moderate, ≥20% & <80%, 0% excluded from CI
weak, 0-19%, 0% excluded from CI
no protection, 0% included in CI
harm (reversed protection), <0% & 0 excluded from CI

Quality of evidence*
High ⊕⊕⊕⊕
Moderate ⊕⊕⊕
Low ⊕⊕
Very low ⊕

How to read table 1. We distinguish seven main end points arranged by rows: VIN2+/VaIN2+, VIN2+ and VaIN2+ associated with HPV16/18 or HPV16/18/6/11(rows 1-3); Any VIN2+/VaIN2+, VIN2+ and VaIN2+ associated with HPV16/18 (rows 4-6); Incident vulvar HPVHPV16/18 infection (row 7). The first seven rows concern young women aged 15-26 year. The next seven rows (8-14) concern mid-adult women aged 14-45 years. We further distinguish 5 exposure groups arranged by columns: women being at baseline hrHPV-negative at baseline, having received at least one dose (col A);Women being at baseline negative for HPV16/18 at baseline, having received at least 1 dose (col B) or all 3 doses (col C); Women regardless of HPV status at base line, having received at least one dose (col D); Women had serological evidence of prior vaccine type HPV infection at baseline (col E).

*The quality of evidence is assessed according to the GRADE guideline (ref).

⊕ ⊕ ⊕ ⊕ means high quality: we are very confident that the true effect lies close to that of the estimate of the effect.

⊕ ⊕ ⊕ means moderate quality: we are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.

⊕ ⊕ means low quality: our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect.

⊕ means very low quality: we have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect.

[1] Quality of evidence downgraded due to serious imprecision in effect estimate (width 95% CI around VE>60%).

[2] Quality of evidence downgraded one level when only one study was retrieved for the outcome.

Table 3.4 Summary of findings of HPV vaccine protection effects against high-grade vulvar and vaginal diseases

Outcomes/ enrollment status	Age group (years)	No. of doses administered	Anticipated absolute effects (95% CI)		Relative effect (95% CI)	Vaccine efficacy (95% CI)
			Risk with placebo	Risk with vaccine		
In women who were hrHPV-negative at baseline						
VIN2+/VaIN2+ associated with HPV16/18	15-26	≥1 dose	42 per 10,000	2 per 10,000 (0 to 16)	0.05 (0.01 to 0.38)	95% (62 to 99%)
VIN2+/VaIN2+ irrespective of HPV type	15-26	≥1 dose	65 per 10,000	15 per 10,000 (6 to 34)	0.23 (0.10 to 0.52)	77% (48 to 90%)
In women who were negative for HPV vaccine type at baseline						
VIN2+/VaIN2+ associated with HPV16/18	15-26	≥1 dose	32 per 10,000	2 per 10,000 (1 to 9)	0.05 (0.01 to 0.27)	95% (73 to 99%)
VIN2+/VaIN2+ associated with HPV16/18	15-26	3 doses	19 per 10,000	1 per 10,000 (0 to 9)	0.06 (0.01 to 0.47)	94% (53 to 99%)
VIN2+ associated with HPV6/11/16/18	15-26	3 doses	16 per 10,000	1 per 10,000 (0 to 10)	0.04 (0.00 to 0.62)	96% (38 to 100%)
VaIN2+ associated with HPV6/11/16/18	15-26	3 doses	13 per 10,000	1 per 10,000 (0 to 11)	0.05 (0.00 to 0.81)	95% (19 to 100%)
In all women regardless of baseline HPV status						
VIN2+/VaIN2+ associated with HPV16/18	15-26	≥1 dose	36 per 10,000	11 per 10,000 (5 to 21)	0.29 (0.14 to 0.60)	71% (40 to 86%)
VIN2+/VaIN2+ irrespective of HPV type	15-26	≥1 dose	58 per 10,000	30 per 10,000 (17 to 53)	0.52 (0.29 to 0.92)	48% (8 to 71%)
VIN2+ irrespective of HPV type	15-26	≥1 dose	36 per 10,000	17 per 10,000 (9 to 32)	0.48 (0.27 to 0.88)	52% (12 to 73%)

3.3.2 Efficacy in women who were hrHPV-negative or negative for HPV vaccine types at baseline

VEs in women who were hrHPV-negative at baseline were reported in one pooled analysis of the FUTURE I and FUTURE /II trials evaluating 4vHPV vaccine [151] and the results were presented in column A of **Table 3.3** and part a of **Table 3.4** Excellent protection against VIN2+/VaIN2+ associated with HPV16/18 was observed in young women who received at least 1 dose (VE=95%, 95% CI: 62 to 99%); HPV vaccines reduce the risk of VIN2+/VaIN2+ from 42 to 2 per 10,000. When the protection against any high-grade vulvar/vaginal lesions irrespective of HPV types is considered, we observed a lower but still significant protection (VE=77%, 95% CI: 48 to 90%; absolute risk reduced from 65 to 15 per 10,000). However, we judged these to be moderate quality of evidence because only one report was retrieved for the outcome.

Outcomes for women negative for HPV vaccine types at baseline (HPV16/18/6/11 or HPV16/18) were more often reported, both for women who received all three doses and for women who received at least one dose (See column B and column C in **Table 3.3** and part b in **Table 3.4**). In young women aged 15-26 years who received all three vaccine doses, risk of VIN2+/VaIN2+ associated with HPV16/18 reduced from 19 to 1/10,000 with a VE of 94% (95% CI: 53-99%; high-quality evidence) (**Figure 3.2**). Protection against separate endpoint of VIN2+ and VaIN2+ in young women was reported in one pooled analysis of three 4vHPV trials, the VE was 96% (95% CI: 38 to 100%) and 91% (95% CI: 26 to 99%) for VIN2+ and VaIN2+ associated with vaccine HPV types respectively. In young women who received at least one dose of vaccine, HPV vaccines reduce the risk of VIN2+/VaIN2+ associated with HPV16/18 from 32 to 2/10,000 with a pooled VE of 95% (95% CI: 73 to 99%, high-quality evidence).

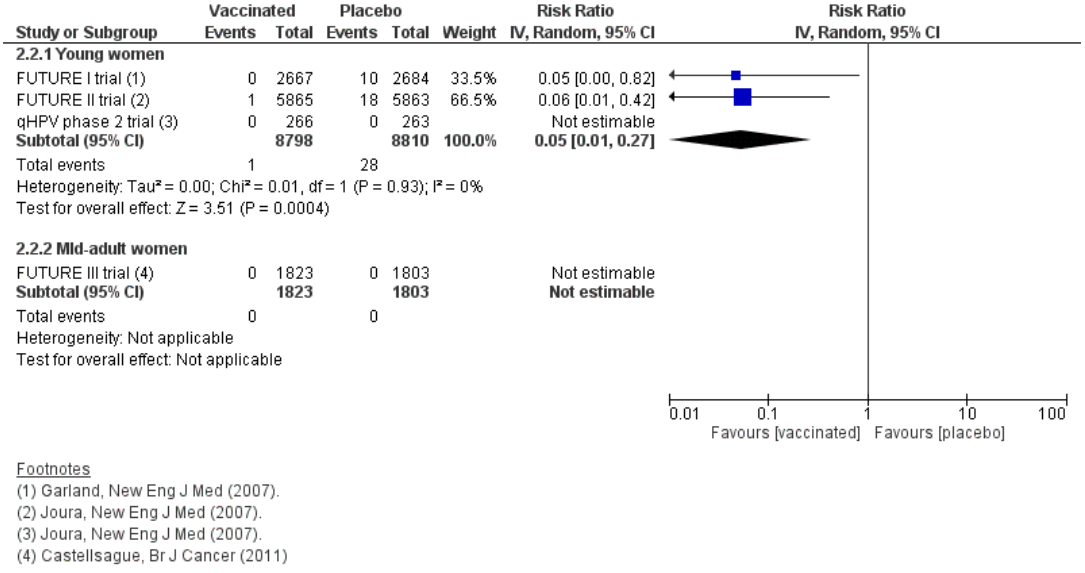


Figure 3.2 Relative risk to develop VIN2+/VaIN2+ associated with HPV16/18 or HPV16/18/6/11 infection in women vaccinated with at least one dose of 4vHPV vaccine vs control women, restricted to women who were HPV16/18 negative at enrolment, stratified by age group

3.3.3 Efficacy of all women regardless of baseline HPV status

Data on protection induced by HPV vaccination against high-grade lesions in all enrolled women regardless of HPV DNA status at enrollment are reported only for those who received at least one dose of vaccines (See column D in **Table 3.3** and part c in **Table 3.4**). In adolescent girls and women aged 15 to 26 years, the risk reduction of VIN2+/VaIN2+ associated with HPV16/18 was lower than in the hrHPV-naïve or HPV vaccine types negative at baseline groups, but the protection was still significant and consistent across three trials (VE=71%, 95% CI: 40 to 86%; the absolute risk reduced from 36 to 11 per 10,000; high-quality evidence). Vaccination with the 4vHPV vaccine of mid-adult women aged 24-45 years, was not protective (**Figure 3.4**) but we judged this to be low-quality evidence. Limited protection against VIN2+ and VaIN2+ irrespective of HPV type was shown for the 4vHPV vaccines only in younger women aged 15 to 26 years (VE= 48%, 95% CI: 8 to 71%; the absolute risk reduced from 58 to 30 per 10,000; moderate-quality evidence). No data were reported for mid-adult women (**Figure 3.5**.).

Incident vulvar HPV16/18 infection was reported only in one 2vHPV vaccine trial with a VE of 54% (95% CI: 5 to 78%; low-quality evidence). No data for persistent infection was reported in the included studies.

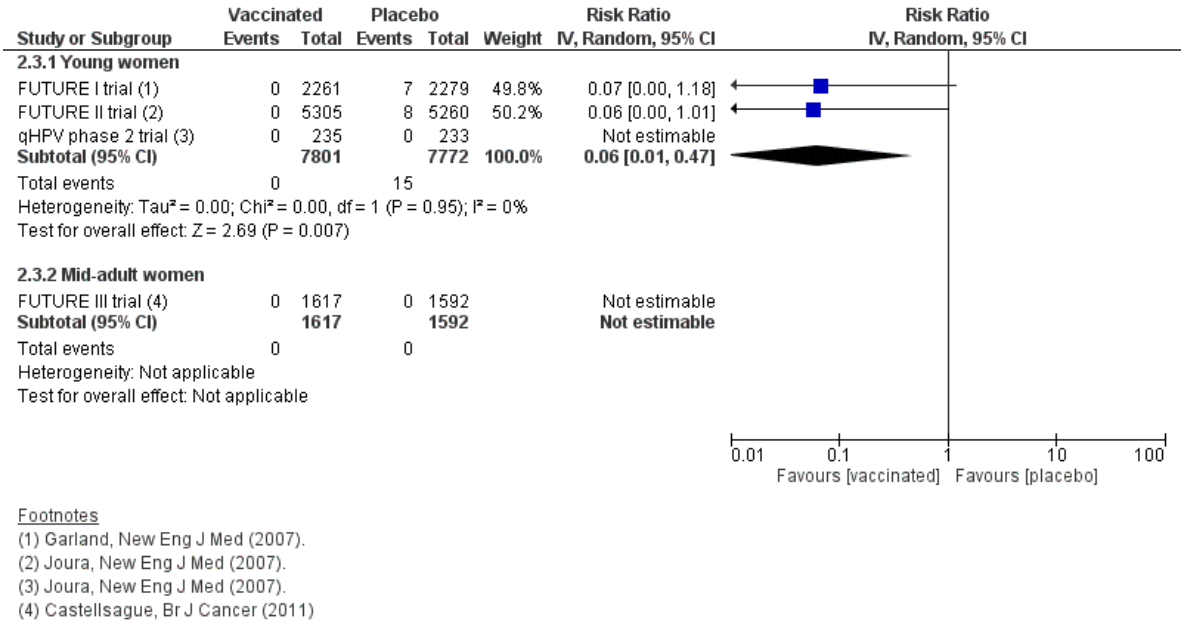
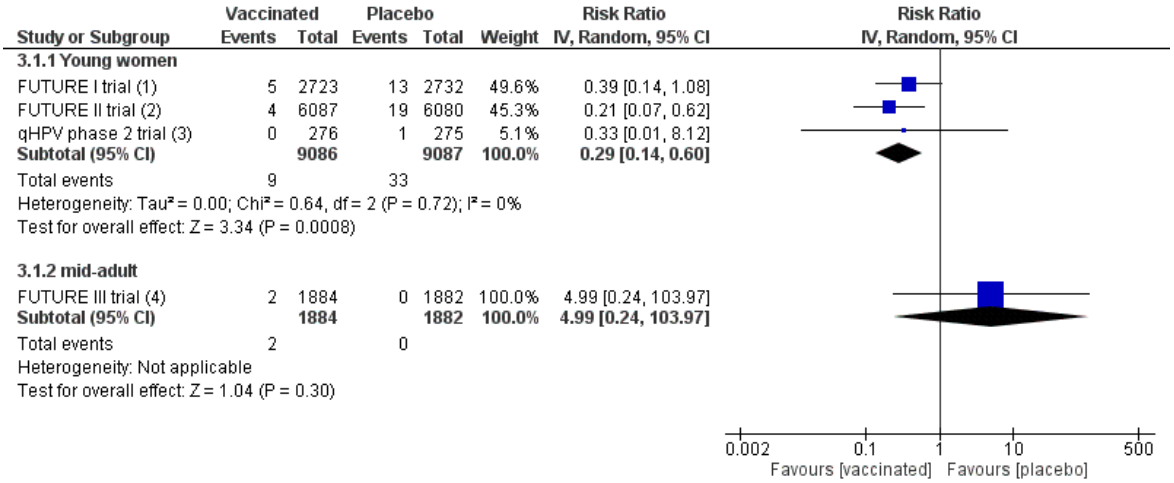
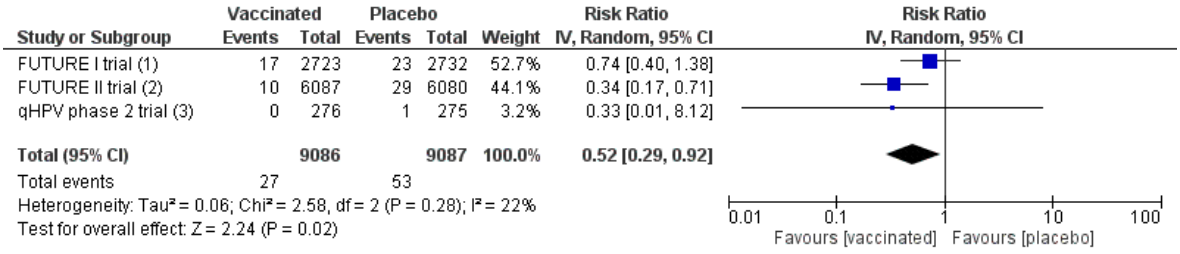


Figure 3.3 Relative risk to develop VIN2+/VaIN2+ associated with HPV16/18 or HPV16/18/6/11 infection in women vaccinated with three doses of 4vHPV vaccine vs control women, restricted to women who were HPV16/18 negative at enrolment, stratified by age group



Footnotes
(1) Garland, New Eng J Med (2007).
(2) Joura, New Eng J Med (2007).
(3) Joura, New Eng J Med (2007).
(4) Castellsague, Br J Cancer (2011).

Figure 3.4 Relative risk to develop VIN2+/VaIN2+ associated with HPV16/18 or HPV16/18/6/11 infection in women vaccinated with at least one dose of 4vHPV vaccine vs control women, including all women regardless of HPV DNA status at enrolment, stratified by age-group.



Footnotes
(1) Garland, New Eng J Med (2007).
(2) Joura, New Eng J Med (2007).
(3) Joura, New Eng J Med (2007).

Figure 3.5 Relative risk to develop any VIN2+/VaIN2+ irrespective of infection with HPV types in young women vaccinated with at least one dose of 4vHPV vaccine vs control women, including all women regardless of HPV DNA status at enrolment.

3.3.4 Efficacy of the 9vHPV vaccine

The efficacy of the 9vHPVvaccine was compared with the 4vHPV vaccine in one study that enrolled females aged 16 to 26 years. No group in this trial received placebo, therefore, the result cannot be integrated in the previous meta-analyses, where women in the control group did not receive an HPV vaccine. For women who were negative for all nine vaccine HPV types at baseline, good protection against the composite endpoint of VIN2+/VaIN2+ associated HPV 31/33/45/52/58 from the 9vHPV vaccine was observed for women who received 3 doses of vaccines (VE=86%, 95% CI:-318 to 99%; moderate-quality evidence), but the protection is not

significant. Because 4vHPV vaccine offers cross-protection against HPV31, HPV33 and HPV45[21], the VE estimate of 9vHPV against high-grade vulvar and vaginal lesions associated with HPV 31/33/45/52/58 expected to be higher if the control group would have received a control intervention without HPV antigen.

3.4 Conclusion

This systematic review and meta-analysis of randomized trials evaluated the efficacy of licensed HPV vaccines against vulvar and vaginal cancers and their precursors. The vaccines demonstrated excellent protection against high-grade vulvar and vaginal lesions caused by HPV types included in the vaccines among young women who had no evidence of HPV infection at enrollment with hrHPV types or with types protected for in the vaccines. These findings provide evidence that HPV vaccines prevent HPV-related vulvar and vaginal precancers in those unexposed groups, very relevant for girls or young women before onset of sexual activity. Protection was moderate when young women who were already exposed to HPV were also included in the analysis (often referred to as the intention-to-treat population in the trials), and these findings provide evidence relevant for vaccination programs in the catch-up population particularly as not all will be exposed to all types covered and protected by the vaccines. In women younger than 26, protection induced by HPV vaccines against any VIN2+ or VaIN2+ is lower than against HPV16/18 associated lesions as expected. Among mid-adult women (24-45 years), no protection against high-grade vulvar and vaginal lesions associated with HPV16/18 was observed. The quality of the evidence for vaccine efficacy in this age group is very low because there is only one trial (FUTURE III trial) showing imprecise results (very broad confidence intervals).

3.5 Expert opinion and five-year view

The safety and clinical efficacy of 2vHPV and 4vHPV vaccines against cervical infection pre-cancer lesions has been assessed previously. In the current review we focused on protection against vulvar and vaginal precancerous lesions associated with the HPV types included in the licensed vaccines with data obtained from randomized trials.

In this meta-analysis, VE against high-grade vulvar or vaginal intraepithelial neoplasia has been documented only for the 4vHPV and 9vHPV vaccines, because these disease outcomes were not included in the trial protocols of the 2vHPV vaccine[189]. However, reduced prevalence of vulvar HPV16/18 infection was observed in the Costa Rica 2vHPV vaccine trial [171]. Moreover, in a post-hoc analysis of the PATRICIA trial, VE of the 2vHPV vaccine against VIN2+/VaIN2+ associated with HPV16/18 was estimated as 54.5 % (95% CI: -42.0 to 87.6%) for all women in the total vaccinated cohort [190, 191]. However, it is worth to mention that visual inspection of the vagina and vulva were only performed in women referred for colposcopy with abnormal Pap cytology and VIN/VaIN lesions were biopsied or excised when needed.

Evidence suggests that one or two doses of the HPV vaccine provides protection against cervical HPV16/18 infections and lesions similar to the three-doses schedule[100, 137]. Using

a similar post-hoc analysis as explained in Arbyn et al[100], we can compute a a posterior VE against the composite outcome of VIN2+/VaIN2+ associated with HPV16/18 for women having received only one or two doses and being initially HPV 16/18 DNA negative. Protection with statistical significance was observed (VE=88%, 95% CI: 36 to 98%) from the post-hoc analysis (Data can be provided upon request). The current review provides evidence that prophylactic HPV vaccines prevent HPV-related vulvar and vaginal precancers in young women aged 15 to 26 years, although trials were primarily designed to demonstrate efficacy against cervical precancer. The impact of HPV vaccination on the burden of vulvar and vaginal cancer and precancer should be evaluated in countries or regions with good linkage of HPV vaccination data and cancer registries and will take decades before it can be observed since the peak incidence of these cancers occur on average at older ages than cervical cancer.

For women who had serological evidence of prior vaccine type infection, the VE for VIN2+/VaIN2+ associated with HPV16/18/6/11 was reported in a pooled 4vHPV study with an estimate of 79%[187]. VE was similar (8% higher than the VE against the same endpoint in all enrolled women regardless of HPV DNA status at enrollment) corroborating the conclusion of the Cochrane review that HPV serology status hardly influences observed vaccine protection.

The adverse events after vaccination and pregnant outcomes in women vaccinated for 2vHPV and 4vHPV vaccines has been reviewed in a previously published Cochrane review on the safety and clinical efficacy of prophylactic HPV vaccines against HPV infection and associated cervical cancer and its precursors[100]. The efficacy and safety of 9vHPVvaccine was compared to the 4vHPV vaccine [33]. The frequency of adverse events related to the injection site was higher in the 9vHPV group[54], whereas general systemic, including serious adverse effects and pregnancy related adverse outcomes were balanced between the vaccine groups.

There is less data reported regarding HPV vaccine and vulvar and vaginal precancerous lesions and cancers, however available data from the trials seem to demonstrate similar protection against vulvar and vaginal disease as observed against cervical disease. Excellent protection in HPV naïve groups may reflect the expected impact in routine vaccination in adolescent girls (12-14 years). Protection against high-grade vulvar and vaginal lesions for mid-adult women was poorly documented.

Safety data assessed from randomized trials were limited to the study periods and are not able to capture rare events and case-fatality. Therefore, careful monitoring of long-term safety must be set up including linkage between morbidity/maternity registries and vaccination databases[192, 193]. There is an objective need to produce regular updates of vaccine effectiveness against all HPV related pre-cancer and cancer, where protection against cervical HPV infection and precancer outcomes can be derived from linkage between HPV vaccination and screening registries. Vaccine protection against HPV-related cancers will require joining population-based cancer registries with comprehensive HPV vaccination registries. Future systematic reviews may include more study designs such as registry linkage studies and trend analyses.

Key issues

- HPV vaccines generate excellent protection against high-grade vulvar and vaginal intraepithelial neoplasia caused by HPV types included in the vaccines in females who had no evidence of prior HPV infection. These findings confirm that girls and young women before onset of sexual activity is the first target of HPV vaccination program.
- Protection of HPV vaccines was moderate in young women already exposed to HPV.
- Vaccine efficacy against any VIN2+ or VaIN2+ irrespective of HPV type is lower than against HPV16/18 associated lesions.
- No protection was observed in mid-adult women (24-45 years), however the quality of this evidence is very low because there is only one trial showing imprecise results.
- HPV vaccines show similarly high efficacy against vuval/vaginal disease as previously noted for cervical disease.

PART II

SECONDARY PREVENTION: CLINICAL VALIDATION OF HPV GENOTYPING ASSAYS FOR PRIMARY SCREENING

CHAPTER 4

4. Assessment of the Roche Linear Array HPV Genotyping Test within the VALGENT framework

Adapted from:

Xu L, Ostrebenk A, Poljak M, Arbyn M. Assessment of the Roche Linear Array HPV Genotyping Test within the VALGENT framework. Journal of Clinical Virology 2018; 98: 137-42.

Abstract

Background: Cervical cancer screening programs are switching from cytology-based screening to high-risk (hr) HPV testing. Only clinically validated tests should be used in clinical practice.

Objectives: To assess the clinical performance of the Roche Linear Array HPV genotyping test (Linear Array) within the VALGENT-3 framework.

Study design: The VALGENT framework is designed for comprehensive comparison and clinical validation of HPV tests that have limited to extended genotyping capacity. The Linear Array enables type-specific detection of 37 HPV types. For the purpose of this study, Linear Array results were designated as positive only if one of the 13 hrHPV types also included in the Hybrid Capture 2 (HC2) was detected. The VALGENT-3 framework comprised 1,600 samples obtained from Slovenian women (1,300 sequential cases from routine cervical cancer screening enriched with 300 cytological abnormal samples). Sensitivity for cervical intra-epithelial neoplasia of grade 2 or worse (CIN2+) (n=127) and specificity for <CIN2 (n=1,216) were assessed for Linear Array and for HC2 and non-inferiority of Linear Array relative to HC2 was checked. In addition, the prevalence of separate hrHPV types in the screening population, as well as the concordance for presence of HPV16, HPV18 and other hrHPV types between Linear Array and the Abbott RealTime High Risk HPV test (RealTime) were assessed.

Results: The clinical sensitivity and specificity for CIN2+ of the Linear Array in the total study population was 97.6% (95% CI, 93.3-99.5%) and 91.7% (95% CI, 90.0-93.2%), respectively. The relative sensitivity and specificity of Linear Array vs HC2 was 1.02 [95% CI, 0.98 to 1.05, ($p<0.001$)] and 1.02 [95% CI, 1.01 to 1.03, ($p<0.001$)], respectively The overall prevalence of hrHPV using the Linear Array in the screening population was 10.5% (95% CI, 8.9 to 12.3%) with HPV16 and HPV18 detected in 2.3% and 0.9% of the samples, respectively. Excellent agreement for presence or absence of HPV16, HPV18 and other hrHPV between Linear Array and RealTime was observed.

Conclusions: Linear Array showed similar sensitivity with higher specificity to detect CIN2+ compared to HC2. Detection of 13 hrHPV types with Linear Array fulfills the clinical accuracy requirements for primary cervical cancer screening.

4.1 Background and objectives

In 2012, the International Agency for Research on Cancer (IARC) concluded that at least 12 high-risk human papillomavirus (hrHPV) types (HPV16, HPV18, HPV31, HPV33, HPV35, HPV3, HPV45, HPV51, HPV52, HPV56, HPV58 and HPV59), were carcinogenic to humans for the development of cervical cancer (IARC-2009 hrHPV types)[194]. Furthermore, it has been demonstrated through several randomized controlled trials that hrHPV DNA testing is more effective than cervical cytology in primary screening of women aged 30 years or older [105, 195]. Thus, several countries are currently in the process of introducing primary hrHPV based

screening for cervical cancer. The first two hrHPV DNA assays that had demonstrated high-quality evidence on efficacy with respect to prevention of cervical cancer in large randomized trials with longitudinal follow-up [86, 105] were the Hybrid Capture 2 assay (HC2; Qiagen, Hilden, Germany) [80-82, 195] and GP5+/6+ PCR-based enzyme immunoassay (GP5+/6+-EIA; Diassay, Rijwijk, the Netherlands) [83-85]. Hence, they represent standard comparator assays in the clinical evaluations of alternative HPV DNA tests [196].

The number of commercially available HPV assays has increased significantly over the last decade with prominent differences in their technology, targeted viral genes, HPV types detected, and level of automation. Unfortunately, many of HPV tests currently on the market lack clinical performance evaluation and are without a single peer-reviewed publication [79, 197, 198]. In 2009, international criteria were developed for alternative hrHPV DNA assays, which must be fulfilled in order to be accepted by HPV academic community as clinically validated for the use in primary cervical cancer screening settings[86]. Thus, alternative hrHPV DNA assay should demonstrate good reproducibility together with non-inferior sensitivity and specificity for detecting cervical intraepithelial neoplasia of grade 2 or worse (CIN2+) compared to the HC2 or GP5+/6+-EIA [86]. A list of hrHPV DNA assays that fully or partially fulfil international validation criteria for use in primary cervical cancer screening was published recently[79], most of them targets 12 IARC-2009 hrHPV types plus HPV66 and/or HPV68 [11].

In this study, we assessed the performance of the Linear Array HPV Genotyping Test (Linear Array; Roche Molecular Diagnostics, Branchburg, NJ, USA) through the VALidation of HPV GENotyping Tests (VALGENT) framework. VALGENT is an international study framework aimed at comprehensive comparison and validation of hrHPV DNA tests in primary cervical cancer screening settings. HPV DNA assays evaluated through VALGENT have limited, extended or full genotyping capacity. VALGENT is iterative, using panels collated in different countries. Thus far, two VALGENT panels have been completed [87], using samples collected from Belgium[199-201] and Scotland) [202-205]. VALGENT-3 is using specimens obtained from women participating in the Slovenian national cervical cancer screening programme [82].

Using the VALGENT-3 sample collection, we have evaluated the clinical performance of the Linear Array in comparison to the standard comparator test (HC2) and verified whether the Linear Array fulfils minimal clinical requirements for use in cervical screening. Additionally, we compared the analytical performance of the Linear Array for partial HPV partial genotyping (i.e., using only 14 hrHPV genotypes) with that of Abbott RealTime High Risk HPV test (RealTime; Abbott, Wiesbaden, Germany), another clinically validated hrHPV DNA assay [79].

4.2 Study design

4.2.1 Sample collection

The collation of specimens used for the present iteration of VALGENT-3 project was performed in Slovenia, as previously described [82, 206]. Briefly, throughout December 2009 and August 2010, a total of 1,300 consecutive cervical samples were obtained from women aged 25-64

years who participated in the national cervical cancer screening programme (screening population). Additionally, according to the VALGENT protocol [87], 300 cytological abnormal samples were collected between January 2014 and May 2015, which included 100 women with atypical squamous cervical cells of undetermined significance (ASC-US), 100 women with low-grade squamous intraepithelial lesion (LSIL) and 100 women with high-grade squamous intraepithelial lesion (HSIL) (enrichment population). Ethical approval for the study was obtained from the Medical Ethics Committee of the Republic of Slovenia (consent numbers: 83/11/09 and 109/08/12).

Conventional cytology smears were obtained in compliance with the standard routine gynaecological practice in Slovenia and categorised according to the 2001 Bethesda System[24]. In order to perform HPV DNA testing, a second sample was obtained and placed into ThinPrep vial (ThinPrep PreservCyt solution, Hologic, Marlborough, MA, USA). Coded ThinPrep vials were transported each week to the Laboratory for Molecular Microbiology of the Faculty of Medicine, University of Ljubljana. The 1,600 ThinPrep specimens were labelled with anonymous study number and split into several aliquots immediately upon arrival at the laboratory. Two of the aliquots were used for testing with HC2 (4ml) and RealTime (500 uL). Testing of the screening and the enrichment population was performed in 2010 and 2014, respectively. The remaining aliquots were stored at -70°C and were used for other HPV DNA tests included in the VALGENT-3 framework [82]. In 2016, the Linear Array testing was performed using using 50 uL of DNA extracted from 1 mL of the original ThinPrep aliquot.

4.2.2 HPV testing

The Linear Array is a HPV genotyping test, which enables identification of 37 high- and low-risk HPV types (HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 44, 45, 51, 52, 53, 54, 56, ,58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, 89, and IS39) [197, 198]. Linear Array is qualitative test which uses biotinylated primers sets PGM09/PGMY11 and PC04/GH20 for simultaneous amplification of a 450 bp and 268 bp fragments of the HPV L1 gene and human beta-globin gene, respectively. Following PCR amplification, genotyping is performed using a single nylon strip coated with HPV type-specific and human beta-globin-specific oligonucleotide probes [198]. Testing was performed in accordance with the manufacturer's instructions.

HC2 detects 13 hrHPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and was used as the standard comparator test for the assessment of the clinical performance of the Linear Array. For the purpose of this study, hrHPV positivity for Linear Array was defined as the presence of one or more of the 13 HPV types also detected by HC2 unless otherwise specified.

The RealTime test is an automated multiplex real-time PCR-based assay, which enables concurrent individual detection of HPV16 and HPV18 and pooled detection of 12 other hrHPV genotypes (HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68).

4.2.3 Clinical outcomes and performance assessment

The algorithm of cytological assessment and referral for colposcopy has been described in detail previously [82]. Briefly, all cervical specimens were evaluated by certified cytologists who were blinded to HPV results. Women with atypical squamous cells, cannot exclude high-grade lesion (ASC-H) or worse were referred to immediate colposcopy according to the Slovenian national screening guidelines [207]. Women who were HPV16 and/or HPV18 positive were referred to colposcopy, regardless of their cytology results. Colposcopy-directed punch biopsies were obtained from areas that were suspicious for high-grade lesions, and pathologists, who were unaware of the HPV results, performed histopathological assessment.

We considered histologically confirmed CIN2+ as the clinical disease outcome. Because women negative for intraepithelial lesion or malignancy (NILM) were not referred to colposcopic verification, we considered them as subjects without disease if they had two or more consecutive NILM cytological results (at enrolment and ≥ 1 year within 36-48 months of follow-up). This group was used to compute the clinical specificity for ≤CIN1. The clinical sensitivity and specificity of the Linear Array for CIN2+ or CIN3+ were calculated. Clinical performance was assessed separately for the total study population and for women >30 years. Using a non-inferiority statistics with a relative sensitivity threshold of 90% and a relative specificity threshold of 98%, we compared the clinical performance of the Linear Array to that of the HC2 [86, 208]. The McNemar test was used in order to compare differences between matched proportions [209]. The level of statistical significance for the non-inferiority test and McNemar was set at a value of 0.05. All analyses were performed using STATA version 14 (College Station, TX, USA).

As a secondary objective, we also compared the clinical sensitivity and specificity of hrHPV testing with the Linear Array and with the RealTime, considering the 14 genotypes targeted by RealTime as hrHPV positive. The agreement between the Linear Array and the RealTime was also assessed, separately for HPV16, HPV18, non-16/18 hrHPV and hrHPV using the Kappa statistic [210].

4.3 Results

4.3.1 Study population characteristics

The demographics and cytopathological results of the study population have already been described[206]. Briefly, the average age of women in the total study population (screening and enrichment population) was 39 years (range, 20-77), with 18.4% of the population <30 years. In the screening population (n=1,300), the cytological stratification was as follows[206]:: NILM (95.2%), ASC-US (2.4%), ASC-H (0.2%), atypical glandular cells (AGC, <0.1%), LSIL (1.0%), and HSIL (1.1%). When the total study population (n=1,600) was considered, 45 histologically confirmed CIN2 and 82 CIN3+ were identified; however, the majority of CIN2+ cases (107/127; 84.2%) were identified within the enrichment population. As shown in **Table 4.1**, a total of 1,216 (76.0%) women had two consecutive negative cytology results (NILM).

Table 4.1 Clinical outcomes used for the computation of clinical sensitivity and specificity from the two study populations: 1,300 consecutive women participating in the national cervical cancer screening program (screening population) and 300 women with abnormal cytology (enrichment population).

Clinical outcomes	Screening population	Enriched population	Total study population
Non-disease outcome			
Two consecutive negative cytology results (with rescreening after 36-48 months)	1,216	0	1,216
Disease outcomes			
CIN2	9	36	45
CIN3	9	69	78
CIS a	2	0	2
Squamous Cancer	0	1	1
Adenocarcinoma	0	1	1

CIS: Carcinoma in situ

4.3.2 Prevalence of hrHPV types

Of the 1,300 consecutive samples obtained from women included in the Slovenian HPV Prevalence Study [82] (the screening population), 10.5% of women (137/1300) tested positive for the presence of any of the 13 hrHPV types using Linear Array. When 14 hrHPV types included in the RealTime (13 hrHPV types in HC2 plus HPV66) were considered, the test positivity rate in the screening population was 10.9% (141/1300). The hrHPV prevalence decreased with age and was 23.7% in age group 20-29 years, 10.4% in age group 30-39, 6.7% in age group 40-49, and 5.0% in age group 50-59 (Figure 4.1, top left graph). We observed similar trends for all individual hrHPV types as well ($p<0.01$). The type-specific prevalence was 2.3% (30/1300) for HPV16, 0.9% (12/1300) for HPV18, 2.5% (33/1300) for HPV31, 0.9% (11/1300) for HPV33, 0.2% (3/1300) for HPV35, 0.9% (11/1300) for HPV39, 0.4% (5/1300) for HPV45, 1.5% (19/1300) for HPV51, 0.9% (11/1300) for HPV52, 0.4% (5/1300) for HPV56, 0.4% (6/1300) for HPV58, 0.9% (11/1300) for HPV59, 0.3% (4/1300) for HPV68 and 0.9 (12/1300) for HPV66. The overall and type-specific prevalence of hrHPV according to 10-year age groups is shown in **Figure 4.1**.

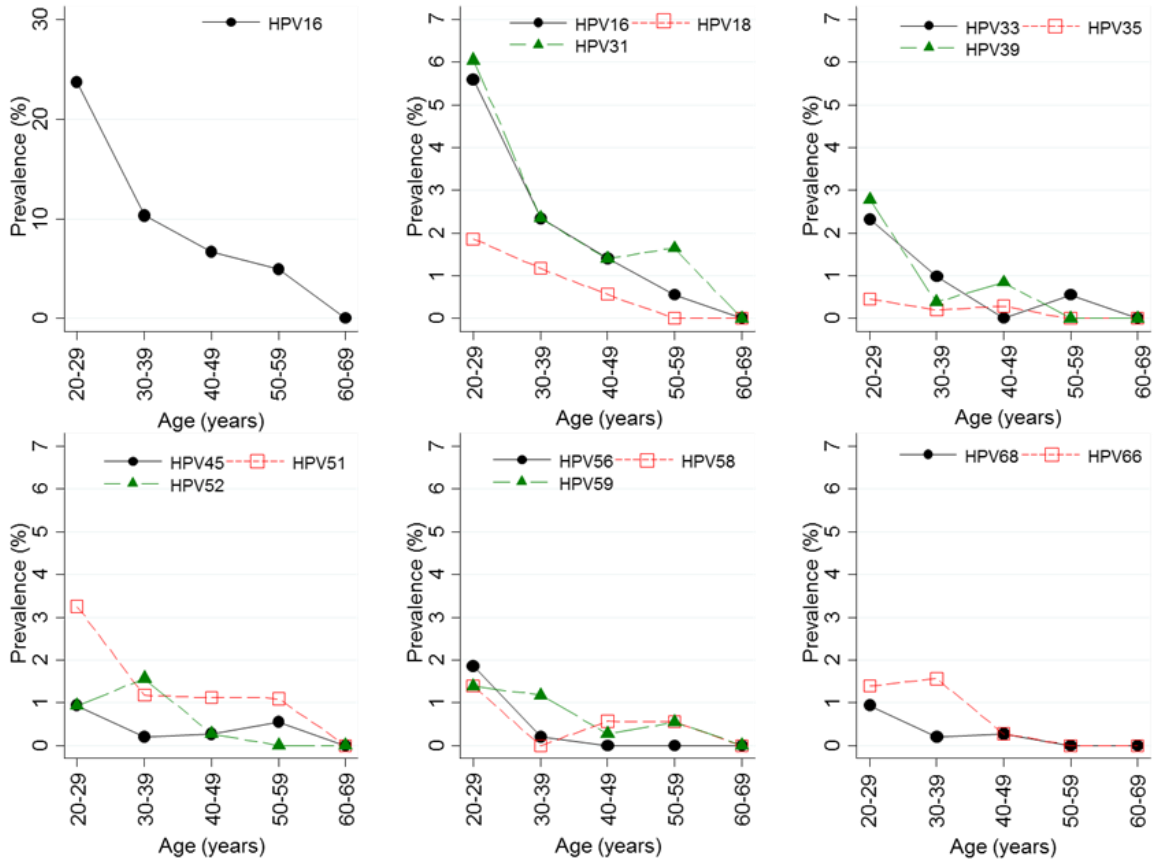


Figure 4.1 The overall and type-specific prevalence of 13 hrHPV types per 10-year age groups in the screening population.

4.4 Clinical performance of the LA compared to HC2

Comparison of the Linear Array and HC2 results stratified for CIN2+, CIN3+ and ≤CIN1 in the total study population and in women >30 years is presented in **Table 4.2**. In the total study population, the clinical sensitivity of the Linear Array and HC2 for detection of CIN2+ was 97.6% (95% CI, 93.3— 99.5%) and 96.1% (95% CI, 91.1— 98.7%), respectively, and for CIN3+ 100% (95% CI, 95.6—100%) and 97.6% (95% CI, 91.5—99.7%), respectively. When the analysis was restricted to women >30 years, the clinical sensitivity of the Linear Array and HC2 for detection of CIN2+ was 98.0% (95% CI, 92.8—99.8%) and 95.9% (95% CI, 89.9—98.9%), respectively, and for CIN3+ 100% (95% CI, 94.6—100%) and 97.0% (95% CI, 89.5—99.6%), respectively. The clinical specificity for ≤CIN1 of the Linear Array and HC2 was 91.7% (95% CI, 90.0—93.2%) and 90.1% (95% CI, 88.3—92.3%) in the total study population and 94.3% (95% CI, 92.7—95.6%) and 92.7% (95%CI, 90.9—94.2%) in women >30 years old, respectively.

Table 4.2 Comparison of the Linear Array and HC2 for the identification of 13 hrHPV in women with CIN2+, CIN3+ and ≤CIN1. Analysis was performed separately for the total study population and for women >30 years old.

Clinical outcome	HC2 result	No. of women with Linear Array result			Clinical performance parameters % (95% CI)	
		+a	-	Total	HC2	Linear Array
Total study population						
CIN2+	+	121	1	122	Sensitivity (CIN2+):	Sensitivity (CIN2+):
	-	3	2	5	96.1 (91.1 – 98.7)	97.6 (93.3 -99.5)
	Total	124	3	127		
CIN3+	+	80	0	80	Sensitivity (CIN3+):	Sensitivity (CIN3+):
	-	2	0	2	97.6 (91.5-99.7)	100 (95.6 -100)
	Total	82	0	82		
≤CIN1	+	91	29	120	Specificity:	Specificity:
	-	10	1,086	1,096	90.1 (88.3-91.8)	91.7 (90.0-93.2)
	Total	101	1,115	1,216		
Women >30 years old						
CIN2+	+	93	1	94	Sensitivity (CIN2+):	Sensitivity: (CIN2+)
	-	3	1	4	95.9 (89.9-98.9)	98.0 (92.8-99.8)
	Total	96	2	98		
CIN3+	+	64	0	64	Sensitivity (CIN3+):	Sensitivity (CIN3+):
	-	2	0	2	97.0 (89.5-99.6)	100 (94.6-100)
	Total	66	0	66		
≤CIN1	+	51	23	74	Specificity:	Specificity:
	-	7	932	939	92.7 (90.9–94.2)	94.3 (92.7-95.6)
	Total	58	955	1,013		

a. A positive Linear Array results represents detection of at least one of the following 13 hrHPV types included in the HC2: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and HPV 68.

The relative clinical sensitivity and specificity of the Linear Array compared to HC2 is shown in **Table 4.3**. The Linear Array had a relative sensitivity of 1.02 (95% CI, 0.98–1.05; P_{McN}=0.625 and $P_{n.inf}$=0.0001) for CIN2+ and 1.03 (95% CI, 0.99-1.06; P_{McN}=0.500 and Pn.inf =0.0004) for CIN3+. The relative specificity of the Linear Array for ≤CIN1 was 1.02 (95% CI, 1.01-1.03; P_{McN}=0.0034 and $P_{n.inf}$ =0.0000). Similar results were obtained if the analysis was restricted to women >30 years (**Table 4.3**).

The absolute sensitivity and specificity of Linear Array for CIN2+ was hardly affected if 14 HPV types (IARC-2009 hrHPV types plus HPV66 and HPV68) were used to define hrHPV positivity. As shown in the Supplementary information, Table 4.S1, the sensitivity for CIN2+ was 98.4% (94.4-99.8%) and specificity was 91.4% (89.7-93.0%) in the total study population. The relative accuracy estimates of Linear Array compared to HC2 were also very similar (Table 4.S1).

Table 4.3 Relative sensitivity for CIN2+ and CIN3+ and relative specificity for ≤CIN1 of the Linear Array compared to HC2.

Study population	Parameter	Clinical outcome	Relative accuracy (95% CI)	PMcNa	Pn.infb
	Relative sensitivity	CIN2+	1.02 (0.98-1.05)	0.6250	0.0001
Total study population	Relative sensitivity	CIN3+	1.03 (0.99-1.06)	0.5000	0.0004
	Relative specificity	≤CIN1	1.02 (1.01-1.03)	0.0034	0.0000
	Relative sensitivity	CIN2+	1.02 (0.98-1.06)	0.6250	0.0008
Women >30 years old	Relative sensitivity	CIN3+	1.03 (0.99-1.08)	0.5000	0.0000
	Relative specificity	≤CIN1	1.02 (1.01-1.03)	0.0052	0.0000

a. P for the McNemar test for a difference between matched proportions and PMcN>0.05 indicates that the sensitivity or specificity of the Linear Array assay are not significantly different from that of the HC2.
b. P for the test for non-inferiority. A sensitivity threshold of at least 90% and a specificity threshold of at least 98% relative to that of the HC2 were applied in a non-inferiority score test. Pn.inf. <0.05 means that the sensitivity or specificity of the Linear Array is not significantly lower than that of the HC2.

4.5 Comparison of the Linear Array and RealTime

4.5.1 Clinical relative accuracy

The results of the clinical performance of the Linear Array compared to RealTime are summarized in **Table 4.4**. The Linear Array had a relative sensitivity of 1.02 (95% CI, 0.98–1.06; P_{McN}=0.1573 and Pn.inf <0.0001) for CIN2+ and 1.03 (95% CI, 0.99–1.04; P_{McN}=0.3173 and Pn.inf =0.0007) for CIN3+. The relative specificity of the Linear Array for ≤CIN1 was 1.00 (95% CI, 0.97–1.02; P_{McN}=0.1025 and $P_{n.inf}$ =0.0001). Similar results were obtained when the analysis was restricted to women >30 years (**Table 4.4**).

Table 4.4 Relative sensitivity for CIN2+ and CIN3+ and relative specificity for ≤CIN1 of the Linear Array compared to RealTime.

Study population	Parameter	Clinical outcome	Relative accuracy (95% CI)	P_{McN}[a]	$P_{n.inf}$[b]
	Relative sensitivity	CIN2+	1.02 (0.98-1.06)	0.1573	<0.001
Total study population	Relative sensitivity	CIN3+	1.03 (0.99-1.04)	0.3173	0.0007
	Relative specificity	≤CIN1	1.00 (0.97-1.02)	0.1025	0.0001
	Relative sensitivity	CIN2+	1.02 (0.98-1.06)	0.1573	0.0001
Women >30 years old	Relative sensitivity	CIN3+	1.02 (0.99-1.05)	0.3173	0.0017
	Relative specificity	≤CIN1	1.00 (0.97-1.02)	0.0455	0.0002

a. P for the McNemar test for a difference between matched proportions and PMcN>0.05 indicates that the sensitivity or specificity of the Linear Array assay are not significantly different from that of the RealTime.
b. P for the test for non-inferiority. A sensitivity threshold of at least 90% and a specificity threshold of at least 98% relative to that of the RealTime were applied in a non-inferiority score test. Pn.inf. <0.05 means that the sensitivity or specificity of the Linear Array is not significantly lower than that of the RealTime.

4.5.2 Analytical comparison

The analytical agreement between the Linear Array and RealTime was assessed separately for HPV16, HPV18, non-16/18 hrHPV positive and hrHPV positive result (**Table 4.5**). The concordance between the Linear Array and RealTime were 99.9%, 99.7%, 99.3%, and 99.0% for HPV16, HPV18, non-16/18 hrHPV positive, and hrHPV positive result, respectively, and corresponding Kappa values were 0.99, 0.92, 0.98, and 0.96, respectively.

Table 4.5 The concordance between the Linear Array and RealTime for HPV16, HPV18, non-16/18 hrHPV positive, and hrHPV positive result, and corresponding Kappa values for the total study population

HPV type	LA+/RT+	LA+/RT-	LA-/RT+	LA-/RT-	Concordance	Kappa (95% CI)
HPV 16	113	1	1	1,485	99.9%	0.991 (0.977-1.000)
HPV 18	30	4	1	1,565	99.7%	0.922 (0.853-0.990)
non-HPV16/18 hrHPV+a	228	12	4	1,356	99.0%	0.960 (0.841-0.980)
hrHPV+b	332	9	2	1,257	99.3%	0.979 (0.967-0.992)

LA+ = positive Linear Array, LA- =negative Linear Array, RT+ =RealTime positive, RT- =RealTime negative.
a. non-HPV16/18 hrHPV+: positive for HPV31,33,35,39,45,51,52,56,58,59,66 and 68.
b. hrHPV+: positive for HPV16,18,31,33,35,39,45,51,52,56,58,59,66 and 68.

4.6 Discussion

The Linear Array is a frequently used HPV genotyping tests, which enables consensus and type-specific detection of 37 HPV types. Although the Linear Array was not intended for the use in primary HPV-based cervical cancer screening, it is often used in epidemiological studies and as a test to verify type coverage and cross-reactivity of hrHPV DNA assays with untargeted types [211-213]. For the purpose of this study, Linear Array was considered positive if one or more of the 13 hrHPV types targeted by HC2 was detected, since primary aim of the study was the clinical evaluation where only a subset of clinically most relevant HPV types is usually considered in the screening settings. To the best of our knowledge, the Linear Array has not been validated previously to the standards set forth in international guidelines for evaluation of candidate HPV tests in cervical cancer screening settings [79].

In the present study, we evaluated the clinical performance of the Linear Array compared to HC2 within the VALGENT-3 framework. Regardless of the clinical outcome (CIN2+ or CIN3+) and study population (total study population and women >30 years), the clinical sensitivity and specificity of the Linear Array was consistently high, ranging from 97.6% to 100% and 91.7% to 94.3%, respectively. The Linear Array demonstrated similar sensitivity and higher specificity for detection of CIN2+ compared to standard comparator test HC2, indicating its fulfilment of the equivalency criteria set in the guidelines for the use of HPV DNA tests in primary cervical cancer screening. In addition, the clinical accuracy estimates of the Linear Array and RealTime were similar. Excellent analytical agreement of the Linear Array compared to RealTime was observed for HPV16, HPV18, non-16/18 hrHPV positive and hrHPV positive results.

Linear Array testing was performed 2 to 6 years subsequent to HC2 and RealTime testing. However, our findings show that prolonged storage at -70°C before LA testing did not affect its performance compared to the other two tests performed on fresh samples earlier. Our study confirms previous observations that when appropriately aliquoted and stored, archival ThinPrep samples can be safely used for evaluation of performance of HPV DNA several years after sample collection allowing retrospective cohort studies with long and continuous follow-up [214, 215].

Due to the HPV16 and HPV18 being responsible for approximately 70% of cervical cancer, partial HPV genotyping is frequently used in the triage of hrHPV-positive women [216, 217]. Consequently, several commercially available hrHPV tests have added partial genotyping for HPV16 and 18. However, recent data suggest that wider genotyping for other selected hrHPV types (particularly HPV31 and HPV33) may also be clinically valuable [218]. Based on the results of the Predictors 2 study, classification of hrHPV genotypes into high- (requiring separate readouts), intermediate- and low-risk groups could therefore improve risk stratification and orient new management algorithms [196],[218].

For validation of hrHPV assays, international guidelines propose HC2 or GP5+/6+-EIA as standard comparator tests [86]. Thus far, for HPV tests with limited to full genotyping capacity, no consensus standard comparator has been set up to determine the genotyping accuracy. Given the fact that the analytical performance of the Linear Array has repeatedly been evaluated in the WHO HPV LabNet Proficiency panels demonstrating high proficiency performance and based on our clinical validation, the Linear Array shows a potential to be considered as a reference test for other HPV genotyping tests intended for clinical use [219, 220].

Analysis of discordant genotyping results of clinically validated HPV tests is sometimes very challenging. Often, accurate genotyping tests, but with disadvantageously high analytical sensitivity are used in such discordant analysis causing potential misclassification and bias. Based on our clinical validation and proven high genotyping accuracy, it seems that Linear Array could also be safely used as a reference test in discordant analysis of conflicting results of clinically validated HPV tests.

To conclude, the clinical performance of the Linear Array is non-inferior to the HC2 based on the evaluation in Valgent-3 framework. The Linear Array fulfils the proposed international criteria for HPV DNA tests used in primary cervical cancer screening settings. Linear Array shows great potential to be used as comparator test for existing and newly developed clinical HPV tests with limited to full genotyping capacity.

Conflict of interest

MA’s and LX's institution has received support from VALGENT (1-4) projects, as described previously in details in methodological VALGENT protocol paper (Arbyn2016JClinVirol[87]).

MP's and AO's institution received research grants from Abbott Molecular. Manufacturers of the evaluated HPV tests were not involved in the study design, data collection, data analysis and interpretation, or writing the manuscript.

Acknowledgement

All authors are supported by the seventh framework program of DG Research of the European Commission, through the COHEAHR Network (grant No. 603019).

Supplementary information

Table 4.S1. Performance of Linear Array in comparison to HC2 in women with CIN2+ and ≤CIN1 in the total study population.

Total study population	Linear Array result	HC2 result			Accuracy (95% CI)	
		+	-	Total	Linear Array	HC2
CIN2+ (n=127)	+[a]	121	4	125	Sensitivity	
	-	1	1	2	98.4% (94.4-99.8%)	96.1% (91.1 – 98.7%)
					Relative sensitivity Linear Array/HC2	
	Total	122	5	127	1.02 (0.98 – 1.07) $p_{n.inf}$=0.0004, P_{McN}=0.1797	
≤CIN1 (n=1,215)	+[a]	94	10	104	Specificity	
	-	26	1,086	1,112	91.4% (89.7-93.0%)	90.1% (88.3-91.8%)
					Relative specificity Linear Array/HC2	
	Total	120	1,096	1,215	1.01 (0.99 – 1.04) $p_{n.inf}$<0.0001, P_{McN}=0.0077	

a. A positive Linear Array results represents detection of at least one of the following 14 hrHPV types: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and HPV 68.

CHAPTER 5

5. Clinical evaluation of INNO-LiPA HPV Genotyping Extra II Assay using the VALGENT framework

Adapted from:

Xu L, Padalko E, Ostribenk A, Poljak M, Arbyn M. Clinical evaluation of INNO-LiPA HPV Genotyping Extra II Assay using the VALGENT framework. International Journal of Molecular Sciences 2018; 19: 2704.

Abstract

In this diagnostic test validation study, we assessed the clinical accuracy and HPV genotyping performance of the INNO-LiPA HPV Genotyping Extra II (INNO-LiPA) within the VALGENT-3 framework. VALGENT is designed to assess the analytical and clinical performance of HPV tests with genotyping capacity. The VALGENT-3 panel comprised 1,300 consecutive cervical cell specimens enriched with 300 samples with abnormal cytology obtained from women attending the Slovenian cervical cancer screening programme. The INNO-LiPA allows type-specific detection of 32 HPV types; however, for the clinical accuracy assessment we considered it as high-risk (hr)HPV positive when at least one of the following HPV types was present: HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, and HPV68). Clinical accuracy for detection of cervical intraepithelial neoplasia grade 2 or worse (CIN2+) was compared between INNO-LiPA and Hybrid Capture 2 (HC2), which is a standard comparator test for HPV tests used in cervical cancer screening. In addition, hrHPV and type-specific detection HPV types was compared between INNO-LiPA and Linear Array HPV Genotyping Test (Linear Array). The prevalence of hrHPV determined by INNO-LiPA was 17.1% (95%CI: 15.0-19.2%) in the screening population. HrHPV testing with INNO-LiPA had a sensitivity for CIN2+ of 96.9% (95%CI: 92.1-99.1%) which was non-inferior to HC2 (relative sensitivity of 1.01; 95%CI, 0.97-1.04; pn.inf =0.0002]) and a specificity for ≤CIN1 of 85.3% (95%CI: 83.2-87.3%) which was inferior to HC2 (relative specificity of 0.95; 95%CI, 0.93-0.97; pn.inf =0.9998). Genotyping agreement between INNO-LiPA and Linear Array was excellent for hrHPV, HPV16, HPV18, HPV35, HPV45, HPV58 and HPV59, but good or fair for other HPV types. To conclude, INNO-LiPA demonstrated non-inferior clinical sensitivity but lower specificity compared to HC2 in addition to excellent concordance compared to Linear Array for hrHPV and some genotypes.

5.1 Introduction

Over 200 human papillomavirus (HPV) types have been identified and classified based on their nucleotide sequences, with new HPV types being characterized at an increasing rate [155]. Among them, 12 high-risk HPV (hrHPV) types (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58 and HPV59) are causally linked with cervical cancer and their immediate precursors [194]. In addition, eight more HPV types have been associated with some rare cases of cervical cancer (HPV26, HPV53, HPV66, HPV67, HPV68, HPV70, HPV73 and HPV82) [11].The recognition of the strong etiological association between persistent hrHPV infection and cervical cancer has led to the development of novel HPV tests to enhance secondary prevention of the cervical cancer [105, 221]. Furthermore, randomized controlled trials (RCTs) have demonstrated that HPV-based screening is more effective than cervical cytology in reducing the incidence of invasive cervical carcinoma in primary screening for cervical cancer for women aged 30 years or older [64, 105]. Thus, a number of countries are currently in the process of switching from cervical cytology to HPV based primary screening for cervical cancer [222].

Many HPV tests are available on the market but only few have been clinically validated for use in primary screening settings [197]. The Hybrid Capture 2 assay (HC2; Qiagen, Hilden, Germany) [64, 80-82] and GP5+/6+ PCR-based enzyme immunoassay (GP5+/6+-EIA; Diassay, Rijwijk, the Netherlands) [83-85] are HPV DNA assays that had been clinically validated for primary screening based on longitudinal evidence obtained from large RCTs. Therefore, HC2 and GP5+/6+-EIA are accepted as the standard comparator tests in evaluations of alternative HPV tests [86]. Several other HPV tests have been fully or partially validated and demonstrate non-inferior clinical sensitivity and specificity for detection of cervical intraepithelial neoplasia grade 2 or worse (CIN2+) compared to the standard comparator tests and high inter-and intra-laboratory reproducibility [79, 86]. Majority of validated HPV tests target 13 or 14 hrHPV types in aggregate, but some have limited (partial genotyping for HPV16 and HPV18 only), extended (separate genotyping of HPV16, HPV18 and other hrHPV types) and full (type-specific genotyping of all included types) genotyping ability [79]. Since HPV16 and HPV18 are responsible for approximately 70% of cervical cancer, partial HPV genotyping for these two types is frequently used in the triage of HPV-positive women [216, 217]. Although the usefulness of full genotyping of hrHPV types is not yet established, a recent study showed that in addition to HPV16, HPV31 and HPV33 are more carcinogenic than other hrHPV types, suggesting wider genotyping may also be clinically valuable [218].

INNO-LiPA HPV genotyping assay, based on the principle of reverse hybridization after highly sensitive PCR amplification with SPF10 primers, have been used for HPV genotyping over two decades [223, 224]. During this timeframe, the original assay has undergone several modifications, resulting in a few different versions. The INNO-LiPA HPV Genotyping Extra II assay (INNO-LiPA; Fujirebio Europe, Ghent, Belgium) evaluated in the current study is the most recent assay launched by the company in 2015, targeting 32 types, four types more than the previous version. This new version contains genotype specific probes for more decisive genotyping results, an upgraded SPF10 primer set resulting in improved sensitivity (comparable for all hrHPV types), improved human DNA control primers and provides a ready-to-use amplification reagent [225].

In the present study, the VALidation of HPV GENotyping Tests (VALGENT) framework was used to evaluate the clinical accuracy of INNO-LiPA in comparison with HC2. For the first time it was verified whether hrHPV testing with INNO-LiPA fulfils the minimal requirements for use in primary cervical cancer screening [86]. In addition, type-specific concordance was compared between the INNO-LiPA and the Linear Array HPV Genotyping Test (Linear Array; Roche Molecular Diagnostics, Branchburg, NJ, USA). HrHPV testing with the latter test was recently clinically validated through the VALGENT network as well and has been proposed as a standard analytical HPV genotyping comparator test to resolve discordant typing results of clinically validated HPV assays [226].

5.2 Results

The characteristics of the VALGENT-3 study population, including demographics, cytological and histological results have been described previously [206, 226]. Of the 1,600 samples

analyzed by INNO-LiPA, four samples showed no signal for human HLA-DPB1 gene control line. These four samples were considered as invalid and therefore excluded for further analysis. Of the 1,296 valid samples obtained from screening population, 17.1% women (221/1,296) tested positive for the presence of any of the 13 hrHPV types by INNO-LiPA. The overall and type-specific prevalence of 13 hrHPV types in the total study population determined by INNO-LiPA is summarized in **Table 5.1**. The hrHPV prevalence was 15.2% in women with NILM and increased to 42.8%, 69.0% and 86.0% in women diagnosed with ASC-US, LSIL and HSIL, respectively. The risk ratio of HSIL compared to women with NILM was highest (RR>8.00) in women infected with in HPV16, HPV33, HPV18 and HPV45.

Table 5.1 Overall prevalence of hrHPV (aggregate of 13 types) and of individual hrHPV types detected by INNO-LiPA in the total study population according to baseline cytology

HPV type	HrHPV prevalence (No. and %) by cytology results				Ratio prevalence HSIL/NILM
	NILM (N=1,234)	ASC-US (N=131)	LSIL (N=113)	HSIL (N=114)	
13 hrHPV*	187 (15.2%)	56 (42.8%)	78 (69.0%)	98 (86.0%)	5.7
HPV16	32 (2.6%)	12 (9.2%)	27 (23.9%)	56 (49.1%)	18.9
HPV18	12 (1.0%)	4 (3.1%)	9 (8.0%)	10 (8.8%)	8.8
HPV31	54 (4.4%)	22 (16.8%)	19(16.8)	23 (20.2%)	4.6
HPV33	11 (0.9%)	5 (3.8%)	9 (8.0%)	11 (9.7%)	10.8
HPV35	3 (0.2%)	1 (0.8%)	0 (0.0%)	2 (0.9%)	4.5
HPV39	16 (1.3%)	1 (0.8%)	5 (4.4%)	2 (1.8%)	1.4
HPV45	6 (0.5%)	5 (3.8%)	4 (3.5%)	5 (4.4%)	8.8
HPV51	31 (2.5%)	4 (3.1%)	9 (8.0%)	5 (4.4%)	1.8
HPV52	27 (2.2%)	10 (7.6%)	11 (9.7%)	7 (6.1%)	2.8
HPV56	11 (0.9%)	2 (1.5%)	7 (6.2%)	5 (4.4%)	4.9
HPV58	9 (0.7%)	3 (2.3%)	7 (6.2%)	5 (4.4%)	6.3
HPV59	11 (0.9%)	3 (2.3%)	4 (3.5%)	0 (0.0%)	0
HPV68	16 (1.3%)	5 (3.8%)	7 (6.2%)	5 (4.4%)	3.4

NILM, negative for intraepithelial lesion or malignancy; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.* A positive hrHPV result represents detection of at least one of the 13 hrHPV types included in the HC2: HPV16, HPV18, HPV 31, HPV 33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59 and HPV 68. Women infected with multiple HPV types were counted only once.

5.2.1 Clinical performance of the INNO-LiPA

The accuracy data for the INNO-LiPA and HC2 for the outcomes CIN2+, CIN3+ and ≤CIN1 are shown in **Table 5.2** for the total study population and for women aged 30 years or older. When the whole study population was considered, INNO-LiPA detected 123 of 127 CIN2+ cases and 82 of 82 CIN3+ cases, which corresponds to a sensitivity of 96.9% (95% CI, 92.1-99.1) and 98.8% (93.4 – 100), respectively. The specificity for ≤CIN1 of INNO-LiPA (1,034/1,212) was 85.3% (95% CI, 83.2 – 87.3). Similar results were obtained for women aged 30 years or older.

The relative sensitivity of INNO-LiPA compared to HC2 was 1.01 (95% CI, 0.97─1.04; pmcn=0.6547; pn.inf=0.0002) for CIN2+ and 1.01 (95% CI, 0.97─1.06; pmcn=0.5637; pn.inf =0.001) for CIN3+. The relative specificity of INNO-LiPA for ≤CIN1 was 0.95 (95% CI, 0.93─0.97; pmcn=0.0000; pn.inf =0.0000). Similar results were obtained if the analysis was restricted to women ≥30 years (**Table 5.3**).

5.2.2 Genotyping agreement between INNO-LiPA and Linear Array

In the total study population, concordance between INNO-LiPA and Linear Array was assessed at type-specific level and overall for 13 hrHPV types (**Table 5.4**). Overall concordance of the two assays for 13 hrHPV types was 93.0% and the corresponding Kappa value was 0.805 (95% CI, 0.757-.0854), indicating excellent agreement between INNO-LiPA and Linear Array. Similarly, the level of agreement was also excellent for detection of HPV16, HPV18, HPV35, HPV45, HPV58 and HPV59. However, for the identification of other individual types, level of agreement ranged from good to poor between the two assays (**Table 5.4**). In addition, INNO-LiPA detected more positive cases than Linear Array for all individual types common to both assays.

Table 5.2 Sensitivity of INNO-LiPA and HC2 for detection of CIN2+ and CIN3+ and specificity of both assays for detection of ≤CIN1. Analysis was performed separately for the total study population and for women ≥30 years old.

Assay, study population and clinical outcome	**Sensitivity**			**Specificity**		
	n/N	%	95%CI	n/N	%	95%CI
INNO-LiPAa						
Total study population						
CIN2+	123/127	96.9	(92.1 – 99.1)			
CIN3+	81/82	98.8	(93.4 – 100)			
≤CIN1				1,034/1,212	85.3	(83.2 – 87.3)
Women >30 years old						
CIN2+	95/98	96.9	(91.3-99.4)			
CIN3+	65/66	98.5	(91.8-100)			
≤CIN1				887/1,009	87.9	(85.7-89.9)
HC2						
Total study population						
CIN2+	122/127	96.1	(91.1-98.7)			
CIN3+	80/82	97.6	(91.5-99.7)			
≤CIN1				1,092/1,212	90.1	(88.3-91.8)
Women >30 years old						
CIN2+	94/98	95.9	(89.9-98.9)			
CIN3+	64/66	97.0	(89.5-99.6)			
≤CIN1				935/1,009	92.7	(90.9–94.2)

a. Positive INNO-LiPA results represents detection of at least one of the following 13 hrHPV types included in the HC2: HPV16, HPV18, HPV 31, HPV 33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59 and HPV 68.n, number of cases; N, total number of cases; CI, confidence interval.

Table 5.3 Relative sensitivities for detection of CIN2+ and CIN3+ and relative 123/127 for detection of ≤CIN1 of INNO-LiPA versus HC2. Analysis was performed separately for the total study population and for women ≥30 years old.

INNO-LiPA vs HC2	Relative sensitivity	Relative specificity	p_{mcn}[a]	$p_{n.inf}$[b]
Total study population				
CIN2+	1.01 (0.96-1.06)		0.6547	0.0002
CIN3+	1.01 (0.97-1.06)		0.5637	0.001
≤CIN1		0.95 (0.93-0.97)	<0.001	0.9998
Women >30 years old				
CIN2+	1.01 (0.96-1.06)		0.6547	0.001
CIN3+	1.02 (0.96-1.07)		0.5637	0.003
≤CIN1		0.95 (0.92-0.98)	<0.001	0.999

a p for the McNemar test for a difference between matched proportions and pmcn>0.05 indicates that the sensitivity or specificity of the INNO-LiPA assay are not significantly different from that of the HC2.
b p for the test for non-inferiority. A sensitivity threshold of at least 90% and a specificity threshold of at least 98% relative to that of the HC2 were applied in a non-inferiority score test. pn.inf <0.05 means that the sensitivity or specificity of the INNO-LiPa is not significantly lower than that of the HC2.

Table 5.4 Agreement (concordance and kappa values) between the INNO-LiPA and the Linear Array for overall hrHPV positivity and for 29 individual HPV types common to both assays in the total study population

HPV type	I+/L+	I+/L-	I-/L+	I-/L-	Concordance	Kappa (95% CI)	p_{mcn}[a]
13 hrHPVb	318	103	9	1,166	93.0%	0.805 (0.757 - 0.854)	<0.001
HPV16	112	16	2	1,466	99.0%	0.920 (0.871– 0.969)	0.001
HPV18	31	6	3	1,556	99.4%	0.870 (0.822 – 0.920)	0.3173
HPV31	68	50	1	1,417	96.8%	0.712 (0.664 – 0.759)	<0.001
HPV33	24	13	1	1,558	99.2%	0.770 (0.722 – 0.818)	0.0013
HPV35	5	0	0	1,591	100.0%	1.000 (0.951 – 1.049)	1.0000
HPV39	15	9	2	1,570	99.1%	0.728 (0.680 – 0.777)	0.0348
HPV45	14	6	0	1,576	99.6%	0.822 (0.774 – 0.870)	0.0143
HPV51	33	16	1	1,546	98.9%	0.790 (0.742 – 0.838)	<0.001
HPV52	30	26	2	1,538	94.6%	0.674 (0.633 – 0.714)	0.8840
HPV56	16	9	7	1,564	99.0%	0.662 (0.613 – 0.712)	0.6171
HPV58	19	5	0	1,572	99.7%	0.882 (0.833 – 0.931)	0.0253
HPV59	17	2	2	1,575	99.8%	0.894 (0.845 – 0.943)	1.0000
HPV68	7	26	0	1,563	98.4%	0.345 (0.308 – 0.382)	<0.001
HPV26c	0	0	0	1,596	100.0%	-	1.0000
HPV53	43	23	1	1,529	98.5%	0.774 (0.726 – 0.822)	<0.001
HPV66	29	16	1	1,550	98.9%	0.768 (0.720 – 0.816)	<0.001
HPV70	11	10	0	1,575	99.4%	0.685 (0.638 – 0.731)	0.0016
HPV73	19	7	2	1,570	99.4%	0.788 (0.739 – 0.837)	0.0956
HPV82	5	4	1	1,586	99.7%	0.665 (0.617 – 0.713)	0.1797
HPV06	7	14	1	1,574	99.1%	0.479 (0.435 – 0.523)	0.0008
HPV11	2	3	0	1,591	99.8%	0.571 (0.526 – 0.615)	0.0833
HPV40	1	4	0	1,591	99.8%	0.333 (0.296 – 0.369)	0.0455
HPV42	3	5	9	1,579	99.1%	0.296 (0.248 – 0.344)	0.2850
HPV54	10	14	12	1,560	98.4%	0.427 (0.378 – 0.476)	0.6949
HPV61	16	17	6	1,557	98.6%	0.575 (0.527 – 0.623)	0.0218
HPV62	15	12	9	1,560	98.7%	0.582 (0.533 – 0.632)	0.5127
HPV67	3	5	1	1,587	99.6%	0.498 (0.452 – 0.543)	0.1025
HPV81	3	3	1	1,589	99.8%	0.599 (0.551 – 0.647)	0.3173
HPV83	0	6	3	1,587	99.4%	-0.003 (-0.049 -0.044)	0.3173

I+= INNO-LiPA positive; I-= INNO-LiPA negative; L+=Linear Array positive; L-=Linear Array negative.
Color legend (adapted from Landis and Koch for the levels of agreement[227]): dark green (1.00 ≥ κ >0.80): excellent; light green (0.80 ≥ κ > 0.60): good; yellow (0.60 ≥ κ > 0.40): moderate; orange (0.40 ≥ κ > 0.20): fair; red (0.20 ≥ κ > 0.00): poor.
a. p for the McNemar test for a difference between matched proportions and pmcn <0.05 indicates that the HPV positivity detected by INNO-LiPA is significantly different from that of the Linear Array. b. 13 hrHPV types:

HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59 and HPV 68.c. No HPV26 positive cases detected by both assays, kappa value not applicable.

5.3 Discussion

The INNO-LiPA provides full genotyping capability for 32 HPV types. For the purpose of this study, INNO-LiPA was considered positive if at least one of the 13 hrHPV types targeted by HC2 was detected.

To the best of our knowledge, the INNO-LiPA has not been validated previously according to the international guidelines for evaluation of new HPV tests in primary cervical cancer screening settings. Here, we present the first study to evaluate the clinical performance of the INNO-LiPA compared to HC2 using samples from the VALGENT-3 panel. In the whole study population, the INNO-LiPA showed a sensitivity for the detection of CIN2+ and CIN3+ of 97% and 99%, respectively, which was similar to HC2. However the clinical specificity for ≤CIN1 was only 85% which was 5% (95% CI 3-7%) lower than the comparator test.

INNO-LiPA is a SPF10 PCR that targets a short highly conserved region in the L1 gene [224, 228]. The small size of the amplicon makes the test analytically very sensitive. However, at the same time, discrimination of the individual types is challenging and complex and it is therefore not so surprising that the clinical specificity is lower compared to HPV tests targeting longer DNA sequences [229]. The small size of the amplicon makes INNO-LiPA particularly useful for testing of archived cell preparations or formaline-fixed-paraffin-embedded tissue blocks stored over long periods where parts of the viral genome can be fragmented [214].

INNO-LiPA provides for each HPV type a qualitative output which is translated into a positive/negative result. The appreciation of presence or absence of blue lines is not quantifiable. Therefore, adaptation of the cut-off, which may allow a more optimal balance between clinical sensitivity and specificity, is in case of INNO-LiPA not possible.

Excellent analytical agreement between INNO-LiPA and Linear Array was observed for 13 hrHPV types overall, HPV16, HPV18, HPV35, HPV45, HPV58 and HPV59. In addition, INNO-LiPA detected more positive cases than Linear Array for all individual HPV types that are common to both assays, with positivity rate of hrHPV determined by INNO-LiPA statistically significantly higher than that determined by Linear Array (pmcn<0.001) in the total study population. The significant difference of positivity rate for hrHPV with these two genotyping methods is consistent with the tests' clinical performances with regard to relative specificity compared to HC2.

In conclusion, in our study INNO-LiPA exhibits lower clinical specificity, however this is most likely due to detection of HPV infections with low concentrations and therefore can play an important role in evaluation of viral infection outcomes of vaccination trials and in monitoring the impact of HPV vaccination. Moreover, INNO-LiPA may be useful in epidemiological studies to investigate the prevalence and distribution of HPV types and in studies of the natural history of HPV infection at the type-specific level.

5.4 Materials and methods

5.4.1 Clinical specimens

The VALGENT framework is designed to assess the comparative analytical and clinical performance of HPV tests that offer limited to full genotyping capability [87]. VALGENT is iterative, using specimens collected in different countries. The first two VALGENT panels have been completed, using samples collected from Belgium [199-201] and Scotland [202-205]. The third study panel (VALGENT-3) was collated in Slovenia as previously described [82, 206, 226]. Briefly, 1,300 consecutive cervical samples were collected from women who participated in the organised Slovenian national cervical cancer screening program between December 2009 and August 2010 (screening population). The study panel was enriched with 300 cytologically abnormal specimens collected between January 2014 and May 2015 (enrichment population). As required in the VALGENT protocol [87], the enrichment population included 100 women with atypical squamous cervical cells of undetermined significance (ASC-US), 100 women with low-grade squamous intraepithelial lesion (LSIL) and 100 women with high-grade squamous intraepithelial lesion (HSIL).

The sample collection, aliquoting procedure and storage details have been described in detail in previous VALGENT-3 manuscripts [206, 226]. Briefly, in July 2016 Ghent University (Belgium) received 1,600 samples of extracted DNA. DNA was extracted from original samples collected into ThinPrep PreservCyt solution (Hologic, Marlborough, MA, USA). Prior to DNA extraction, 1 mL aliquot of original ThinPrep sample was centrifuged at 13,000 g for 15 minutes with supernatant being discarded and cellular pellet resuspended in 200 µl PBS buffer. DNA extraction was performed using QIAamp MinElute Media Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Bound DNA was finally eluted with 50 µl of ATE buffer and stored at -70°C prior to further testing. According to the manufacturer's instructions we have used 10 µl of extracted DNA for INNO-LiPA testing. Similarly, from the second aliquot of original ThinPrep DNA was extracted for Linear Array testing, where 50 µl of extracted DNA was used for further testing.

5.4.2 HPV testing

5.4.2.1 INNO-LiPA HPV Genotyping Extra II

The INNO-LiPA is a line probe assay based on the principle of reverse hybridization for qualitative detection and identification of 32 different HPV types, including 13 hrHPV (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59 and HPV68), 6 possible hrHPV (HPV26, HPV53, HPV66, HPV70, HPV73 and HPV82), 9 low-risk HPV (HPV6, HPV11, HPV40, HPV42, HPV43, HPV44, HPV54, HPV61 and HPV81) plus 4 other HPV genotypes (HPV62, HPV67, HPV83 and HPV89). INNO-LiPA uses the biotinylated consensus primers (SPF10) to amplify a 65-bp region within the L1 region of multiple alpha HPV types. The resulting biotinylated amplicons are then denatured and hybridized with specific oligonucleotide probes. A primer set for the amplification of the human HLA-DPB1 gene is included to monitor sample quality and extraction. The INNO-LiPA assay (sample incubation,

stringent wash and color development) was performed fully automated using the AutoBlot 3000H (Bio-Rad Laboratories Inc, Hercules, CA, USA). Interpretation of the developed strips was done by scanning and automated interpretation using with the LiRAS for LiPA HPV software (Fujirebio Europe, Ghent, Belgium). The test was performed in accordance with the manufacturer's instructions.

5.4.2.2 HC2

HC2 detects 13 hrHPV types (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, and HPV68) and is accepted as a standard comparator test for the clinical validation of hrHPV DNA assays which may be used for primary cervical cancer screening [86]. For the purpose of the present study, hrHPV positivity for INNO-LiPA was defined as the presence of one or more of the 13 hrHPV targeted by HC2.

5.4.2.3 Linear Array

The Linear Array is HPV test with full genotyping capacity, which detects 37 high- and low-risk HPV types (HPV6, HPV11, HPV16, HPV18, HPV26, HPV31, HPV33, HPV35, HPV39, HPV40, HPV42, HPV44, HPV45, HPV51, HPV52, HPV53, HPV54, HPV56, HPV58, HPV59, HPV61, HPV62, HPV64, HPV66, HPV67, HPV68, HPV69, HPV70, HPV71, HPV72, HPV73, HPV81, HPV82, HPV83, HPV84, HPV89, and IS39) that is frequently used in virological and epidemiological research. In the present study, Linear Array is used as a comparator test to evaluate the analytical genotyping accuracy of the INNO-LiPA.

5.4.2.4 Clinical outcome and INNO-LiPA performance assessment

As described in previous VALGENT-3 reports [206, 226], cytological assessment and referral of patients with abnormal cytology results to colposcopy were done according to the Slovenian national screening guidelines [207] which are in agreement with European guidelines [110]. Colposcopy-directed punch biopsies were obtained from suspicious areas for final histopathological assessment.

Women with histologically confirmed CIN2+ results were considered as diseased subjects. Due to the fact that women with normal cytological result of negative for intraepithelial lesion or malignancy (NILM) were not referred to colposcopy verification in our study, we considered them as subjects without disease only if they had two or more consecutive NILM cytological results (at enrolment and at subsequent screening between 12 to 48 months later). We used this group of women to compute the clinical specificity for ≤CIN1.

The clinical sensitivity and specificity of the INNO-LiPA for CIN2+ and CIN3+ were calculated. We compared the clinical accuracy of INNO-LiPA to HC2 for CIN2+ and CIN3+, using non-inferiority statistics with a relative sensitivity threshold of 90% and a relative specificity threshold of 98% [208]. The McNemar statistic was used in order to compare the differences between matched proportions [209]. For both statistics, the level of significance was set at 0.05. All analyses were performed using STATA version 14 (College Station, TX, USA).

Separate and consensus genotyping agreement for the types common to INNO-LiPA and Linear Array was assessed using Kappa [230] and McNemar statistics [209]. Kappa values from 0.0 to 0.20, 0.21 to 0.40, 0.41 to 0.60, 0.61 to 0.80 and 0.81 to 1.0 indicate poor, fair, moderate, good and excellent level of agreement between two assays. A McNemar p-value <0.05 indicates significant discordance between genotyping results determined by the two assays.

Funding: L.X., A.O., M.P. and M.A. were supported by the COHEAHR Network (grant No. 603019), which was funded by the 7th Framework Programme of DG Research and Innovation, European Commission (Brussels, Belgium).

Acknowledgment: Fujirebio Europe provided INNO-LiPA HPV genotyping Extra II kits to the Department of Clinical Chemistry, Microbiology and Immunology, Ghent University Hospital.

Conflicts of Interest: M.A.’s and L.X.'s institution has received support from VALGENT projects, as described previously in the VALGENT protocol paper [87]. M.P.’s and A.O.'s institution received research grants from Abbott Molecular. E:P ‘s institution received reagents for testing INNO-LiPA HPV genotyping Extra II assay free of charge from the manufacturer for the duration of the testing.

CHAPTER 6

6. Using the VALGENT-3 framework to assess the clinical and analytical performance of RIATOL qPCR HPV Genotyping assay

Adapted from:

Benoy I*, **Xu L*,** Vanden Broeck D, Poljak M, Ostrebenk A, Arbyn M, Bogers J. Using the VALGENT-3 framework to assess the clinical and analytical performance of RIATOL qPCR HPV Genotyping assay. Journal of Clinical Virology 2019; 120: 57-62.
*Joint first author

Abstract

Background and objective: The VALGENT framework is developed to assess the clinical performance of HPV tests that offer genotyping capability. Samples from the VALGENT-3 panel are used to identify an optimal viral concentration threshold for the RIATOL qPCR HPV genotyping assay (RIATOL qPCR) to assure non-inferior accuracy to detect high-grade cervical intraepithelial neoplasia (CIN), compared to Qiagen Hybrid Capture 2 (HC2), a standard comparator test validated for cervical cancer screening.

Study design: The VALGENT-3 panel comprised 1,300 samples from women participating in the Slovenian cervical cancer screening programme, enriched with 300 samples from women with abnormal cytology. In follow-up, 126 women were diagnosed with CIN2+ (defined as diseased) and 1,167 women had two consecutive negative Pap smears (defined as non-diseased).

All 1,600 samples were analysed with the RIATOL qPCR. Viral concentration was expressed as viral log10 of the number of copies/ml. A zone of viral concentration cut-offs was defined by relative ROC analysis where the sensitivity and specificity were not inferior to HC2.

Results: The RIATOL qPCR had a sensitivity and specificity for CIN2+ of 97.6% (CI: 93.2-99.5%) and 85.1% (CI: 82.9-87.1%), respectively, when the analytical cut off was used. At a cut off of 6.5, RIATOL qPCR had a sensitivity of 96.0% (CI: 91.0-98.7%) and a specificity of 89.5% (87.6-91.2%). At optimized cut off, accuracy of the qPCR was non-inferior to the HC2 with a relative sensitivity of 1.00 [CI: 0.95-1.05 (p=0.006)] and relative specificity of 1.00 [CI: 0.98-1.01 (p=0.0069)].

Conclusions: The RIATOL qPCR has a high sensitivity and specificity for the detection of CIN2+. By using a fixed cut-off based on viral concentration, the test is non-inferior to HC2. HPV tests that provide viral concentration measurements or other quantifiable signals allow flexibility to optimize accuracy required for cervical cancer screening.

6.1 Background and objectives

Human papillomaviruses (HPV), common sexually transmitted viruses with more than 200 genotypes, are the principal cause of cervical cancer. Twelve high-risk HPV (hrHPV) genotypes (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58 and HPV59) are recognized by the International Agency for Research on Cancer (IARC) as class I or IIa carcinogens[194].

Current cervical cancer screening recommendations are being revised towards primary HPV testing[105, 195]. Many HPV assays, that are currently used in clinical practice identify hrHPV genotypes as a group with limited genotyping capacity (often with separate identification of HPV16 and HPV18 only). HPV full genotyping assays (separate identification of all hrHPV genotypes) can be important in risk-based management of screen-positive women, as marker

for the detection of minimal residual disease after treatment for cervical lesions and to monitor vaccination effects.

Several HPV assays generate a signal which increases in strength with the amount of virus present in the sample and use this signal to generate a dichotomous result. However, the quantitative value of the signal can be used to adapt the optimal clinical threshold to assure satisfactory accuracy for detection of high-grade cervical intraepithelial neoplasia (CIN) lesions in a screening population. Exact quantitative values are rarely used for reporting HPV results since their clinical/prognostic value is still a matter of ongoing debate[231-236].

Given the multitude of commercially available HPV tests, validated assays which assure high-quality screening needs to be identified. A collaborative framework VALidation of HPV GENotyping Tests (VALGENT)[87] allows verification of minimal criteria that needs to be fulfilled for the use of any HPV assays in primary cervical cancer screening. In clinical practice, only clinically validated assays should be used and continuous monitoring of test performance is necessary to assure optimal safety of HPV-based screening programs[79, 194, 237].

The RIATOL qPCR HPV genotyping assay is a laboratory developed test (RIATOL qPCR, Antwerp, Belgium)[238] which has been routinely used in Algemeen Medisch Laboratorium (AML , Sonic healthcare, Antwerp, Belgium) for more than 12 years. It has been accredited (ISO15189) and validated according to the international criteria for HPV DNA tests to be used in primary cervical cancer screening settings[79, 86]. Besides a qualitative result for 14 hrHPV genotypes (class I and IIA carcinogenic HPV genotypes plus HPV66 and HPV68), the assay also quantifies viral concentration (VC) of each targeted HPV genotype. In this study, we assessed the clinical performance of RIATOL qPCR through the third instalment of VALGENT project (VALGENT-3) and to identify the optimal clinical VC cut-offs to assure that the test fulfils the required accuracy performance criteria for primary cervical cancer screening.

6.2 Study design

6.2.1 Sample collection

The collection of specimens used for the present iteration of VALGENT-3 was performed in Slovenia, as previously reported.[206, 226, 239-241]. In brief, from December 2009 to August 2010, 1,300 consecutive cervical samples were collected from women aged 25-64 years who participated in the Slovenian national cervical cancer screening programme (screening population). Additionally, from January 2014 to May 2015, this collection was enriched with 300 cytological abnormal specimens (enrichment population), which included 100 women with atypical squamous cervical cells of undetermined significance (ASC-US), 100 women with low-grade squamous intraepithelial lesion (LSIL) and 100 women with high-grade squamous intraepithelial lesion (HSIL). Ethical approval was obtained from the Medical Ethics Committee of the Republic of Slovenia (consent numbers: 83/11/09 and 109/08/12).

All samples were stored in ThinPrep PreservCyt solution (Hologic, Marlborough, MA, USA) at -70°C with aliquots disseminated in the Laboratory for Molecular Microbiology of the Faculty of Medicine, University of Ljubljana, Slovenia to participating laboratories for testing with different HPV assays.

In January 2016, samples of 1ml of the original ThinPrep aliquot was sent on dry ice to AML, Department of Molecular Diagnostics, Antwerp, Belgium. Samples were handled with care to avoid contamination during storage, aliquoting, transfer to and reception at AML. Upon arrival at AML, samples were stored at -80°C until further processing.

6.2.2 Cytology

Conventional cytology was performed in accordance with the standard routine gynaecological practice in Slovenia and in agreement with the European guidelines[242]. Results were classified according to the 2001 Bethesda system[24].

6.2.3 Hybrid Capture 2 testing

Hybrid Capture 2 (HC2) testing was done according to the manufacturer's instructions. HC2 detects 13 hrHPV genotypes in aggregate ((class I and IIA carcinogenic HPV genotypes plus HPV68) and was used as the standard comparator test for the assessment of the clinical performance of the RIATOL qPCR.

6.2.4 RIATOL qPCR

The RIATOL qPCR is a fully automated, clinically validated laboratory developed HPV test[238, 243]. Processing of the samples was performed in batches of 91 samples. After thawing, samples were vortexed rigorously and transferred manually to a 96 deep-well block. DNA was extracted from the cervical samples using the Cervista MTA system (Hologic, Bedford, MA, USA), in combination with the Genfind DNA extraction kit. Subsequently, the DNA was amplified using a series of real-time qPCR reactions on the LightCycler 480 type I (Roche Molecular Systems, Pleasanton, California, USA). The presence of 14 different hrHPV genotypes is determined using TaqMan based real-time PCR reactions targeting type specific)[238] sequences of viral genes (HPV16 E7, HPV18 E7, HPV31 E6, HPV33 E6, HPV35 E6, HPV39 E7, HPV45 E7, HPV51 E6, HPV52 E7, HPV56 E7, HPV58 E6, HPV59 E7, HPV66 E6, HPV68 E7). The PCR reactions are done in ultra-low volume (6µl) and are performed in 8 multiplex reactions. Cellularity control is performed on every sample by amplification of the beta-globin gene.

Quantification of the amount of HPV in a sample was determined from type specific standard curves constructed from serial dilutions of known quantities of type specific synthetic gene constructs (g-block, Integrated DNA Technologies(IDT), Coralville, Iowa, USA). These results, expressed as the number of copies per µl extracted DNA were used to calculate the VC of type specific HPV copies per ml ThinPrep suspension using the following equation:

$VC = C_{dna} \times V_{elu} / V_{inp}$

Where:

VC = viral concentration (copies HPV per ml sample)

C_{dna} = copies HPV per µl DNA (copies/µl)

V_{elu} = elution volume DNA extraction (µl)

V_{inp} = input volume sample for DNA extraction (ml)

The lower level of detection (LOD) for the Riatol qPCR assay is 4 $\log_{10}$ copies/ml. Results were reported as hrHPV negative, hrHPV positive or inconclusive. Based on the beta-globin standard curve, DNA concentration (ng/µl) was determined in every sample. Samples with a DNA concentration below 0.12 ng/µl were considered as invalid and reported as inconclusive. This cut-off was chosen based on extensive analyses demonstrating that, below this cut-off, consistency is not guaranteed. An inconclusive result included no or insufficient material/cells for analysis. A sample was considered analytically HPV negative if none of the 14 hrHPV tests showed a positive signal and the beta-globin DNA concentration was above 0.12 ng/µl. HrHPV positivity was defined using two types of cut-off: 1) an analytical cut-off, which corresponded with the lowest threshold yielding a measurable signal, and 2) an optimised clinical cut-off, yielding the best compromise in clinical accuracy, as explained below. For all positive samples, VCs were expressed as the $\log_{10}$(copies/ml). In samples with multiple hrHPV infections, only the concentration of the hrHPV genotype with the highest concentration was used for further analysis.

6.2.5 Clinical outcome and performance measurements

Follow-up and management of the patients with abnormal cytology result was done according to the Slovenian national guidelines[207], and the detailed algorithm has been described in previously published reports[82, 206, 226, 239-241].

The histologically confirmed cervical intraepithelial neoplasia grade 2 or worse (CIN2+) were considered as the clinical disease outcome and used for the computation of clinical sensitivity. We considered Women with two consecutive cytological results of negative for intraepithelial lesion or malignancy (NILM) (at enrolment and subsequent screening 12-48 months later) were grouped as non-diseased and used for the computation of clinical specificity for ≤CIN1.

6.2.6 Statistical analysis

The clinical sensitivity and specificity of RIATOL qPCR for CIN2+ and CIN3+ were computed and compared to HC2 using the non-inferiority score test[86, 208], accepting 0.90 and 0.98 as benchmarks for relative sensitivity and relative specificity, respectively[86]. Statistically significant non-inferiority was accepted when the one-sided p value was < 0.05[208]. All analyses were performed using STATA version 14 (College Station, TX, USA).

ROC curve analysis was performed to assess the trade-off between sensitivity and specificity as a function of the VC. Subsequently, the range of VC was identified where the clinical sensitivity and specificity was not inferior to that of the HC2. Statistically this translated to find the minimal and maximal VC, where the lower 90% confidence interval (CI) around the relative

sensitivity exceeded 0.90 and the lower 90% CI around the relative specificity exceeded 0.98. Ninety percent CIs were used since this correspond approximately with 0.05 confidence level for one-sided non-inferiority testing[79].

6.3 Results

6.3.1 RIATOL qPCR HPV analytical genotyping prevalence

The characteristics of the VALGENT-3 population in terms of demographics, cytological and histological results has been reported previously[82, 206, 226, 239-241]. Of the 1,600 VALGENT-3 samples analysed with the RIATOL qPCR, 56 (3.5%) had a human DNA concentration below the cut-off of 0.12 ng/µl. These samples were considered as invalid and excluded from further analysis, although these group comprises one CIN2+ case .Of the 1,544 remaining samples, 217 (17.4%) women in the screening population (N=1,249) and 80 (27.1%) women in the enrichment population (N=295) tested positive for the presence of hrHPV. The overall and type-specific prevalence of 14 hrHPV genotypes stratified according to the baseline cytology is shown in **Table 6.1**. HPV was more prevalent in women with abnormal cytology compared to women with normal cytological results. When samples were tested with RIATOL qPCR considering the 13 hrHPV genotypes targeted by HC2, the prevalence of hrHPV in NILM, ASC-US, LSIL and HSIL were 13.9%, 45.3%, 72.7% and 84.5%, respectively.

6.3.2 Accuracy of the RIATOL qPCR using the analytical cut-off

The RIATOL qPCR and HC2 results stratified for the outcomes CIN2+, CIN3+ and ≤CIN1 using the analytical cut-off are presented in Table 2. The clinical sensitivity of the RIATOL qPCR using the analytical cut-off was 97.6% (95%CI, 93.2-99.5%) and 100.0% (95%CI, 95.5-100%) for CIN2+ and CIN3+, respectively. When the analysis is restricted to women >30 years, absolute sensitivities were similar (Supplementary information, Table6.S1). The specificity of the RIATOL qPCR for ≤CIN1 was 85.1% (95%CI, 82.9-87.1%) in the total study population and slightly higher when analysis was restricted to women >30 years, 87.5% (95%CI, 85.3-89.5%).

The relative clinical performance of the RIATOL qPCR compared to HC2 is presented in **Table 6.2**. When using the analytical cut-off, the RIATOL qPCR had a relative clinical sensitivity of 1.02 (95%CI, 0.97 to 1.06, $P_{n.inf}$=0.0001) for CIN2+ and 1.03 (95%CI, 0.99 to 1.06, $P_{n.inf}$<0.0001) for CIN3+. The relative clinical specificity of the RIATOL qPCR assay for ≤CIN1 was 0.95 (95%CI, 0.92 to 0.98, $P_{n.inf}$=0.9998).

Table 6.1 Overall and type-specific prevalence of hrHPV genotypes in the total study population by baseline cytology result, using HC2 and RIATOL qPCR (with analytical cut-off).

Assay and HPV genotypes	No (%) with cytological result: NILM (N= 1,189)[a]	ASC-US (N = 128)	LSIL (N= 110)	HSIL+ (N= 113)
HC2				
hrHPV [b]	125 (10.5)	63 (49.2)	85 (77.3)	97 (85.8%)
RIATOL qPCR				
hrHPV (13 genotypes)[c]	165 (13.9)	58 (45.3)	80 (72.7)	95 (84.1)
hrHPV (14 genotypes)[d]	182 (15,3)	64 (50)	85 (77,3)	99 (87,6)
HPV 16	25 (2.1)	11 (8.6)	28 (25.5)	56 (49.6)
HPV 18	12 (1.0)	8 (6.3)	9 (8.2)	11 (9.7)
HPV 31	37 (3.1)	18 (14.1)	23 (20.9)	19 (16.8)
HPV 33	11 (0.9)	6 (4.7)	7 (6.4)	7 (6.2)
HPV 35	3 (0.3)	1 (0.8)	1 (0.9)	1 (0.9)
HPV 39	18 (1.5)	3 (2.3)	5 (4.6)	3 (2.7)
HPV 45	5 (0.4)	5 (3.9)	4 (3.6)	6 (5.3)
HPV 51	24 (2.0)	4 (3.1)	9 (8.2)	6 (5.3)
HPV 52	18 (1.5)	9 (7.0)	8 (7.3)	7 (6.2)
HPV 56	11 (0.9)	6 (4.7)	11 (10.0)	6 (5.3)
HPV 58	11 (0.9)	3 (2.3)	11 (10.0)	8 (7.1)
HPV 59	21 (1.8)	3 (2.3)	4 (3.6)	2 (1.8)
HPV 66	21 (1.8)	11 (8.6)	9 (8.2)	6 (5.3)
HPV 68	26 (2.2)	7 (5.5)	9 (8.2)	4 (3.5)
hrHPV (14genotypes)[e]	128 (10.8)	55 (43.0)	81 (73.6)	96 (85.0)

[a] Cytological negative samples (NILM) are only from the screening population; [b] Positive for at least one of 13 hrHPV genotypes (HPV16, HPV18, HPV31, HPV33, HPv35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV68); [c] Positive for at least one of the 13 hrHPV genotypes targeted by HC2 with the RIATOL qPCR; [d] Positive for at least one of 14 hrHPV genotypes with the RIATOL qPCR (13 genotypes targeted by HC2 + HPV66); [e] considering the optimised clinical cut-off; NILM, negative for intraepithelial lesion or malignancy; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.

Table 6.2 Relative sensitivity for CIN2+ and CIN3+ and relative specificity for ≤CIN1 of the RIATOL qPCR assay versus HC2 in the total study population (using the analytical cut-off).

Clinical outcome	HPV test	HC2+	HC2-	Total
CIN2+ (N=126)	RIATOL qPCR+	120	3	123
	RIATOL qPCR-	1	2	3
	Total	121	5	126
	Relative sensitivity RT/HC2 for CIN2+: 1.02 (0.97-1.06), pn.inf=0.0001			
CIN3+ (N=81)	RIATOL qPCR +	79	2	81
	RIATOL qPCR -	0	0	0
	Total	79	2	81
	Relative sensitivity RT/HC2 for CIN3+: 1.03 (0.99-1.06), pn.inf<0.0001			
≤CIN1 (N=1,167)	RIATOL qPCR+	100	74	174
	RIATOL qPCR-	20	973	993
	Total	120	1047	1167
	Relative specificity RT/HC2 for ≤CIN1: 0.95 (0.92-0.98), pn.inf=0.9998			

* $p_{n.inf.}$ <0.05 means that the sensitivity or specificity of the RIATOL qPCR assay are not significantly lower than that of the HC2, accepting the benchmarks of 0.90 and 0.98 for relative sensitivity and relative specificity, respectively.

Table 6.3 Relative clinical sensitivities for CIN2+ and CIN3+ and relative clinical specificities for ≤CIN1 of the RIATOL qPCR versus HC2 in the total population (using the optimized clinical cut-off of 6.493 log_{10} copies/ml)

Clinical outcome	HPV test	HC2 +	HC2 -	Total
CIN2+ (N=126)	RIATOL qPCR+	118	3	121
	RIATOL qPCR -	3	2	5
	Total	121	5	126
	Relative sensitivity RT/HC2 for CIN2+: 1.00 (0.95-1.05), pn.inf=0.0006			
CIN3+ (N=81)	RIATOL qPCR+	77	2	79
	RIATOL qPCR -	2	0	2
	Total	79	2	81
	Relative sensitivity RT/HC2 for CIN3+: 1.00 (0.95-1.05), pn.inf=0.0045			
≤CIN1 (N=1,167)	RIATOL qPCR +	94	28	122
	RIATOL qPCR-	26	1019	1045
	Total	120	1047	1167
	Relative specificity RT/HC2 for ≤CIN1: 1.00 (0.98-1.01), pn.inf=0.0069			

* $p_{n.inf.}$ <0.05 means that the sensitivity or specificity of the RIATOL q-PCR assay are not significantly lower than that of the HC2, accepting the benchmarks of 0.90 and 0.98 for relative sensitivity and relative specificity, respectively.

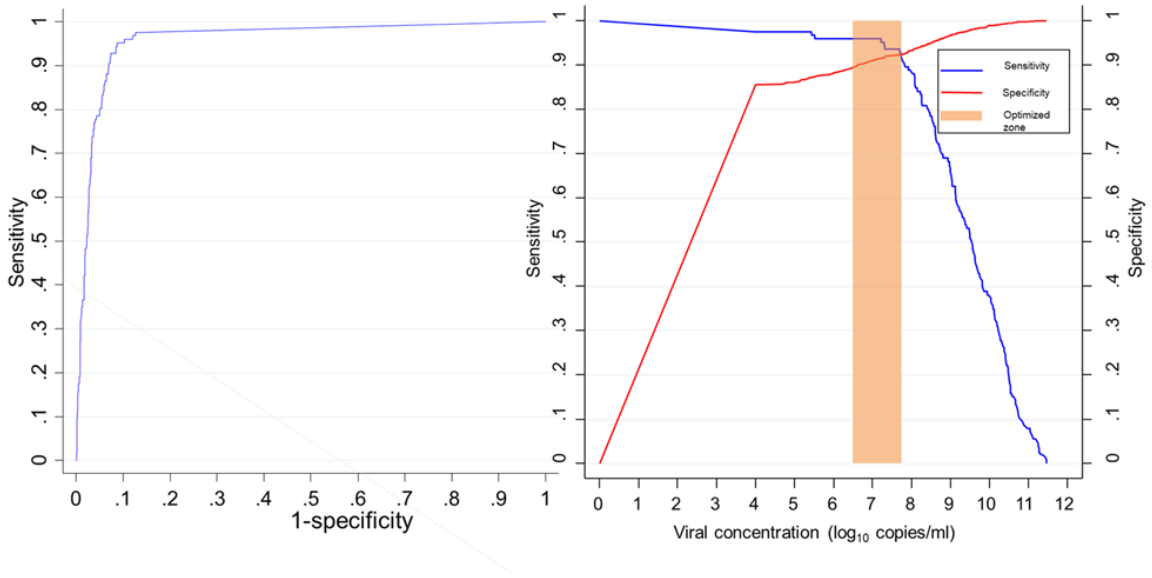


Figure 6.1 Part a. ROC curve of the sensitivity as a function of the false-positivity rate (1-specificity) of the RIATOL qPCR to detect CIN2+. Part b: Variation in sensitivity and specificity for CIN2+ as a function of the viral concentration expressed as log_{10} (HPV copies/ml of sample). In case of infection with multiple genotypes, the HPV type with the highest concentration was chosen. Within the orange bar (range: 6.493.-7.747), international accuracy requirements for cervical cancer screening tests are fulfilled.

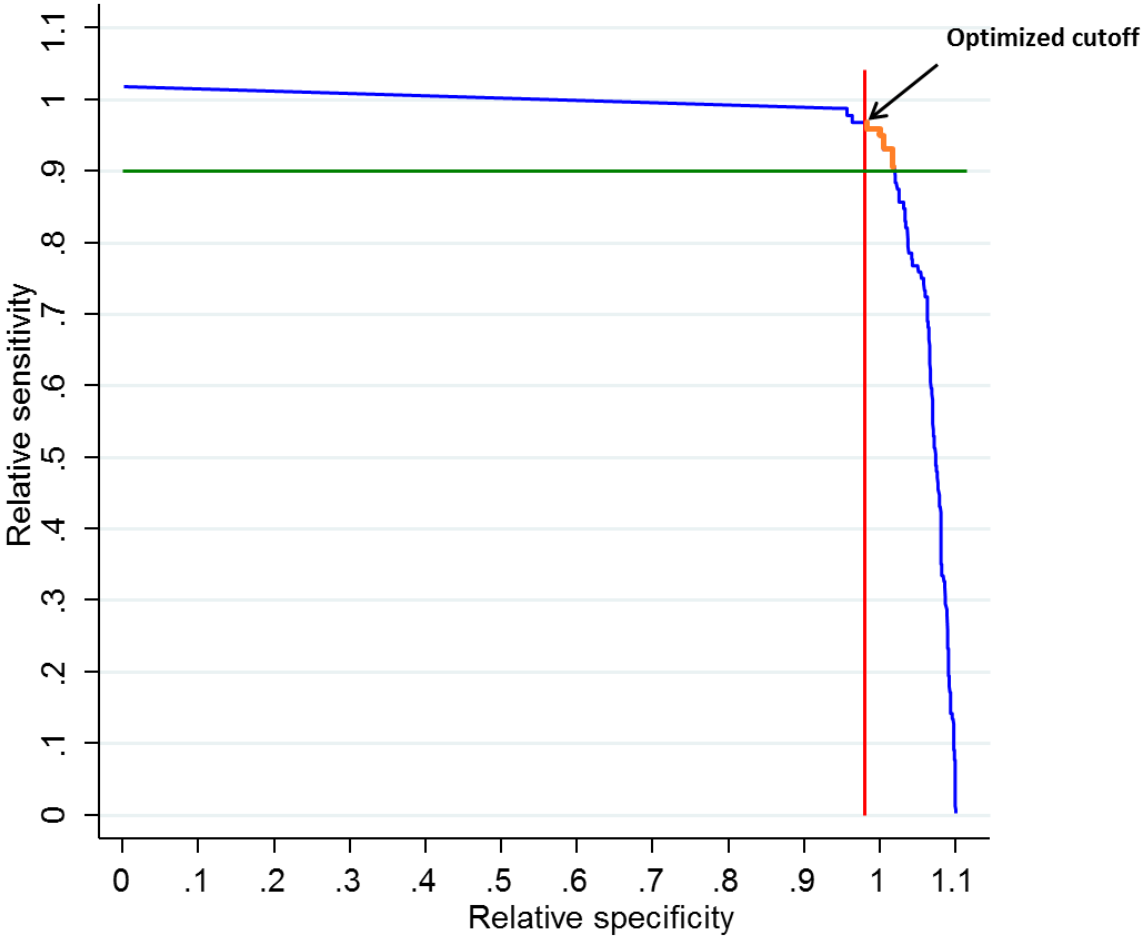


Figure 6.2 Plot of the left confidence interval bound around the relative sensitivity for CIN2+ against the relative specificity of the RIATOL qPCR vs HC2 corresponding to viral concentration cut-off points. The orange zone of the line (upper right corner) indicates the range where the requirement of non-inferior accuracy compared to HC2 is fulfilled (viral concentration expressed as $\log_{10}$[copies/ml]) ≥6.493 and <7.747). Optimized cut-off = 6.493.

6.3.3 Clinical performance of the RIATOL qPCR with the optimized cut-off

Since non-inferiority was not reached for clinical specificity when using the analytical cut-off, an algorithm was developed to calculate a clinically relevant cut-off, which would result in non-inferior clinical accuracy when compared to HC2. A ROC curve of the sensitivity as a function of the false-positivity rate of the RIATOL qPCR to detect CIN2+ was constructed (Figure 6.1a). Subsequently, the variation in sensitivity and specificity for CIN2+ was plotted as a function of the VC (**Figure 6.1**b). Next, the range of VC was identified where the clinical sensitivity and specificity where not inferior to HC2 (**Figure 6.1**b and **Figure 6.2**).

The range where the requirements of non-inferior accuracy of the RIATOL qPCR compared to HC2 are fulfilled was between 6.493 and 7.747. As optimized clinical cut-off, the VC in this range with the highest sensitivity is chosen for further analysis at 6.493.

Using the optimized cut-off, the absolute clinical sensitivity of RIATOL qPCR for CIN2+ and CIN3+ in the total study population was 96.0% (95%CI, 91.0-98.7%) and 98.0% (95%CI, 91.4-99.7%), respectively (**Table 6.3**), while the absolute clinical specificity for ≤CIN1 was 90.0% (95CI, 87.6-91.2%). Comparable results are found when the analysis was limited to women aged 30 years or older (Supplementary information, Table 6.S1).

The relative clinical sensitivity and specificity of the RIATOL qPCR compared to HC2 is presented in **Table 6.3**. When using the optimized clinical cut-off, the RIATOL qPCR had a relative sensitivity of 1.00 for CIN2+ (95%CI, 0.95 to1.05) and 1.00 for CIN3+ (95%CI 0.95 to 1.05) with a $p_{n.inf}$ of 0.0006 (CIN2+) and 0.0045 (CIN3+), and therefore considered as non-inferior to HC2. The relative specificity of the RIATOL qPCR assay for ≤CIN1 was 1.00 (95%CI, 0.98 to 1.01) and also non-inferior to HC2 ($p_{n.inf}$=0.0069). Similar results were obtained when the analysis was restricted to women >30 years (Supplementary information, Table 6.S2) (**Table 6.4**).

Table 6.4 bsolute sensitivity and specificity of hrHPV testing with HC2 and the RIATOL qPCR with different cut-offs to detect CIN2+ and the relative accuracy compared to HC2.

Cut-off (log_{10} copies/ml)	Sensitivity RIATOL qPCR, %	Relative sensitivity of RIATOL qPCR/HC2 (90% CI)	$p_{n.inf}$	Specificity RIATOL qPCR, %	Relative specificity of RIATOL qPCR/HC2 (90% CI)	$p_{n.inf}$
Analytical	97.6	1.02 (0.97 – 1.06)	0.0001	85.1	0.95 (0.92 – 0.98),	0.9998
6.493	96.0	1.00 (0.95-1.05)	0.0006	89.5	1.00 (0.98-1.01)	0.0069
7.747	91.3	0.95 (0.90-1.00)	0.0399	92.5	1.03 (1.01-1.06)	<0.001

* $p_{n.inf.}$ <0.05 means that the sensitivity or specificity of the RIATOL q-PCR assay are not significantly lower than that of the HC2, accepting the benchmarks of 0.90 and 0.98 for relative sensitivity and relative specificity, respectively.

6.4 Discussion

In the present study, the clinical performance of the RIATOL qPCR was compared to HC2 within the VALGENT-3 project. At the analytical cut-off, non-inferiority criteria for screening, as defined by Meijer et al[86], was not reached for the clinical specificity. Therefore, a clinically relevant viral concentration cut-off were analysed post-hoc, balancing both sensitivity and specificity to meet the defined criteria. A zone of VCs was identified by relative ROC curve analysis, where the accuracy of the RIATOL qPCR was non-inferior to HC2. Within the defined range, the minimum cut-off value was chosen as threshold. This calculated threshold for screening purposes yields the highest sensitivity with the specificity that still fulfils the proposed criteria.

In cases where multiple HPV infections were present, VC of the hrHPV type with the highest concentration was used for further analyses. When the analysis was performed with the cumulative hrHPV concentration, defined as the logarithm of the sum of the genotype-specific concentrations of all present hrHPV genotypes, comparable results were found (data not shown). Little is known about the influence of an individual HPV genotype in the presence of multiple genotypes on the carcinogenicity[244], therefore further research is necessary to understand the complexity of multiple HPV infections. Since we reported the VC for each HPV genotype separately, for the convenience of the clinician a cut-off per genotype will facilitate the interpretation of the results. For this reason, we choose to calculate the cut-off based on the highest concentration. We are aware of this possible limitation.

Since HPV genotyping information of the biopsies was not available, it was impossible to determine a genotype-specific cut-off. In addition, the cohort used in this study was too small to get reliable and statistically significant results per each targeted hrHPV genotype. However, the authors agree that this would be the ideal situation to have a clinical cut off value per HPV genotype. More methodological research is needed to find feasible clinical and or analytical validation concepts for HPV genotyping tests at the type-specific level.

Riatol qPCR has been clinically validated based on a fixed Ct value (Ct ≤34.00)[243]. This is the first study to calculate an optimized clinical cut-off defined in terms of VC within the VALGENT-3 study. Although there is an international WHO standard available for HPV 16 and

18, comparison of viral load measurements with other quantitative assays is difficult. Consensus quantification strategies for calculation of the amount of HPV particles present in a liquid based cytology sample have not yet been reached and currently different calculation options are used.

The presence of a large sample set and well documented follow-up database, makes VALGENT study well suited for clinical validation of multiple HPV genotyping test at the same time. To insure correct interpretation of CT values, an additional QC standard must be included in the assay.

VALGENT contributes in defining the list of HPV tests, which fulfil the defined minimal requirements of HPV genotyping assays for use in primary cervical cancer screening.

Our study demonstrates that HPV tests, providing viral concentrations (or other quantifiable signals) allow flexibility to optimize the clinical accuracy required for primary cervical cancer screening. This technique is already applied in the paper of Viti et al[240] where a modification of the EUROArray HPV cut-off for HPV 16 makes the test non inferior to HC2.

In the future, HPV assays that generate quantified outputs might have an advantage compared to assays only producing a qualitative output because of their adaptability for particular specimens (vaginal self-samples, urine samples, other non-cervical specimens) or specified clinical settings (vaccinated women, follow-up after treatment) or for certain surveillance or research purposes (HPV vaccination trials, epidemiological studies, post vaccination surveillance of HPV infections). Our team strongly supports the application of different cut-off values, predefined according to specific situations/needs, i.e. primary screening, follow-up, sample type, etc. Insights are based on historical routine data, suggesting to be superior versus exploiting a fixed cut-off (unpublished data). Future research is needed to confirm this.

In conclusion, by using the optimised cut-off based on viral concentration, the RIATOL qPCR test shows non-inferior sensitivity and specificity for CIN2+ compared to the HC2 and fulfils the international accuracy criteria for primary cervical cancer screening.

Acknowledgments

Special thanks go to the AML study department where Lies De Baere and Kristel Vanderlick greatly assisted the research. We also thank lab technicians, Brenda Gabriels, Dorien Vanroy, Sarah Hamels, Lieve Degruyter and Tamara Mombaerts for their excellent technical support.

Conflict of interests

IB and JB are clinical pathologist working in the private laboratory AML. DVB is a molecular biologist working in the private laboratory AML. AML has received research support in the form of free kits, reduced prices or funding from Abbott, Hologic, Cepheid, Roche, Becton Dickinson, Seegene, Biomérieux, Rover Medical devices, Aprovix and My Sample. IB and JB have also received travel grants to attend symposia, conferences and meetings from Hologic and Abbott.

MA, LX, MP and AO were supported by the COHEAHR Network, funded by the 7th Framework Programme of DG Research and Innovation, European Commission, Brussels, Belgium (grant No. 603019) and from VALGENT.

Supplementary Table 6.S1. Relative sensitivities for CIN2+ and CIN3+ and relative specificities for ≤CIN1 of the RIATOL qPCR assay versus HC2 in the total population (using the analytical cut-off) in women aged 30 years or older.

Clinical outcome	HPV test	HC2 positive	HC2 negative	Total
CIN2+ (≥30 yrs) (N=97)	RIATOL qPCR +	92	3	95
	RIATOL qPCR -	1	1	2
	Total	93	4	97
	Relative sensitivity RT/HC2 for CIN2+: 1.02 (0.97-1.07), $p_{n.inf}$=0,0002			
CIN3+ (≥30 yrs) (N=65)	RIATOL qPCR +	63	2	65
	RIATOL qPCR -	0	0	0
	Total	63	0	65
	Relative sensitivity RT/HC2 for CIN3+: 1.03 (0.99-1.08), $p_{n.inf}$=0.0008			
≤CIN1 (≥30 yrs) (N=969)	RIATOL qPCR +	56	65	121
	RIATOL qPCR -	18	830	848
	Total	74	895	969
	Relative specificity RT/HC2 for ≤CIN1: 0.95 (0.92-0.98), $p_{n.inf}$=0.9470			

Supplementary Table 6.S2. Relative sensitivities for CIN2+ and CIN3+ and relative specificities for ≤CIN1 of the RIATOL qPCR assay versus HC2 in the total population (using the optimized clinical cut-off of 6.493 log copies/ml) in women aged 30 years or older.

		HC2 positive	HC2 negative	Total
CIN2+ (≥30 yrs) (N=97)	RIATOL qPCR +	90	3	93
	RIATOL qPCR -	3	1	4
	Total	93	4	97
	Relative sensitivity RT/HC2 for CIN2+: 1.00 (0.94-1.06), $p_{n.inf}$=0.0022			
CIN3+ (≥30 yrs) (N=65)	RIATOL qPCR +	61	2	63
	RIATOL qPCR -	2	0	2
	Total	63	2	65
	Relative sensitivity RT/HC2 for CIN3+: 1.00 (0.92-1.09), $p_{n.inf}$=0.0070			
≤CIN1 (≥30 yrs) (N=969)	RIATOL qPCR +	52	26	78
	RIATOL qPCR -	22	869	891
	Total	74	895	969
	Relative specificity RT/HC2 for ≤CIN1: 1.00 (0.97-1.02), $p_{n.inf}$=0.0232			

CHAPTER 7

7. Evaluation and optimization of the clinical accuracy of Hybribio's 14 High-risk HPV with 16/18 Genotyping assay within the VALGENT-3 framework

Adapted from:

Xu L, Ostrebenk A, Poljak M, Arbyn M. Evaluation and optimization of the clinical accuracy of Hybribio's 14 High-risk HPV with 16/18 Genotyping assay within the VALGENT-3 framework. Journal of Clinical Microbiology 2020. 58(6).

Abstract

Hybribio's 14 High-risk HPV with 16/18 Genotyping Real-time PCR (HBRT-H14) is a human papillomavirus (HPV) assay with approval from the China Food and Drug Administration widely used in China. VALGENT (VALidation of HPV GENotyping Tests) is an established framework for evaluating HPV tests' clinical performance relative to validated comparators. The aim of this study was to assess the clinical accuracy of HBRT-H14 following international validation criteria. Within VALGENT-3, clinical performance of HBRT-H14 was compared with the Hybrid Capture 2 (HC2), Linear Array HPV Genotyping Test (Linear Array) and Cobas 4800 HPV test (Cobas). VALGENT-3 comprised 1,300 consecutive samples and 300 abnormal cytological samples from the Slovenian cervical cancer screening program. Disease was defined as histologically confirmed CIN2+ and CIN3+, and two negative cytology results in a row were a proxy for non-disease. In the total study population, relative sensitivity and specificity of HBRT-H14 versus HC2 for detecting CIN2+ were 0.98 (95% CI, 0.94–1.03; *p* non-inferiority[ni] < 0.01) and 0.97 (95% CI, 0.96–0.99; p_{ni} = 0.78), respectively. Applying an optimized *a posteriori* cutoff, defined using Linear Array and Cobas as bridging tests, yielded relative values of 0.98 (95% CI, 0.94–1.03; p_{ni} < 0.01) and 1.01 (95% CI, 1.00–1.03; p_{ni} < 0.01), respectively. In conclusion, HBRT-H14 was as sensitive but less specific than HC2 for detecting cervical precancer at the predefined cutoff. However, HBRT-H14 fulfilled international accuracy criteria for cervical cancer screening when using an optimized cutoff and might be attractive in low-resource settings given its low cost.

7.1 Introduction

Cervical cancer screening with human papillomavirus (HPV) testing offers better protection against invasive cervical carcinoma than cytology and longer intervals between multiple screening rounds [64, 105]. Thus, HPV testing is gradually being implemented in organized screening programs for cervical cancer worldwide with the condition that only clinically validated tests for HPV should be used. Commercial HPV assays are abundant, but few have undergone clinical validation and fulfil the international consensus criteria to be used in settings for primary cervical cancer screening [79, 86, 197, 198]. Thus, the VALidation of HPV GENotyping Tests (VALGENT) framework was launched, seeking comprehensive comparison and clinical validation of HPV assays with genotyping capacity [87]. VALGENT has been through several iterative instalments using samples from women participating in screening for cervical cancer from Belgium (VALGENT-1) [199-201], Scotland (VALGENT-2) [202-205], Slovenia (VALGENT-3) [206, 226, 239-241, 245] and Denmark (VALGENT-4) [246, 247] .

This study first evaluated the clinical accuracy of Hybribio's 14 High-risk HPV with 16/18 Genotyping Real-time PCR assay (HBRT-H14; Hybribio, Chaozhou, China) within the VALGENT-3 panel relative to the standard comparator test, the Hybrid Capture 2 HPV DNA assay (HC2; Qiagen, Gaithersburg, MD, USA) [79, 86, 87] using the manufacturer-defined cutoff. Then, in case of insufficient accuracy was observed, a cutoff optimisation assessment was set up, as performed in previous VALGENT studies [240, 245]. Since HBRT H14 detects

separately HPV16, HPV18 and 12 other hrHPV types in aggregate, we used two HPV assays as bridging tests providing the same or full genotyping results: the Linear Array HPV Genotyping Test (Linear Array; Roche Molecular Diagnostics, Branchburg, NJ, USA) [226] and the clinically validated FDA-approved assay with similar genotyping capacity Cobas 4800 HPV test (Cobas; Roche Molecular Systems, Alameda, CA, USA) [241] .

7.2 Materials and methods

7.2.1 VALGENT-3 panel study population

Altogether, 1,600 samples were collated in Slovenia for the VALGENT-3 panel as previously described [206, 226, 239-241, 245]. From the study of HPV prevalence in Slovenia [82], 1,300 consecutive samples were obtained from women age 25 to 64 participating in the national screening program for cervical cancer (screening population). Three hundred samples came from women referred for colposcopy due to abnormal cytology per the VALGENT protocol [87], in particular atypical squamous cervical cells of undetermined significance (ASC-US, 100 women), low-grade squamous intraepithelial lesions (LSILs, 100 women) and high-grade squamous intraepithelial lesions (HSILs, 100 women; the enrichment population). Two cervical specimens came from each woman: one for conventional cytology and another for HPV DNA testing, placed in ThinPrep medium (ThinPrep PreservCyt solution, Hologic, Marlborough, MA, USA). ThinPrep samples were divided into several aliquots and stored at −70 °C for testing with an HPV assay in the VALGENT-3 framework.

The Slovenian Medical Ethics Committee provided ethical approval (consent nos. 83/11/09 and 109/08/12).

7.2.2 HPV testing

All the samples were tested with HBRT-H14, HC2, Linear Array and Cobas. All the assays were carried out following the manufacturer's instructions at the Institute of Microbiology and Immunology at the University of Ljubljana's Faculty of Medicine. DNA for HBRT-H14 and Linear Array testing was extracted from original samples collected in ThinPrep using QIAamp MinElute Media Kit (Qiagen) following the manufacturer's instructions.

HBRT-H14 is a TaqMan-based real-time PCR assay targeting the *E6* and *E7* regions of 14 hrHPV types, allowing concurrent separate genotyping for HPV16 and HPV18 from the 12 other hrHPV types (HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). Signals are detected on four channels with spectrally unique dyes, yielding data for HPV16, HPV18, 12 other hrHPVs and human β-globin gene [248]. Per the manufacturer's instructions, samples were deemed HPV positive if the cycle threshold (C_t) was ≤ 40 for all channels. The samples were tested with HBRT-H14 between March 2017 and May 2018.

HC2 is a semi-quantitative US FDA–approved test allowing detection of 13 hrHPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) used as the standard comparator test to assess the clinical performance of HBRT-H14 based on its clinical validity proven in large

randomized trials that have a longitudinal follow-up [86, 105]. As described previously [206, 226, 239], HC2 testing was performed within 2 weeks after obtaining the sample: thus the samples were tested in 2010 for the screening population and in 2015 for the enrichment population.

Linear Array is a qualitative HPV assay with full genotyping capability. It targets the L1 region of 37 high-risk and low-risk types of HPV (HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 44, 45, 51–54, 56, 58, 59, 61, 62, 64, 66–73, 81, 82–84, 89, and IS39) and the β-globin gene as an internal control [226]. Linear Array was performed in 2016. This study defined hrHPV positivity as the presence of at least one of the 14 hrHPV types that was also detected by HBRT-H14.

Cobas is a fully automated multiplex real-time PCR assay targeting the L1 region of 14 hrHPV types allowing partial genotyping of HPV16 and HPV18 as well as pooled detection of 12 other hrHPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). Internal control of the sample cellularity was carried out on all samples by amplifying the human β-globin gene. Cobas testing was performed in 2015.

7.2.3 Clinical outcomes and statistical analysis

Following the ZORA (the Slovenian National Cervical Cancer Screening Programme) criteria, women with atypical squamous cells for which high-grade lesions (ASC-H) or worse could not be excluded were referred for colposcopy and women with LSIL or ASC-US were invited for a repeat Pap smear and, with repeated ASC-US or worse, referred for colposcopy. HPV16- and/or HPV18-positive women were directly referred for colposcopy without regard to cytological results. Colposcopy-directed punch biopsies were taken from areas suspected for high-grade lesions, and pathologists blinded to the HPV results carried out histopathological assessment.

Women with cervical intraepithelial neoplasia scoring grade 2 or worse (CIN2+) and CIN grade 3 or worse (CIN3+) confirmed by histology were deemed diseased, and they were included as the denominator for the clinical sensitivity estimation. For clinical specificity estimates, we considered women controls (≤ CIN1) if they had two or more consecutive negative scores for cytology results for intraepithelial lesion or malignancy (NILM) upon enrolment and at the next screening after 12 to 48 months. We calculated clinical sensitivity scores for CIN2+ and CIN3+ as well as clinical specificity for ≤ CIN1 of HBRT-H14, HC2, Linear Array and Cobas. We assessed clinical performance separately for the entire study population and for women over 30. The McNemar test (McN) was used to compare differences between matched proportions [209]. A matched non-inferior statistic (ni) with a 90% relative sensitivity threshold and 98% relative specificity threshold was used when comparing clinical performance of HBRT-H14 to HC2 [86, 208]. The level of statistical significance for both statistics (p_{MnC} and p_{ni}) was set at 0.05. All the analyses were carried out using STATA version 14 (College Station, TX, USA).

Moreover, we assessed possible cutoff adaptation by changing the C_t thresholds of the HBRT-H14 and performed cutoff optimization analysis separately for HPV16, HPV18 and other hrHPV channels using two bridging tests: Linear Array and Cobas. Because testing for 14 hrHPV types

with Linear Array has demonstrated non-inferior clinical accuracy to that of HC2 within VALGENT-3 [226] and its full genotyping capability, Linear Array was employed as a first bridging test and Cobas as a second one [241]. The purpose of the HPV16 cutoff optimization was to lower the corresponding C_t to maximize the number of ≤ CIN1 cases with negative HBRT-H14 result while maintaining a maximum of CIN2+ cases that were HPV16+ for both HBRT-H14 and the bridging test. A similar algorithm was applied for HPV18 and other hrHPV.

7.3 Results

Because HC2 has no internal control, specimen validity for HC2 could not be assessed; however, all 1,600 samples had valid Linear Array test results. Six and nine samples had invalid results with HBRT-H14 and Cobas, respectively. **Figure 7.1** presents a flow chart showing the process from the panel collation of samples, and the HPV testing to the final endpoint ascertainment for diseased and non-diseased groups. For assessing clinical accuracy, six samples that tested invalid with HBRT-H14 were excluded from further analysis. Of 1,594 samples with valid HPV results in the entire study population, two consecutive normal cytology results were obtained from 1,211 women; they were considered controls and used as the denominator for computing clinical specificity. We used 127 women with CIN2+ and 82 with CIN3+ for the clinical sensitivity assessment.

Table 7.S1 in the supplementary information summarizes HBRT-H14, HC2, Linear Array and Cobas results of the VALGENT-3 panel. In the screening population, HPV positivity rate detected by HBRT-H14, HC2, Linear Array and Cobas was 14.4%, 12.1%, 10.7% and 10.8%, respectively, and 67.0%, 71.3%, 66.7% and 68.7% in the enrichment population, respectively.

7.3.1 Absolute clinical accuracy of HBRT-H14, HC2, Linear Array and Cobas

In the entire study population, 120/127 CIN2+ and 78/82 CIN3+ cases were positive with HBRT-H14, corresponding to an absolute CIN2+ and CIN3+ clinical sensitivity of 94.5% (95% CI: 89.0–97.8%) and 95.1 (95% CI: 88.0–98.7%), respectively (**Table 7.1**). Out of 1,211 women with ≤ CIN1, 1,065 tested HPV-negative with HBRT-H14, corresponding to an absolute clinical specificity of 87.9% (95% CI: 86.0–89.7%). Similar results for women ≥ 30 years are presented in **Table 7.1.** Absolute sensitivity for detecting CIN2+ and CIN3+ as well as specificity for ≤ CIN1 of HC2, Linear Array and Cobas are presented in **Table 7.1**, where results are reported for the total population and for women age 30 and older.

7.3.2 Relative sensitivity and specificity of HBRT-H14 in comparison to HC2

Table 7.2 presents the clinical performance of HBRT-H14 in comparison to HC2 with regard to detection of CIN2+, CIN3+ and ≤ CIN1 in the overall study population and in women age 30 and older. Within the entire study population, HBRT-H14 has a relative sensitivity of 0.98 (95% CI: 0.94–1.03) for CIN2+ and 0.98 (95% CI: 0.92–1.04) for CIN3+. The relative specificity of HBRT-H14 for ≤ CIN1 is 0.97 (95% CI: 0.96–0.99). The sensitivity of HBRT-H14 for CIN2+ was non-inferior to HC2 (p_{ni} = 0.003), whereas the specificity of HBRT-H14 for ≤ CIN1 was inferior

to HC2 (p_{ni} = 0.777 and relative specificity < 1, with 95%CI excluding unity). Similar results were found when restricting the analysis to women age 30 and older.

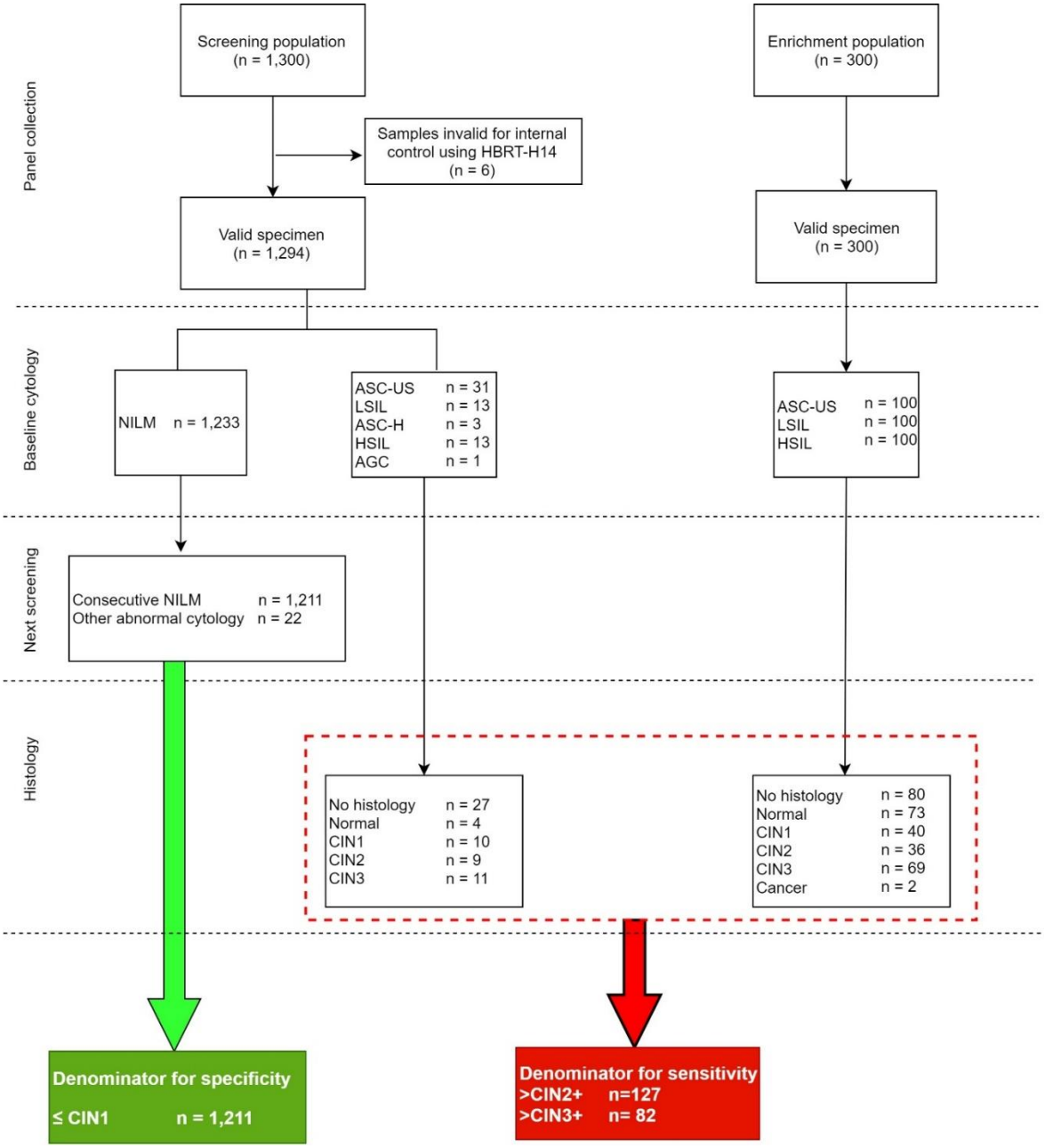


Figure 7.1 Flow chart for panel sample collation, baseline cytology, cytology in the next screening round and final histology results. Women with histologically confirmed CIN2+ and CIN3+ were used as the denominator for sensitivity, and women with two consecutive negative cytology results (≤ CIN1) were used as the denominator to compute specificity. The next screening round was for the screening population, not the enrichment population.

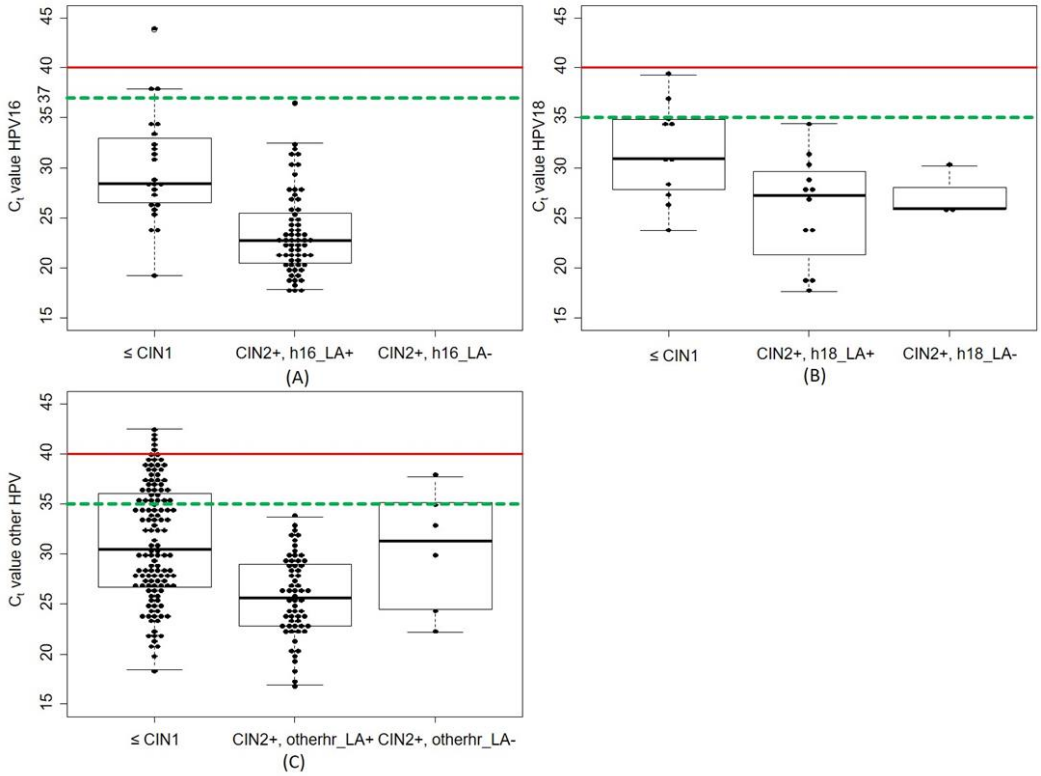


Figure 7.2 Box plots showing the distribution of C_t values for HPV16 (A), HPV18 (B) and other hrHPV types (C) using HBRT-H14 (*y* axis) in women with ≤ CIN1, women with CIN2+ and LinearArray positive for the HPV type considered (e.g. CIN2+, h16_LA+) and women with CIN2+ but LinearArray negative for the HPV type considered (e.g. CIN2+,h16_LA-). The predefined cutoff for each type was set at 40 (solid red lines). By comparing with LinearArray we could optimize and set the C_t values to the dashed green lines for HPV16 to 37, for HPV18 to 35 and for other hrHPV to 35, respectively (dashed green lines).

Because the non-inferiority criteria were not fulfilled for the clinical specificity of HBRT-H14 when using the manufacturer-defined cutoff, a straightforward cutoff optimization analysis was performed to identify clinically relevant cutoffs, which would lead to non-inferior clinical specificity of HBRT-H14 while maintaining non-inferior sensitivity for CIN2+ and CIN3+ in comparison to HC2. The boxplots in **Figure 7.2** show that reduction of cutoffs of Ct for HPV16, HPV18 and other hrHPVs from the original values of 40 to 37, 35 and 35, respectively, results in satisfactory clinical accuracy. Similar cutoff reduction results were obtained when using Cobas as the bridging test (Supplementary Figure 7.S1).

Table 7.2 also presents the relative clinical performance of HBRT-H14 in comparison to HC2 after cutoff optimization. HBRT-H14 had a relative sensitivity of 0.98 (95% CI: 0.94–1.03, p_{ni} = 0.003) for CIN2+ and 0.98 (95% CI: 0.92–1.04, p_{ni} = 0.026) for CIN3+. The relative specificity of HBRT-H14 for ≤ CIN1 was 1.01 (95% CI: 1.00–1.03, p_{ni} < 0.001). Restricting the analysis to women older than 30 yielded comparable results.

Table 7.1 Clinical sensitivity for detection of CIN2+ and CIN3+ and clinical specificity for ≤ CIN1 of HBRT-H14, HC2 and Cobas in the total study population and in women ≥ 30 years old.

	Clinical sensitivity						**Clinical specificity**		
Study group and test	CIN2+			CIN3+			≤ CIN1		
	n/N	%	95% CI	*n/N*	%	95% CI	*n/N*	%	95% CI
Total study population									
HBRT-H14	120/127	94.5%	(89.0–97.8%)	78/82	95.1%	(88.0–98.7%)	1,065/1,211	87.9%	(86.0–89.7%)
HC2	122/127	96.1%	(91.1–98.7%)	80/82	97.6%	(91.5–99.7%)	1,093/1,211	90.3%	(89.9–91.9%)
Linear Array	125/127	98.4%	(94.4–99.8%)	82/82	100.0%	(95.6–100.0%)	1,109/1,211	91.6%	(88.4–93.1%)
Cobas*	122/127	96.1%	(91.1–98.7%)	80/82	97.6%	(91.5–99.7%)	1,102/1,204	91.5%	(89.8–93.0%)
Women ≥ 30 years									
HBRT-H14	93/98	94.9%	(88.5–97.8%)	63/66	95.5%	(87.3–99.1%)	917/1,009	90.9%	(88.9–92.6%)
HC2	94/98	95.9%	(89.8–98.9%)	64/66	97.0%	(89.5–99.6%)	937/1,009	92.9%	(91.1–94.4%)
Linear Array	97/98	99.0%	(94.4–100.0%)	66/66	100.0%	(94.6–100.0%)	952/1,009	94.4%	(92.7–95.7%)
Cobas*	95/98	96.9%	(91.3–99.4%)	64/66	97.0%	(89.5–99.6%)	943/1,009	94.1%	(92.5–95.5%)

*Seven samples tested invalid by Cobas were excluded from the analysis.

Table 7.2 Relative sensitivities for CIN2+ and CIN3+ and relative specificities for ≤ CIN1 of the HBRT-14 versus the HC2 in the total study population and in women ≥ 30 years old, (a) using the manufacturer-defined cutoff and (b) using the optimized cutoff.

	Relative sensitivity (95% CI)	**Relative specificity (95% CI)**	P_{McN}[a]	P_{ni}[b]
a. using manufacturer-defined cutoff				
Total study population				
CIN2+	0.98 (0.94–1.03)		0.480	0.003
CIN3+	0.98 (0.92–1.04)		0.688	0.026
≤ CIN1*		0.97 (0.96–0.99)	0.001	0.777
Women ≥ 30 years				
CIN2+	0.99 (0.94–1.05)		1.000	0.006
CIN3+	0.98 (0.92–1.05)		1.000	0.023
≤ CIN1*		0.98 (0.96–0.99)	0.006	0.568
b. using optimized cutoff				
***Total* study population**				
CIN2+	0.98 (0.94–1.03)		0.727	0.003
CIN3+	0.98 (0.92–1.04)		0.688	0.026
≤ CIN1*		1.01 (1.00–1.03)	0.016	< 0.001
Women ≥ 30 years				
CIN2+	0.99 (0.94–1.05)		1.000	0.025
CIN3+	0.98 (0.92–1.05)		1.000	0.023
≤ CIN1*		1.02 (1.00–1.03)	0.005	< 0.001

*Two consecutive negative cytology results.
[a]*P* for the McNemar test for a difference between matched proportions.
[b]*P* for the test for non-inferiority.

7.4 Discussion

HBRT-H14 is a real-time PCR-based assay designed for HPV-based screening for primary cervical cancer. It is capable of simultaneous differentiation of HPV16 and HPV18 from the pool of other 12 hrHPV types. The China Food and Drug Administration approved HBRT-H14 in 2012, and it has been widely used in Chinese hospitals and laboratories [249]. However, only a few data on clinical performance have been published in peer-reviewed literature [249] [249]. In 2013, Bian et al. [248]evaluated the clinical accuracy of HBRT-H14 and HC2 on 424 SurePath samples (Becton, Dickinson and Company, Sparks, MD, USA) derived from a Chinese screening population. However, due to the non-matched design of Bian et al.'s study, which randomly divided samples into two groups and tested them with either HBRT-H14 or HC2, non-inferior statistics could not be calculated. Nevertheless, HBRT-H14 demonstrated good absolute sensitivity and specificity for detecting CIN2+ (96.3% and 78.2%, respectively) similar to that of HC2 (78.0% and 79.4%, respectively).

As far as we know, no study has assessed the clinical performance of HBRT-H14 according to the international validation criteria for HPV tests used for cervical cancer screening [86]. This study evaluated the clinical performance of HBRT-H14 for detecting high-grade CIN within the

VALGENT-3 framework for the first time. Compared to the standard HC2 comparator test, HBRT-H14 demonstrated non-inferior sensitivity for detecting CIN2+ and CIN3+, but regardless of the population studied (the entire study population or women age 30 and older) it demonstrated inferior specificity for ≤ CIN1. Therefore, an optimization analysis was performed *post hoc*, and a new cutoff was identified, allowing a gain in specificity without loss of sensitivity. By using the *post hoc* defined cutoff, HBRT-H14 met the non-inferiority screening criteria that Meijer et al. defined in 2009 [86].

Our study used both Linear Array and Cobas as a bridging test in the cutoff optimization analysis and generated similar new cutoffs. This suggests that other validated assays with limited genotyping such as Cobas can also be used as bridging test for clinical performance optimization of alternative HPV tests using genotyping capacity in the future, when Linear Array is no longer commercially available. Namely, the manufacturer of Linear Array (Roche) announced discontinuation of the production of Linear Array on March 18th, 2019, and Linear Array is no longer commercially available since January 1st, 2020.

In a recent study, Xue et al. compared performance of HBRT-H14 to that of Cobas for detecting CIN2+ using a total of 214 samples from a colposcopy referral population in China [249]. HBRT-H14 had a clinical sensitivity of 94.6% (95% CI: 86.9–97.9%) and specificity of 66.0% (95% CI: 56.6–74.4%) and was considered non-inferior to Cobas. Although the non-inferior statistic was not reported in the original paper, we were able to calculate it *a posteriori* based on reported data. Interestingly, Xue et al. considered samples HPV positive if C_t values were ≤ 36 instead of the ≤ 40 predefined by the manufacturer. Although the rationale of using an alternative cutoff was not explained in Xue's study, it is relatively close to the optimized cutoff generated and suggested in our study, and it reaffirms the necessity of cutoff optimization for HBRT-H14 clinical use in cervical cancer screening. For HPV tests that generate quantitative signal strengths and allow flexibility for use in various clinical settings (primary screening, triage of women with borderline cytology or treatment follow-up), comprehensive analysis and further studies are needed to achieve the best clinical value.

Previously published VALGENT reports and cervical cytology biobank studies have showed that archived aliquoted ThinPrep liquid-based specimens stored at -70°C can be safely used several years after initial collection to evaluate the clinical performance of HPV DNA tests [226, 241, 250]. Since influence of prolonged specimen storage on the clinical performance of HBRT-H14 was not assessed in our study, this can be considered as a study limitation, however based on our experience with evaluation of other HPV tests in VALGENT-3 framework we strongly believe that evaluation on fresh ThinPrep samples would have yielded accuracy values equal to those observed here. Another potential limitation of our study is that HBRT-H14 was evaluated in single domesticated population of cervical samples. However, as presented in the VALGENT protocol paper [87], the absolute specificity of any HPV assay varies substantially between specimens taken from populations with different indications for HPV testing (e.g. primary screening, triage of women with borderline cytology or treatment follow-up), but the relative specificity of HPV assays compared to the standard comparator assay was similar in

different settings (e.g. population of patients from different countries), indicating that the relative accuracy is a robust validation parameter for the assessment of diagnostic test accuracy.

HBRT-H14 has several advantages: it has high throughput and is a technically undemanding assay providing relatively quick results (2–3 hours), it can be operated on the great majority of real-time PCR platforms at low cost [249]. Moreover, the analytical performance of HBRT-H14 was evaluated in the 2013 and 2014 WHO HPV LabNet international proficiency studies, showing high proficiency performance (100% proficient on all four datasets) [251].

To conclude, at the predefined cutoff of test positivity, HBRT-H14 was as sensitive as but less specific than HC2 for detecting CIN2+. However, with an *a posteriori* optimized cutoff, HBRT-H14 fulfilled the international criteria for use in screening for cervical cancer.

7.5 Supplementary information

Table 7.S1. HBRT-H14, HC2, LinearArray and Cobas results in the screening and enrichment population within the VALGENT-3 panel.

	Screening population		**Enrichment population**		**Total**	
Characteristic	***n***	**%**	***n***	**%**	***n***	**%**
HBRT-H14 (using the manufacturer-defined cutoff)*						
hrHPV+	186	14.4%	201	67.0%	387	24.3%
HPV16+	35	2.7%	83	27.7%	118	7.4%
HPV18+	17	1.3%	21	7.0%	38	2.4%
otherhrHPV+	151	11.7%	138	46.0%	289	18.1%
hrHPV−	1,108	85.6%	99	33.0%	1,207	75.7%
HBRT-H14 (using the optimized cutoff)*						
hrHPV+	138	10.7%	196	65.3%	334	21.0%
HPV16+	32	2.5%	81	27.0%	113	7.1%
HPV18+	13	1.0%	20	6.7%	33	2.1%
otherhrHPV+	108	8.4%	128	42.7%	236	14.8%
hrHPV−	1,156	89.3%	104	34.7%	1,260	79.1%
HC2*						
hrHPV+	156	12.1%	214	71.3%	370	23.2%
hrHPV−	1,138	87.9%	86	28.7%	1,224	76.8%
Linear Array*						
hrHPV+	138	10.7%	200	66.7%	338	21.2%
HPV16+	29	2.2%	84	28.0%	113	7.1%
HPV18+	11	0.9%	22	7.3%	33	2.1%
otherhrHPV+	105	8.1%	116	38.7%	221	13.9%
hrHPV−	1,156	89.3%	100	33.3%	1,256	78.8%
Cobas**						
hrHPV+	139	10.8%	206	68.7%	345	21.7%
HPV16+	30	2.3%	84	28.0%	114	7.2%
HPV18+	10	0.8%	24	8.0%	34	2.1%
otherhrHPV+	112	8.7%	140	46.7%	252	15.9%
hrHPV−	1,148	89.2%	94	31.3%	1,242	78.3%

HPV = human papillomavirus; hrHPV = high-risk HPV; other hrHPV = HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68; +/− = positive or negative; *n* = number of cases.

*Six samples tested invalid with HBRT-H14 were excluded from the analysis.
**Seven samples tested invalid by Cobas were excluded from the analysis.

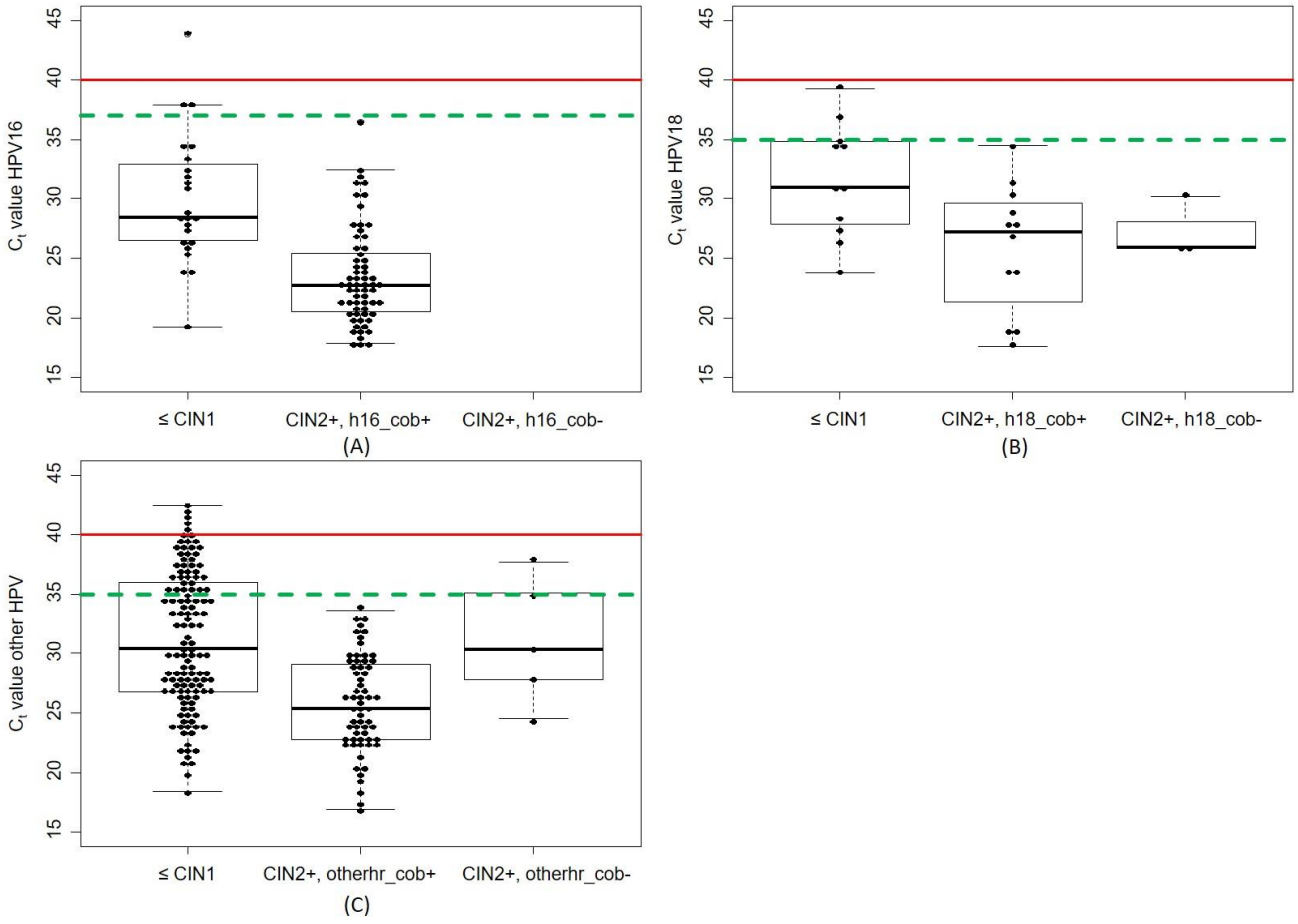


Figure 7.S1. Box plots showing the distribution of C_t values for HPV16 (A), HPV18 (B) and other hrHPV types (C) using HBRT-H14 (*y* axis) in women with ≤ CIN1, women who were CIN2+ and Cobas positive for the HPV type considered (e.g. CIN2+,h16_cob-) and women with CIN2+ but Cobas negative for the HPV type considered (e.g. CIN2+,h16_cob-). The predefined cutoff for each type was set at 40 (solid red lines). By comparing with Cobas, we could optimize and set the C_t values to the dashed green lines for HPV16 to 37, for HPV18 to 35 and for other hrHPV to 35, respectively (dashed green lines).

PART III

SECONDARY PREVENTION: TRIAGE OF WOMEN WITH ATYPICAL CYTOLOGY

CHAPTER 8

8. Accuracy of genotyping for HPV16 and 18 to triage women with low-grade squamous intraepithelial lesions: a pooled analysis of VALGENT studies

Adapted from:

Xu L, Benoy I, Cuschieri K, Poljak M, Bonde J, Arbyn M. Accuracy of genotyping for HPV16 and 18 to triage women with low-grade squamous intraepithelial lesions: a pooled analysis of VALGENT studies. Expert Review of Molecular Diagnostics 2019; 19(6):443-451.

Abstract:

Background: Genotyping for the most carcinogenic HPV types (HPV16/HPV18) can identify high risk of underlying cervical precancer and guide further management.

Research design and methods: A pooled analysis was performed of the clinical accuracy of high-risk HPV testing and HPV16/18 genotyping in triage of women with low grade squamous intraepithelial lesions (LSIL). Data regarding 24 assays evaluated in four VALGENT validation panels were used.

Results: In women with LSIL, hrHPV had a pooled sensitivity for CIN2+ of 95.5% (95% CI: 91.0-97.8%) and a specificity of 25.3% (95% CI: 22.2-28.6%). HPV16/18 genotyping had a sensitivity and specificity for CIN2+ of 52.9% (95% CI: 48.4-57.4%) and 83.5% (95% CI: 79.9-86.5%), respectively. The average risk of CIN2+ was 46.1% when HPV16/18-positive, 15.5% in women who were HPV16/18-negative but positive for other hrHPV types and 4.3% for hrHPV-negative women.

Conclusions: Triage of women with LSIL with HPV16/18 genotyping increases the positive predictive value compared to hrHPV testing but at the expense of lower sensitivity. Arguably, women testing positive for HPV16/18 need further clinical work-up. Whether colposcopy referral or further surveillance is recommended for women with other hrHPV types may depend on the post-test risk of precancer and the local risk-based decision thresholds.

8.1 Introduction

In women with minor cytological abnormalities, atypical squamous cells of undetermined significance (ASC-US), triage with high-risk human papillomavirus (hrHPV) testing is recommended in many cervical cancer screening protocols worldwide [105, 106]. For moderate cytological abnormalities, low-grade squamous intraepithelial lesions (LSIL), triage with hrHPV testing is less informative due to the high prevalence of HPV [106, 252], and the management of LSIL positive women is divergent across settings[106, 252]. To avoid immediate referral of all LSIL patients to colposcopy and the adverse effects of overtreatment, triage tools are needed to identify the minority of women with LSIL with underlying or incipient high-grade lesions. Partial genotyping for HPV16 and HPV18 has been proposed as a candidate triage marker. A previous systematic review and meta-analysis[108], published in 2017, indicated that although genotyping for HPV16 and HPV18 has poor sensitivity, it may be useful as an additional triage tool in LSIL hrHPV positive women in a two-step triage scenario. Here, a risk-based decision-making tool based on post-test risk is presented[108].

The number of commercially available HPV assays enabling HPV16 and HPV18 typing capabilities has increased substantially in the past five years as clinical focus is changing from simple detection of hrHPV towards extended or full genotyping[87, 105, 167, 197, 253]. Current evidence on the usefulness of HPV16 and HPV18 typing as a triage for the management of women with LSIL is here updated using new accuracy data obtained from the international VALidation of HPV GENotyping Tests (VALGENT) framework. VALGENT aims for the comparison and validation of HPV genotyping tests for clinically relevant outcomes using sample-populations relevant for primary cervical cancer screening[87]. An important objective of VALGENT is to document the accuracy of genotyping for the triage of women with minor cytological abnormalities and the contribution of more than twenty new accuracy datasets on the triage of women with LSIL will complete the current evidence base[108].

In the current pooled analysis of four individual VALGENT studies, we assess the accuracy of genotyping for HPV16/18 in triage of women with LSIL cytology to identify women with underlying cervical intraepithelial neoplasia of grade 2 or worse (CIN2+). The pre-test and post-test risks of CIN2+ were computed to suggest management decisions based on agreed threshold levels[108].

8.2 Materials and Methods

8.2.1 VALGENT framework and sample collection

The VALGENT framework contains several iterative sample panels collated in different countries. Up to now, three VALGENT panels have been completed with a fourth ongoing. VALGENT-1 were provided by the AML laboratory using SurePath collected samples (Antwerp, Belgium)[199-201, 204, 254], VALGENT-2 by the Scottish HPV Reference Laboratory using ThinPrep collected samples (Edinburgh, Scotland)[202, 203, 205], VALGENT-3 were performed using ThinPrep collected samples from the Laboratory for Molecular Microbiology of

University of Ljubljana (Ljubljana, Slovenia)[206, 226, 239-241], and VALGENT-4 using fresh SurePath collected samples by the Molecular Pathology Laboratory of Copenhagen University Hospital (Copenhagen, Denmark) [247]. In each VALGENT panel, the study population comprised a continuous series of 1,000 or 1,300 cervical specimen (archived or fresh) from women participating in the local cervical cancer screening programme supplemented with 300 abnormal pathological samples (100 ASC-US, 100 LSIL and 100 high-grade squamous intraepithelial lesions [HSIL]) [87]. Detailed information about each panel collection, processing and manipulation can be found in previously published VALGENT reports [199-206, 226, 239-241, 247, 254].

8.2.2 Clinical outcomes and performance measurement

According to the VALGENT protocol [87], we considered the presence of histologically confirmed CIN2+ as main disease outcome identified through follow-up and management according to national guidelines. Colposcopy was triggered by abnormal cytology in VALGENT-2 [202, 203], and by abnormal cytology and/or positive hrHPV testing in VALGENT-1, -3 and -4 [82, 87, 247]. For the purpose of current pooled analysis of triage accuracy, we use the number of women found with LSIL cytology and subsequently detected CIN2+ as the denominator for the computation of clinical sensitivity. Specificity was evaluated on women with LSIL index cytology and with normal colposcopy/histology findings and/or a negative cytology outcome through the follow-up period.

8.2.3 Evaluated tests in VALGENT studies

The full name, abbreviated name and the type specific remit of the evaluated assays, delivered in the VALGENT testing laboratories are summarised in **Table 8.1**. Throughout the rest of the paper, assays will be labelled by their shortened name. In total, twenty-four different HPV assays were evaluated in this pooled analysis. Five assays have limited HPV genotyping capacity, which can identify HPV16 and HPV18 separately (Cobas, Abbott, HPVRisk, HybribioHR and Harmonia); two has extended genotyping capacity (Onclarity and Xpert) and seventeen were full genotyping assays identifying at least 14 hrHPV types (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58,HPV59, HPV66 and HPV68) separately (Riatol qPCR, BSGP5 +/6+ MPG, GP5+/6+ LMNX, TS-E7-MPG, PapilloCheck, Linear Array, AnyplexHR, Anyplex HPV28, Innolipa, Euroarray, Papiloplex, Genoarray, LifeRiver Venus, CLART, Massarray, Modified GP5+/6+ and BGI).

The evaluated index tests were HPV assays identifying HPV16 alone or HPV16 and HPV18 jointly (HPV16/18). A positive HPV16/18 test was considered positive if HPV16 and/or HPV18 were present and negative when both types are absent. The comparator tests were hrHPV testing with GP5+/6+ PCR EIA (VALGENT-2 and VALGENT-4) and HC2 (VALGENT-3). These two tests are accepted as the standard comparator tests in the validation of new HPV assays with potential application for primary cervical cancer screening [86]. In VALGENT-1, the aggregate of 14 hrHPV types identified with the RIATOL qPCR was used as comparator test since no results generated by HC2 or GP5+/6+ PCR-EIA were available. For Euroarray, the

optimised cut-off proposed in the previously published VALGENT report was used for current analysis[240]. The cut-off set by the individual assay manufacturer was used without modification for all the rest evaluated assays.

To triage women with LSIL in order to detect CIN2+, the following questions on test accuracy of the different HPV genotyping assays were addressed: 1) what is the absolute clinical accuracy (sensitivity and specificity) of genotyping for HPV16 alone or for HPV16 and HPV18 combined (HPV16/18); 2) what is the relative clinical accuracy of genotyping for HPV16/18 compared to general hrHPV testing; 3) what is the relative accuracy of combined HPV16/18 genotyping compared to HPV16 genotyping alone; and 4) what is the clinical accuracy of HPV16/18 genotyping when considered as a second triage step in the management of hrHPV-positive LSIL women.

Table 8.1 Characteristics of the different HPV tests evaluated in VALGENT framework

	Assay (**abbreviated name**; manufacturer)	Performed by	Evaluated in Valgent No:	Test genotyping capacity
Standard comparator tests				
*	Hybrid Capture 2 High-Risk HPV DNA Test (**HC2**; Qiagen, Gaithersburg, MD, USA)	Laboratory for Molecular Microbiology, Faculty of Medicine, University of Ljubljana, Slovenia	3	No separate genotyping. Detects 13 hrHPV types in aggregate: 16,18,31,33,35,39,45,51,52,56,58,59 and 68.
*	GP5+/6+ PCR- EIA (**GP5+/6+ EIA**; Diassay B.V., Rijkswijk, the Netherlands)	DDL Diagnostic Laboratory, Rijswijk, The Nederland	1,2,4	No separate genotyping. Detections in bulk 14 hrHPV types in aggregate: same as HC2 plus 66.
		Evaluated tests		
1	Riatol qPCR assay (**Riatol qPCR**; lab in-house assay)	AML laboratory, Antwerp, Belgium	1,2,3	Individual detection of: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68; Detects also phr type 53 and lr types 6 & 11.
2	BSGP 5+/6+-PCR/MPG assay (**BSGP5 +/6+ MPG**; lab in-house assay)	Department of Genome Modifications and Carcinogenesis, DKFZ, Heidelberg, Germany	1	Individual detection of: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68b;pHr types 26, 53, 67, 70, 73, 82, 6, 11, 30, 42, 43, 44, 7, 13, 32, 34, 40, 54, 61, 62, 71, 72, 74, 81, 83, 84, 85, 86, 87, 89, 90, 91, 97, 102, 106, 55, 64 and 68a.
3	GP5+/6+ PCR Luminex genotyping kit (**GP5+/6+ LMNX**; Diassay B.V., Rijkswijk, the Netherlands)	DDL Diagnostic Laboratory, Rijswijk, The Nederland	1,2,4	Individual detection of 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68; Detects also phr types: 26,53,73,82.
4	TS-E7-MPG assay (**TS-E7-MPG**; in-house assay)	IARC, Lyon, France	1	Individual detection of 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82.
5	BD Onclarity HPV assay (**Onclarity**; BD Diagnostics, Sparks, MD, USA)	SHRL, Edinburg, Scotland & Hvidovre Hospital, Dept. Pathology, Hvidovre, Denmark	2, 4	Individual detection of 16, 18, 31, 45, 51, 52, 33/58, 56/59/66 and 35/39/68.
6	Xpert HPV (**Xpert**; Cepheid, Sunnyvale, CA, USA)	SHRL, Edinburg, Scotland	2	16, 18/45 and 11 other hrHPV types (See GP5+/6+ PCR-EIA).
7	PapilloCheck HPV-screening (**PapilloCheck**; Greiner Bio-One, Frickenhausen, Germany)	French HPV Reference Laboratory, Institut Pasteur, Paris, France.	2	Individual detection of 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82, 6, 11, 40, 42, 43, 44.
8	Linear Array HPV genotyping (**Linear Array**; Roche Molecular Diagnostics, Branchburg, NJ, USA)	Laboratory for Molecular Microbiology, Faculty of Medicine, University of Ljubljana, Slovenia	3	Individual detection of 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82, 6, 11, 40, 42, 54, 61, 70, 72, 81, CP6108, 55, 62, 64, 67, 69, 71, 83, 84, and IS39.
9	Cobas 4800 HPV test (**Cobas**; Roche Molecular System, Pleasanton, CF, USA)	Laboratory for Molecular Microbiology, Faculty of Medicine, University of Ljubljana, Slovenia	3	16,18 and 12 other hr types (See GP5+/6+ PCR- EIA)
10	Abbott RealTime High Risk HPV test (**Abbott**; Abbott, Wiesbaden, Germany)	Laboratory for Molecular Microbiology, Faculty of Medicine, University of Ljubljana, Slovenia	3	16,18 and 12 other hr types (See GP5+/6+ PCR- EIA)
11	Anyplex II HPV HR assay (**AnyplexHR**; Seegene, Seoul, South Korea)	Laboratory for Molecular Microbiology, Faculty of Medicine, University of Ljubljana, Slovenia	3	Individual detection of 14 hrHPV types: 16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66, 68

	Assay (**abbreviated name**; manufacturer)	Performed by	Evaluated in Valgent No:	Test genotyping capacity
12	Anyplex HPV28 detection assay (**Anyplex28**; Seegene, Seoul, South Korea)	Laboratory for Molecular Microbiology, Faculty of Medicine, University of Ljubljana, Slovenia	3	Separate identification of 28 types: 14 hr types (16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66, 68); 6 phr types (26, 53, 69, 70, 73, 82); 8 lr types (6, 11, 40, 42, 43, 44, 54, 61).
13	INNO-LiPA Extra II HPV Genotyping assay (**Innolipa**; Fujirebio Europe, Ghent, Belgium)	Ghent University Hospital, Ghent, Belgium	3,4	Separate identification of 28 different HPV types, 14 hr types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68); 5 phr types (26,53,73, 70,82); 6 lr types (6, 11, 40, 43, 44, 54); 3 types with undetermined risk (69, 71, 74).
14	EUROArray HPV (**Euroarray**; EUROIMMUN; Lübeck, Germany)	SHRL, Edinburg, Scotland	3	Separate identification of 30 types: 14 hr types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68); 4phr types (26, 53, 73, 82); 12 lr or ur types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, 89).
15	HPV-risk array (**HPVRisk**; Self-screen BV, Amsterdam, The Netherlands)	Cancer Center Amsterdam, VU University Medical Center, Amsterdam, The Netherlands	3,4	HPV16, 18 and 13 other hr types ((See GP5+/6+ PCR- EIA plus 67)
16	Hybribio 21 HPV Genoarray diagnostic kit (**Genoassay**; Hybribio, HongKong, China)	Laboratory for Molecular Microbiology, Faculty of Medicine, University of Ljubljana, Slovenia	3	Separate identification of 21 types: 14 hr types (16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66, 68); 1 phr types (53); 6 lr types (6, 11, 42, 43, 44, 81).
17	Hybribio 14 High-risk HPV with 16/18 Genotyping Real-time PCR kit (**HybribioHR**; ; Hybribio, HongKong, China)	Laboratory for Molecular Microbiology, Faculty of Medicine, University of Ljubljana, Slovenia	3	16,18 & 12 other hr types (See GP5+/6+ PCR- EIA)
18	Papilloplex High Risk HPV (**Papiloplex;** GeneFirst, Oxfordshire, UK)	SHRL, Edinburg, Scotland	4	Individual detection of 14 hr types (See GP5+/6+ PCR-EIA)
19	Liferiver Harmonia HPV assay (**Harmonia**; Liferiver, Shanghai, China)	SHRL, Edinburg, Scotland	4	16,18 & 12 other hr types (See GP5+/6+ PCR- EIA)
20	Liferiver Venus HPV assay (**LiferiverVenus**; Liferiver, Shanghai, China)	SHRL, Edinburg, Scotland	4	Separate identification of 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 & 82
21	CLART HPV4 assay (**CLART**; Genomica, Madrid, Spain)	Hvidovre Hospital, Dept. Pathology, Hvidovre, Denmark	4	Individual detection of : 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68
22	Agena HPV MassArray assay (**Massarray**; Agena Bioscience, Hamburg, Germany)	Hvidovre Hospital, Dept. Pathology, Hvidovre, Denmark	4	Individual detection of: 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 73
23	Modified GP5+/6+ PCR (**Modified GP5+/6+**; Karolinska University Hospital, Stockholm, Sweden)	International HPV Reference Center, Karolinska University Hospital, Stockholm, Sweden	4	Individual detection of 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68; Detects also phr types: 26,53,73,82.
24	BGI SENTIS HPV test (**BGI**; Shenzhen, China)	BGI Institute, HongKong, China	4	Individual detection of 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68; Detects also lr types: 6 & 11.

hrHPV: high-risk HPV types; phr types: probably/possibly carcinogenic HPV types; lr types: low-risk HPV types.

8.2.4 Statistical analysis

A bivariate normal model for the logit transformations of sensitivity and specificity were used to compute the pooled absolute sensitivity and specificity [255, 256]. The relative sensitivity and specificity of the evaluated index tests versus the comparator test were computed by including the test as a covariate in the bivariate normal model [75, 257]. Summary receiver-operating characteristics (sROC) plots were used to illustrate the joint overall and study-specific sensitivity and specificity of genotyping for HPV16 and HPV16/18 and hrHPV testing for triage of women with LSIL. Statistical analyses were conducted with STATA 14 (Stata Corp., College Station, Tex) using metandi [256] and a user-written procedure allowing multivariate diagnostic test accuracy meta-regression.

8.3 Results

8.3.1 Characteristics of study populations in different VALGENT panels

In VALGENT-1, 122 women with LSIL at enrolment (mean age: 33, range: 15-65) had follow-up outcomes with 15 CIN2+ cases identified. The total number of LSIL patients at enrolment with valid clinical outcomes in VALGENT-2 and -3 were 98 (mean age: 30.5, range: 19-62) and 47 (mean age: 35.4, range: 20-65]), respectively. According to the VALGENT protocol and the routinely indicated follow-up and management procedure in Scotland and Slovenia, in total, 20 and 22 confirmed CIN2+ cases were identified respectively. The VALGENT-4 study is on-going, with follow-up data until March 2018; from this 62 LSIL patients (mean age: 39.0, range: 30-57) and 14 CIN2+ were identified.

8.3.2 Absolute accuracy of genotyping for HPV16 and HPV16/18 compared to hrHPV testing

HPV16/18 genotyping identified, on average, 52.9% (95% CI: 48.4-57.4%) of CIN2+ while the pooled specificity to exclude CIN2+ was 83.5% (95% CI: 79.9-86.5%) (**Figure 8.1**). The pooled absolute accuracy measures for the genotyping of HPV16 and HPV16/18 and the hrHPV testing in triage of women with LSIL to detect underlying CIN2+ with 95% confidence intervals (CIs) are reported in **Table 8.2**. The sROC plot displays the sensitivity and specificity of the hrHPV testing (red), of genotyping for HPV16 (blue) and of HPV16/18 (green) in the triage of women with LSIL to detect CIN2+ (**Figure 8.2**).

Table 8.2 Pooled absolute sensitivity and specificity of genotyping for HPV16 and HPV1618 and hrHPV testing in triage of women with LSIL to detect underlying CIN2+

Genotyping	Outcome	No of studies/tests	Pooled value, in %	
			Sensitivity (95% CI)	Specificity (95% CI)
HPV16	CIN2+	30	46.9 (41.6-52.3)	89.2 (86.0-91.8)
HPV16/18	CIN2+	29	52.9 (48.4-57.4)	83.5 (79.9-86.5)
HrHPV	CIN2+	32	95.5 (91.0-97.8)	25.3 (22.2-28.6)

8.3.3 Relative accuracy of HPV16 and HPV16/18 compared to each other and compared to hrHPV testing

Genotyping of HPV16 and HPV16/18 demonstrated substantially higher specificity for the detection of CIN2+, compared with testing for hrHPV types. The pooled specificity ratios were 3.40 (95% CI: 2.97-3.89; p<0.0001) and 3.20 (95% CI: 2.81-3.64; p<0.0001), respectively (**Table 8.4**). The sensitivity of the two partial genotyping methods was lower compared with the hrHPV testing for detecting CIN2+. The pooled ratios were 0.50 (95% CI: 0.45-0.57; p<0.0001) for HPV16 genotyping and 0.57 (95% CI: 0.51-0.63; p<0.0001) for HPV16/18 genotyping. HPV16/18 genotyping detected on average 13% more CIN2+ than HPV16 genotyping, although this was not statistically significant (p=0.06), whereas the specificity to exclude CIN2+ was on average 6% lower which reached significance (95% CI: 3-9%; p<0.0001).

Table 8.3 Pooled analysis of the relative sensitivity and relative specificity of A) genotyping for HPV16 compared to hrHPV testing; B) genotyping for HPV16/18 compared to hrHPV testing and C) genotyping for HPV16/18 versus genotyping for HPV16 only, in triage of women with LSIL to detect CIN2+.

Outcome	**Number of comparisons**	**Relative sensitivity**	**P**	**Relative specificity**	**p**
A) Comparison HPV16 vs hrHPV					
CIN2+	30	0.50 (0.45-0.57)	<0.0001	3.40 (2.97-3.89)	<0.0001
B) Comparison HPV16/18 vs hrHPV					
CIN2+	29	0.57(0.51-0.63)	<0.0001	3.20 (2.81 –3.64)	<0.0001
C) Comparison HPV16/18 vs HPV16					
CIN2+	32	1.13 (0.99-1.28)	0.06	0.94 (0.91-0.97)	<0.0001

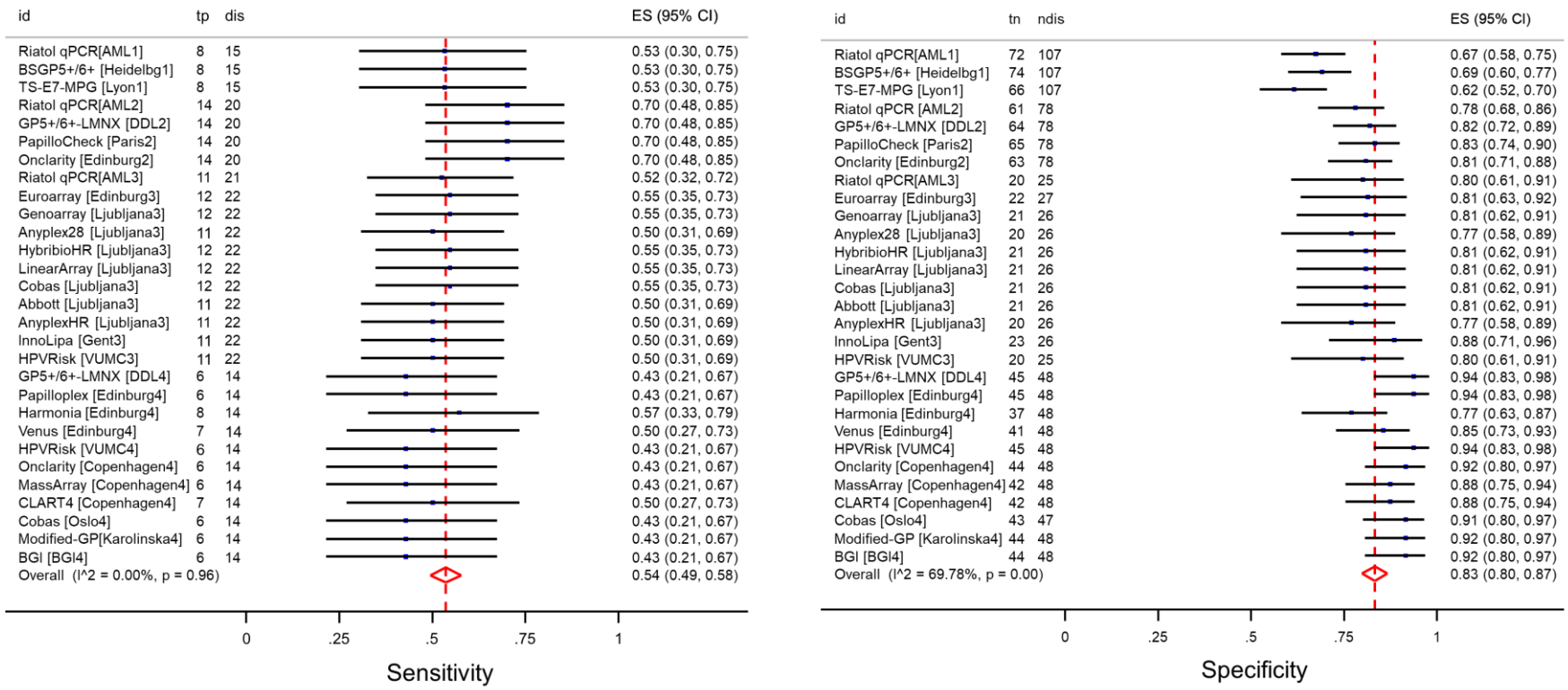


Figure 8.1 Pooled analysis of the sensitivity (left) and specificity (right) of genotyping for HPV16/18 to detect CIN2+ in women with LSIL.
CIN: cervical intra-epithelial neoplasia; HPV: human papillomavirus; LSIL: low-grade squamous intraepithelial lesion.

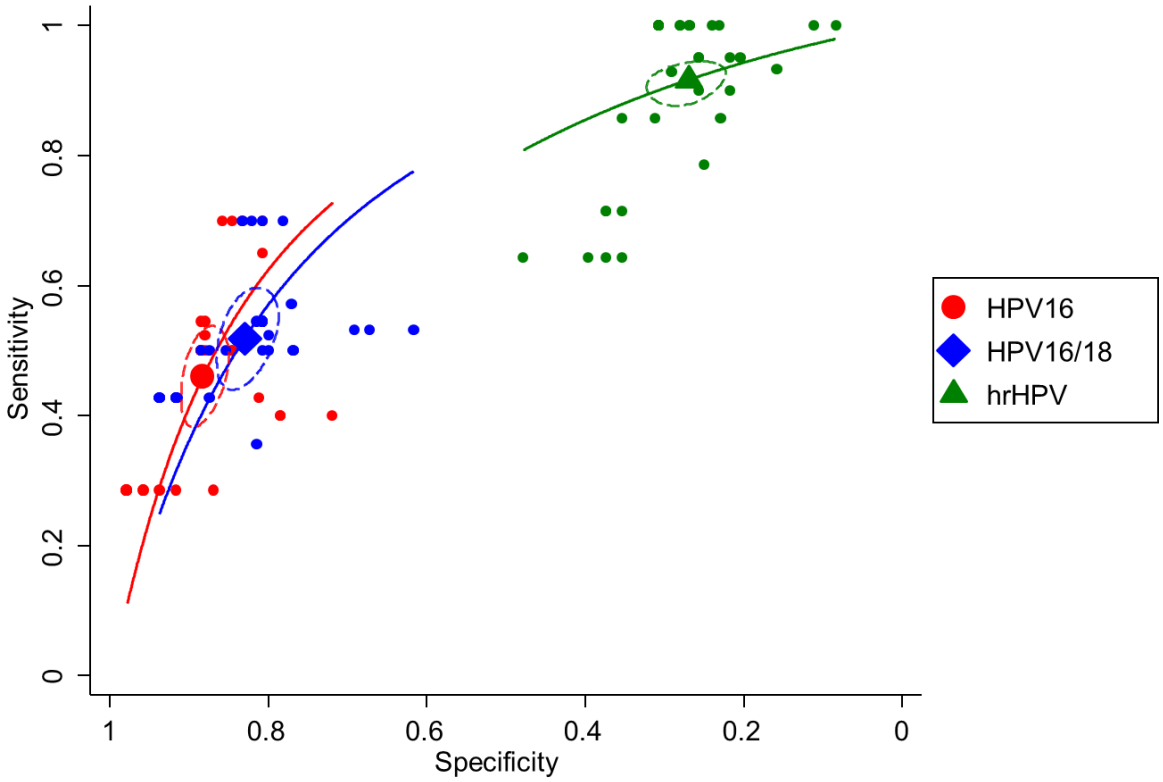


Figure 8.2 Summary receiver operation characteristic plot of the sensitivity as a function of the specificity of hrHPV and genotyping for HPV16/18 and HPV16 alone to detect CIN2+ in women with LSIL.
CIN: cervical intra-epithelial neoplasia; HPV: human papillomavirus; hr: high-risk; LSIL: low-grade squamous intraepithelial lesion.

8.3.4 Pre-test and post-test risk of cervical precancer

In **Table 8.4**, the pre- and post-test probabilities of CIN2+ of two triage strategies are presented. The average risk of CIN2+ before triaging test (pre-test risk) was derived from a previously published meta-analysis[108].The post-test probabilities can be computed with the pre-test risk and the sensitivity and specificity derived from the current pooled analysis (**Table 8.2**). Women with LSIL had on average a risk of CIN2+ of 21% before triaging test, triage with a hrHPV test stratifies this risk to 25.4% if hrHPV-positive and 4.3% if hrHPV-negative. Triage testing with HPV16/18 positive increased the risk of CIN2+ to 46.1%. Women testing HPV16/18 negative contain two subgroups: a) women who are hrHPV negative and b) those who are positive for other hrHPV types. They have a risk of CIN2+ of 13.0%.

A new pooled-analysis was performed to evaluate the accuracy of triage with HPV16/18 genotyping for CIN2+ restricted to hrHPV positive women (**Table 8.3**). The pooled sensitivity and specificity were 58.1% (95% CI: 53.1-62.5%) and 76.5% (95% CI: 72.5-80.0%), respectively. The post-test risk of CIN2+ is shown in the last row in **Table 8.4**; here the pre-test risk of CIN2+ corresponds with the post-test risk for triage with hrHPV testing (25.4%) with risks of 45.2% (if HPV16/18 positive) and 15.5% (if HPV16/18-negative, but other hrHPV-positive).

Table 8.4 Pre-test and post-test probabilities of CIN2+ of triage with hrHPV testing or HPV16/18 genotyping among women with LSIL. Data for triage with HPV16/18 genotyping among women hrHPV+ LSIL is shown in the grey row (two-step triage).

Triage Group	Test	Pre-test risk*	Post-test risk	
			if test+	if test-
			PPV	cNPV
LSIL	hrHPV	21%*	25.4%	4.3%
	HPV16/18	21%	46.1%	13.0%
LSIL&hrHPV+	HPV16/18	25%	45.2%	15.5%

*Pre-test risk based on pooled prevalence from previously published meta-analysis (AIM2016). For triage of hrHPV-positive LSIL patient, the pre-test risk corresponds with the post-test risk after hrHPV testing.

8.4 Discussion

This pooled analysis demonstrates the utility and the limitations of genotyping for HPV16 and HPV16/18 in triage of women with LSIL cytology. We found that HPV16 genotyping detects around half of women with LSIL cytology and underlying CIN2+. The addition of HPV18 to HPV16 only genotyping increased the sensitivity for CIN2+ with 13% but decreased the specificity with 6%. Due to the substantially larger number of <=CIN1 cases than CIN2+ cases, differences were significant for the specificity but not statistically significant for sensitivity. The pooled specificity of HPV16/18 genotyping to exclude CIN2+ is 84%, which was, as expected, substantially more specific but less sensitive than testing for all hrHPV types.

The underlying risk of cervical precancer should determine management[258]. A good triage test to manage LSIL patients should have good discriminatory power to indicate colposcopy referral when the triage test is positive and a return to either routine screening or re-test at a defined interval when the triage test is negative. Based on results of test accuracy obtained from current pooled analysis and the knowledge of CIN2+ prevalence in women with LSIL from the previously published meta-analysis[108], the post-test probabilities of CIN2+ of different triage strategies could be computed and translated into patient management algorithms.

A one-step HPV16/18 triage strategy clearly is not clinically acceptable since it does not allow to distinguish two groups (hrHPV negative and other than HPV16/18 positive) with clearly different risks. When HPV16/18 genotyping is applied in a two-step triage scenario to hrHPV-positive, its sensitivity is slightly higher, and its specificity is slightly lower compared to HPV16/18 when applied as a single triage.

The average risk of underlying CIN2+, pooled from the VALGENT studies, was 46% if HPV16/18-positive, 16% in women who were positive for other hrHPV types but negative for HPV16/18 and 4% for LSIL hrHPV-negative women. In European settings, a risk of CIN2+ (or positive predictive value) of >20% has been proposed as a threshold to indicate colposcopy[66, 259, 260]; an interval for surveillance testing 6-12 months later could be proposed if the risk of

CIN2+ is between 2-20%, and an interval for routine testing three years later is proposed if the risk for CIN2+ is <2%. In contrast, in the USA, if the risk of CIN2+ exceeds 10.2%, colposcopy referral is proposed[261]. Summarizing, use of HPV16 and/or 18 positive outcomes in women with LSIL could be considered useful in a European setting, effectively selecting those women who would have a colposcopy referral versus those with other hrHPV types who can be referred for re-testing at a defined interval.

With genotyping capability being an increasing feature of HPV testing platforms, our findings of the current study may help inform patient management pathways for women with LSIL through description of the underlying risks associated with HPV16/18 positivity.

In total, we included 24 HPV tests, which performed overall similarly with respect to clinical sensitivity and specificity in the different triage scenarios. However, limitations should be noted. First, in VALGENT, cross-sectional and short-term longitudinal endpoints are defined in agreement with the local follow-up guidelines within a single screening round. The follow-up periods for screen-positive women varied between 0 and 20 months. Short-term outcomes do not provide insights on the risk of developing high-grade disease over time. How the sensitivity and specificity of HPV16/18 genotyping change between cross-sectional or longitudinal endpoint has been assessed in a previously published meta-analysis [4]. The absolute longitudinal sensitivity of genotyping for HPV16/18 (>=2 years after the index finding of LSIL) was 12-15 percent lower whereas the longitudinal specificity was 1-2 percent higher than the respective cross-sectional accuracy measures. Another limitation was the small number of LSIL women who had a final diagnosis of CIN3+ in each of the VALGENT panels (always <10). Although it would be better to use histologically confirmed CIN3+ as main disease outcome, the low number of CIN3+ would make the sensitivity estimations unstable and imprecise. Therefore, we had to restrict our analysis to the endpoint of CIN2+.

The pooled results are comparable with the evidence in the previously published meta-analysis of genotyping with HPV16/18 in women with low grade cervical lesions[108]. It suggests that partial genotyping tests can be used to risk stratify precancer in hrHPV-positive women and to inform about need for immediate colposcopy or re-test within a defined interval. However, the clinical utility of HPV16/18 genotyping in LSIL patients is moderate, since negative triage results do not bring down the risk to a sufficiently low level allowing for a safe relieve to routine screening. Therefore, research for more performant triage markers should be continued. Since LSIL reflects a prevalent HPV infection, finding appropriate triage markers might be a relevant setting to discover triage tests that are also useful for the management of hrHPV-positive women in a context of primary HPV-based screening.

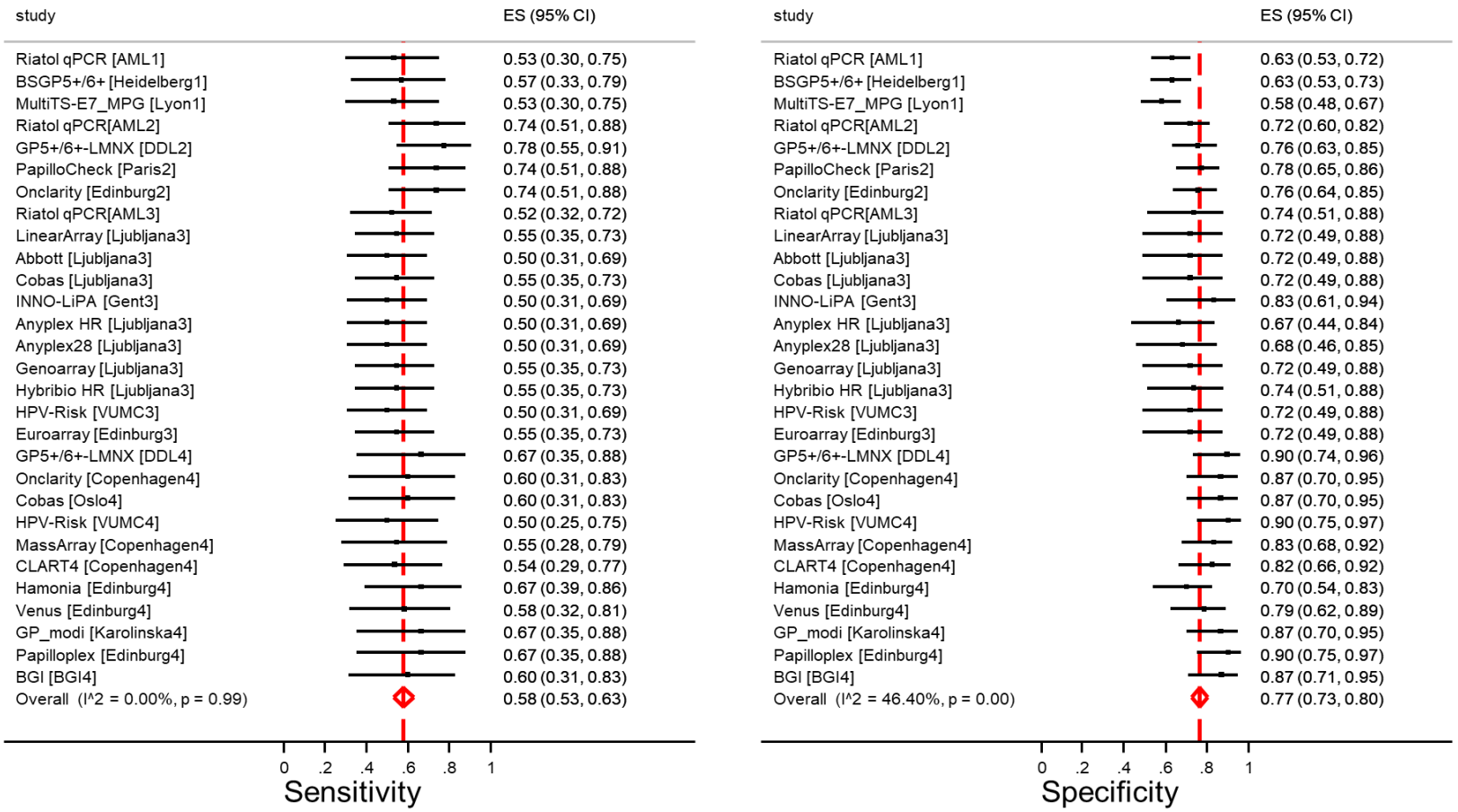


Figure 8.3 Pooled analysis of the sensitivity (left) and specificity (right) of genotyping for HPV16/18 to detect CIN2+ in women with LSIL who were hrHPV-positive. CIN: cervical intraepithelial lesion. HPV: human papillomavirus; hr: high-risk; LSIL: low-grade squamous intraepithelial lesion.

8.5 Conclusion

In Conclusion, triage of women with LSIL with partial genotyping of HPV16/18 increases the positive predictive value compared to detection of all hrHPV types but at the expense of loss in sensitivity. Women testing positive for HPV16/18 need further diagnostic and/or therapeutic work-up. Women testing HPV16/18-negative but positive for other hrHPV types may also be referred to colposcopy or kept under further surveillance depending on local decision thresholds. HrHPV-negative LSIL patients may be kept under surveillance or released to routine screening also depending on local decision thresholds. Further development and optimization of triage markers is needed to manage women with LSIL beyond limited genotyping.

8.6 Five-year view

Triage of minor cytology will stay important in several countries as long as cytology remains the primary cervical cancer screening test. Additional triage options beyond genotyping for HPV16 and 18 which allow better management are still needed. However, it is expected that within 5 years, many countries will have switched to HPV-based cervical cancer screening. In the future, triage of HPV-positive women will become a major topic for new meta-analytical work. However, defining evidence-based algorithms for management will continue to be driven by the assessment of risks of significant disease and how these risks change by screening and triage tests.

Key issues

- Triage of women with LSIL by HPV16/18 genotyping in a one-step triage strategy is poorly sensitive and not clinically acceptable.
- Genotyping for HPV16/18 to triage women with LSIL may be useful as a second triage test for women testing hrHPV-positive.
- Women with LSIL testing positive for HPV16/18 can be referred to colposcopy directly.
- Women with LSIL testing positive for other hrHPV types may also be referred to colposcopy or maintained under surveillance depending on local decision thresholds.

Declaration of interest

LX was supported by COHEAHR Network (grant No. 603019), coordinated by the Free University of Amsterdam (The Netherlands), funded by the 7th Framework Programme of DG Research and Innovation, European Commission (Brussels, Belgium). Sciensano the employer of LX has received support from VALGENT as explained in Arbyn et al J Clin Virol 2016 PM:26522865 and in Bonde et al J Clin Virol 2018 PM:30253376. LX has not received financial advantages from test manufacturers for work conducted in the framework of VALGENT.

IB is a clinical pathologist working in the private laboratory AML. AML has received research support in the form of free kits, reduced prices or funding from Abbott, Hologic, Cepheid, Roche, Becton Dickinson, Seegene, Biomérieux, Rover Medical devices, Aprovix and My Sample. IB has also received travel grants to attend symposia, conferences, and meetings from Hologic and Abbott. IB, nor AML, has received any financial advantage from test manufacturers nor any payment for work and tests conducted in the framework of VALGENT.

KC has no conflicts of interest to declare, KC's institution has received research funding and or associated gratis consumable from the following in the last 3 years: Hologic, Cepheid, Qiagen, Becton-Dickinson, Euroimmun, SelfScreen, LifeRiver, Genomica, Genefirst.

MP was supported by COHEAHR Network (grant No. 603019), coordinated by the Free University of Amsterdam (The Netherlands), funded by the 7th Framework Programme of DG Research and Innovation, European Commission (Brussels, Belgium). Faculty of Medicine, University of Ljubljana, Slovenia, the employer of MP has received free-of-charge reagents from test manufacturers involved in VALGENT as explained in Arbyn et al J Clin Virol 2016 PM:26522865 and in Bonde et al J Clin Virol 2018 PM:30253376. MP has not received financial advantages from test manufacturers for work conducted in the framework of VALGENT.

JB attended meetings with various HPV test manufacturers. JB has received honoraria from Hologic, Roche, Qiagen, Genomica, and BD Diagnostics for lectures, and is the principal investigator on studies partly funded and/or received reagents at reduced or no cost by BD diagnostics, Biocartis, and Genomica SAU. The employer of JB has received free-of-charge reagents from test manufacturers involved in VALGENT as explained in Arbyn et al J Clin Virol 2016 PM:26522865 and in Bonde et al J Clin Virol 2018 PM:30253376. JB has not received financial advantages from test manufacturers for work conducted in the framework of VALGENT.

MA was supported by COHEAHR Network (grant No. 603019), coordinated by the Free University of Amsterdam (The Netherlands), funded by the 7th Framework Programme of DG Research and Innovation, European Commission (Brussels, Belgium). Sciensano the employer of MA has received support from VALGENT as explained in Arbyn et al J Clin Virol 2016 PM:26522865 and in Bonde et al J Clin Virol 2018 PM:30253376. MA has not received financial advantages from test manufacturers for work conducted in the framework of VALGENT.

CHAPTER 9

9. Triage of ASC-H: a meta-analysis of the accuracy of high-risk HPV testing and other markers to detect cervical precancer

Adapted from:

Xu L, Verdoodt F, Wentzensen N, Bergeron C and Arbyn M. Triage of ASC-H: a meta-analysis of the accuracy of high-risk HPV testing and other markers to detect cervical precancer. Cancer Cytopathology. 2016; 124(4): 261-272.

Abstract

Background: Women with a cytological diagnosis of atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion (ASC-H) are usually immediately referred to colposcopy. However, triage may reduce the burden of diagnostic work-up and avoid over-treatment.

Methods: A meta-analysis was conducted to assess the accuracy of hrHPV testing, and testing for other molecular markers to detect CIN of grade II or III or worse (CIN2+ or CIN3+) in women with ASC-H. An additional question assessed was whether triage is useful given the relatively high pre-triage probability of underlying precancer.

Results: The pooled absolute sensitivity and specificity for CIN2+ of HC2 (derived from 19 studies) was 93% (95% CI: 89-95%) and 45% (95% CI: 41-50%), respectively. The $p16^{INK4a}$ staining (only 3 studies) has similar sensitivity (93%, 95% CI:75-100%) but superior specificity (specificity ratio: 1.69) to HC2 for CIN2+. Testing for PAX1 gene methylation (only 1 study) showed a superior specificity of 95% (specificity ratio: 2.08). The average pre-test risk was 34% for CIN2+ and 20% for CIN3+. A negative HC2 result decreased this to 8% and 5%, whereas a positive result upgraded the risk to 47% and 28%.

Conclusions: Due to the high probability of precancer in ASC-H, the utility of triage is limited. The usual recommendation to refer women with ASC-H to colposcopy is not altered by a positive triage test, whatever the test used. A negative hrHPV DNA or $p16^{INK4a}$ test may allow for repeat testing but this recommendation will depend on local decision thresholds for referral.

Keywords: cervical cancer; cervical intraepithelial neoplasia; triage; atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion; ASC-H; HPV; meta-analysis; diagnostic test accuracy

9.1 Introduction

By repetitive high-quality cytological screening and treatment of cervical precancerous lesions, the incidence of and mortality from cervical cancer can be reduced substantially. However, still about halve a million of women are diagnosed every year with cervical cancer.[118] The cytological diagnosis of ASC-H is a relatively new cytological classification and is a subset of atypical squamous cells (ASC) formally introduced in the 2001 Bethesda System (2001).[24] ASC-H has cytological characteristics that are intermediate between atypical squamous cells of undetermined significance (ASC-US) and high-grade intraepithelial lesion (HSIL). As an uncommon cytological interpretation, ASC-H is reported infrequently and the prevalence of Pap smears interpreted as ASC-H varies significantly among laboratories with a mean reporting rate of 0.43% (5^{th}-95^{th} percentile: 0-2%) in the USA.[262] The finding of ASC-H is often associated with a high HPV positive rate (10^{th} -50^{th}-90^{th} percentile: 0%-54%-79%) [263] and a relatively high risk of underlying intraepithelial neoplasia of grade II or III or worse (CIN2+, CIN3+), ranging from 13%[264] to 66%[265] and 11%[266] to 35% [267], respectively. In comparison, ASC-US is a far more prevalent cytological category which has lower likelihood of underlying CIN2+ (pooled average of 12%) and CIN3+ (7%).[105, 106]

Management of cervical cytological lesions depends on the severity of the lesion and its inherent underlying or future risk of high-grade cervical intraepithelial neoplasia or cancer.[109, 110] Consistent evidence is available supporting the recommendation to use high-risk HPV (hrHPV) testing to triage women ASC-US[105, 106] and general consensus exist regarding the recommendation to refer all women with HSIL directly to colposcopy.[110, 268] However, divergent recommendations are found in the international literature regarding the management with intermediately severe cytological abnormalities such as *low-grade intraepithelial lesion* (LSIL), *atypical glandular cells* (AGC) and ASC-H.

American and European guidelines recommend immediate colposcopy for women with ASC-H.[109-111] The ASCCP (American Society of Colposcopy and Cervical Pathology) consensus guideline in 2006 was primarily based on data from the Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesion (ASCUS/LSIL) Triage Study (ALTS), which indicated that ASC-H had a significantly greater hrHPV positivity (84%) and underlying risk of high-grade CIN compared with ASCUS.[269] The 5-year cancer risk among 467 women of the Kaiser Permanente Northern California (KPNC) cohort with HPV-negative ASC-H was 2% , which is judged too high to justify observation[270]. Therefore, the ASCCP 2012 updated guidelines continues to recommend immediate colposcopy regardless of HPV result for women with ASC-H cytology[109] even though the level of evidence of this recommendation was graded as moderate. However, a retrospective study carried out at University of Pittsburg Medical Center (UPMC) identified 885 HPV-negative ASC-H patients with available follow-up results. In an average follow-up period of 29 months, only 14 (1.6%) patients showed evidence of CIN2+ and no case of invasive cervical cancer was diagnosed.[271] These data suggest that HPV triage in the management of women with ASC-H may be useful.

To reduce the burden of diagnostic work-up and to avoid over-treatment and adverse effects associated with excision of lesions,[113, 272] it is appropriate to identify markers which may increase safety and efficiency of management procedures for women with ASC-H. We therefore conducted a systematic review and meta-analysis to assess the accuracy not only of hrHPV testing but also of other molecular markers to predict presence or development of cervical precancer.

9.2 Material and Methods

9.2.1 Clinical question

This meta-analysis evaluates the test accuracy of HPV testing and other molecular markers to triage women with a cytological result of ASC-H to predict the presence of CIN2+ or CIN3+. The following clinical questions were addressed: A) what is the absolute accuracy (sensitivity and specificity) of hrHPV testing with the Hybrid Capture-2 assay (HC2) and B) what is the absolute accuracy of other hrHPV assays and other molecular markers? and C) what is the relative accuracy of these other assays and markers compared to HC2?

Because of the high underlying risk of cervical precancer associated with ASC-H, the review also assesses whether a negative triage test could downgrade the risk sufficiently to avoid immediate diagnostic work-up. The Population-Index Test-Comparator Test-Outcomes-Studies (PICOS) components of the clinical questions are explained in the Supplementary information.

9.2.2 Inclusion criteria and search strategy

Studies were eligible if the following criteria were met: (1) women had cytological report of ASC-H (2) hrHPV testing was performed by HC2 and/or other assays and/or triage with other molecular markers (3) women were subsequently submitted to a reference test to verify presence or absence of CIN2+ or CIN3+ and (4) the separate accuracy data (number of false- and true-positive and negative results) were reported, computable or could be requested. Outcome assessment including colposcopy and directed biopsy, with or without endocervical curettage was considered as the reference standard. Only studies enrolling at least 20 women with ASC-H were selected. All retrospective or prospective studies that evaluated the accuracy of triage testing in women with ASC-H to predict the presence of CIN2+ or CIN3+ were eligible for this meta-analysis.

Twelve eligible studies had been identified through a previously performed literature search for a meta-analysis on hrHPV testing in triage of ASC-US and LSIL and for which details on the search strategy are described elsewhere.[105, 106] To update and extend retrieval of references, a new search string which focused on reports of ASC-H was performed in three databases, MEDLINE, EMBASE and CENTRAL. The search strategy used two terms only, “ASC-H” and “cannot exclude high grade intraepithelial lesion”, combined by the OR Boolean operator. No language restrictions were applied. Additionally, the references of included papers and citation lists of previous meta-analyses and other key studies were browsed using

www.scopus.com. Eligibility of studies was evaluated by L.X. and F.V. In case of discordance, M.A. was consulted for final decision on in- and exclusion.

9.2.3 Evaluated tests

The main evaluated index test was HC2 test since it is the most widely studied hrHPV assay. HC2 targets DNA sequences of HPV types 16,18,31,33,35,38,39,45,51,52,56,58,59 and 68, using the standard cut off (signal strength, relative light units ≥ 1, expressing semi-quantitatively the viral load compared to a control sample with 1 pg of HPV DNA per millilitre). Other tests including other HPV assays identifying nucleic acid sequences of hrHPV types jointly or HPV assays detecting a limited number of HPV types were also included as well as other molecular markers (overexpression of proteins or methylation of certain viral or human genes) were also evaluated. The cut-off proposed by the manufacturer of each assay was accepted as the positivity criterion.

9.2.4 Reference standard

All women had to be submitted to verification with colposcopy, colposcopy-directed biopsies, possibly supplemented with endocervical curettage. The histological interpretation of biopsies was considered as the outcome, accepting negative colposcopy as sufficient ascertainment for the absence of disease, when no biopsies were taken in case of normal satisfactory colposcopic findings. Two levels of disease outcome were considered: CIN2+ and CIN3+.

9.2.5 Data extraction and statistical analysis

For all included studies, information on the design and characteristics of the study were abstracted by L.X. and F.V. and evaluated by M.A. The quality of each included study was evaluated using the second version of the check list for Quality Assessment of Diagnostic Accuracy Studies (pri-2).[273] A score was given to four domains (participant selection, triage test, reference standard, and flow & timing), based on a set of signalling questions assigned to each domain.

When four or more studies were available, the absolute sensitivity and specificity of the tests were estimated jointly with a bivariate normal model for the logit transforms of sensitivity and specificity.[274] Summary receiver-operating-characteristics (sROC) plots were drawn to show the joint overall and study-specific sensitivity and specificity.[256] When less than four studies were available, sensitivity and specificity were pooled separately using random-effect models for pooling proportions and ratios of proportions.[275, 276]

The relative sensitivity and specificity of other tests compared to HC2 were computed using *metadas*, a SAS macro for meta-analysis of diagnostic accuracy studies that allows the inclusion of test as a covariate making comparison of tests possible (See Supplementary Material Chapter 4).[277] The influence of other study characteristics, such as the QUADAS-2 items, on test accuracy were also assessed with *metadas*.

Post-test probabilities of CIN2+ and CIN3+ were computed by applying the pooled sensitivity and specificity estimates on the observed average prevalence (=pre-test probabilities). The post-test risk if triage is positive corresponds with the positive predictive value (PPV), whereas the post-test risk in triage-negative women corresponds with the complement of the negative predictive value (cNPV=1-NPV). A positive triage was considered as efficient as the PPV exceeded 20% (for CIN2+) and 10% (for CIN3+) whereas a negative triage was considered as safe when cNPV was lower than 2% (for CIN2+) and 1% (for CIN3+).

9.3 Results

9.3.1 Selection of studies

Twelve eligible studies had already been identified through a previously performed literature search for a meta-analysis on hrHPV testing in triage of ASC-US and LSIL.[105, 106] An additional systematic literature search in MEDLINE, EMBASE and CENTRAL, performed on March 31, 2015, resulted in 405 unique articles. After a primary eligibility check of the titles, 303 references were excluded. 88 manuscripts were excluded based on further evaluation of the abstracts and/or the full manuscripts. Among which two studies[278, 279] were excluded because no histologically confirmed CIN2+ were identified in the group with a negative triage test, resulting in zero true negatives or a specificity of 0%. From the KPNC cohort,[280, 281] only the report with complete cross-sectional accuracy data was chosen[281]. As a result, a total of 25 studies were selected. Two studies from an American laboratory were both included,[282, 283] since the proportion of overlapping participants was small. The process of study selection and the reason of exclusion of studies is shown in the PRISMA flow chart in **Figure 9.1**

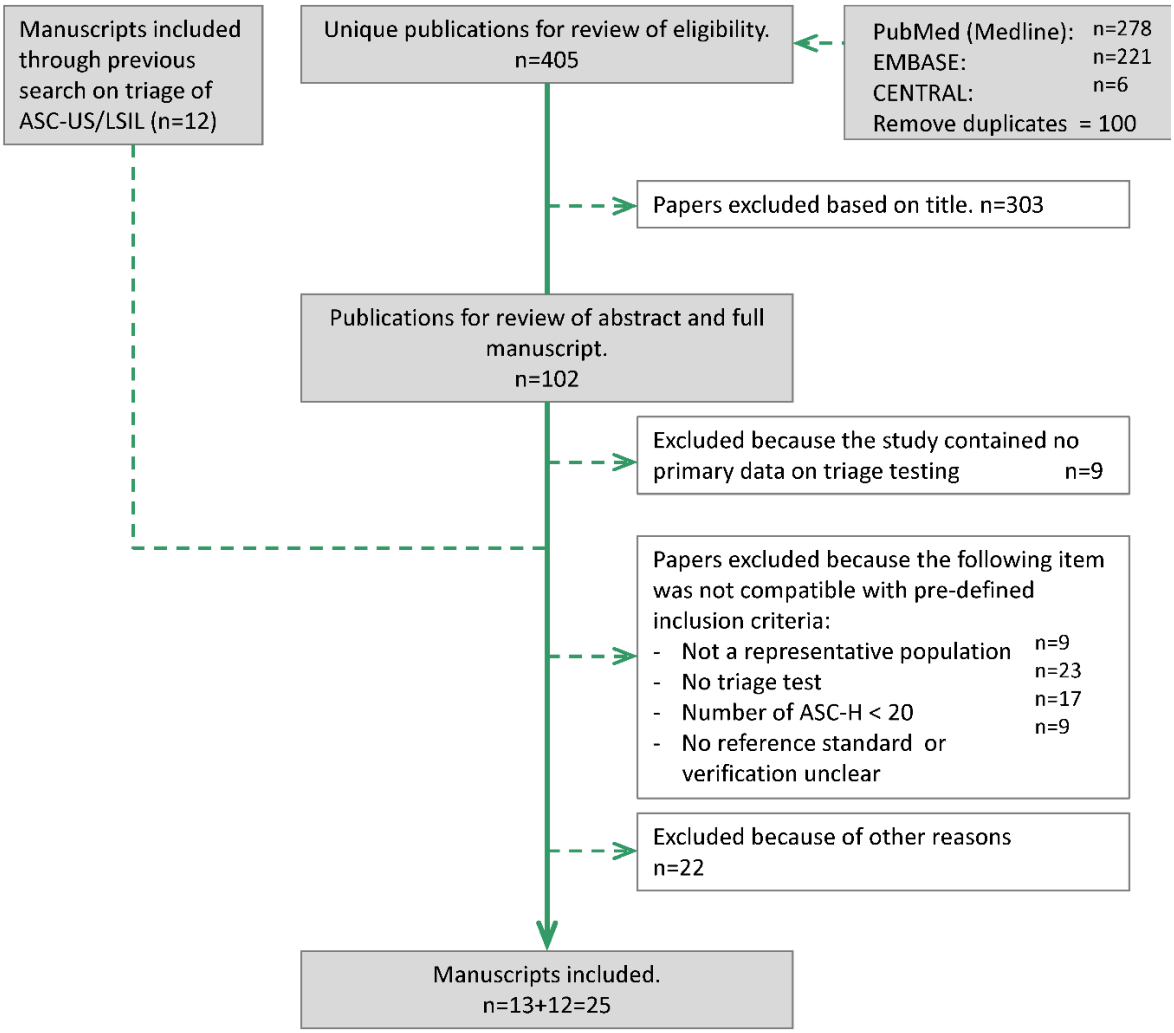


Figure 9.1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses32 flow chart showing the selection process for the retrieved studies. ASC-H indicates atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion; ASCUS, atypical squamous cells of undetermined significance; CENTRAL, Cochrane Central Register of Controlled Trials; LSIL, low-grade squamous intraepithelial lesion.

9.3.2 Study characteristics

An overview of study design, population and test characteristics of included studies is listed in Table 9.S1. Eight reports had a prospective design [264, 266, 267, 283-287] and seventeen reports had a retrospective design.[265, 269, 281, 282, 288-300] Eighteen studies included women with ASC-H identified through primary screening [265, 266, 281-284, 287-291, 294-300] and seven studies recruited subjects in colposcopy clinics where women were referred to because of prior cytological result of ASC-H. [264, 267, 269, 285, 286, 292, 293] Most included studies presented cross-sectional data. One study included women from a randomized controlled trial in which 110 ASC-H cases were retrospectively identified at cytology review by 4 pathologists [269].

Nineteen studies reported accuracy data for the HC2 (Digene Corp., Gaithersburg, USA) assay [264-267, 269, 281-284, 286-292, 295, 297, 300]. Four studies reported accuracy data of other hrHPV DNA testing, and among which, two with Linear Array® HPV Genotyping (Roche Molecular Diagnostics, Almede, USA) [285, 301], one with the Cervista HPV HR test (Hologic, Marlborough, USA),[298] and one with Multiplex PCR with genotype-specific primers (Eurofins MWG Operon, Huntsville, USA).[293] For two studies accuracy data were obtained for HPV16/18 genotyping.[267, 285] For $p16^{INK4a}$ staining [293, 294, 299] and $p16^{INK4a}$/Ki-67 dualstaining,[285, 296, 298] six studies were retrieved. In one study, accuracy of over-methylation of the paired boxed gene 1 (PAX1) gene was assessed.[266].

All 25 included studies contributed accuracy data for CIN2+ but only 7 for CIN3+. The evaluation of the quality of the included studies is summarized in Supplementary Table 9.S2. Risks of selection bias were low to moderate, except for three studies [288, 291, 293] where women who had no biopsy data were not included. Concerns of bias regarding the reference standard were low to moderate. For more than half of the included studies (14/25), it was unclear whether the results of the reference test were masked towards the triage test or not. Complete verification with a valid reference standard was provided in 23/25 (92%) studies, and incorporation bias was avoided in 24/25 (96%) studies and was unclear in only 1 study.[287] The delay between triage testing and verification outcome was not sufficiently documented in 8/25 (32%) studies. Withdraws were not well explained in 9/25 (36%) studies. Un-interpretable results for the triage test and reference test were not reported in 11/25 (44%) studies.

Table 9.1 Pooled sensitivity and specificity of HC2 to detect CIN2+ and CIN3+, and pooled disease rate in women diagnosed with ASC-H on cytology.

Outcome	Nb of studies	Sensitivity, %		Specificity, %		Disease rate, %	
		Pooled estimate (95% CI)	Range	Pooled estimate (95% CI)	Range	Pooled estimate (95% CI)	Range
CIN2+	19	93 (89-95)	68-100	45 (41-50)	26-72	34(28-40)	13-66
CIN3+	5	91(81-96)	71-100	42 (34-51)	32-65	20(14-28)	11-36

Abbreviations: CIN2+, grade two cervical intraepithelial neoplasia or worse; CIN3+, grade three CIN or worse. HC2, Hybrid Capture-2 assay.

9.3.3 Absolute accuracy of HC2

The pooled absolute sensitivity, specificity, and the pooled disease rate and their 95% confidence intervals (CIs) are listed in **Table 9.1**. The pooled absolute sensitivity was 93% (95% CI: 89-95%) and 91% (95% CI, 81-96) to detect CIN2+ and CIN3+, respectively. The pooled absolute specificity for excluding CIN2+ and CIN3+ was 45% (95% CI: 41-50%) and 42% (95% CI: 34-51%), respectively. (**Table 9.1** and **Figure 9.2**). The corresponding SROC plots for outcome of CIN2+ and CIN3+ shown in **Figure 9.3**, display the variation of test accuracy in the individual studies as well as the overall pooled accuracy. The average hrHPV positivity rates was 67% (95% CI: 63-72%), ranging from 51% to 83%. The average prevalence of CIN2+ and CIN3+ among women triaged with HC2 was in 34% (95% CI: 28-40%, range:13-66%) and 20% (95% CI: 14-28%, range: 11-36%), respectively.

The variation of the accuracy of HC2 to detect CIN2+ according to study quality (assessed by the QUADAS items) and other covariates are shown in Supplementary Tabel 9.S3 and Table 9.S4**.** Study design (prospective or retrospective, psensi=0.90, pspeci=0.33), study setting (primary screening or referred population, p_{sensi}=0.88, p_{speci}=0.92) and study size (<100 vs ≥100 cases of ASC-H, p_{sensi}=0.81, p_{speci}=0.36) did not significantly affect the accuracy of HC2 in triage of women with ASC-H. A significantly higher sensitivity of HC2 was noted when the risk of inappropriate exclusion of patients was high compared to low risk studies. The sensitivity was significantly higher when the following reasons of concern regarding study quality were noted: a) the reference was not clearly described, and b) avoidance of incorporation bias was unclear. When un-interpretable results were not reported for both triage and reference test, specificity was significantly lower.

Table 9.2 Pooled absolute sensitivity and specificity of all triage tests and relative accuracy of other tests compared to HC2 to detect CIN2+ in women with ASC-H.

Triage test	#	**Sensitivity, %**		**Sensitivity ratio (95% CI)**	**Specificity, %**		**Specificity ratio (95% CI)**
		Estimate (95% CI)	Range		Estimate (95% CI)	Range	
HC2	19	93 (89-95) £	57-100	1.00	45 (41-50) £	27-91	1.00
Other hrHPV tests€	4	96 (88-100) £	82-100	1.02 (0.95-1.10)	35 (20-52) £	17-50	0.76 (0.53-1.10)
HPV16/18 genotyping	2	50 (35-65) †	50-50	0.50 (0.31-0.82) *	74 (60-86) †	72-76	1.72 (1.41-2.10) *
p16INK4a	3	93 (75-100) †	82-100	0.99 (0.87-1.12)	77 (65-88) †	68-83	1.69 (1.39-2.06) *
p16INK4a/Ki-67	3	94 (84-99) †	88-94	1.00 (0.92-1.10)	50 (27-74) †	31-68	1.12 (0.81-1.54)
Methylation	1	81 (65-93) ‡	--	1.01 (0.92-1.10)	95 (90-99) ‡	--	2.08 (1.85-2.32) *

£Pooled estimate of sensitivity & specificity computed jointly with the bivariate model, € Other hrHPV tests comprise Linear Array, Cervista and Multiplex PCR, † pooled estimate of sensitivity and specificity computed separately with a random effect model, * significant likelihood ratio test which assess whether the relative accuracy is statistically different from unity (p<0.05), ‡ estimate from one study,-- not applicable for only one study. Abbreviations: ASC-H, atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion; CIN2+, grade two cervical intraepithelial neoplasia or worse; CIN3+, grade three CIN or worse; HC2, Hybrid Capture-2 assay.

Only three studies provided age-stratified accuracy data for HC2 [281, 291, 300] (See Supplementary Figure 9.S1). In all studies, prevalence of CIN2/3+, test positivity rate and PPV for CIN2/3+ decreased by age. For the risk of CIN2+ and CIN3+ among test negative subjects, no relation with age was observed.

9.3.4 Accuracy of other triage tests

The pooled absolute accuracy, as well as the relative accuracy of other triage tests compared to the HC2 assay, for the detection of CIN2+, are shown in **Table 9.2**. The forest plot (**Figure 9.4**) shows the study-specific and pooled sensitivity and specificity for CIN2+ of the other triage tests. Given absence of significant heterogeneity in the sensitivity (I^2=8.6% and p=0.35) and presence moderate but non-significant heterogeneity in the specificity (I^2=56.0%% and p=0.08),

pooled accuracy estimates could be computed for triage with hrHPV testing with other assays (Linear Arrary, Cervista and Multiplex PCR). The pooled absolute sensitivity and specificity for CIN2+ were 96% (95% CI: 88-100%) and 35% (95% CI: 20-52%), respectively. The relative sensitivity and specificity of other hrHPV tests versus HC2 for CIN2+ was 1.02 (95% CI: 0.95-1.10) and 0.76 (95% CI: 0.53-1.10), respectively. HPV16/18 genotyping identified on average 50% (95% CI: 35-65%) of CIN2+ and correctly excluded 74% (95% CI: 60-86%) of women without CIN2+. Genotyping for HPV16/18 was less sensitive (ratio of 0.50, 95% CI: 0.31-0.82) but more specific (ratio of 1.72. 95% CI: 1.41-2.10) than HC2.

For $p16^{INK4a}$ staining, the pooled absolute sensitivity and specificity for CIN2+ were 93% (95% CI: 75-100%) and 77% (95% CI: 65-88%) respectively. The $p16^{INK4a}$ was as sensitive for detecting CIN2+ as HC2 is (ratio= 0.99, 95% CI: 0.87-1.12) but was substantially more specific (ratio =1.69; 95% CI: 1.39-2.06). For $P16^{INK4a}$/Ki-67 dual staining, the pooled absolute sensitivity and specificity for CIN2+ were 94% (95% CI: 84-99%) and 50% (95% CI: 27-74%) respectively. Compared to HC2, $P16^{INK4a}$/Ki-67 dual staining had similar sensitivity (ratio of 1.00, 95% CI: 0.92-1.10) and not significantly higher specificity (ratio of 1.12, 95% CI: 0.81-1.54).

With only one available study, the sensitivity of methylation of *PAX1* to detect CIN2+ in women with ASC-H was 81% (95% CI: 65-93%), while its specificity was 95% (95%CI: 90-99%). Compared to HC2, the specificity of methylation to exclude CIN2+ is substantially higher with a ratio of 2.08 (95% CI: 1.85-2.32).

9.3.5 Post-test probabilities

The pre- and post-test probabilities of CIN2+ and CIN3+ after triage testing are presented in **Table 9.3**. Women with ASC-H have on average a pre-test risk of CIN2+ and CIN3+ of 34% (95% CI: 29-39%) and 20% (95% CI:16-25%), respectively. Triage with the hrHPV testing of HC2 upgrades this risk (post-test risk), to 47% (for CIN2+) and 28% (for CIN3+) if HPV-positive and downgrades the risk to 8% (for CIN2+) and 5% (for CIN3+) if HPV-negative. After genotyping for HPV16/18, the risk of CIN2+ increases to 50% (for CIN2+) and 40%(for CIN3+) if HPV16/18-positive and decreases to 26% (for CIN2+) and 9% (for CIN3+) if HPV16/18-negative. Triage with $p16^{INK4a}$ staining stratifies the post-test risk of CIN2+ to 68% (if test+) and 5% (if test-). Triage of women with ASC-H using methylation of *PAX1* lifts up the risks of CIN2+ to 89% and CIN3+ to 96% if test positive and brings them down to 9% (for CIN2+) and 2% (for CIN3+) if triage negative.

Table 9.3 Sensitivity, specificity, likelihood ratios, pre- and post-test probabilities of CIN2+ and CIN3+ of triage with HC2 and other tests among women with ASC-H.

Triage test	Outcome	#	Average pre-test risk,% (95% CI)	Pooled Sensitivity,% (95% CI)	Pooled specificity,% (95% CI)	PLR	NLR	Test+,% (95% CI)	Pooled post-test risk if test+ PPV,% (95% CI)	Pooled post-test risk if test- 1-NPV,% (95% CI)
HC2	CIN2+	19	34 (29-39)	93 (89-95)	45 (41-50)	1.69	0.16	67 (63-72)	47 (41-52)	8 (6-9)
	CIN3+	5	20 (16-25)	91 (81-96)	42 (34-51)	1.57	0.22	67 (63-72)	28 (23-34)	5 (4-7)

HPV16/18 genotyping	CIN2+	2	34 (29-39)	50 (35-65)	74 (60-86)	1.92	0.68	38 (28-48)	50 (44-55)	26 (22-30)
	CIN3+	2	20 (16-25)	73 (53-91)	73 (62-83)	2.70	0.37	38 (28-48)	40 (34-48)	9 (7-11)
p16INK4a	CIN2+	3	34 (29-39)	93 (75-100)	77 (65-88)	4.04	0.09	48 (36-61)	68 (62.4-72.2)	5 (4-6)
Methylation	CIN2+	1	34 (29-39)	81 (65-93)	95 (90-99)	16.20	0.20	23 (17-31)	89 (87-91)	9 (8-11)
	CIN3+	1	20 (16-25)	93 (69-99)	99 (95-100)	93.00	0.07	23 (17-31)	96 (95-97)	2 (1-3)

Abbreviations: #, number of studies; CIN2+, grade two cervical intraepithelial neoplasia or worse; CIN3+, grade three CIN or worse; PLR: positive likelihood ratio, NLR: negative likelihood ratio, test+: triage test positivity rate. HC2, Hybrid Capture-2 assay.

As shown in Supplementary **Table 9.S5**, in women younger than 50, the average pre-test risk of CIN2+ and CIN3+ is 33% (95% CI: 17-48%) and 25% (95% CI: 22-28%), respectively. Women 50 or older have on average a pre-test risk of 14% (95% CI: 3-26%) and 13% (95% CI: 10-16%), respectively. The post-test probabilities of CIN2+ after triage with HC2 are: 47% (if test+ and <50 years), 27% (if test+ and ≥50 years), 6% (if test- and <50 years) and 4% (if test- and ≥50 years). Triage with hrHPV testing of HC2 upgrades the post-test risk of CIN3+, to 51% (women <50 years) and 53% (women ≥50 years) if HPV-positive and downgrades the risk to 6% (women <50 years) and 3% (women ≥50 years) if HPV-negative.

In Supplementary **Table 9.S6**, we estimated the number of useful referrals (TP), missed cases (FN), unnecessary referrals (FP), and correctly reassured cases or colposcopies avoided (TN) when a given triage method is applied on 1,000 women with ASC-H. These numbers are computed from the meta-analytically pooled sensitivity and specificity assuming an average, low or high background risk of CIN2+ and CIN3+ observed from the range of studies included in the systematic review. Triage with HC2 of an average ASC-H population (pre-test probability of CIN2+ of 34%) would identify 316 women needing treatment, would miss 24 cases of CIN2, would generate 363 unnecessary referrals and would avoid 297 colposcopies.

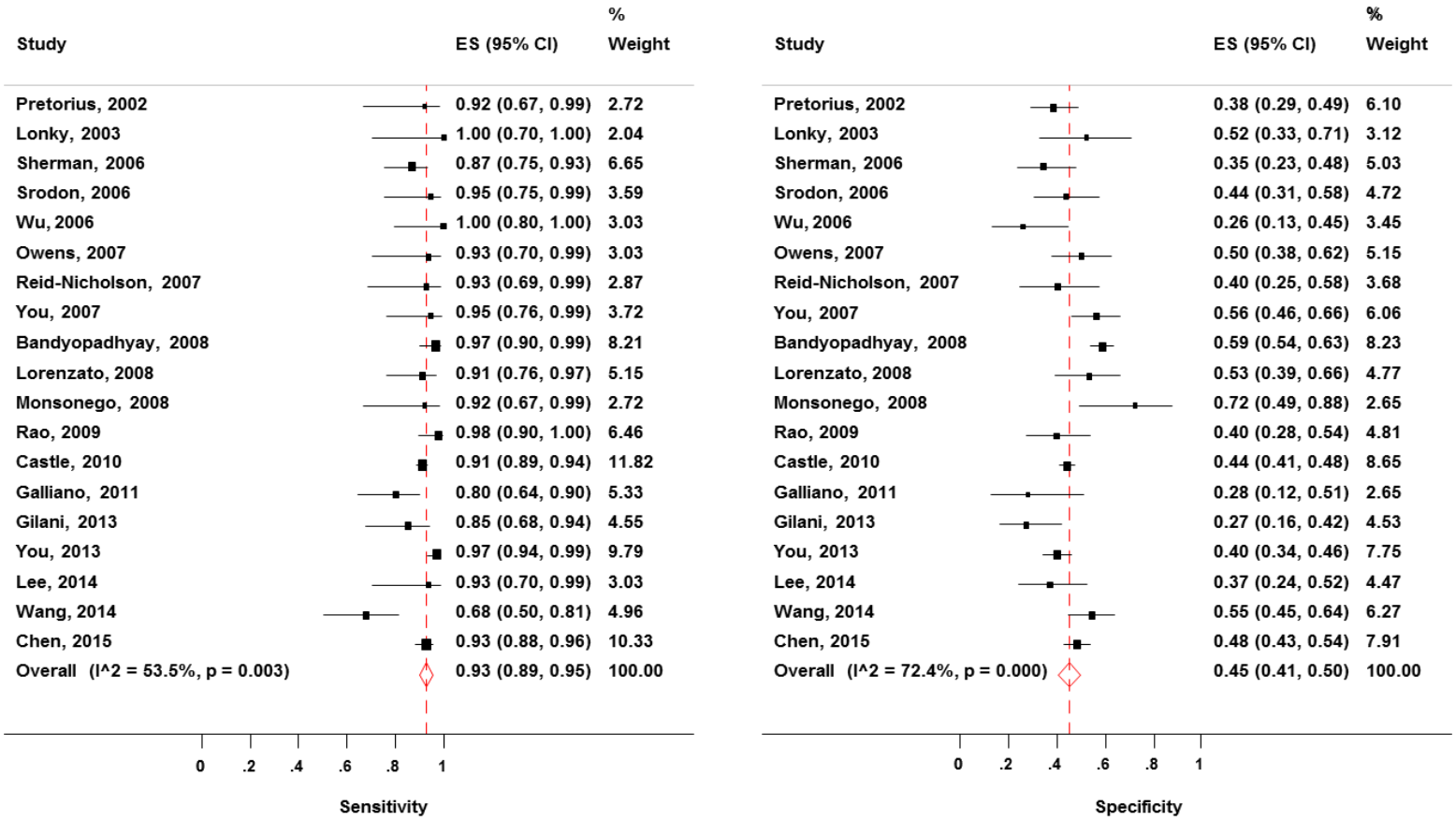


Figure 9.2 Meta-analysis of the sensitivity (left) and specificity (right) of HC2 to detect CIN2+ in women with ASC-H. The pooled values are computed with a bivariate model.

Abbreviations: ASC-H, atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion; CIN2+, grade two cervical intraepithelial neoplasia or worse; CIN3+, grade three CIN or worse; HC2, Hybrid Capture-2 assay.

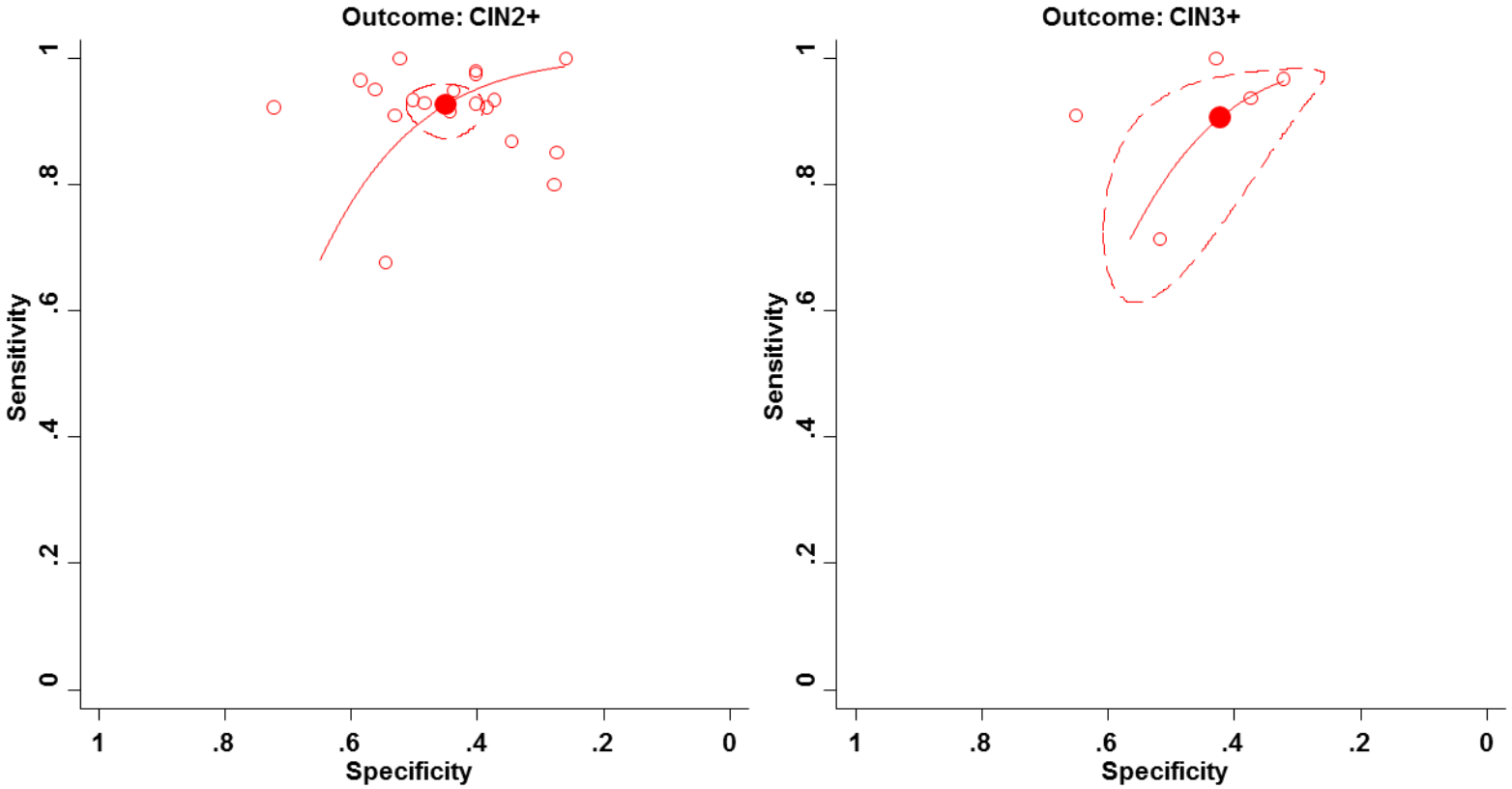


Figure 9.3 Meta-analysis of the sensitivity and specificity of the HC2 assay to triage women with ASC-H to detect CIN2+ (left) and CIN3+ (right). Filled circles indicate the summary point; hollow circles, individual studies; solid line, summary receiver-operator curve; dashed line, 95% confidence ellipse.

Abbreviations: ASC-H, atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion; CIN2+, grade two cervical intraepithelial neoplasia or worse; CIN3+, grade three CIN or worse; HC2, Hybrid Capture-2 assay.

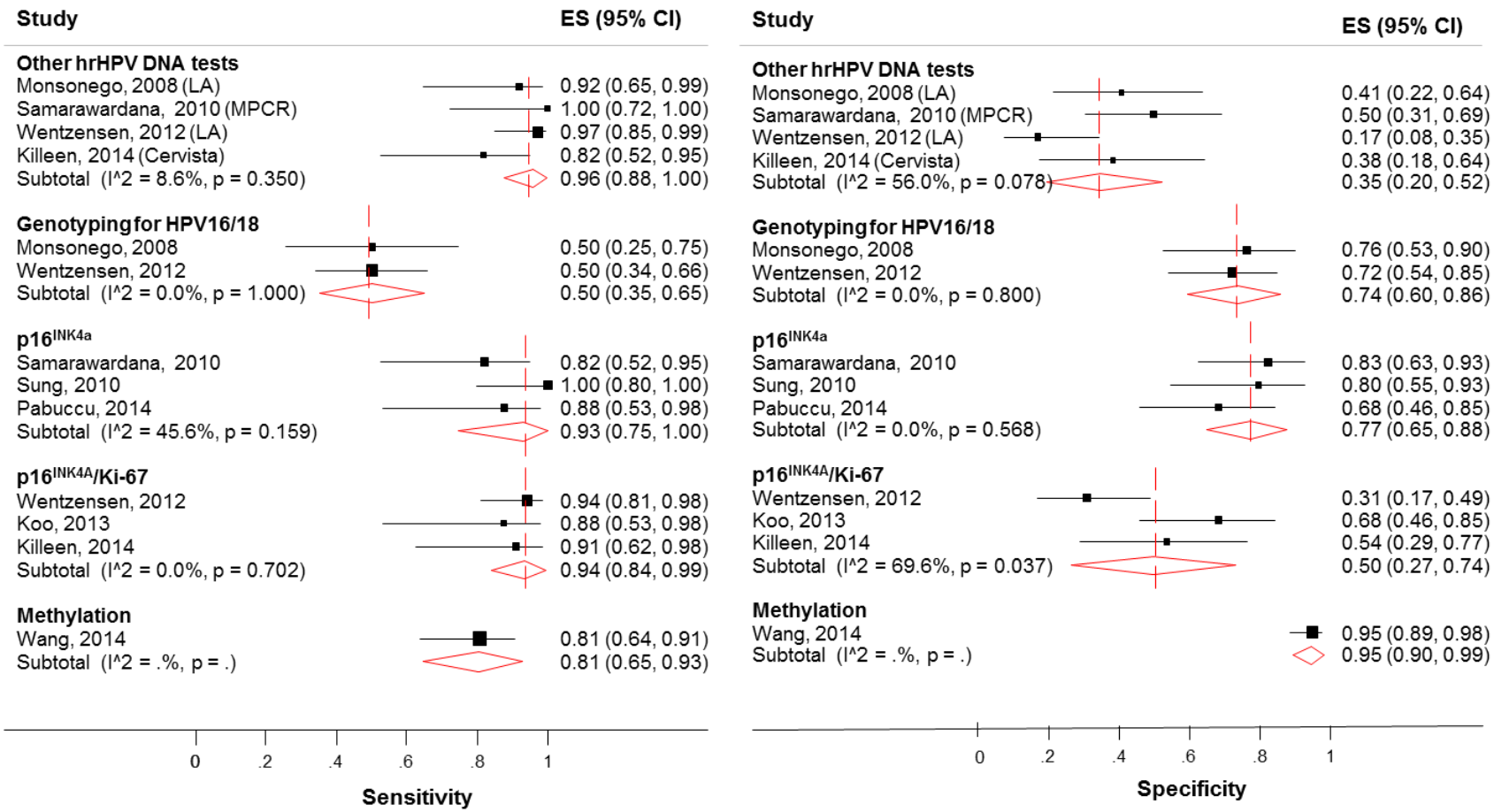


Figure 9.4 Subgroup meta-analysis of the sensitivity (left) and specificity (right) of other triage tests to detect CIN2+ in women with ASC-H. The pooled estimates of sensitivity and specificity are computed separately with a random effect model. Abbreviations: ASC-H, atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion; CIN2+, grade two cervical intraepithelial neoplasia or worse; LA, Linear Array; MPCR, Multiplex PCR.

9.4 Discussion

Across all 25 included studies, over 4,000 women with a diagnosis of ASC-H were identified. Among those women, CIN2+ and CIN3+ was detected in 34% (95%CI: 29-39%, range: 13-66%) and 20% (95% CI: 16-25%, range: 11-36%) of the cases. Although disease rates vary among different studies, the pooled values are in line with those of a previous systematic review.[262]

In this meta-analysis, testing for hrHPV infection by HC2 demonstrated good sensitivity of 93% but a rather low specificity of 45%. In comparison, in triage of ASC-US a similar average sensitivity (90%, 95% CI: 88–92) but higher specificity (58%, 95% CI: 54–63) have been observed for HC2.[105, 106] The underlying pre-triage risk of CIN2+ is considerably higher in women with ASC-H (on average 34%) compared to with those with ASC-US (on average 12%)[105, 106] Whereas it is accepted that a women with ASC-US and a negative hrHPV test may be released to routine screening, this recommendation seems not permitted for hrHPV negative women with ASC-H.[104, 110]. Indeed, in women who were hrHPV-negative the risk is 8% for CIN2+ and 5% for CIN3+, which is much lower than that in hrHPV-positive women with ASC-H, but still is too high to withdraw them from follow-up. The post-test risk of CIN3+ among hrHPV-negative women aged 50 or older was lower (3%) than among younger women (5%), but the plausible recommendation of repeat testing would not be different (risk of CIN3+ in both age categories>1%[66]).

The large range of hrHPV positivity (51-90%) and underlying range of prevalence of CIN2+ (13-66%) and CIN3+(11-35%), observed in our meta-analysis, illustrates the subjectivity of the cytological diagnosis of ASC-H. In practice, the variation of hrHPV positivity is even larger (10^{th}-90^{th} percentile range of 0-79% in a recent survey in the USA)[263]. We postulate that the low rates of hrHPV positivity in ASC-H may reveal cytological overcalling in certain cytological laboratories. In the ALTS study, where the cytological diagnosis of ASC-H was based on expert quality control review, the hrHPV positivity was 84%. In Supplementary **Figure 9.S2**, we have pooled the hrHPV positivity and underlying prevalence of CIN2+ and assessed the correlation between both in a scatter plot (See Supplementary **Figure 9.S3**).The clear positive trend between both parameters can be observed (Slope=0.53 , 95% CI: 0.26-0.80, p=0.0001, R^2= 0.46). It suggests that the hrHPV positivity rate in ASC-H as well as in other cytology categories may be used as an indicator for the quality of cervical cytology interpretation.[302] Monitoring of the hrHPV positivity rate in women with ASC-H and correlating this to the subsequent risk of cervical precancer according to HPV status (assessed routinely or for reasons of surveillance) may be a good procedure to improve the quality of cytological interpretation as well as to challenge local guidelines for triage.

We included studies with diagnostic follow-up of less than 2 years, so these risks are cross-sectional rather than longitudinal in nature. In European guidelines, a PPV for CIN2+ ≥20% or PPV for CIN3+ ≥10%, and cNPV for CIN2+ ≤ 2% or for CIN3+ ≤1% are often accepted as decision thresholds to define management.[66, 258, 259] Since the PPV of hrHPV testing in women with ASC-H clearly exceeds the positive triage criterion, the recommendation to refer

immediately to colposcopy is clear, whatever the used hrHPV assay. However, the hrHPV negative women with ASC-H show a risk which is within the 1%-10% and 2-20% benchmarks for CIN3+ and CIN2+ respectively, therefore, a recommendation for repeat testing 6-12 months later would be acceptable in an European context.

In Supplementary **Table 9.S5**, we provide a framework for clinicians and decision makers to judge whether triage of women with ASC-H is useful or acceptable in a given setting (low, average or high pre-test probability of CIN2+ and CIN3+). In a European context (where referral is acceptable if risk of CIN3+ exceeds 10%), repeat testing, would be appropriate when the pre-test risk is low or average. In the USA, however, where referral thresholds may be lower[303], only in a low background risk situation (for instance in the UPMC study [291]), retesting after 6-12 months would be acceptable.

Several other authors also suggest that hrHPV testing for the triage of women with ASC-H would be useful for selecting patients with ASC-H who should undergo immediate colposcopic examination.[283, 288, 289, 304, 305] However, the use of HPV DNA triage testing in the primary work-up of ASC-H is not recommended in the ASCCP guidelines.[109] This consensus guideline in 2012[109] is based on the data from KPNC cohort study, in which 44 (10.6%) of 414 patients with HPV-negative ASC-H results were diagnosed of CIN2+ and who were followed-up for at least 5 years.[303] However, a large cohort study carried out in UPMC had a larger group of 885 HPV-negative ASC-H women, of whom only 14 (1.6%) of 885 patients developed high-grade CIN and no case of invasive cancer were diagnosed during a mean follow-up period of 29 months.[271] The authors considered the risk of CIN2+ among HPV-negative women is sufficiently low to recommend women from this group to repeat Pap and HPV testing after 1 year. However, given the rather low hrHPV rate (51%, 95% CI: 47-55%) in this ASC-H study population, we suspect a certain degree of overcall in cytological interpretation. A prior meta-analysis of the proportion of HC2 positivity in ASC-US showed an average estimate of 39% (95% CI: 39-46%, range 26-74%).[306] So we assume that in this UPMC study, a substantial proportion of ASC-H might be classified as ASC-US in other laboratories.

Genotyping for the most important carcinogenic HPV types (HPV16 and 18) results in tremendous gain of specificity (74%) but also large loss of sensitivity (relative sensitivity: 0.50) compared to HC2 in excluding patients with CIN2+, suggesting that HPV16/18 genotyping has limited utility in the management of women with ASC-H. The $p16^{INK4a}$ staining has superior specificity (55%) but similar sensitivity (95%) to HC2 and seems therefore more useful in management of patients with ASC-H. PAX1 gene methylation[266] showed excellent specificity (95%) but its sensitivity was significantly lower than HC2. As shown in Supplementary **Table 9.S5**, risk of CIN2+ and CIN3+ after a negative triage result with $p16^{INK4a}$ and methylation markers is sufficiently downgraded to accept repeat testing instead of referral but the level of evidence is low given the small number of studies.

For a patient with an ASC-H and a normal or an unsatisfactory colposcopy, hrHPV DNA testing or $p16^{INK4a}$ cyto-immunochemistry could play a role in the follow-up decisions. However, the

improved risk stratification for a small group of screen-positive women needs to be weighed against the increased complexity of triage and management algorithms.

The included studies were of moderate to good methodological quality according to QUADAS-2 criteria, providing us reasonable confidence in the reliability of the meta-analysis. We found rather precise estimates of the accuracy to detect CIN2+ (rather narrow confidence intervals) but substantial heterogeneity in the accuracy estimates of HC2. However, for the outcome of CIN3+, the 95% confidence ellipse in SROC plots was rather wide, due to the small number of studies, which downgrades the quality of evidence. For the other triage tests, even smaller number of studies could be retrieved resulting in a low level evidence for the use of these molecular markers in clinical practice[307]

Another limitation of this meta-analysis is the lack of long-term longitudinal outcomes for women with ASC-H. The predictive values of triage tests in this meta-analysis are based on short-term colposcopic and histologic examination while the cumulative risk of high grade CIN and cancer increases over time[270]. Limitations are also connected with the infrequent occurrence of ASC-H in screening, often resulting in retrospective study designs that are generally limited by the difficulty to obtain complete clinical and virology data for participants with sufficiently similar characteristics.

9.5 Conclusion

Our meta-analysis shows that a cytological result of ASC-H is associated with a high risk of cervical precancer, which justifies immediate referral for colposcopy. However, our results support a certain utility of hrHPV DNA testing and in particular of $p16^{INK4a}$ cyto-immunochemistry. A positive triage result does not alter the decision to refer, but those testing negative could be recalled for a repeat test 6-12 months later in countries with a conservative follow-up policy. Nonetheless, in countries with a low decision threshold for colposcopy referral, triage of ASC-H would be considered as not useful to orient diagnostic work-up.

Supplementary information

9.5.1 S1. Clinical questions

What is the accuracy of HC2 to identity women with atypical squamous cells—cannot exclude high-grade squamous intraepithelial lesion (ASC-H) who need referral to colposcopy? Is HC2 a good triage test to manage women with ASC-H?

Are other hrHPV tests and tests for other molecular markers as accurate as HC2 to identify women with ASC-H who need referral to colposcopy? Is it better than HC2? In other words: is any of the other tests a good triage test to manage women with ASC-H?

What is the average post-test risk of CIN2+ and CIN3+after a positive (=PPV) or negative triage test (1-NPV)?

In particular, is the post-test risk after a negative triage test sufficiently low to allow repeat testing and avoid immediate referral for colposcopy and biopsy?

9.5.2 S2. PICOS components

P: women participating in cytological screening for cervical cancer or referred to colposcopy showing a cytology result of ASC-H
I: index test: HC2
C: comparator tests: other hrHPV assays and molecular markers
O: accuracy to detect underlying disease (=CIN2+, CIN3+/AIS+):

- Absolute accuracy and relative accuracy (sensitivity, specificity) for all studies with complete design (outcome verified for all subjects)
- Risk of CIN2+ and CIN3+ (underlying prevalence=pretest risk); test-positivity rate; PPV and 1-NPV (post-test risk if test-positive or negative)

S:

- Complete diagnostic studies
- RCTs

9.5.3 S3. Search string in PUBMED-MEDLINE

(ASC-H) or ("cannot exclude high grade intraepithelial lesion")

Same search string was used in EMBASE and Cochrane central library

9.5.4 S4. SAS log file using the macro metadas *to calculate the relative accuracy of other tests compared to HC2

```
proc import out=work.ASCH

 datafile = "D:\metaASCHforsas_quadas.dta"

 dbms = dta replace;

run;

data work.ASCH;

 set ASCH;

 where outcome=2 and tgroup=14;

run;

proc print;

run;

%include 'D:\metadas_v13.sas';

%metadas(import=n, dsname=ASCH, logfile='D:\ASCH.log',

 subject=study, debug=y, keepds=y, predict=y, bothmodels=n, method=b, cvsummorder=level,cvref=12, covariate=testfam);

run;
```

* The *metadas* macro can be downloaded from:

http://dta. cochrane. org/sites/dta. cochrane. org/ files/ uploads/METADAS_v1.3_txt.txt

9.5.5 S5. Study characteristics

Table 9.S1 Population and study characteristics

Study Country	#	Study design	Population setting	Inclusion/exclusion	Mean age (range)	Triage tests (cut-off)	Gold standard verification	Outcome
Pretorius 2002 USA	104	Prospective cohort study with cross-sectional data.	Follow-up (colposcopy clinic)	Incl: women referred to colposcopy because of ASC-H.	31* (12-80)	HC2 (RLU>1)	Colposcopy + colposcopy-directed cervical and/or vaginal biopsy. ECC/LEEP/conisation/hysterectomy when needed.	CIN2+
Lonky 2003 USA	32	Prospective cohort study with cross-sectional data.	Screening	Incl: women were referred to colposcopy because of ASC-H.	29 (21-38)	HC2 (RLU>1)	Colpospcopy + colposcopy-directed cervical biopsy. ECC in case of unsatisfactory colposcopy.	CIN2+ CIN3+
Sherman 2006 USA	108	Retrospective sample selected from RCT trial.	Follow-up	Incl: women with a consensus Pap smear result of ASC-H.	25*	HC2 (RLU>1)	Colposcopy referral for HSIL and LEEP for CIN2+.	CIN2+ CIN3+
Srodon 2006 USA	67	Prospective cohort study. Timing of FU <8m.	Screening	Incl: women with a Pap smear result of ASC-H. Excl: women without histologic follow-up data or cytologic follow-up only.	N.D.	HC2 (N.D.)	All women had histological verification consisted of colposcopy, cervical biopsy, ECC, LEEP, cone biopsies and hysterectomy.	CIN2+
Wu 2006 USA	42	Retrospective cohort study with cross-sectional data.	Screening (high-risk population)	Incl: women with ASC-H were retrieved from the pathology database. Excl: women without follow-up biopsies.	36 (15-85)	HC2 (RLU>1)	All women had follow-up biopsies.	CIN2+
Owens 2007 USA	73	Retrospective cohort study. Timing of FU:2yr.	Screening	Incl: women with a Pap smear result of ASC-H in the pathology database.	34 (15-70)	HC2 (RLU>1)	All women had histologic follow-up results.	CIN2+
Reid-Nicholson 2007 USA	44	Retrospective cohort study with cross-sectional data.	Screening	Incl: women with a Pap smear result of ASC-H were retrieved from the database. Excl: women with no biopsies follow-up.	45 (16-87)	HC2 (N.D.)	All women had colposcopy and biopsy with no details mentioned.	CIN2+

Study Country	#	Study design	Population setting	Inclusion/exclusion	Mean age (range)	Triage tests (cut-off)	Gold standard verification	Outcome
You 2007 China	109	Retrospective cohort study	Screening	Incl: women with a Pap smear result of ASC-H.	38 (18-81)	HC2 (RLU>1)	All women had colposcopy + colposcopy-directed biopsy.	CIN2+ CIN3+
Bandyopadhyay 2008 USA	505	Retrospective cohort study. Timing of follow-up <4m.	Screening	Incl: women with a Pap smear result of ASC-H. Excl: women had no histologic follow-up data, had repeat cytology data only, or ECC only.	34 (14-88)	HC2 (RLU>1)	All women had colposcopy +colposcopy-directed cervical biopsy, ECC and/or cervical conization by LEEP/cold knife conization.	CIN2+
Lorenzato 2008 France	82	Prospective cohort study with cross-sectional data.	Follow-up (colposcopy clinic)	Incl: women with a Pap smear result of ASC-H .	/	HC2 (RLU>1)	All women had histologic control, no further information provided.	CIN2+
Monsonego 2008 France	31	Prospective cohort study/colposcopy clinics and pathology laboratory	Follow-up (colposcopy clinic)	Incl: women referred to colposcopy because of ASC-H.	35 (17-71)	HC2 (RLU>1) LA & HPV16/18 genotyping	All women had colposcopy+colposcopy directed biopsy, LEEP, cone biopsy and punch biopsy when needed.	CIN2+ CIN3+
Rao,2009, Australia	100	Retrospective cohort study with cross-sectional data	Follow-up (colposcopy clinic)	Incl: women referred to colposcopy because of ASC-H. Excl: previous dysplasia, lack of HPV testing and/or follow-up data, pregnant.	32 (18-69)	HC2 (N.D.)	All women had colposcopy +colposcopy-directed biopsy, LEEP or cone biopsy if necessary.	CIN2+
Castle 2010 USA	1345	Retrospective cohort study	Screening	Incl: women with a Pap smear result of ASC-H.	N.D.	HC2 (RLU>1)	All women underwent routine colposcopy and histology. If colposcopy showed a CIN2+ lesion, LEEP was performed.	CIN2+ CIN3+ CC
Samarawardana 2010 USA	34	Retrospective cohort study. Timing of follow-up:<3m.	Follow-up (colposcopy clinic)	Incl: women with a Pap smear result of ASC-H. Excl: no colposcopy, insufficient residual cytology specimens.	N.D.	P16Ink4a Multiplex PCR	All women had colposcopy+ colposcopy-directed biopsy, and/or ECC when clinically indicated. Accept negative colposcopy as having no evidence of CIN2+.	CIN2+
Sung 2010 Korea	30	Retrospective cohort study with	Screening	Incl: women referred to colposcopy because of ASC-H.	N.D.	HC2 (1 RLU) P16Ink4a	All women had colposcopy + colposcopy-directed cervical biopsies or cervical conizations.	CIN2+

Study Country	#	Study design	Population setting	Inclusion/exclusion	Mean age (range)	Triage tests (cut-off)	Gold standard verification	Outcome
		cross-sectional data						
Galliano 2011 USA	53	Retrospective cohort study with unknown follow-up time.	Sceening	Incl: women with a Pap smear result of ASC-H. Excl: no histology follow-up.	34 (18-84)	HC2 (N.D.)	All women had colposcopy + colposcopy-directed biopsy, LEEP or hysterectomy when needed.	CIN2+
Wentzensen 2012 USA	63	Prospective cohort study/cross-sectional data/	Follow-up (colposcopy clinic)	Incl: women referred to colposcopy because of previous abnormal cytology and ASC-H at study cytology. Excl: previous treatment for cervical disease, prior chemotherapy or radiation treatment for cervical neoplasia, pregnancy, HIV infection.	26 (18-67)	P16/ki67 LA & HPV16/18 genotyping	All women had colposcopy +colposcopy-directed biopsies and additional random biopsies. LEEP when needed. Histology outcome based on worst biopsy result during the colposcopy or LEEP.	CIN2+ CIN3+
Gilani 2013 USA	71	Retrospective cohort study with cross-sectional data.	Screening	Incl: women with ASC-H whose histologic follow-up were available were retrieved from the pathology database.	32.53 (18-90)	HC2 (N.D.)	All women had histology follow-up from cervical biopsies, or endocervical curretages, or cervical conizations or vaginal biopsies.	CIN2+
Koo 2013 Korea	27	Retrospective cohort study with cross-sectional data.	Screening	Incl: women with ASC-H, had both HPV genotyping and histological examination. Excl: pregnancy, HIV-infected, hysterectomy, prior treatment for CIN.	N.D. (20-65)	P16/ki-67 HPV DNA kit HPV16/18 genotyping	All women had colposcopy+ colposcopic-directed biopsies and additional random biopsies. If CIN2+, then treated with CKC or LEEP.	CIN2+ CIN3+
You 2013 China	410	Prospective cohort study	Screening	Incl: women with a Pap smear result of ASC-H.	39.7 (N.D.)	HC2 (N.D.)	All women had colposcopy+colposcopy-directed biopsy and/or ECC.	CIN2+

Study Country	#	Study design	Population setting	Inclusion/exclusion	Mean age (range)	Triage tests (cut-off)	Gold standard verification	Outcome
Lee 2014 Korea	58	Retrospective cohort study with cross-sectional data.	Screening	Incl: women with a Pap smear result of ASC-H, co-testing with hrHPV and followed by colposcopy	45.4 (15-80)	HC2 (N.D.)	All women had colposcopy+ colposcopy-directed biopsy. No further information provided in the paper.	CIN2+
Killeen 2014 USA	24	Retrospective cohort study with cross-sectional data.	Screening	Incl: women who were ASC-H with hr-HPV testing and biopsy results. Excl: age <21 yrs, prior hysterectomies, insufficient residual cytology specimens.	35.82 (21-86)	P16/ki67 Cervista	All women had colposcopy+ colposcopy-directed biopsy.	CIN2+
Pabuccu 2014 Turkey	27	Retrospective cohort study with cross-sectional data.	Screening	Incl: women with a Pap smearresult of ASC-H were retrieved from a pathology database.	42.6 (N.D.)	P16	All women had colposcopy +colposcopy-directed biopsy. No further information provided in the paper.	CIN2+
Wang 2014 China	130	Prospective cohort study with cross-sectional data.	Screening	Incl: women with a Pap smear result of ASC-H . Excl: pregnancy, previous cancer and history of immune compromise disease.	46 (25-68)	HC2 (N.D.) PAX1 methylation	All women had colposcopy +colposcopy-directed biopsy. No further information provided in the paper.	CIN2+ CIN3+ CC
Chen 2015 USA	493	Retrospective cohort study with unknown timing of follow-up.	Screening and follow-up cytology	Incl: women with a Pap smear result of ASC-H retrieved from the pathology database. Excl: concurrent diagnosis of LSIL+ASC-H, with ASC-H+AGC.	39 (18-73)	HC2 (N.D.)	All women had colposcopy +colposcopy-directed biopsy, LEEP or conization or surgical resections.	CIN2+

*median age.

Abbreviations: ASC-H, atypical squamous cells, cannot exclude HSIL; CC, cervical cancer (including invasive squamous cell carcinoma, adenocarcinoma, adenosquamocarvinooma); ECC, endocervical curettage; HC2, Hybrid Capture 2; LEEP, loop electrical excision procedure; CKC, cold knife curettage; N.D., Not Documented; PCR, polymerase chain reaction

9.5.6 S6. QUADAS-2 table

Table 9.S2 QUADAS-2 items

	Patients		Triage tests		Reference test			Flow and timing						Concerns of applicability: risk of bias		
	P1	P2	T1	T2	R1	R2	R3	F1	F2	F3	F4	F5	F6	Patient	Triage tests	Reference
Pretorius,2002	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	Low	Low	Low
Lonky,2003	Y	U	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Moderate	Low	Low
Sherman,2006	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	Low	Low	Low
Srodon,2006	Y	U	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	Moderate	Low	Low
Wu,2006	Y	N	N†	Y	U	U	Y	U	Y	Y	N	N	N	High	Low	Moderate
Owens,2007	Y	U	Y	Y	Y	U	Y	Y	Y	Y	N	N	N	Moderate	Low	Moderate
Reid-Nicholson,2007	Y	U	N†	Y	Y	U	Y	U	U	U	Y	Y	Y	Moderate	Low	Moderate
You,2007	Y	Y	Y	Y	Y	U	Y	U	Y	Y	Y	Y	Y	Low	Low	Moderate
Bandyopadhyay,2008	Y	N	Y	Y	Y	U	Y	Y	Y	Y	Y	Y	Y	High	Low	Moderate
Lorenzato,2008	Y	U	Y	Y	Y	U	Y	Y	Y	Y	Y	Y	Y	Moderate	Low	Moderate
Monsonego,2008	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Low	Low	Low
Rao,2009	Y	U	N†	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Moderate	Low	Low
Castle,2010	Y	Y	Y	Y	Y	Y	Y	U	Y	Y	N	N	N	Low	Low	Low
Samarawardana,2010	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	High	Low	Low
Sung,2010	Y	Y	Y	Y	Y	U	Y	U	Y	Y	Y	Y	Y	Low	Low	Moderate
Galliano,2011	Y	Y	N†	U	Y	U	Y	Y	N	N	Y	N	N	Low	Moderate	Moderate
Wentzensen,2012	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	Low	Low	Low
Galani,2013	Y	U	N†	Y	Y	U	Y	Y	Y	Y	N	N	N	Moderate	Low	Low
Koo,2013	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Low	Low	Low
You, 2013	Y	Y	N†	U	U	U	U	U	U	U	N	U	U	Low	Moderate	Moderate
Lee,2014	Y	Y	N†	Y	Y	U	Y	Y	Y	Y	Y	Y	Y	Low	Low	Low
Killeen,2014	Y	Y	Y	U	Y	U	Y	Y	U	Y	N	Y	Y	Low	Moderate	Moderate
Pabuccu,2014	Y	Y	Y	Y	Y	U	Y	Y	Y	Y	Y	Y	Y	Low	Low	Moderate
Wang,2014	Y	Y	Y	Y	Y	Y	Y	U	Y	Y	Y	Y	Y	Low	Low	Low
Chen,2015	Y	U‡	N†	Y	Y	U	Y	U	Y	Y	N	N	N	Moderate	Low	Moderate

Risk of bias item: P1= acceptable enrolment method; P2= inappropriate exclusions avoid, T1= pre-specified test cut-off. T2=results of the index test and the comparator are masked towards each other and both the index and comparator tests are masked towards the reference test, R1=acceptable reference test, R2=results of the reference test are masked towards the index and comparator tests. R3= incorporation bias avoided, F1=acceptable delay between triage tests and reference test, F2=partial verification avoided,F3=differential verification avoided. F4=withdrawals explained, F5=un-interpretable results reported for triage tests, F6=un-interpretable results reported for reference test. Each quality item is judged with the following: Y=fulfilled, U=unclear, N=not fulfilled.

*Women had no histologic follow-up data were not included in the final analysis.

† The cut-off for HC2 was not documented, but risk for bias was considered low.‡Eligible population is mixed with primary screening and follow-up cytology data, women were excluded if they were with concurrent diagnosis of LSIL+ASC-H or with ASC-H+AGC.

9.5.7 S7 Influence of QUADAS items

Table 9.S3 Meta-analysis of the relative sensitivity and specificity of HC2 to detect CIN2+ in studies where risk of bias was not avoided (QUADAS score=N) and studies where risk of bias was unclear (QUADAS score=U) compared to studies where risk of bias was avoided.

	Relative sensitivity (95% CI)				Relative specificity (95% CI)			
	No vs. Yes		Unclear vs. Yes		No vs. Yes		Unclear vs. Yes	
QUADAS items	Estimate	p	Estimate	p	Estimate	p	Estimate	p
P1	-	-	-	-	-	-	-	-
P2	1.08 (1.01-1.15)	0.0159	1.04 (0.98-1.11)	NS		NS	1.0 (0.85-1.16)	NS
T1	-	-	-	-	-	-	-	-
T2	-	-	1.00 (0.88-1.15)	NS	-	-	0.83 (0.65-1.06)	NS
R1	-	-	1.07 (1.02-1.12)	0.0041	-	-	0.79 (0.54-1.13)	NS
R2	-	-	1.02 (0.95-1.10)	NS	-	-	0.97 (0.80-1.19)	NS
R3	-	-	1.06 (1.01-1.13)	0.0235	-	-	0.88 (0.66-1.18)	NS
F1	-	-	1.00 (0.94-1.08)	NS	-	-	1.03 (0.86-1.24)	NS
F2	0.87 (0.71-1.07)	NS	1.05 (0.99-1.11)	NS	0.59 (0.23-1.52)	NS	0.85 (0.67-1.10)	NS
F3	0.87 (0.71-1.07))	NS	1.05 (0.99-1.11)	NS	0.59 (0.23-1.52)	NS	0.86 (0.67-1.10)	NS
F4	1.02 (0.96-1.09)	NS	-	-	0.87 (0.70-1.07)	NS	-	-
F5	0.96 (0.91-1.02)	NS	1.04 (0.98-1.11)	NS	0.75 (0.64-0.87)	0.0001	0.74 (0.60-0.91)	0.0040
F6	0.96 (0.91-1.02_	NS	1.04 (0.98-1.11)	NS	0.75 (0.64-0.87)	0.0001	0.74 (0.60-0.91)	0.0040

QUADAS items[273]: (P1) acceptable enrolment method, (P2) inappropriate exclusions avoided, (T1) pre-specified cut-off, (T2) results of screen and reference test blinded towards hrHPV test, (R1) acceptable reference test, (R2) results of hrHPV test blinded towards reference test, (R3) incorporation avoided, (F1) acceptable delay between tests, (F2) partial verification avoided, (F3) differential verification avoided, (F4) withdrawals explained, (F5) un-interpretable results reported for triage test and (F6) reference test. NS: no significant difference (95% CI of relative estimate contains unity), -: no data.

9.5.8 S8 Influence of other study characteristics

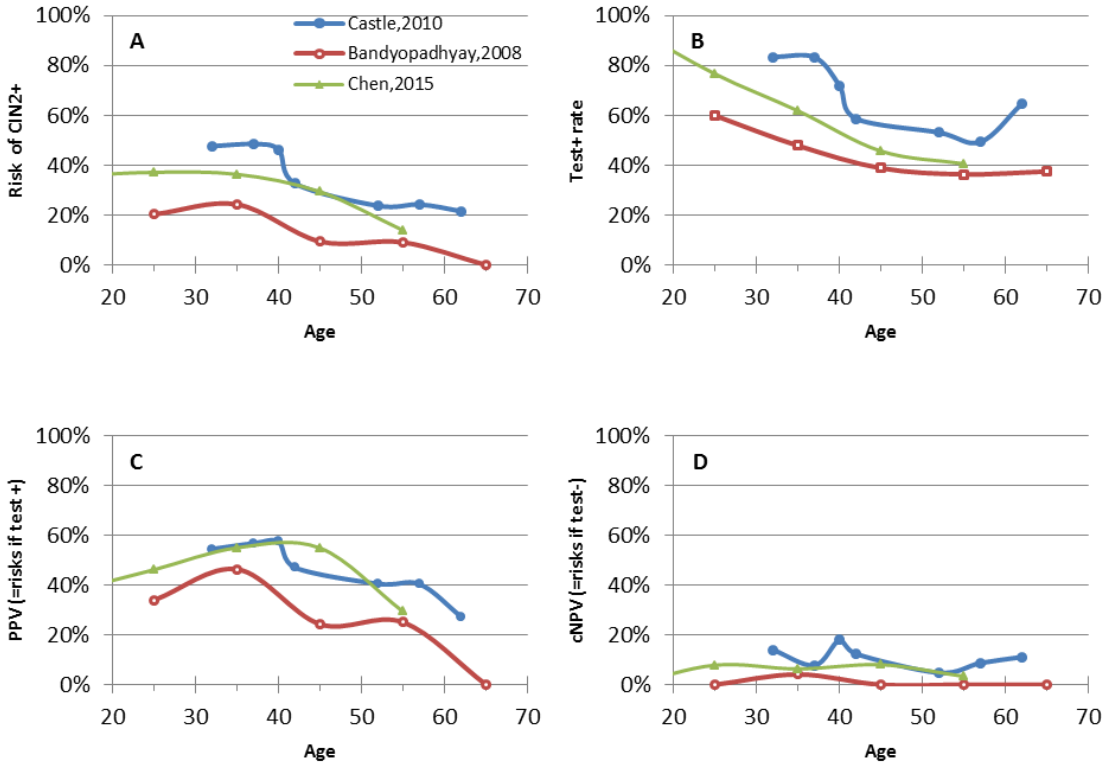
Table 9.S4 Meta-analysis of the relative sensitivity and specificity of HC2 to detect CIN2+ compared by study characteristics.

Study characteristics	Relative Sensitivity (95% CI)	P value	Relative Specificity (95% CI)	P value
Prospective vs. retrospective	1.00 (0.94-1.07)	0.90	0.90 (0.72-1.12)	0.33
Primary screening vs. referral	1.01 (0.94-1.08)	0.88	0.99 (0.78-1.26)	0.92
Small vs large study*	1.01 (0.94-1.08)	0.81	1.10 (0.90-1.34)	0.36

*Studies including less than 100 women with ASC-H case are considered as small , while studies recruiting 100 ASC-H cases of more are considered large.

9.5.9 S9 Influence of age

Figure 9.S1 Accuracy of HC2 in ASC-H triage by age, derived from 2 studies[281, 291] (Castle,2010: blue circles; Bandyopadhyay,2008: red circles; Chen, 2015: green triangles). A: prevalence of CIN2+; B: HC2 test positivity rate; C: risk of CIN2+ if HC2 is positive; D: risk of CIN2+ if HC2 is negative; E: prevalence of CIN3+; F: HC2 test positivity rate; G: risk of CIN3+ if HC2 is positive; H: risk of CIN3+ if HC2 is negative



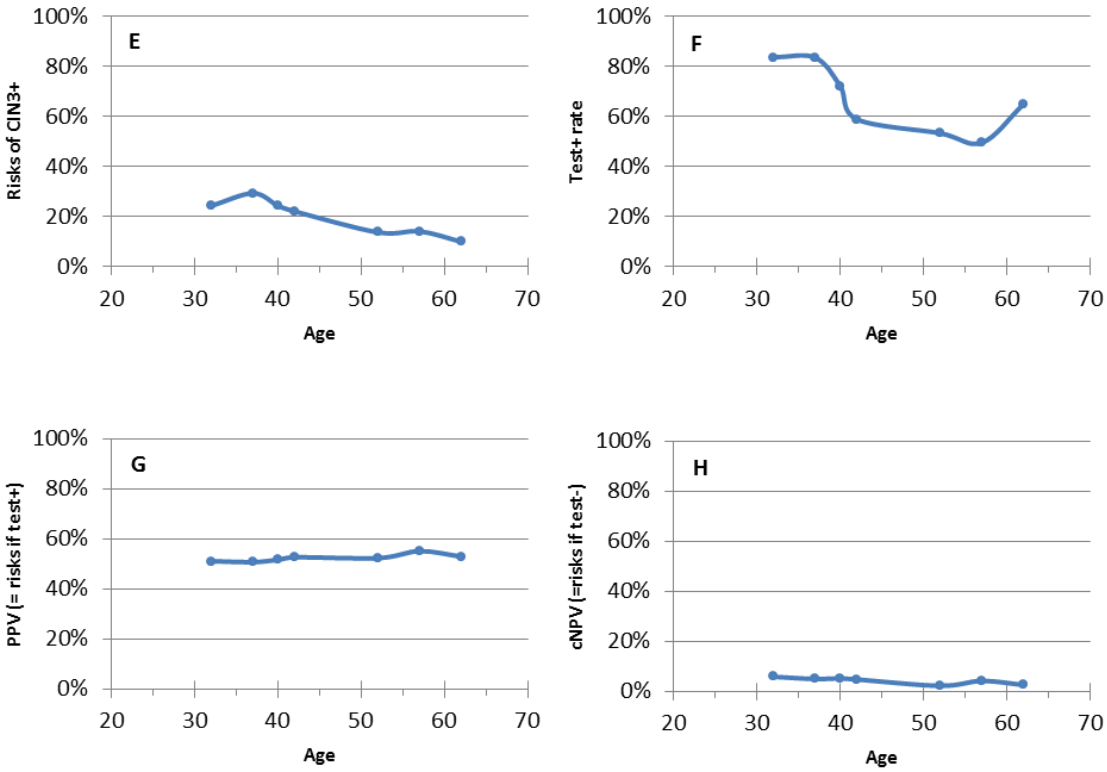

E
Risks of CIN3+
100%
80%
60%
40%
20%
0%
20
30
40
50
60
70
Age
F
Test+ rate
100%
80%
60%
40%
20%
0%
20
30
40
50
60
70
Age
G
PPV (= risks if test+)
100%
80%
60%
40%
20%
0%
20
30
40
50
60
70
Age
H
cNPV (=risks if test-)
100%
80%
60%
40%
20%
0%
20
30
40
50
60
70
Age

Table 9.S5 Pre- and post-test probabilities of CIN2+ and CIN3+ of triage with HC2 among women in different age groups with ASC-H.

Age group	Outcome	Nb of studies	Average pre-test risk,% (95% CI)	Pooled Sensitivity,% (95% CI)	Pooled specificity,% (95% CI)	PLR	NLR	Test+, % (95% CI)	Pooled post-test risk	
									if test+	if test-
									PPV,% (95% CI)	1-NPV,% (95% CI)
<50	CIN2+	3	33 (17-48)	94 (92-96)	47 (36-58)	1.77	0.13	66 (53-78)	47 (27-62)	6 (3-11)
	CIN3+	1	25 (22-28)	95 (91-97)	51 (46-56)	1.92	0.10	46 (43-49)	51 (47-56)	5 (3-9)
≥50	CIN2+	3	14 (3-26)	86 (79-92)	63 (53-73)	1.69	0.16	44 (31-57)	27 (7-45)	4 (1-7)
	CIN3+	1	13 (10-16)	89 (77-96)	82 (76-86)	4.62	0.14	21 (17-25)	53 (43-64)	3 (1-7)

PLR: positive likelihood ratio, NLR: negative likelihood ratio, PPV: negative predictive value, NPV: negative predictive value, CIN: cervical intraepithelial neoplasia.

9.5.10 S10 Risk of cervical precancer after a positive or negative screening test

Sensitivity and specificity are test characteristics that rather stable within a given spectrum of disease. Therefore, in systematic reviews and meta-analyses, sensitivity and specificity are the test measures that are pooled to synthesize knowledge on test performance. However, patients, clinicians, and decision makers defining policies for good clinical practice, are in the first place interested in the probability of disease when a test is positive (positive predictive value: PPV) and the risk of disease when a test is negative (complement of the negative predictive value: 1-NPV=cNPN). The PPV provides information on the risk of underlying pre-cancer and consequently on the efficiency of referral for further management. The inverse of the PPV (1/PPV) corresponds with the number needed to refer [colposcopy/biopsy] to find 1 case of cervical precancer. The NPV provides assurance on the safety that a women does not have (pre)-cancer and will have a very low risk to develop (pre-) by the next screening round.

Below, we computed for a plausible series of background prevalence of CIN2+ and CIN3+ (possible pretest probabilities) for women with ASC-H derived from our meta-analysis. The predictive values, computed for a given setting/area, allow decision making regarding the use of a test in this setting/area. The risk of underlying precancer or cancer (CIN2+ and/or CIN3+) should be sufficiently low to reassure women and to refer them back to the normal screening schedule. Whereas the risk of CIN2+ and/or CIN3+ should be sufficiently high if the screening test is positive (=PPV). If the PPV is not high enough a triage test is needed.

We considered the following range of background risk of cervical CIN2+:

- Low: 17%
- Intermediate: 34%
- High: 50%.

We accepted the following cutoffs for the measures of efficiency (PPV) and safety (cNPV), considering prevalent CIN2+ as targeted prevalent disease:

- PPV: >20%
- cNPV: <2%.

We considered the following range of background risk of cervical CIN3+:

- Low: 11%
- Intermediate: 20%
- High: 33%.

We accepted the following cutoffs for the measures of efficiency (PPV) and safety (cNPV), considering prevalent CIN3+ as targeted prevalent disease:

- PPV: >10%

cNPV:<1%

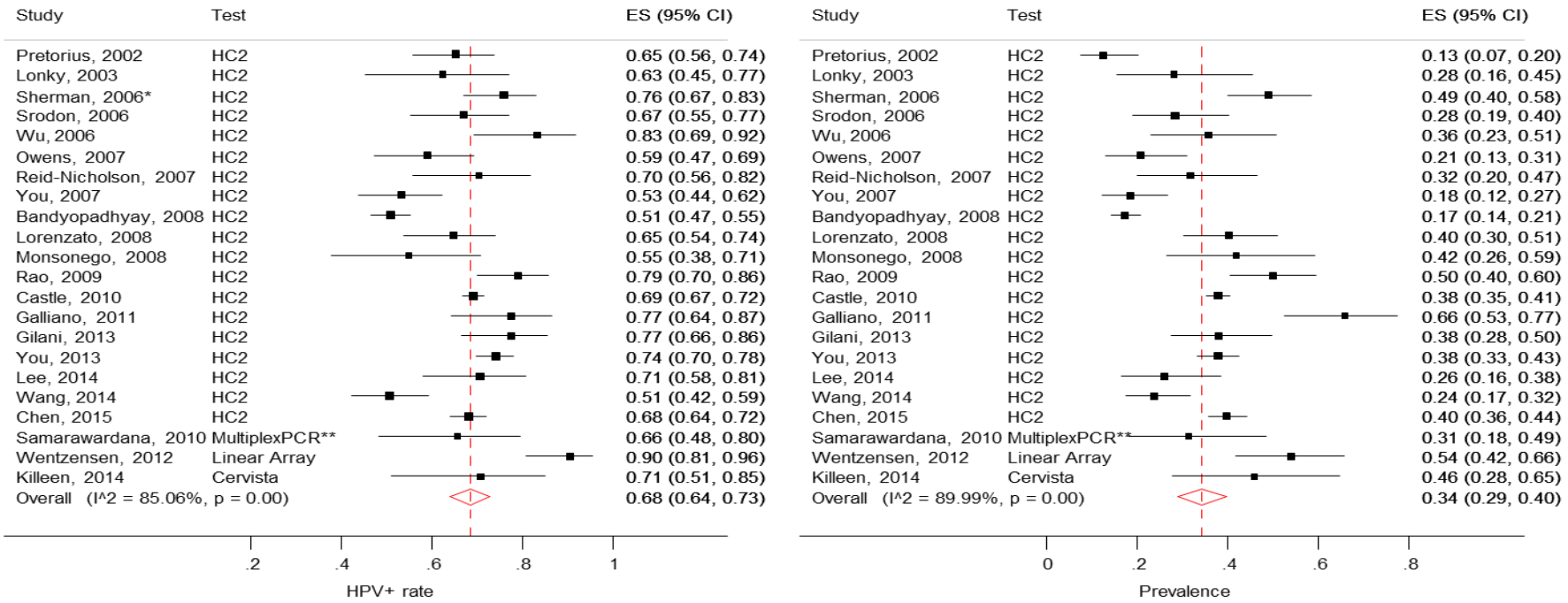
Table 9.S6 Number of true- and false positives and negatives among 1,000 women with ASC-H, post-test probabilities of CIN2+ or CIN3+ in case of a positive (PPV) or negative (1-NPV) at screening using 4 triage tests: HC2, HPV16/18 genotyping, p16 staining and methylation, applied in 3 situations of low, medium and high back-ground risk (prevalence=pre-test probabilities of CIN2+ = 17%, 34% or 50% and of CIN3+= 11%, 20% or 33%).

					At enrolment				Post-test risk	
Test		Sensitivity/ Specificity*	Prevalence	%test+	Useful referrals	Missed cases	Unnecessary referrals	True reassurance	If test+	If test-
(sample)					TP	FN	FP	TN	PPV	1-NPV
					Outcome of CIN2 or worse					
HC2	SE	93%	17%	61%	158	12	457	374	26%	3%
	SP	45%	34%	68%	316	24	363	297	47%	8%
			50%	74%	465	35	275	225	63%	14%
HPV16/18 genotyping	SE	50%	17%	30%	85	85	216	614	28%	12%
	SP	74%	34%	34%	170	170	172	488	50%	26%
			50%	38%	250	250	130	370	66%	40%
P16	SE	93%	17%	35%	158	12	191	639	45%	2%
	SP	77%	34%	47%	316	24	152	508	68%	5%
			50%	58%	465	35	115	385	80%	8%
Methylation	SE	81%	17%	18%	138	32	42	789	77%	4%
	SP	95%	34%	31%	275	65	33	627	89%	9%
			50%	43%	405	95	25	475	94%	17%
					Outcome of CIN3 or worse					
HC2	SE	91%	11%	62%	100	10	516	374	16%	3%
	SP	42%	20%	65%	182	18	464	336	28%	5%
			33%	69%	300	30	389	281	44%	10%
HPV16/18 genotyping	SE	73%	11%	32%	80	30	240	650	25%	4%
	SP	73%	20%	37%	146	54	216	584	40%	9%
			33%	42%	241	89	181	489	57%	15%
Methylation	SE	93%	11%	11%	102	8	9	881	92%	1%
	SP	99%	20%	20%	186	14	8	792	96%	2%
			33%	31%	307	23	7	663	98%	3%

* derived from the meta-analysis; TP: number true positives, FN: number of false-negatives; FP: number of false-positives; TN: number of true negatives; PPV: positive predictive value; NPV: negative predictive value; HC2: Hybrid Capture-2 assay; hrHPV: assay detecting high-risk types of human papillomavirus; ASC-US: atypical squamous cells of unspecified significance; LSIL: low-grade squamous intraepithelial lesion.

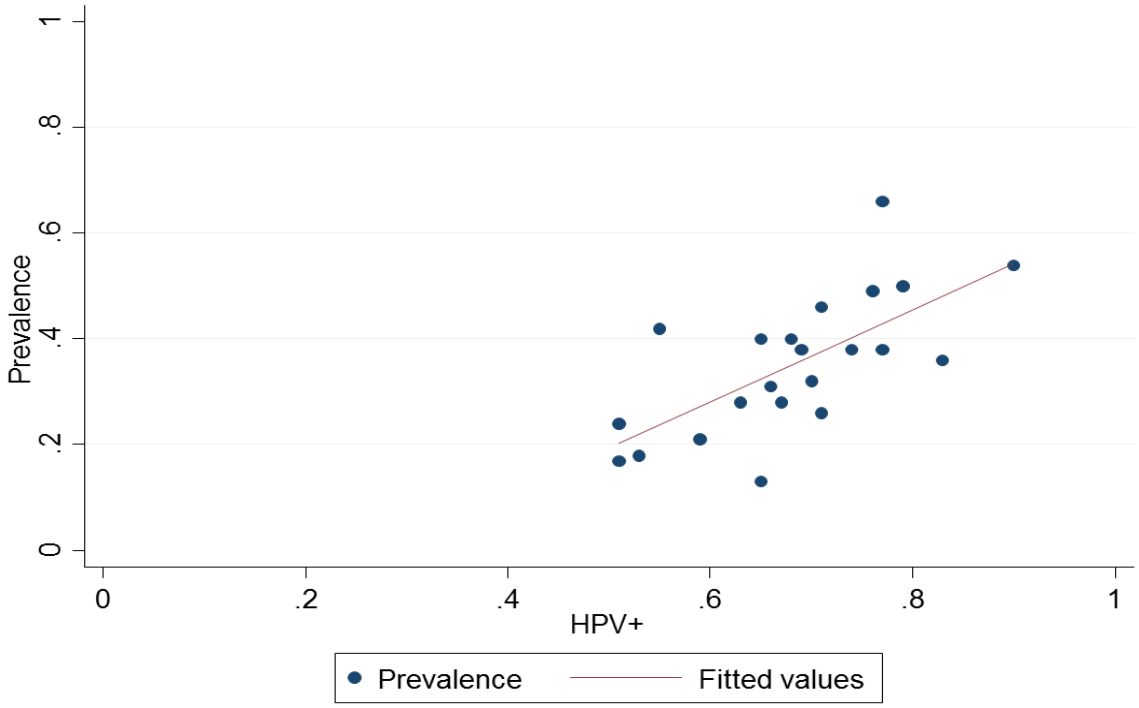
9.5.11 S11 Relation between hrHPV positivity and prevalence of underlying CIN2+ among women with ASC-H

Figure 9.S2 Meta-analysis of the underlying prevalence of CIN2+ (left) and hrHPV positivity rate (right) in women with ASC-H. The pooled values are computed with a random-effect model.



*Used data for which outcome assessment was available, the HPV+ rate is 76% here but 84% reported by the author.
**PCR using HPV multiplex primer mix detecting 16 HPV genotypes.

Figure 9.S3 Scatter plot of prevalence of underlying CIN2+ (Y) against hrHPV positive rate (X) of each included study with the regression line plotted in red.



R-squared=0.4613, $P_{f\text{-}test}$=0.0005, Slope estimate=0.5322,95% CI:0.26-0.80, p-value=0.0001

CHAPTER 10

10. General discussion and proposition for future research

In this thesis, we synthesized and extended evidence relevant in primary and secondary prevention of cervical cancer and other HPV-related gynaecological cancers by conducting high quality systematic reviews and meta-analyses. In the meantime, we also performed several clinical validation studies of HPV genotyping assays usable for primary cervical cancer screening. A relevant discussion is included in each chapter. In this chapter, we would like to present a general discussion relevant to previous chapters and propose several future research perspectives.

10.1 Other important comments on prophylactic HPV vaccines

The Cochrane review in Chapter 2, addressed safety and protection of prophylactic HPV vaccines against cervical precancer and cancer, whereas the review in Chapter 3 concerned the protection against vulvar and vaginal precancers. In addition to the main results reported in the thesis, there are several other important comments that we would also like to include in the discussion.

10.1.1 Differences in efficacy and safety between the 2vHPV and (4vHPV vaccine

Based on subgroup analysis by vaccine type we found some evidence that 2vHPV vaccine was more efficacious than the 4vHPV vaccine against any CIN2+ and CIN3+ irrespective of HPV types among women who were hrHPV DNA negative at baseline and against any CIN3+ regardless of HPV DNA status at baseline. Possible explanations for these differences include different populations with different serological status enrolled in the clinical trials or better cross-protection of the 2vHPV vaccine against other hrHPV types.

In the meantime, differences in safety between the 2vHPV vaccine and 4vHPV vaccine were observed in our review in Chapter 2. A significantly higher rate of local adverse events (e.g. pain and swelling at the injection site) for women who received the 2vHPV vaccine, which was also observed in a head-to-head trial comparing immunogenicity and safety of the 2vHPV and 4vHPV vaccines [308]. A statistically non-significant higher frequency of medically significant conditions was also noted among recipients of the 2vHPV versus the 4vHPV vaccine.

10.1.2 Efficacy of the 9vHPV vaccine

The 9vHPV vaccine contains seven hrHPV types (HPV16, HPV18, HPV31, HPV33, HPV45, HPV52 and HPV58) together with two lrHPV types (HPV6 and HPV11). The seven hrHPV types included in the 9vHPV vaccine are causally related to about 90% of all cervical cancer cases worldwide [309, 310]. The efficacy of the 9vHPV vaccine was compared to the 4vHPV vaccine in the RCT. By lack of comparison to a control group not containing HPV VLPs, the efficacy data of the 9vHPV vaccine cannot be integrated in the previous meta-analyses.

The results from the RCT with follow-up of 6 years demonstrated that the 9vHPV vaccine prevented infection and cervical lesions related to the five extra hrHPV types, HPV31, HPV33, HPV45, HPV52, HPV58 (HPV31/33/45/52/58), included in the vaccines. No statistically significant protection could be observed against the vulvar and vaginal high-grade lesions

associated with HPV31/33/45/52/58. However, given to the fact that 4vHPV vaccine offers cross-protection, the vaccine efficacy estimates presented in the RCT might be higher if a placebo without HPV particles were used in the control group. In terms of immunogenicity, the 9vHPV vaccine and 4vHPV vaccine had a similar profile with respect to the HPV types included in the 4vHPV vaccine [54, 55].

10.1.3 Post marketing surveillances of HPV vaccines

The RCTs were not designed to evaluate cervical cancer and the duration of the studies was too short to determine the effects of HPV vaccination on invasive cancer outcomes. The observation of a reduced incidence of cervical cancer and other HPV-related cancers in vaccinated cohorts will have to be obtained from country-wide population-based surveillance by linking cancer and vaccination registries [100, 311].

Effectiveness of HPV vaccines in the real world can be observed from trend analyses and linkage studies joining cervical cancer screening databases and vaccination registries. Different studies demonstrated a significant reduction in the prevalence of HPV vaccine types, cervical cytological abnormalities and CIN2+ lesions in countries where HPV vaccination has been introduced and where a considerable coverage has been achieved [157, 158, 166, 312-319]. The vaccination effects in the general population has been assessed in a meta-analysis comparing prevalence of HPV infection before and after the introduction of HPV vaccination [165]. For girls and young women aged 13 to 19 years, a statistically significant reduction of HPV16/18 infection and of infection with HPV31, HPV33 and HPV45 was observed. However, no significant differences were observed in the group of women older than 20 years. The vaccination effects increased by vaccination coverage and years since vaccination. No differences by vaccine brand were observed. These findings are in line with the findings from the randomized trials. Herd immunity was reported from surveillance studies, in which lower prevalence of HPV vaccine types in the vaccinated and non-vaccinated groups belonging to the same birth cohorts in the pre- versus post-vaccination period were observed in Scotland [158] and Australia [167].

10.1.4 Safety profile of HPV vaccines

In this thesis, all estimates of adverse effects and efficacy reported were restricted to those reported from randomised trials. Safety of HPV vaccines is evaluated at population scale in post-licensure surveillances studies from different safety monitor systems which have shown that HPV vaccines have a favourable safety profile [192, 320-325].

Up to now, no safety concerns, in pregnant women when inadvertent vaccine doses were administered, have come to light in RCTs or in post-licensure surveillance studies. WHO also concluded that inadvertent administration of HPV vaccine during pregnancy has no known adverse outcomes in either mother or infant.

10.1.5 Efficacy of fewer than three doses of HPV vaccine

Because for several trials included in the Cochrane review, results were provided for the same outcome among women being initially HPV16/18 negative and having received all three doses and at least one dose. This allowed us to compute, in a post-hoc analysis, by simple subtraction the number of events and women at risk having received only one or two doses. The Cochrane review demonstrated for the first time that less than three doses protected against precancer associated with HPV16/18 infection in young women being HPV16/18 negative at enrolment [100].

The efficacy of fewer than three doses of 2vHPV vaccines has been evaluated in post-hoc pooled analyses of two large scale clinical trials [137, 326], no significant difference were found whether two doses or one dose were administered, compared to the three-dose schedule.

10.1.6 Cost-effectiveness of adding males to female-only vaccination programmes

According to the recently released European Centre for Disease Prevention and Control (ECDC) guidance on HPV vaccination in EU countries, the cost-effectiveness of adding males to female-only vaccination programmes depends on the prior objective of the HPV vaccination programme [70]. If the priority is to prevent cervical illness in women, then adding males to current programme becomes increasingly cost-effective in presence of persistently lower vaccination coverage among females with reducing vaccine price. If the main objective is to prevent HPV-related disease, a universal/gender neutral HPV vaccination is more cost-effective [70]. However, interventions to increase vaccination coverage among girls where it is low may still be more cost-effective than adding vaccination of boys [327].

10.1.7 Implications for practices and research

The meta-analyses showed that both HPV vaccines offered excellent protection against high-grade cervical, vulvar and vaginal precancer associated with HPV16/18 in young women who were free of hrHPV or HPV16/18 infection at baseline. HPV vaccine efficacy was lower when participants with previous HPV infection were also included. For all endpoints and exposure groups, VE was lower in mid-adult women aged 24 to 45 years when data were available. In all mid-adult women, with or without HPV infection at enrolment, no protection against cervical, vulvar, or vaginal precancer was observed. The meta-analyses also showed that fewer than three doses may offer protection against HPV16/18 endpoints in young women. The RCTs were not designed to evaluate invasive cancers and the duration of the studies was too short and not powered to determine the protection against invasive cancers.

Whereas the efficacy of the 2vHPV and 4vHPV vaccines against cervical precancer associated with HPV16 or 18 is similar, protection of the 2vHPV vaccine against any cervical precancer irrespective of HPV types appears to be higher.

Although the trials were large and no safety concerns were established, long-term surveillance and registry-based research (linking vaccination databases with screening, cyto-histopathology,

cancer registries and biobanks; and linking with morbidity, mortality and birth/maternity registries) are needed to establish vaccine efficacy and safety over time. This will help also to assess type replacement, cross-protection, duration of protection associated with three or fewer doses and vaccine safety in pregnant women.

10.2 Clinical validation of HPV assays for primary screening

10.2.1 HPV genotyping assays evaluated within VALGENT framework

A systematic review was conducted in 2015 to answer the question which criteria defined in the Meijer guideline based on reproducibility and equivalent accuracy [79]. Besides the two standard comparator tests HC2 and GP5+/6+-EIA, the Cobas 4800 HPV test, the Abbott RealTime High Risk HPV test, the PapilloCheck HPV-screening test, BD Onclarity HPV assay and the HPV-Risk assay were considered as fully validated for cervical screening. Other tests which partially fulfil the Meijer guidelines are the following: Cervista HPV HR Test, GP5+/6+-LMNX, Riatoal qPCR HPV genotyping assay and MALDI-TOF.

Up to now, four VALGENT panels have been completed and generated about 20 publications evaluating the clinical and analytical performance of HPV genotyping assays [87, 199-202, 204-206, 226, 239-241, 245-247, 254, 328-331]. The list of the validated hrHPV assays fulfil criteria for use in primary cervical cancer screening maybe extended in subsequent VALGENT assessments. VALGENT has become a world standard for HPV test validation.

The clinical validity of BD Onclarity HPV assay, PapilloCheck HPV-screening test, HPV-Risk assay, Anyplex II HPV HR, Riatol qPCR and Alinity mHPV were confirmed again while being evaluated in the VALGENT framework. The Xpert HPV assay and the Linear Array assay were two newly validated hrHPV DNA assays could be added to the list of validated hrHPV DNA assays. After cutoff optimisation, the Eurroarray, Hybribio's 14 hrHPV assay and Liferiver HarmoniaHPV assay could also be added to the list.

From the systematic review work on evidence regarding application of new methods of cervical cancer prevention, conducted in Sciensano's Unit of Cancer Epidemiology, certain domains where good quality primary studies were identified. Whereas previous meta-analyses clearly demonstrate higher efficacy of cervical cancer screening with one of two particular HPV DNA assays compared to cytology, insufficient information was available regarding the possible alternative HPV assays. Therefore, criteria were formulated for HPV tests usable in primary screening and a protocol for validation of new HPV assays was developed (the VALGENT protocol). The existence of continuously updated lists of validated HPV assay facilitates decision making for health authorities involved in the set-up of new HPV-based screening programmes.

10.2.2 Cost-effectiveness of cervical cancer screening with HPV testing

The cost-effectiveness of implementing a public health intervention depends on the country. In Belgium, it has been concluded in 2015 that HPV-based screening at 5-year intervals was cost-

effective and cost-saving compared to cytology-based screening at 3-year intervals. Since costs for HPV tests continue to decrease substantially over recent years, the HPV-based screening is expected to be even more cost-effective [332].

Co-testing, is a screening strategy with two tests (microscopic examination of cervical cells and HPV testing on these cells), could be slightly more effective that HPV testing along but the level of evidence for higher effectiveness is low [333]. Co-testing is substantially more expensive than HPV testing alone and not considered as a cost-effective strategy [334].

Table 10.1 List of HPV DNA assays validated for cervical cancer screening, evaluated in the VALGENT framework.

Evaluated assay	Study	Evaluated assay		Comparator assay	Evaluated/comparator assay				Non-inferiority test*	
		Absolute			Absolute		Relative			
		sensitivity	specificity		sensitivity	specificity	sensitivity	specificity	p_{sens}	p_{spec}
PapilloCheck	Heard, 2016 [205]	96.1%	89.7%	GP5+/6+ EIA	94.1%	90.4%	1.02	0.99	0.0002	0.0970
Riatol qPCR	Benoy, 2019 [245]	96.0%	89.5%	HC2	96.0%	89.7%	1.00	1.00	0.0006	0.0069
BD Onclarity	Cuschieri, 2015 [202]	96.7%	89.6%	HC2	98.4%	89.9%	0.98	1.00	0.0245	0.0155
BD Onclarity	Bonde, 2019 [246]	92.6%	92.6%	GP5+/6+ EIA	92.6%	89.2%	1.00	1.04	<0.0001	<0.0001
HPV-Risk assay	Polman, 2018 [206]	93.7%	91.8%	HC2	96.1%	89.9%	0.98	1.02	<0.001	<0.001
HPV-Risk assay	Heideman, 2019 [329]	93.4%	92.6%	GP5+/6+ EIA	92.6%	89.1%	1.01	1.04	0.0006	<0.0001
Anyplex II HPV HR	Ostrbenk, 2018 [241]	96.9%	94.1%	HC2	95.9%	92.7%	1.01	1.01	0.001	<0.0001
Xpert HPV	Cuschieri, 2016 [203]	94.1%	90.3%	GP5+/6+ EIA	94.1%	90.3%	1.00	1.00	0.0171	0.0269
Linear Array	Xu, 2018 [226]	98.0%	94.3%	HC2	96.1%	90.1%	1.02	1.02	0.0076	<0.0001
EUROArray*	Viti, 2018 [240]	93.7%	89.9%	HC2	96.1%	90.1%	0.98	1.00	0.0076	0.0070
HBRT-H14*	Xu, 2020 [331]	94.5%	91.5%	HC2	96.1%	90.3%	0.98	1.01	0.0034	<0.0001
Liferiver Harmonia*	Bhatia, 2020 (In submission)	93.4%	89.3%	GP5+/6+ EIA	92.6%	89.2%	1.01	1.00	0.0003	0.0052

10.3 Triage of women with atypical cervical cytology

Cytology-based primary screening has been used for more than half a century and is still employed by the majority of organized screening programs worldwide. Although the future will evolve towards primary HPV-based screening, it will probably take at least a decade before it will be implemented in most countries with a cervical cancer screening programme. Therefore, during the transition period, triage of women with atypical cervical cytology remains a clinically relevant issue. In addition to the discussed points in previous chapter, there are several other important comments worth mentioning in the general discussion.

10.3.1 The usefulness of hrHPV testing in the management of women with ASC-H

In the meta-analysis in Chapter 9, we observed substantial inter-study heterogeneity in hrHPV positivity in ASC-H cases among the 25 included studies. This finding is in line with the 2012 survey of the College of American Pathologists in which a wide range of hrHPV test positivity in ASC-H patients among US laboratories was also observed [263]. Among included studies in the meta-analysis, we found that the underlying risk of CIN2+ increased significantly with hrHPV positivity. For example, the study from the University of Pittsburgh Medical Center (UPMC) reported the lowest hrHPV rate (51%) and the lowest underlying risk of CIN2+ (17%) [291]. In laboratories as UPMC that have a low threshold for ASC-H, there appears to be a benefit of hrHPV triage as it is generally the case for ASC-US [106]. However, in many other studies with higher hrHPV positivity rates in ASC-H, the triage utility is limited since hrHPV-negative women with ASC-H still need referral to colposcopy (because the risk of CIN2+ still is higher than the safety threshold of 2% [108]). We would propose also for ASC-H to consider the cyto-virological correlation as a basis for quality control to homogenize cytological interpretation among laboratories [105, 306].

10.3.2 The usefulness of Pretest-Posttest-Probabilities plot

In Chapter 8, a risk-based decision-making tool based on pretest and posttest risk for cervical precancer was used subsequently to the estimate of the pooled accuracy estimates [108]. The purpose of a triage test is to stratify risk and to facilitate decision making according to benchmarks defined by experts or local guidelines. In our case of triage of women with LSIL, CIN2+ was considered as the endpoint and the European and US benchmarks were discussed. In Figure 10.1 [108], we see that negative triage results do not bring down the risk of precancer to a sufficiently low level (<2%, green zone) allowing for a safe release to routine screening. Therefore, women with LSIL still must be kept under surveillance, in spite of negative HPV16/18. Research for further development and optimization of triage markers should be continued.

With more triage markers available and increasing request for appropriate screen & management options for different scenarios, more options will become available for cervical cancer prevention requiring tools for assessment. The pretest-posttest-probabilities plot offers an opportunity to answer questions whether a specific screening or triage option is useful in a

particular setting and allows for easy communication with experts, stakeholders and decision makers.

10.3.3 Management of women with atypical cervical cytology

In the context of cytology-based screening, a summary of recommendations for triage of women with atypical cervical cytology is presented in this section (Figure 10.2).

For the management of women with ASC-US, triage with hrHPV DNA or RNA tests is recommended. For those with positive HPV triage test, immediate colposcopy is recommended. Immediate colposcopy is also recommended if HSIL, AGC and AIS are reported [106, 335]. Recommendations for triage of women with LSIL vary among countries, some may be referred to colposcopy without triage test.

The pooled results suggest that clinical utility of genotyping with HPV16/18 in women with LSIL may be useful in a two-step triage setting. Women testing HPV16/18 positive need immediate referral to colposcopy. Depending on decision thresholds of different countries or regions: 1) women testing HPV16/18-negative but positive for other hrHPV types may be referred to colposcopy or kept under surveillance; and hrHPV-negative LSIL patients may be kept under surveillance or released to routine screening.

Due to the high risk of precancer in women with ASC-H, together with the findings in the meta-analyses in Chapter 9, the usual recommendation for referring women with ASC-H for colposcopy is remained.

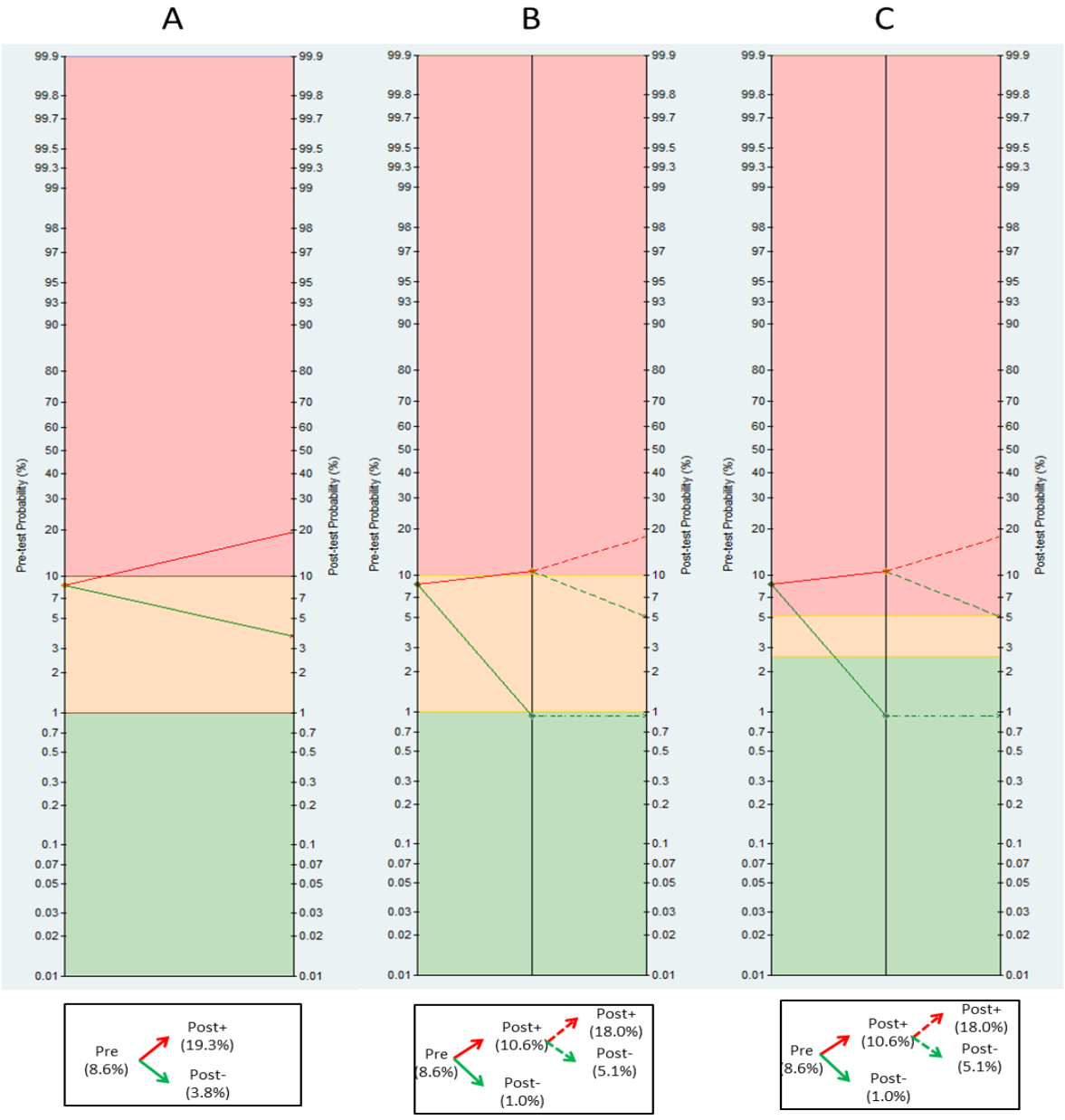


Figure 10.1 Pretest (left Y axis) and posttest probabilities (right Y axis) of CIN3+ after triage in women with LSIL using HPV16/18 genotyping as a single triage test (plot A) or using a two-step triage with hrHPV testing followed by HPV16/18 genotyping if hrHPV+ (plots B & C). Benchmarks are defined at risk levels 1% and 10%, often applied in Europe (Plots A & B), and at risk levels 2.6% and 5.2%, applied in the US (Plot C).

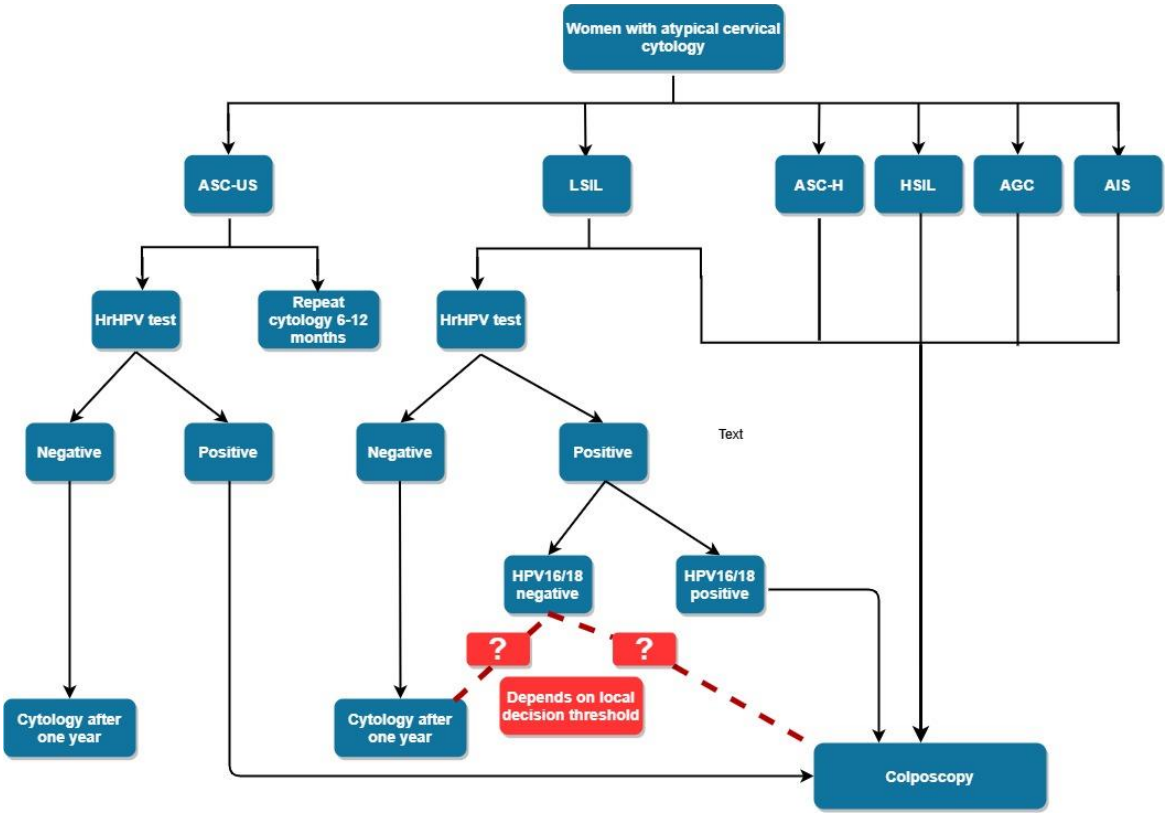


Figure 10.2 Management of women with atypical cervical cytology.

10.4 Limitation and strength

The current thesis manuscript includes the most comprehensive systematic review of the safety and efficacy of the licensed 2vHPV and 4vHPV vaccines against cervical precancer and HPV16/18 infection in adolescent girls and women. This review provides a template for reporting future results of vaccination trials according to different outcomes (HPV infections or cervical high grade lesions, either associated with infection with vaccine types or irrespective of HVP infection) for different exposure groups (absence of hrHPV, absence of the HPV types targeted by the HPV vaccines, or regardless of HPV infection at enrolment).The thesis also includes the first systematic review on efficacy of licensed HPV vaccines against vulvar and vaginal endpoints. A general limitation is related to meta-analysis of aggregated data extracted from publish studies, therefore, there is a limited number of potentially informative covariates that could be accounted for in the meta-analysis, such as age, time since sexual debut and number of sexual partners.

Through the international recognized VALGENT framework, we could keep the list the clinical validated HPV assays usable in primary cervical cancer screening updated, which may facilitate decision making of public health authorities who are considering to set up the HPV-based screening. However, we must realize that all the HPV assays have been included in VALGENT are evaluated in laboratories with high academic level. The validity of these assays in low-resources settings has not yet been assessed in VALGENT, which can be considered as a

limitation. Realizing this limitation, we have decided to setup a new panel of VALGENT-5 study, which will be mainly focused on HPV genotyping assays usable in low-resources settings.

10.5 Future perspective and propositions

10.5.1 Future vaccine development.

In 2018, then WHO Director General called for global actions towards cervical cancer elimination [336].To eliminate cervical cancer as a public health problem, which means bringing the world age-standardised incidence down to 4/100,000 women-years, countries should try to vaccinate 90% of girls before they reach the age of 15 years, screen 70% of women at least twice with a high precision tests in the age range 35-45 years and treat at least 90% of screen-detected lesions [337]. As of December 2019, 102 countries had introduced HPV vaccination into their national routine immunization programmes covering about 36% of the target population worldwide [338].

Currently, only two manufactures, MSD and GSK, produce licenced HPV vaccines. It is an issue which is addressed by the WHO as a warning that the HPV vaccine supply availability currently assured by the two manufactures cannot meet the global demand. With more countries planning the introduction of HPV vaccines, the demand-supply imbalance is forecasted to grow. Several other manufactures in China and India are developing HPV vaccines.China's first domestically made vaccine, Cecolin (Innovax, inc.), licensed by China drug regulator on January 02, 2020, is currently under review for WHO prequalificaiton. The research and approval of more HPV vaccines other than the three currently on the global market are in the pipeline. The current evidence on the safety, efficacy and effectiveness of HPV vaccines against HPV-related diseases should be updated with data from new studies.

10.5.2 Cervical cancer screening of cohorts vaccinated against HPV

The first birth cohorts of women vaccinated against HPV are entering the target age range for cervical cancer screening. Recent research suggests that in a (partially) vaccinated population, less intensive screening programmes (a later start age, longer time interval and less invasive primary test) may provide similar or higher benefits at lower cost (and lower harm as measured by the colposcopy rate) than maintaining current screening guidelines [339, 340]. However, Kim et al. [341] warn that a one-size-fit-all screening policy that aims to target the average risk profile in a population may lead to inefficiencies and loss of health benefits. Therefore, it is essential to assess the real impact of a less intensive screening programme for the unvaccinated population.

It is believed that the implementation of primary HPV screening [339, 342], together with the development of new technologies for triage [343], will reform the general approach to the prevention of HPV-related disease over the coming years [344]. Future research to update evidence and surveillance of early effects in countries implementing HPV-based screening in particular among young HPV-vaccinated cohorts will orient future guidance on how to screen vaccinated women.

10.5.3 Future assessment of new test assays

In the era of HPV-based screening and the magnitude of available HPV tests on the market, it is essential to keep the list of clinical validated tests eligible for primary screening regularly updated. The increased availability of bio-specimens archived in biobanks, including also cervical specimens used for screening, generates enormous possibilities for biobank-based research. Well preserved cervical cell archives can be used for the clinical evaluation and validation of new assays and biomarkers potentially usable for screening or triage. The linkage with pathology registries capturing the outcome of screening, triage and treatment enable efficient prospective study designs making studies shorter and cheaper. This research framework will facilitate and shorten the validation of new diagnostic methods needed for for people from middle or low-resources regions. This can help to tackle the ultimate goal of cervical cancer elimination as well. To this end, VALGENT framework is a good example of public-private scientific collaboration in which strict legal regulations were stated and the independence of the scientific work is guaranteed.

10.5.4 HPV testing on self-samples

Meta-analyses have shown that hrHPV testing using a PCR-based assay has similar accuracy for detecting CIN2+ and CIN3+ on self-collected and on clinician samples. Offering self-sampling kits is generally more efficient in reaching non-screened women than sending invitations [67, 70]. However, the influence of the devices used for self-sampling was not able to evaluate in the meta-analyses. Therefore, the VALHUDES (VALidation of Human papillomavirus assays and collection DEvices for HPV testing on Self-samples and urine samples) protocol was initiated with the purpose to evaluate and compare the clinical accuracy of clinically validated PCR-based assays on vaginal self-samples collected with various devices and on urine samples [345].

Currently, the first VALHUDES has started in Belgium and is in the process of patients' enrolment. Six assays will be evaluated: RealTime High Risk HPV assay, Cobas-4800 and -6800, Onclarity, Xpert HPV and Anyplex II HPV HR. Statistical analyses will be performed at the Unit of Cancer Epidemiology, Belgian Cancer Center, findings will be published subsequently.

10.5.5 Risk-based screening and assessment of screen-positive women

Screening for cervical cancer with a "one-size-fits-all" protocol has been implemented in many countries worldwide. These screening programs only provide suboptimal protection against cancer for women at high risk, lead to suboptimal allocation of resources and substantial screening- and treatment-related harms. Therefore, there is an urgent need for optimisation of cervical cancer screening programs by risk stratification, in particular because cervical cancer is on the rise in several countries, the uptake of screening remains moderate in subpopulations at high risk and costs related to screening are high. Cervical cancer is well suited for development of a risk-stratified screening program since cervical screening has a substantial effect at the population level and many risk factors are strongly predictive. Data on many strong

risk factors are already on file with the screening programs and could readily be used for optimization of programs by risk stratification.

A Horizon 2020 project, RISCC, has started since January 1 2020. In this project, risk-based screening methods will be developed for the prevention of cervical cancer. With modern digital applications, risk-based screening profiles will be seamlessly embedded into screening algorithms and in that way enhance the effectiveness and efficiency of cervical cancer screening. This also includes offering HPV-self sampling to populations at high risk and with low participation in screening programs.

10.5.6 HPV-negative cancers

Recent report highlights the existence of HPV-negative cancers, that had a negative HPV test result preceding the cancer diagnosis [346]. This observation has generated concerns regarding safety of screening based on HPV testing along and claims for co-testing (screening with two tests: cervical cytology and a hrHPV assay).

Currently, there are several hypotheses which may explain the HPV-negative fraction of cervical cancers. The first one is that the majority of HPV- cervical cancers may not originate from squamous or glandular epithelium of the cervix uteri. The possible mechanisms or technical issues explaining existence of HPV-negative cervical cancers originating from squamous/glandular epithelium of the cervix may be due to poor quality or very small amount of tumour cells in the tissue specimen may jeopardize adequate polymerase chain reaction (PCR) amplification; or the integration of viral DNA in the human genome leading to the loss of viral genes including those commonly targeted by conventional hrHPV tests. It is also possible that carcinogenesis may be driven by a very low viral load lower than the analytical cut-off of hrHPV tests.

There are also hypotheses about that cervical cancers may be caused by other HPV types than those currently considered as hrHPV types. Immunochemistry markers, messenger RNA (mRNA), miRNA and methylation reflect better the progression towards malignant transformation of HPV infected cells than cyto-histopathology. Absence of certain markers may support the assumption of carcinogenesis not induced by HPV. Next Generation Sequencing (NGS) analysis might provide more accurate characterisation than histopathology (in terms of prognosis and may reveal alternative therapeutic choices, in particular for HPV-negative cancers).

Future research projects are needed in order to evaluate the above-mentioned hypotheses, research to quantify the burden of HPV-negative cervical cancers is also essential.

Summary

Persistent infection with high-risk human papillomavirus (hrHPV) is a prerequisite for cancer of the cervix, and a proportion of cancers of the vagina, vulvar, anus, penis, and oropharynx as well of precursor lesions of all these cancers. This finding has led to the development of prophylactic HPV vaccines and HPV DNA or RNA tests. The main purpose of this thesis is to synthesize evidence relevant in primary and secondary prevention of cervical cancer and other HPV-related cancers, to keep current evidence in the field updated and extended with new technologies and strategies.

Chapter 1 provides a general introduction on HPV and HPV-mediated carcinogenesis, HPV prevalence in different anogenital sites and the burden of HPV-related anogenital cancers, as well as an explanation on HPV-related cancers prevention strategies. Outline of the thesis is also presented in the first chapter.

The first part of this thesis focuses on the evidence on efficacy and safety of prophylactic HPV vaccines derived from randomized controlled trials.

Chapter 2 evaluates the harms and protection of prophylactic HPV vaccines against cervical precancer and cancer, where the review in chapter 3 concerns the protection of HPV vaccines against vulvar and vaginal precancers. Results from the meta-analyses in chapter 2 and chapter 3 show that HPV vaccines offered excellent protection against high-grade cervical, vulvar and vaginal lesions associated with HPV16/18 in young women who were free of hrHPV or HPV16/18 infection at baseline. HPV vaccine efficacy was lower when participants with previous HPV infection were also included. For all endpoints and exposure groups with available, vaccine efficacy was lower in mid-adult women aged 24 to 45 years. In all mid-adult women, with or without HPV infection at enrolment, no protection against cervical, vulvar or vaginal precancer was observed.

High-quality screening programs are available for cervical cancer at the level of secondary prevention only, therefore, the second part and the third part of this thesis focus on cervical cancer screening strategies in HPV-based and cytology-based scenarios, respectively. Only clinically validated HPV tests should be used in HPV-based primary screening setting. A substantial number of HPV assays is available; however, the clinical performance of these assays differs significantly and few of them have been clinically validated for use. Finding appropriate test specimen representative for primary screening is essential. The VALGENT framework facilitates the comparison and validation of HPV genotyping assays for clinically relevant outcomes using sample from population appropriate for primary cervical cancer screening.

Chapter 4 evaluates the clinical performance of the Roche Linear Array HPV genotyping test (Linear Array) in comparison to the standard comparator test (Hybrid Capture 2 [HC2]) using the VALGENT-3 framework. Results show that Linear Array has similar sensitivities with higher

specificity to detect cervical intraepithelial neoplasia grade 2 or worse (CIN2+) compared to HC2. Consequently, according to international validation criteria, detection of 13 hrHPV types with Linear Array can be considered clinically validated for use in primary screening purposes.

Also using the VALGENT-3 sample collation, in chapter 5, the INNO-LiPA HPV Genotyping Extra II assay (INNO-LiPA) was evaluated and verified whether it fulfills the minimal requirements for use in primary screening. Type-specific concordance is compared between the INNO-LiPA and Linear Array. Results show that INNO-LiPA demonstrated non-inferior clinical sensitivity, but lower specificity compared to HC2 in additional to excellent concordance compared to Linear Array for hrHPV and some genotypes.

RIATOL qPCR HPV genotyping assay (RIATOL qPCR) is a laboratory developed assay being evaluated within the VALGENT-3 framework. The clinical performance of RIATOL qPCR was assessed in chapter 5 and the optimal clinical viral concentration cut-offs was identified assuring RIATOL qPCR fulfills the required accuracy performance criteria for primary cervical cancer screening.

Chapter 6 evaluates the clinical accuracy of the Hybribio's 14 High-risk HPV with 16/18 Genotyping Realtime PCR (HBRT-H14) assay relative to HC2. Results show that at the predefined cut-off, HBRT-H14 is as sensitive but less specific than HC2 for detection cervical precancer. However, the clinical performance of HBRT-H14 was optimized by comparing to the previously clinically validated HPV genotyping assay of Linear Array and Cobas 4800 HPV test. HBRT-H14 fulfils the international accuracy criteria for cervical cancer screening when using the optimized cuf-off.

In the context of cytology-based screening, the management of patients with an abnormal cytological lesion depends on the severity of the lesion and its underlying future risk of high-grade CIN and cancer.

Chapter 8 updates the current evidence on the usefulness of genotyping for HPV16 and HPV18 (HPV16/18) as a triage marker for the management of women with low-grade squamous intraepithelial lesion (LSIL) with accuracy data obtained from the four panels of the VALGENT framework. Results of the pooled analyses show that triage of women with LSIL with HPV16/18 genotyping increases the positive predictive value compared to hrHPV testing but at the expense of lower sensitivity. Women testing positive for HPV16/18 need further clinical work-up. Whether colposcopy referral or further surveillance is recommended for women with other hrHP types may depend on the post-test risk of precancer and the local risk-based decision thresholds.

In chapter 9, a meta-analysis was conducted to assess the accuracy of hrHPV testing and other molecular markers for triaging women with a cytological result of atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion (ASC-H) to predict the presence or development of cervical precancer. Consequently, due to the high probability of precancer with ASC-H, the utility of triage is limited. The usual recommendation for referring women with ASC-

H for colposcopy is not altered by a positive triage test, whatever test is used. A negative hrHPV DNA or $p16^{INK4a}$ test may allow repeat testing, but this recommendation will depend on local risk-based decision thresholds.

Chapter 10 concludes with a general discussion. In this discussion, we provide comments on prophylactic HPV vaccine and summarize the effectiveness and safety profile of HPV vaccines from post marketing surveillances. We also discuss several essential points in both HPV-based and cytology-based primary cervical cancer screening settings. Furthermore, in this thesis, we present perspectives and propositions for future research of primary and secondary prevention of HPV-related diseases strategies.

Sammenvatting

Persisterende infectie met hoog-risico humaan papillomavirus (hrHPV) is een voorwaarde voor bijna alle kankers van de baarmoederhals, en een deel van de vagina, vulva, anus, penis en orofarynx alsook van de voorloper stadia. Deze bevinding heeft geleid tot de ontwikkeling van profylactische HPV-vaccins en HPV-DNA- of RNA-tests. Het belangrijkste doel van dit proefschrift is om bevindingen te synthetiseren die relevant zijn voor de primaire en secundaire preventie van baarmoederhalskanker en andere HPV-gerelateerde gynaecologische kankers, om de huidige kennis in dit gebied up-to-date te houden en uit te breiden met nieuwe technologieën en strategieën.

Hoofdstuk 1 geeft een algemene inleiding over HPV en HPV-geïnduceerde carcinogenese, HPV-prevalentie in de verschillende anogenitale locaties en de belasting van HPV-gerelateerde anogenitale kankers, evenals een uitleg over HPV-gerelateerde strategieën om de ontwikkeling van kanker te voorkomen. Het overzicht van het proefschrift wordt ook gepresenteerd in het eerste hoofdstuk.

Het eerste deel van dit proefschrift richt zich op het bewijs over de werkzaamheid en veiligheid van profylactische HPV-vaccins afkomstig uit gerandomiseerde gecontroleerde onderzoeken.

Hoofdstuk 2 beschrijft de nadelen en bescherming van profylactische HPV-vaccins tegen cervicale (voorstadia van) kanker, terwijl de review in hoofdstuk 3 betrekking heeft op de bescherming die HPV-vaccins bieden tegen vulvaire en vaginale (voorstadia van) kanker. Resultaten van de meta-analyses in hoofdstuk 2 en hoofdstuk 3 laten zien dat HPV-vaccins uitstekende bescherming bieden tegen hooggradige cervicale, vulvaire en vaginale laesies geassocieerd met HPV16 / 18 bij jonge vrouwen die bij vaccinatie vrij waren van hrHPV- of HPV16 / 18-infectie. De werkzaamheid van het HPV-vaccin was lager wanneer deelnemers met een eerdere HPV-infectie ook werden geïncludeerd. Voor alle eindpunten en blootstellingsgroepen, waarvoor data beschikbaar waren was de werkzaamheid van het vaccin lager bij vrouwen van 24 tot 45 jaar. Wanneer gekeken werd naar vrouwen van 24 jaar of ouder, met of zonder HPV-infectie bij inclusie, werd geen bescherming tegen cervicale, vulvaire of vaginale prekanker waargenomen.

Voor baarmoederhalskanker zijn enkel op het niveau van secundaire preventie kwalitatief hoogwaardige screeningprogramma's beschikbaar, daarom richt het tweede deel en het derde deel van dit proefschrift zich op respectievelijk HPV-gebaseerde en cytologie-gebaseerde screeningsstrategieën voor baarmoederhalskanker.

In een op HPV gebaseerde primaire screening mogen enkel klinisch gevalideerde HPV-tests worden gebruikt. Er is een aanzienlijk aantal HPV-testen beschikbaar, maar de klinische prestaties van deze testen verschillen aanzienlijk en slechts enkele ervan zijn klinisch gevalideerd voor gebruik. Het vinden van geschikt testmateriaal dat representatief is voor de primaire screening is essentieel. Het VALGENT-netwerk (Validation of HPV GENotyping Tests) vergemakkelijkt de vergelijking en validatie van HPV-genotyperingstests op vlak van klinisch

relevante resultaten en dit met behulp van een steekproef van een populatie geschikt voor primaire screening op baarmoederhalskanker.

Hoofdstuk 4 evalueert de klinische prestatie van de Roche Linear Array HPV-genotyperingstest (Linear Array) in vergelijking met de standaard comparator test (Hybrid Capture 2 [HC2]) met behulp van het VALGENT-3-netwerk. Resultaten tonen aan dat Linear Array een vergelijkbare gevoeligheid heeft met een hogere specificiteit om cervicale intra-epitheliale neoplasie graad 2 of slechter (CIN2 +) te detecteren in vergelijking met HC2. Voor gebruik bij primaire screeningdoeleinden kan de detectie van 13 hrHPV-types met Linear Array als klinisch gevalideerd worden beschouwd, en dit volgens internationale validatiecriteria.

In hoofdstuk 5 werd, eveneens aan de hand van de VALGENT-3-stalen, de INNO-LiPA HPV Genotyping Extra II assay (INNO-LiPA) geëvalueerd en geverifieerd of deze voldoet aan de minimale vereisten voor gebruik bij primaire screening. Type-specifieke concordantie werd vergeleken tussen de INNO-LiPA en Linear Array. Resultaten tonen aan dat INNO-LiPA een niet-inferieure klinische sensitiviteit vertoonde, maar een lagere specificiteit vergeleken met HC2, dit naast uitstekende concordantie vergeleken met Linear Array voor hrHPV en sommige genotypen.

De RIATOL qPCR HPV-genotyperingstest (RIATOL qPCR) is een in het laboratorium ontwikkelde test die werd geëvalueerd binnen het VALGENT-3-netwerk. De klinische validiteit van RIATOL qPCR werd beoordeeld in hoofdstuk 5 waarbij de optimale klinische virale concentratie-cut-offs werden geïdentificeerd en werd verzekerd dat RIATOL qPCR voldoet aan de vereiste validatiecriteria voor primaire screening op baarmoederhalskanker.

Hoofdstuk 6 evalueert de klinische nauwkeurigheid van de Hybribio's 14 hoog-risico HPV test met 16/18 Genotyping Realtime PCR (HBRT-H14) in vergelijking met HC2. De resultaten laten zien dat HBRT-H14 bij de vooraf gedefinieerde grens even gevoelig maar minder specifiek is dan HC2 voor detectie van cervicale pre-kanker. De klinische validiteit van HBRT-H14 werd echter geoptimaliseerd door vergelijking met de eerder klinisch gevalideerde HPV-genotyperingstest van Linear Array en Cobas 4800 HPV-test. HBRT-H14 voldoet aan de internationale nauwkeurigheidscriteria voor screening op baarmoederhalskanker bij gebruik van de geoptimaliseerde cuf-off.

Ook aan de hand van de VALGENT-3-steekproefverzameling, in hoofdstuk 5, werd de INNO-LiPA HPV Genotyping Extra II test (INNO-LiPA) geëvalueerd en geverifieerd of deze voldoet aan de minimale vereisten voor gebruik bij primaire screening. Type-specifieke concordantie werd vergeleken tussen de INNO-LiPA en Linear Array. Resultaten tonen aan dat INNO-LiPA niet-inferieure klinische gevoeligheid vertoonde, maar een lagere specificiteit vergeleken met HC2 naast uitstekende concordantie vergeleken met Linear Array voor hrHPV en sommige genotypen.

In de context van op cytologie gebaseerde screening hangt de behandeling van patiënten met een abnormale cytologische laesie af van de ernst van de laesie en het onderliggende toekomstige risico op de ontwikkeling van hooggradige CIN en kanker.

Hoofdstuk 8 actualiseertde huidige evidentie over het nut van genotypering voor HPV16 en HPV18 (HPV16 / 18) als een triage marker voor de behandeling van vrouwen met laaggradige squameuze intra-epitheliale laesie (LSIL), dit met nauwkeurigheidsgegevens verkregen uit de vier VALGENT projecten. Resultaten van de gepoolde analyses laten zien dat triage van vrouwen met LSIL met HPV16 / 18-genotypering de positief voorspellende waarde verhoogt in vergelijking met hrHPV-testen, maar ten koste gaat van een lagere gevoeligheid. Vrouwen die positief testen op HPV16 / 18 hebben nood aan verdere diagnostische opvolging. Of vrouwen met andere hrHPV-typen ook moeten opgevolgd worden hangt af van het posttestrisico op pr-kanker en de lokale beslissingsdrempels.

In hoofdstuk 9 werd een meta-analyse uitgevoerd rond de nauwkeurigheid van hrHPV-testen en andere moleculaire merkers in de triage van vrouwen met een cytologisch resultaat van ASC-H om de aanwezigheid of ontwikkeling van cervicale pre-kanker te voorspellen. Omwille van de grote kans op pre-kanker met ASC-H, is de bruikbaarheid van triage beperkt. De gebruikelijke aanbeveling voor het verwijzen van vrouwen met ASC-H voor colposcopie wordt immers niet gewijzigd door een positieve triagetest. Een negatieve hrHPV-DNA- of p16INK4a-test kan kan conservatief opgevolgd worden, maar deze aanbeveling is afhankelijk van lokale op risico gebaseerde beslissingsdrempels.

In hoofdstuk 10 volgt een algemene discussie. In deze discussie geven we commentaar op profylactisch HPV-vaccins en vatten we de effectiviteit en het veiligheidsprofiel van HPV-vaccins samen uit postmarketing studies. We bespreken ook verschillende essentiële punten in zowel op HPV gebaseerde als op cytologie gebaseerde primaire screenings settings voor baarmoederhalskanker. Verder presenteren we in dit proefschrift perspectieven en aanbevelingen voor toekomstig onderzoek naar de primaire en secundaire preventie van HPV-gerelateerde ziektestrategieën.

Acknowledgement

This thesis becomes a reality with the kind support and help of many people. I would like to extend my sincere thanks to all of them.

First and foremost, I thank my supervisors, Dr Marc Arbyn and Professor Steven Weyers, for providing me the opportunity to work on this PhD project.

Marc, thank you for entrusting me with the research position in your team, for your continuous support of me, for your patience, motivation, enthusiasm, and immerse knowledge. I have learned so much with you, both made me go further professionally and personally. I could not have imagined having a better supervisor and mentor for my PhD journey.

Steven, I am sincerely grateful for your valuable guidance and constructive advices to get this thesis done in time. Many thanks for your warm support and always being there no matter how busy your life was, making my life at Ugent so much easier.

For the last five years, I had the honour to work with so many experts from the HPV society. I am grateful for the hard work of my co-authors and their valuable contribution to improve the quality of the studies presented in this thesis. Thanks, Professor Mario Poljak, Dr Anja Oštrbenk, Dr Kate Cuschieri, Dr Ramja Bhatia, Dr Jesper Bonde, Dr Ditte Moller Ejegod, Dr Danielle Heideman, Dr Ina Benoy and Professor Elizaveta Padalko, I hope to be able to work with you again in the future.

This thesis has been critically assessed and approved by an outstanding examination: Professor Oliver Degomme, Professor Philippe Tummers, Professor Koen Van de Vijver, Professor Wei Hong Zhang, Professor Davy Van den Broeck, Professor Gilbert Donders and Dr Johan Van der Heyden. I am grateful for your valuable comments and suggestions, especially for making your time to participate in my internal defense during the summer holidays.

I would like to thank my directors, Professor Herman Van Oyen, Dr Viviane Van Casteren and Dr Marc van den Bulcke, for the opportunity to work at the Sciensano and their encouragement for me to engage in this PhD adventure. I would like to acknowledge my colleagues at the Cancer Centre: Régine, Pauline, Katrien, Laure, Chloé, Aline, Els, Gordana, Wannes and Els D. and Marie-Jo, for the nice work environment and those lovely team building activities; for my colleagues in the HPV team: Vicky, Eliana, Cindy, Joris, Hélène and Iman, I am so happy to have the opportunity to work with you, independently and collaboratively. I learned so much from you, and I enjoyed our professional discussions and lovely chats after work a lot 😊

Special thanks to my dearest friends in Belgium: Yan, Dandi, Momo, Wei, Lan, Jing, Yin, Qiqi and all the other members in our big Gent family, I am grateful for our friendship, for your support, and all the time that we spent together; To Eliana, Suchsia, Remila, Vicky and Renata, for me you are not only colleagues but also friends who are always being positive and supportive, I will

always remember our lovely lunch dates; To Rui and Peishuo, I am deeply grateful for your help during my pregnancy while my husband is in China, that means a lot to our family; Also to Yaxin, thank you for keeping me accompanied while I was alone, for taking care of my dog Lucky while I was away and for all of our inspiring discussions. To all my friends, I wish you all the best in the future!

Most importantly, I would love to give my sincere thanks to my lovely family who had a fundamental role in getting me through the PhD journey successfully: Mom and Dad, you are the best parents in the world, I am deeply indebted for your unconditional love and support, there is no way to thank you for everything you have done for me; To my baby boy, thank you for coming into my life at the perfect time and accompanying mama to finalize this journey; Zhisheng, my husband, thanks for your love, encouragement and confidence in me, you give me hope and strength to continue with the hard work. I could not have done this PhD project without you.我爱你们😊

List of Publications

1. **Xu L**, Ostrebenk A, Poljak M, Arbyn M. Evaluation and optimization of the clinical accuracy of Hybribio's 14 High-risk HPV with 16/18 Genotyping assay within the VALGENT-3 framework. Journal of Clinical Microbiology 2020; 58(6): e00234-20. Published 2020 May 26.
2. **Xu L**, Selk A, Garland SM, Bogliatto F, Kyrgiou M, Weyers S, Arbyn M. Prophylactic vaccination against human papillomaviruses to prevent vulval and vaginal cancer and their precursors. Expert Review of Vaccines 2019;18(11):1157-1166.
3. **Xu L**, Benoy I, Cuschieri K, Poljak M, Bonde J and Arbyn M. Accuracy of genotyping for HPV16 and 18 to triage women with low-grade squamous intraepithelial lesions: a pooled analysis of VALGENT studies. Expert Review of Molecular Diagnostics 2019; 19(6): 543-51.
4. Benoy I*, **Xu L***, Vanden Broeck D, Poljak M, Oštrbenk Valenčak A, Arbyn M, Bogers J. Using the VALGENT-3 framework to assess the clinical and analytical performance of the RIATOL qPCR HPV genotyping assay 2019. Journal of Clinical Virology 120:57–62.

   ***Joint first authors**
5. **Xu L**, Padalko E, Ostrebenk A, Poljak M, Arbyn M. Clinical evaluation of INNO-LiPA HPV Genotyping EXTRA II assay using the VALGENT framework. International Journal of Molecular Sciences 2018;19(9).pii:E2704.
6. Arbyn M*, **Xu L***, Simons C, Martin-Hirsch PPL. Prophylactic vaccination against human papillomaviruses to prevent cervical cancer and its precursors. Cochrane Database of Systematic Reviews 2018, Issue 5. Art. No.: CD009069.

   ***Joint first authors**
7. **Xu L**, Ostrebenk A, Poljak M, Arbyn M. Assessment of the Roche Linear Array HPV Genotyping test within the VALGENT framework. Journal of Clinal Virology 2018; 98:37-42.
8. **Xu L**, Verdoodt F, Wentzensen N, Bergeron C, Arbyn M. Triage of ASC-H: A meta-analysis of the accuracy of high-risk HPV testing and other markers to detect cervical precancer. Cancer Cytopathology 2016;124: 261-72.
9. **Xu L**, Ostrebenk A, Poljak M, Arbyn M. Assessment of the Abbott ALINITY m HR HPV ASSAY within the VALGENT-3 framework. [To be submitted]

10. Ejegod DM, Hansen M, **Xu L**, et al. Clinical validation of the Cobas 4800 HPV assay using cervical samples in SurePath medium under the VALGENT4 framework. Journal of Clinical Virology. 2020;128:104336.
11. Bonde JH, Pedersen H, Quint W, **Xu L**, Arbyn M, Ejegod DM. Clinical and analytical performance of the BD Onclarity HPV Assay with SurePath screening samples from the Danish cervical screening program using the VALGENT framework. Journal of Clinical Microbiology 2020; 58(2):e01518-19.
12. Heideman DAM, **Xu L**, Hesselink AT, Doorn S, Ejegod DM, Pedersen H, Quint WGV, Bonde J, Arbyn M. Clinical performance of the HPV-Risk assay on cervical samples in SurePath medium using the VALGENT-4 panel. Journal of Clinical Virology 2019 121:104201–1.
13. Oštrbenk A, **Xu L**, Arbyn M, Poljak M. Clinical and Analytical Evaluation of the Anyplex II HPV HR Detection Assay within the VALGENT-3 Framework. Journal of Clinical Microbiology 2018; 56.
14. Viti J, Poljak M, Oštrbenk A, Bhatia R, Alcañiz Boada E, Cornall AM, Cuschieri K, Garland S, **Xu L**, Arbyn M. Validation of EUROArray HPV test using the VALGENT framework. Journal of Clinical Virology 2018;108:38–42.
15. Bonde J, Ejegod DM, Cuschieri K, Dillner J, Heideman DAM, Quint W, Pavon Ribas MA, Padalko E, Christiansen IK, **Xu L**, Arbyn M. The Valgent4 protocol: Robust analytical and clinical validation of 11 HPV assays with genotyping on cervical samples collected in SurePath medium. Journal of Clinical Virology 2018; 108:64–71.
16. Arbyn M and **Xu L**, Efficacy and safety of prophylactic HPV vaccines. A Cochrane review of randomised trials. Expert Revierw of Vaccines. 17 (12):1085-1091, 2018.
17. Polman NJ, Ostrbenk A, **Xu L**, et al. Evaluation of the clinical performance of the HPV-Risk assay using the VALGENT-3 panel. Journal of Clinical Microbiology 2017; 55:3544–3551.
18. Arbyn M, **Xu L**, Verdoodt F, et al. Genotyping for human papillomavirus types 16 and 18 in women with minor cervical lesions: a systematic review and meta-analysis. Annals of Internal Medicine 2017;166: 118-27.
19. Arbyn M, **Xu L**, Wentzensen N. HPV test results provide useful risk stratification information in women with ASC-H Pap test findings. Cancer Cytopathology 2016;124: 754-5.

Reference

1. Group, I.M.W., *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol 90: Human Papillomaviruses.* Vol. 90. 2007, Lyon: IARCPress.
2. de Villiers, E.M., *Cross-roads in the classification of papillomaviruses.* Virology, 2013. **445**(1-2): p. 2-10.
3. de Villiers, E.M., et al., *Classification of papillomaviruses.* Virology, 2004. **324**(1): p. 17-27.
4. Bernard, H.U., et al., *Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments.* Virology, 2010. **401**(1): p. 70-79.
5. Woodman, C.B., S.I. Collins, and L.S. Young, *The natural history of cervical HPV infection: unresolved issues.* Nat. Rev. Cancer, 2007. **7**(1): p. 11-22.
6. de Sanjose, S., et al., *Burden of Human Papillomavirus (HPV)-Related Cancers Attributable to HPVs 6/11/16/18/31/33/45/52 and 58.* JNCI Cancer Spectr, 2018. **2**(4): p. ky045.
7. IARC, *IARC monographs on the evaluation of carccinogenic risks to humans*. 2009.
8. Doorbar, J., et al., *Human papillomavirus molecular biology and disease association.* Rev Med Virol, 2015. **25 Suppl 1**: p. 2-23.
9. Schiffman, M.A., et al., *The carcinogenicity of human papillomavirus types reflects viral evolution.* Virology, 2005. **337**(1): p. 76-84.
10. Munoz, N., et al., *Against which human papillomavirus types shall we vaccinate and screen? The international perspective.* Int. J. Cancer, 2004. **111**(2): p. 278-285.
11. Arbyn, M., et al., *Are twenty human papillomavirus types causing cervical cancer?* J. Pathol, 2014. **234**: p. 431-435.
12. De Martel, C., et al., *The global burden of cancers attributable to infections in the year 2008: a review and synthetic analysis.* Lancet Oncol, 2012. **13**(6): p. 607-615.
13. De Martel, C., et al., *Worldwide burden of cancer attributable to HPV by site, country and HPV type.* Int. J. Cancer, 2017.
14. Giuliano, A.R., et al., *EUROGIN 2014 roadmap: differences in human papillomavirus infection natural history, transmission and human papillomavirus-related cancer incidence by gender and anatomic site of infection.* Int J Cancer, 2015. **136**(12): p. 2752-60.
15. Syrjänen, K. and S. Syrjänen, *Epidemiology of human papilloma virus infections and genital neoplasia.* Scand J Infect Dis Suppl, 1990. **69**: p. 7-17.
16. Coupe, V.M., et al., *Age-dependent prevalence of 14 high-risk HPV types in the Netherlands: implications for prophylactic vaccination and screening.* Br J Cancer, 2008.
17. Schiffman, M., et al., *Human papillomavirus and cervical cancer.* Lancet, 2007. **370**(9590): p. 890-907.
18. Bulkmans, N.W., et al., *High-risk HPV type-specific clearance rates in cervical screening.* Br J Cancer, 2007. **96**(9): p. 1419-1424.

19. Bosch, F.X., et al., *Comprehensive control of human papillomavirus infections and related diseases.* Vaccine, 2013. **31 Suppl 8**: p. I1-I31.
20. Steenbergen, R.D., et al., *Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions.* Nat. Rev. Cancer, 2014. **14**(6): p. 395-405.
21. Moscicki, A.B., et al., *Updating the natural history of human papillomavirus and anogenital cancers.* Vaccine, 2012. **30 Suppl 5**: p. F24-F33.
22. Bengtsson, E. and P. Malm, *Screening for cervical cancer using automated analysis of PAP-smears.* Computational and Mathematical Methods in Medicine, 2014. **2014**.
23. Richart, R.M., *Cervical intraepithelial neoplasia.* Pathol. Annu, 1973. **8**: p. 301-323.
24. Solomon, D., et al., *The 2001 Bethesda System: terminology for reporting results of cervical cytology.* JAMA, 2002. **287**: p. 2114-2119.
25. Scheffner, M., et al., *Functions of human papillomavirus proteins.* Curr. Top. Microbiol. Immunol, 1994. **186**: p. 83-99.
26. Moody, C.A. and L.A. Laimins, *Human papillomavirus oncoproteins: pathways to transformation.* Nat. Rev. Cancer, 2010. **10**(8): p. 550-560.
27. Klingelhutz, A.J. and A. Roman, *Cellular transformation by human papillomaviruses: lessons learned by comparing high- and low-risk viruses.* Virology, 2012. **424**(2): p. 77-98.
28. Munger, K. and P.M. Howley, *Human papillomavirus immortalization and transformation functions.* Virus Res, 2002. **89**(2): p. 213-228.
29. McLaughlin-Drubin, M.E. and K. Munger, *Biochemical and functional interactions of human papillomavirus proteins with polycomb group proteins.* Viruses, 2013. **5**(5): p. 1231-1249.
30. Snijders, P.J., et al., *HPV-mediated cervical carcinogenesis: concepts and clinical implications.* J. Pathol, 2006. **208**(2): p. 152-164.
31. Vink, M.A., et al., *Clinical progression of high-grade cervical intraepithelial neoplasia: estimating the time to preclinical cervical cancer from doubly censored national registry data.* Am J Epidemiol, 2013. **178**(7): p. 1161-9.
32. de Sanjose, S., et al., *Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis.* Lancet Infect. Dis, 2007. **7**(7): p. 453-459.
33. Bruni, L., et al., *Cervical HPV prevalence in five continents: meta-analysis on one million women with normal cytology.* J. Infect. Dis, 2010. **202**(12): p. 1789-1799.
34. Bruni, L., et al., *Human Papillomavirus and Related Diseases in the World. ICO Information Centre on HPV and Cancer (HPV Information Centre)*. 2017.
35. de Sanjose, S., et al., *Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study.* Lancet Oncol, 2010. **11**(11): p. 1048-1056.
36. Faber, M.T., et al., *Prevalence and type distribution of human papillomavirus in squamous cell carcinoma and intraepithelial neoplasia of the vulva.* Int. J. Cancer, 2017. **141**(6): p. 1161-1169.

37. De Vuyst, H., et al., *Prevalence and type distribution of human papillomavirus in carcinoma and intraepithelial neoplasia of the vulva, vagina and anus: a meta-analysis.* Int. J. Cancer, 2009. **124**(7): p. 1626-1636.
38. Bornstein, J., et al., *The 2015 International Society for the Study of Vulvovaginal Disease (ISSVD) Terminology of Vulvar Squamous Intraepithelial Lesions.* J Low Genit. Tract Dis, 2016. **20**(1): p. 11-14.
39. Darragh, T.M., et al., *The Lower Anogenital Squamous Terminology Standardization project for HPV-associated lesions: background and consensus recommendations from the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology.* Int. J. Gynecol. Pathol, 2013. **32**(1): p. 76-115.
40. Henson, D. and R. Tarone, *An epidemiologic study of cancer of the cervix, vagina, and vulva based on the Third National Cancer Survey in the United States.* Am J Obstet Gynecol, 1977. **129**(5): p. 525-32.
41. Plummer, M., et al., *Global burden of cancers attributable to infections in 2012: a synthetic analysis.* Lancet Glob. Health, 2016. **4**(9): p. e609-e616.
42. Miralles-Guri, C., et al., *Human papillomavirus prevalence and type distribution in penile carcinoma.* J. Clin. Pathol, 2009. **62**(10): p. 870-878.
43. Giuliano, A.R., et al., *Incidence and clearance of genital human papillomavirus infection in men (HIM): a cohort study.* Lancet, 2011. **377**(9769): p. 932-40.
44. Mehanna, H., et al., *Prevalence of human papillomavirus in oropharyngeal and nonoropharyngeal head and neck cancer - a systematic review and meta-analysis of trends by time and region.* Head Neck, 2013. **35**(5): p. 747-p755.
45. Ndiaye, C., et al., *HPV DNA, E6/E7 mRNA, and p16INK4a detection in head and neck cancers: a systematic review and meta-analysis.* Lancet Oncol, 2014. **15**(12): p. 1319-1331.
46. Arbyn, M., et al., *Estimates of incidence and mortality of cervical cancer in 2018: a worldwide analysis.* Lancet Glob. Health, 2020. **8**(2): p. e191-e120.
47. Serrano, B., et al., *Epidemiology and burden of HPV-related disease.* Best Pract Res Clin Obstet Gynaecol, 2018. **47**: p. 14-26.
48. Fitzmaurice, C., et al., *Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-years for 32 Cancer Groups, 1990 to 2015: A Systematic Analysis for the Global Burden of Disease Study.* JAMA Oncol, 2016. **3**(4): p. 524-548.
49. WHO, *Comprehensive Cervical Cancer Control: A Guide to Essential Practice (2nd edition)*. 2014, Geneva: WHO.
50. Schiller, J.T., X. Castellsague, and S.M. Garland, *A review of clinical trials of human papillomavirus prophylactic vaccines.* Vaccine, 2012. **30 Suppl 5**: p. F123-F138.
51. Paavonen, J., et al., *Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by oncogenic HPV types*

(PATRICIA): final analysis of a double-blind, randomised study in young women. Lancet, 2009. **374**(9686): p. 301-314.

52. Garland, S.M., et al., *Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases.* N. Engl. J. Med, 2007. **356**(19): p. 1928-1943.
53. Group, F.I.S., *Quadrivalent vaccine against human papillomavirus to prevent high-grade cervical lesions.* N. Engl. J. Med, 2007. **356**(19): p. 1915-1927.
54. Joura, E.A., et al., *A 9-Valent HPV Vaccine against Infection and Intraepithelial Neoplasia in Women.* N. Engl. J. Med, 2015. **372**(8): p. 711-723.
55. Huh, W.K., et al., *Final efficacy, immunogenicity, and safety analyses of a nine-valent human papillomavirus vaccine in women aged 16-26 years: a randomised, double-blind trial.* Lancet, 2017. **390**(10108): p. 2043-2059.
56. Giuliano, A.R., et al., *Efficacy of Quadrivalent HPV Vaccine against HPV Infection and Disease in Males.* N. Engl. J. Med, 2011. **364**(5): p. 401-411.
57. Palefsky, J.M., et al., *HPV vaccine against anal HPV infection and anal intraepithelial neoplasia.* N. Engl. J. Med, 2011. **365**(17): p. 1576-1585.
58. Harder, T., et al., *Efficacy, effectiveness and safety of vaccination against human papillomavirus in males: a systematic review.* BMC. Med, 2018. **16**(1): p. 110.
59. WHO, *Weekly epidemiological record*. 2017, World Health Organization: Geneva. p. 939-404.
60. Laara, E., N.E. Day, and M. Hakama, *Trends in mortality from cervical cancer in the Nordic countries: association with organised screening programmes.* Lancet, 1987. **1**: p. 1247-1249.
61. Arbyn, M., et al., *Trends of cervical cancer mortality in the member states of the European Union.* Eur. J. Cancer, 2009. **45**(15): p. 2640-2648.
62. Verdoodt, F., et al., *Triage of women with minor abnormal cervical cytology: Meta-analysis of the accuracy of an assay targeting messenger ribonucleic acid of 5 high-risk human papillomavirus types.* Cancer Cytopathol, 2013. **121**(12): p. 675-687.
63. Arbyn, M., et al., *Virologic versus cytologic triage of women with equivocal Pap smears: a meta-analysis of the accuracy to detect high-grade intraepithelial neoplasia.* J. Natl. Cancer Inst, 2004. **96**: p. 280-293.
64. Ronco, G., et al., *Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials.* Lancet, 2014. **383**(9916): p. 524-532.
65. Hanselaar, A.G., *Criteria for organized cervical screening programs. Special emphasis on The Netherlands program.* Acta Cytol, 2002. **46**(4): p. 619-629.
66. Arbyn, M., et al., *Accuracy of human papillomavirus testing on self-collected versus clinician-collected samples: a meta-analysis.* Lancet Oncol, 2014. **15**(2): p. 172-183.

67. Arbyn, M., et al., *Detecting cervical precancer and reaching underscreened women by using HPV testing on self samples: updated meta-analyses.* BMJ, 2018. **363**: p. k4823.
68. Verdoodt, F., et al., *Reaching women who do not participate in the regular cervical cancer screening program by offering self-sampling kits: A systematic review and meta-analysis of randomised trials.* Eur. J. Cancer, 2015. **51**(16): p. 2375-2385.
69. Basu, P., et al., *Status of implementation and organization of cancer screening in the European Union Member States - summary results from the second European screening report.* Int. J. Cancer, 2018. **142**(1): p. 44-56.
70. ECDC, *Public consultation on draft guidance for introduction of HPV vaccines in EU countries: focus on 9-valent HPV vacine and vacciantion of boyes and people living with HIV*. 2019. p. 1-80.
71. WHO, *Comprehensive cervical cancer control: a guide to essential practice*. 2006, Geneva: WHO.
72. Cibula, D., et al., *The European Society of Gynaecological Oncology/European Society for Radiotherapy and Oncology/European Society of Pathology Guidelines for the Management of Patients With Cervical Cancer.* Int. J. Gynecol. Cancer, 2018. **28**(4): p. 641-655.
73. Khan, K., et al., *Systematic reviews to support evidence-based medicine, 2nd edition*. 2011: CRC press. 224.
74. Higgins, J.P.T. and S. Green, *Cochrane Handbook for Systematic Reviews of Interventions Version 5.1.0 [updated March 2011].* 2011.
75. Deeks, J.J., P.M. Bossuyt, and C. Gatsonis, *Cochrane Handbook for Systematic Reviews of Diagnostic Test Accuracy Version 1.0.0*. 2013: The Cochrane Collaboration.
76. Rothman, K.J. and S. Greenland, *Modern Epidemiology*. 2nd ed. 1998, Philadelphia: Lippincot-Raven.
77. Moher, D., et al., *Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement.* Ann Intern Med, 2009. **151**(4): p. 264-9, W64.
78. Poljak, M., et al., *Commercially available molecular tests for human papillomaviruses: a global overview.* Clin. Microbiol. Infect, 2020.
79. Arbyn, M., et al., *Which high-risk HPV assays fulfil criteria for use in primary cervical cancer screening?* Clin. Microbiol. Infect, 2015. **21**(9): p. 817-826.
80. Sankaranarayanan, R., et al., *HPV screening for cervical cancer in Rural India.* N. Engl. J. Med, 2009. **360**(14): p. 1385-1394.
81. Kitchener, H.C., et al., *HPV testing in combination with liquid-based cytology in primary cervical screening (ARTISTIC): a randomised controlled trial.* Lancet Oncol, 2009. **10**(7): p. 672-682.
82. Poljak, M., et al., *Comparison of Clinical and Analytical Performance of the Abbott RealTime High Risk HPV Test to the Performance of Hybrid Capture 2 in Population-Based Cervical Cancer Screening.* J. Clin. Microbiol, 2011. **49**(5): p. 1721-1729.

83. Hesselink, A.T., et al., *Cross-sectional comparison of an automated hybrid capture 2 assay and the consensus GP5+/6+ PCR method in a population-based cervical screening program.* J Clin. Microbiol, 2006. **44**(10): p. 3680-3685.
84. Naucler, P., et al., *Efficacy of HPV DNA testing with cytology triage and/or repeat HPV DNA testing in primary cervical cancer screening.* J Natl. Cancer Inst, 2009. **101**: p. 88-98.
85. Rijkaart, D.C., et al., *Human papillomavirus testing for the detection of high-grade cervical intraepithelial neoplasia and cancer: final results of the POBASCAM randomised controlled trial.* Lancet Oncol, 2012. **13**(10): p. 78-88.
86. Meijer, C.J.L.M., et al., *Guidelines for human papillomavirus DNA test requirements for primary cervical cancer screening in women 30 years and older.* Int. J. Cancer, 2009. **124**(3): p. 516-520.
87. Arbyn, M., et al., *VALGENT: a protocol for clinical validation of human papillomavirus assays.* J. Clin. Virol, 2016. **76 (Suppl 1)**: p. S14-S21.
88. Ault, K.A., *Effect of prophylactic human papillomavirus L1 virus-like-particle vaccine on risk of cervical intraepithelial neoplasia grade 2, grade 3, and adenocarcinoma in situ: a combined analysis of four randomised clinical trials.* Lancet, 2007. **369**(9576): p. 1861-1868.
89. Arbyn, M., *Effects of quadrivalent human papillomavirus vaccination.* Lancet, 2007. **370**(9592): p. 1031-1032.
90. Harper, D.M., *Currently approved prophylactic HPV vaccines.* Expert Rev. Vaccines, 2009. **8**(12): p. 1663-1679.
91. Harper, D.M., *Current prophylactic HPV vaccines and gynecologic premalignancies.* Curr. Opin. Obstet. Gynecol, 2009.
92. WHO and V. Initiative for Vaccine Research of the Department of Immunization, and Biologicals, *Human Papillomavirus (HPV) Vaccine Background Paper*. 2009: Geneva. p. 1-249.
93. Kahn, J.A., *HPV vaccination for the prevention of cervical intraepithelial neoplasia.* N. Engl. J. Med, 2009. **361**(3): p. 271-278.
94. Kjaer, S.K., et al., *A pooled analysis of continued prophylactic efficacy of quadrivalent human papillomavirus (Types 6/11/16/18) vaccine against high-grade cervical and external genital lesions.* Cancer Prev. Res. (Phila Pa), 2009. **2**(10): p. 868-878.
95. Koutsky, L.A. and D.M. Harper, *Chapter 13: Current findings from prophylactic HPV vaccine trials.* Vaccine, 2006. **24**(Suppl 3): p. 114-121.
96. Lu, B., et al., *Efficacy and Safety of Prophylactic Vaccines against Cervical HPV Infection and Diseases among Women: A Systematic Review & Meta-Analysis.* BMC Infect Dis, 2011. **11**(1): p. 13.
97. Medeiros, L.R., et al., *Efficacy of human papillomavirus vaccines: a systematic quantitative review.* Int J Gynecol Cancer, 2009. **19**(7): p. 1166-1176.

98. Rambout, L., et al., *Prophylactic vaccination against human papillomavirus infection and disease in women: a systematic review of randomized controlled trials.* CMAJ, 2007. **177**(5): p. 469-479.
99. Szarewski, A., *HPV vaccine: Cervarix.* Expert. Opin. Biol. Ther, 2010. **10**(3): p. 477-487.
100. Arbyn, M., et al., *Prophylactic vaccination against human papillomaviruses to prevent cervical cancer and its precursors.* Cochrane Database Syst. Rev, 2018. **5**: p. CD009069.
101. Arbyn, M. and L. Xu, *Efficacy and safety of prophylactic HPV vaccines. A Cochrane review of randomised trials.* Expert Rev. Vaccines, 2018. **17**(12): p. 1085-1091.
102. Miltz, A., et al., *Systematic review and meta-analysis of L1-VLP-based human papillomavirus vaccine efficacy against anogenital pre-cancer in women with evidence of prior HPV exposure.* PLoS. ONE, 2014. **9**(3): p. e90348.
103. Andersson, K., et al., *The interface of population-based cancer registries and biobanks in etiological and clinical research ?current and future perspectives.* Acta Oncol, 2010. **49**(8): p. 1127-1134.
104. Saslow, D., et al., *American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology screening guidelines for the prevention and early detection of cervical cancer.* CA Cancer J. Clin, 2012. **62**(3): p. 147-172.
105. Arbyn, M., et al., *Evidence regarding human papillomavirus testing in secondary prevention of cervical cancer.* Vaccine, 2012. **30 Suppl 5**: p. F88-F99.
106. Arbyn, M., et al., *Human papillomavirus testing versus repeat cytology for triage of minor cytological cervical lesions.* Cochrane Database Syst. Rev, 2013. **3**(CD008054): p. 1-201.
107. Arbyn, M. *European Guidelines for Quality Assurance in Cervical Cancer Screening*. 2006.
108. Arbyn, M., et al., *Genotyping for human papillomavirus types 16 and 18 in women with minor cervical lesions: a systematic review and meta-analysis.* Ann. Intern. Med, 2017. **166**(2): p. 118-127.
109. Massad, L.S., et al., *2012 updated consensus guidelines for the management of abnormal cervical cancer screening tests and cancer precursors.* J. Low Genit. Tract. Dis, 2013. **17**(5 Suppl 1): p. S1-S27.
110. Arbyn, M., et al., *European Guidelines for Quality Assurance in Cervical Cancer Screening. Second Edition - Summary Document.* Ann. Oncol, 2010. **21**(3): p. 448-458.
111. Wright, T.C., Jr., et al., *2006 consensus guidelines for the management of women with abnormal cervical cancer screening tests.* Am. J. Obstet. Gynecol, 2007. **197**(4): p. 346-355.
112. Arbyn, M., et al. *Peri-natal mortality and other severe adverse pregnancy outcomes associated with treatment of cervical intraepithelial neoplasia: a meta-analysis*. 2008.
113. Kyrgiou, M., et al., *Preterm birth after treatment for cervical precancer: an issue yet to be resolved.* BMJ, 2012. **345**: p. e5847.

114. Herbert, A., et al., *European guidelines for quality assurance in cervical cancer screening: recommendations for cervical cytology terminology.* Cytopathology, 2007. **18**(4): p. 213-219.
115. Luff, R.D. and N.C. Institute, *The revised Bethesda system for reporting cervical/vaginal cytological diagnoses. Report of the 1991 Bethesda Workshop.* Acta Cytol, 1992. **36**: p. 273-276.
116. Darragh, T.M., et al., *The Lower Anogenital Squamous Terminology Standardization Project for HPV-Associated Lesions: Background and Consensus Recommendations From the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology.* J. Low Genit. Tract. Dis, 2012. **16**(3): p. 205-242.
117. Collaboration, C., *Does HPV vaccination prevent the development of cervical cancer? Are there harms associated with being vaccinated? Press Conference, London, 7th of May, 2018.* 2018.
118. Arbyn, M., et al., *Worldwide burden of cervical cancer in 2008.* Ann. Oncol, 2011. **22**(12): p. 2675-2686.
119. Stanley, M., L.A. Pinto, and C. Trimble, *Human papillomavirus vaccines - immune responses.* Vaccine, 2012. **30 Suppl 5**: p. F83-F87.
120. Ostor, A.G., *Natural history of cervical intraepithelial neoplasia: a critical review.* Int. J. Gynecol. Pathol, 1993. **12**(2): p. 186-192.
121. Herbert, A., M. Arbyn, and C. Bergeron, *Why CIN3 and CIN2 should be distinguished on histological reports.* Cytopathology, 2008. **19**(1): p. 63-64.
122. Rowhani-Rahbar, A., et al., *Longer term efficacy of a prophylactic monovalent human papillomavirus type 16 vaccine.* Vaccine, 2009. **27**(41): p. 5612-5619.
123. Naud, P.S., et al., *Sustained efficacy, immunogenicity, and safety of the HPV-16/18 AS04-adjuvanted vaccine: Final analysis of a long-term follow-up study up to 9.4 years post-vaccination.* Hum. Vaccin. Immunother, 2014. **10**(8): p. 2147-2162.
124. Koutsky, L.A., et al., *A controlled trial of a human papillomavirus type 16 vaccine.* N. Engl. J. Med, 2002. **347**(21): p. 1645-1651.
125. Konno, R., et al., *Efficacy of human papillomavirus type 16/18 AS04-adjuvanted vaccine in Japanese women aged 20 to 25 years: final analysis of a phase 2 double-blind, randomized controlled trial.* Int. J. Gynecol. Cancer, 2010. **20**(5): p. 847-855.
126. Konno, R., et al., *Efficacy of the human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical intraepithelial neoplasia and cervical infection in young Japanese women.* Hum. Vaccin. Immunother, 2014. **10**(7): p. 1781-1794.
127. Harper, D.M., et al., *Sustained efficacy up to 4-5 years of bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow - up from a randomised control trial.* Lancet, 2006. **367**(9518): p. 1247-1255.

128. Sow, P.S., et al., *Safety and Immunogenicity of Human Papillomavirus-16/18 AS04-Adjuvanted Vaccine: A Randomized Trial in 10-25-Year-Old HIV-Seronegative African Girls and Young Women.* J. Infect. Dis, 2013: p. in-press.

129. Zhu, F., et al., *Immunogenicity and safety of the HPV-16/18 AS04-adjuvanted vaccine in healthy Chinese girls and women aged 9 to 45 years.* Hum. Vaccin. Immunother, 2014. **10**(7): p. 1795-1806.

130. Zhu, F.C., et al., *Efficacy, immunogenicity and safety of the HPV-16/18 AS04-adjuvanted vaccine in healthy Chinese women aged 18-25 years: results from a randomized controlled trial.* Int. J. Cancer, 2014. **135**(11): p. 2612-2622.

131. Zhu, F.C., et al., *Efficacy, immunogenicity, and safety of the HPV-16/18 AS04-adjuvanted vaccine in Chinese women aged 18-25 years: event-triggered analysis of a randomized controlled trial.* Cancer Med, 2017. **6**(1): p. 12-25.

132. Garcia-Sicilia, J., et al., *Immunogenicity and safety of human papillomavirus-16/18 AS04-adjuvanted cervical cancer vaccine coadministered with combined diphtheria-tetanus-acellular pertussis-inactivated poliovirus vaccine to girls and young women.* J. Adolesc. Health, 2010. **46**(2): p. 142-151.

133. Pedersen, C., et al., *Randomized Trial: Immunogenicity and Safety of Coadministered Human Papillomavirus-16/18 AS04-Adjuvanted Vaccine and Combined Hepatitis A and B Vaccine in Girls.* J. Adolesc. Health, 2012. **50**(1): p. 38-46.

134. Schmeink, C.E., et al., *Co-administration of human papillomavirus-16/18 AS04-adjuvanted vaccine with hepatitis B vaccine: randomized study in healthy girls.* Vaccine, 2011. **29**(49): p. 9276-9283.

135. Hildesheim, A., et al., *Efficacy of the HPV-16/18 vaccine: Final according to protocol results from the blinded phase of the randomized Costa Rica HPV-16/18 vaccine trial.* Vaccine, 2014. **32**(39): p. 5087-5097.

136. Lang Kuhs, K.A., et al., *Effect of different human papillomavirus serological and DNA criteria on vaccine efficacy estimates.* Am. J. Epidemiol, 2014. **180**(6): p. 599-607.

137. Kreimer, A.R., et al., *Efficacy of fewer than three doses of an HPV-16/18 AS04-adjuvanted vaccine: combined analysis of data from the Costa Rica Vaccine and PATRICIA trials.* Lancet Oncol, 2015. **16**(7): p. 775-786.

138. Ngan, H.Y., et al., *Human papillomavirus-16/18 AS04-adjuvanted cervical cancer vaccine: immunogenicity and safety in healthy Chinese women from Hong Kong.* Hong. Kong. Med. J, 2010. **16**(3): p. 171-179.

139. Medina, D.M., et al., *Safety and immunogenicity of the HPV-16/18 AS04-adjuvanted vaccine: a randomized, controlled trial in adolescent girls.* J. Adolesc. Health, 2010. **46**(5): p. 414-421.

140. Bhatla, N., et al., *Immunogenicity and safety of human papillomavirus-16/18 AS04-adjuvanted cervical cancer vaccine in healthy Indian women.* J. Obstet. Gynaecol. Res, 2010. **36**(1): p. 123-132.
141. Kim, Y.J., et al., *Vaccination with a human papillomavirus (HPV)-16/18 AS04-adjuvanted cervical cancer vaccine in Korean girls aged 10-14 years.* J. Korean Med. Sci, 2010. **25**(8): p. 1197-1204.
142. Kim, S.C., et al., *Human papillomavirus 16/18 AS04-adjuvanted cervical cancer vaccine: immunogenicity and safety in 15-25 years old healthy Korean women.* J. Gynecol. Oncol, 2011. **22**(2): p. 67-75.
143. Lim, B.K., et al., *Immunogenicity and Safety of the AS04-adjuvanted Human Papillomavirus-16/18 Cervical Cancer Vaccine in Malaysian Women Aged 18-35 years: A Randomized Controlled Trial.* Med. J Malaysia, 2014. **69**(1): p. 2-8.
144. Lehtinen, M., et al., *Overall efficacy of HPV-16/18 AS04-adjuvanted vaccine against grade 3 or greater cervical intraepithelial neoplasia: 4-year end-of-study analysis of the randomised, double-blind PATRICIA trial.* Lancet Oncol, 2012. **13**(1): p. 89-99.
145. Skinner, S.R., et al., *Efficacy, safety, and immunogenicity of the human papillomavirus 16/18 AS04-adjuvanted vaccine in women older than 25 years: 4-year interim follow-up of the phase 3, double-blind, randomised controlled VIVIANE study.* Lancet, 2014. **384**(9961): p. 2213-2227.
146. Wheeler, C.M., et al., *Efficacy, safety, and immunogenicity of the human papillomavirus 16/18 AS04-adjuvanted vaccine in women older than 25 years: 7-year follow-up of the phase 3, double-blind, randomised controlled VIVIANE study.* Lancet Infect. Dis, 2016. **16**(10): p. 1154-1168.
147. Yoshikawa, H., et al., *Efficacy of quadrivalent human papillomavirus (types 6, 11, 16, and 18) vaccine (GARDASIL((R))) in Japanese women aged 18 to 26 years.* Cancer Sci, 2013. **104**: p. 465-472.
148. Kang, S., et al., *Safety and immunogenicity of a vaccine targeting human papillomavirus types 6, 11, 16 and 18: a randomized, placebo-controlled trial in 176 Korean subjects.* Int. J. Gynecol. Cancer, 2008. **18**(5): p. 1013-1019.
149. Villa, L.L., et al., *High sustained efficacy of a prophylactic quadrivalent human papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up.* Br. J. Cancer, 2006: p. 1459-1466.
150. Mugo, N., et al., *Evaluation of safety and immunogenicity of a quadrivalent human papillomavirus vaccine in healthy females between 9 and 26-釋ears of age in Sub-Saharan Africa.* Human Vaccines & Immunotherapeutics, 2015. **11**(6): p. 1323-1330.
151. Munoz, N., et al., *Impact of human papillomavirus (HPV)-6/11/16/18 vaccine on all HPV-associated genital diseases in young women.* J. Natl. Cancer Inst, 2010. **102**(5): p. 325-339.

152. Castellsague, X., et al., *End-of-study safety, immunogenicity, and efficacy of quadrivalent HPV (types 6, 11, 16, 18) recombinant vaccine in adult women 24-45 years of age.* Br. J. Cancer, 2011. **105**(1): p. 28-37.
153. Guyatt, G.H., et al., *GRADE: an emerging consensus on rating quality of evidence and strength of recommendations.* BMJ, 2008. **336**(7650): p. 924-926.
154. Higgins, J.P., et al., *The Cochrane Collaboration's tool for assessing risk of bias in randomised trials.* BMJ, 2011. **343**: p. d5928.
155. Bzhalava, D., et al., *A systematic review of the prevalence of mucosal and cutaneous human papillomavirus types.* Virology, 2013. **445**(1-2): p. 224-231.
156. Hartwig, S., et al., *Estimation of the overall burden of cancers, precancerous lesions, and genital warts attributable to 9-valent HPV vaccine types in women and men in Europe.* Infect. Agent. Cancer, 2017. **12**: p. 19.
157. Arbyn, M., et al., *Surveillance of effects of HPV vaccination in Belgium.* Cancer Epidemiol, 2016. **41**: p. 152-158.
158. Kavanagh, K., et al., *Changes in the prevalence of human papillomavirus following a national bivalent human papillomavirus vaccination programme in Scotland: a 7-year cross-sectional study.* Lancet Infect. Dis, 2017. **17**(12): p. 1293-1302.
159. Pagliusi, S.R. and A.M. Teresa, *Efficacy and other milestones for human papillomavirus vaccine introduction.* Vaccine, 2004. **23**(5): p. 569-578.
160. WHO, *Safety of HPV vaccines (from meeting of 2-3 December, 2015).* Weekly Epidem. Record, 2016. **91**(3): p. 26-28.
161. Phillips, A., et al., *Safety of Human Papillomavirus Vaccines: An Updated Review.* Drug Saf, 2018. **41**(4): p. 329-346.
162. Bonde, U., et al., *Is HPV vaccination in pregnancy safe?* Hum. Vaccin. Immunother, 2016. **12**(8): p. 1960-1964.
163. Arbyn, M., et al., *EUROGIN 2011 roadmap on prevention and treatment of HPV-related disease.* Int. J. Cancer, 2012. **131**(9): p. 1969-1982.
164. Forman, D., et al., *Global burden of human papillomavirus and related diseases.* Vaccine, 2012. **30 Suppl 5**: p. F12-F23.
165. Drolet, M., et al., *Population-level impact and herd effects following human papillomavirus vaccination programmes: a systematic review and meta-analysis.* Lancet Infect. Dis, 2015. **15**(5): p. 565-580.
166. Tabrizi, S.N., et al., *Fall in human papillomavirus prevalence following a national vaccination program.* J. Infect. Dis, 2012. **206**(11): p. 1645-1651.
167. Tabrizi, S.N., et al., *Assessment of herd immunity and cross-protection after a human papillomavirus vaccination programme in Australia: a repeat cross-sectional study.* Lancet Infect. Dis, 2014. **14**(10): p. 958-966.
168. Jemal, A., et al., *Cancer statistics, 2007.* CA Cancer J. Clin, 2007. **57**(1): p. 43-66.

169. Baandrup, L., et al., *In situ and invasive squamous cell carcinoma of the vulva in Denmark 1978-2007-a nationwide population-based study.* Gynecol. Oncol, 2011. **122**(1): p. 45-49.
170. Judson, P.L., et al., *Trends in the incidence of invasive and in situ vulvar carcinoma.* Obstet. Gynecol, 2006. **107**(5): p. 1018-1022.
171. Lang Kuhs, K.A., et al., *Reduced prevalence of vulvar HPV16/18 infection among women who received the HPV16/18 bivalent vaccine: a nested analysis within the Costa Rica Vaccine Trial.* J. Infect. Dis, 2014. **210**(12): p. 1890-1899.
172. Smith, J.S., et al., *Human papillomavirus type-distribution in vulvar and vaginal cancers and their associated precursors.* Obstet. Gynecol, 2009. **113**(4): p. 917-924.
173. de Sanjose, S., et al., *Worldwide human papillomavirus genotype attribution in over 2000 cases of intraepithelial and invasive lesions of the vulva.* Eur. J. Cancer, 2013. **49**(16): p. 3450-3461.
174. Wilkinson, E.J., B. Kneale, and P.J. Lynch, *Report of the ISSVD terminology committee.* J Reprod Med, 1986. **31**(10): p. 973-974.
175. Sideri, M., et al., *Squamous vulvar intraepithelial neoplasia: 2004 modified terminology, ISSVD Vulvar Oncology Subcommittee.* J. Reprod. Med, 2005. **50**(11): p. 807-810.
176. Chao, A., et al., *Human papillomavirus in vaginal intraepithelial neoplasia.* Int. J. Cancer, 2012. **131**(3): p. E259-E268.
177. Liberati, A., et al., *The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration.* PLoS. Med, 2009. **6**(7): p. e1000100.
178. Guyatt, G.H., et al., *GRADE: Going from evidence to recommendations.* BMJ, 2008. **336**(7652): p. 1049-1051.
179. Guyatt, G.H., et al., *GRADE guidelines: 13. Preparing summary of findings tables and evidence profiles-continuous outcomes.* J. Clin. Epidemiol, 2013. **66**(2): p. 173-183.
180. Group, F.I.S., *Prophylactic efficacy of a quadrivalent human papillomavirus (HPV) vaccine in women with virological evidence of HPV infection.* J Infect. Dis, 2007. **196**(10): p. 1438-1446.
181. Dillner, J., et al., *Four year efficacy of prophylactic human papillomavirus quadrivalent vaccine against low grade cervical, vulvar, and vaginal intraepithelial neoplasia and anogenital warts: randomised controlled trial.* BMJ, 2010. **341**: p. c3493.
182. Majewski, S., et al., *The impact of a quadrivalent human papillomavirus (types 6, 11, 16, 18) virus-like particle vaccine in European women aged 16 to 24.* J. Eur. Acad. Dermatol. Venereol, 2009. **23**(10): p. 1147-1155.
183. Joura, E.A., et al., *Effect of the human papillomavirus (HPV) quadrivalent vaccine in a subgroup of women with cervical and vulvar disease: retrospective pooled analysis of trial data.* BMJ, 2012. **344**: p. e1401.

184. Joura, E.A., et al., *Efficacy of a quadrivalent prophylactic human papillomavirus (types 6, 11, 16, and 18) L1 virus-like-particle vaccine against high-grade vulval and vaginal lesions: a combined analysis of three randomised clinical trials.* Lancet, 2007. **369**(9574): p. 1693-1702.

185. Haupt, R.M. and H.L. Sings, *The efficacy and safety of the quadrivalent human papillomavirus 6/11/16/18 vaccine gardasil.* J. Adolesc. Health, 2011. **49**(5): p. 467-475.

186. Munoz, N., et al., *Safety, immunogenicity, and efficacy of quadrivalent human papillomavirus (types 6, 11, 16, 18) recombinant vaccine in women aged 24-45 years: a randomised, double-blind trial.* Lancet, 2009. **373**(9679): p. 1949-1957.

187. Olsson, S.E., et al., *Evaluation of quadrivalent HPV 6/11/16/18 vaccine efficacy against cervical and anogenital disease in subjects with serological evidence of prior vaccine type HPV infection.* Hum. Vaccin, 2009. **5**(10): p. 696-704.

188. Garland, S.M. *Eficacy of a quadrivalent HPV (types6,11,16,18) L1 VLP vacine against external genital disease: FUTURE 1analysis.*

189. Lehtinen, M. and J. Dillner, *Clinical trials of human papillomavirus vaccines and beyond.* Nat. Rev. Clin. Oncol, 2013. **10**(7): p. 400-410.

190. Garland, S.M., et al., *Prior human papillomavirus-16/18 AS04-adjuvanted vaccination prevents recurrent high grade cervical intraepithelial neoplasia after definitive surgical therapy: Post-hoc analysis from a randomized controlled trial.* Int. J. Cancer, 2016. **139**(12): p. 2812-2826.

191. Garland, S., *Efficacy of the HPV16/18 AS04-adjuvanted vaccine against vulvar/vaginal intraepithelial neoplasia.* In: IPV Conference, Berlin, 2011, 2011.

192. Slade, B.A., et al., *Postlicensure safety surveillance for quadrivalent human papillomavirus recombinant vaccine.* JAMA, 2009. **302**(7): p. 750-757.

193. Naleway, A.L., et al., *Absence of venous thromboembolism risk following quadrivalent human papillomavirus vaccination, Vaccine Safety Datalink, 2008-2011.* Vaccine, 2016. **34**(1): p. 167-171.

194. Humans, I.W.G.o.t.E.o.C.R.t., *Biological Agents: A Review of Human Carcinogens.* IARC Monogr. Eval. Carcinog. Risks Hum, 2012. **100B**: p. 1-475.

195. Ronco, G., et al., *Efficacy of human papillomavirus testing for the detection of invasive cervical cancers and cervical intraepithelial neoplasia: a randomised controlled trial.* Lancet Oncol, 2010. **11**(3): p. 249-257.

196. Cuzick, J., et al., *Individual detection of 14 high risk human papilloma virus genotypes by the PapType test for the prediction of high grade cervical lesions.* J Clin Virol, 2014. **60**(1): p. 44-49.

197. Poljak, M., et al., *Commercially available molecular tests for human papillomaviruses (HPV): 2015 update.* J. Clin. Virol, 2016. **76 (Suppl 1)**: p. S3-S9.

198. Poljak, M. and B.J. Kocjan, *Commercially available assays for multiplex detection of alpha human papillomaviruses.* Expert. Rev. Anti. Infect. Ther, 2010. **8**(10): p. 1139-1162.

199. Schmitt, M., et al., *Multiple HPV infections with high viral loads are associated with cervical lesions but do not differentiate grades of cervical abnormalities.* J. Clin. Microbiol, 2013. **51**(5): p. 1458-1464.

200. Schmitt, M., et al., *Prevalence and viral load of 51 genital human papillomavirus types and 3 subtypes.* Int. J. Cancer, 2013. **132**(10): p. 2395-2403.

201. Schmitt, M., et al., *Viral load of high-risk human papillomaviruses as reliable clinical predictor for the presence of cervical lesions.* Cancer Epidemiol. Biomarkers Prev, 2013. **22**(3): p. 406-414.

202. Cuschieri, K., et al., *Clinical and analytical performance of the Onclarity HPV assay using the VALGENT framework.* J Clin. Microbiol, 2015. **53**(10): p. 3272-3279.

203. Cuschieri, K., et al., *Performance of a cartridge based assay for the detection of clinically significant HPV infection - lessons from VALGENT (Validation of HPV Genotyping Tests).* J Clin. Microbiol, 2016. **54**(9): p. 2337-2347.

204. Geraets, D., et al., *Clinical evaluation of a GP5+/6+-based Luminex assay having full high-risk HPV genotyping capability and an internal control.* J. Clin. Microbiol, 2014. **52**(11): p. 3996-4002.

205. Heard, I., et al., *Clinical and analytical performance of the PapilloCheck HPV-Screening assay using the VALGENT framework.* J. Clin. Virol, 2016. **81**: p. 6-11.

206. Polman, N.J., et al., *Evaluation of the clinical performance of the HPV-Risk assay using the VALGENT-3 panel.* J. Clin. Microbiol, 2017. **55**(1): p. 3544-3551.

207. (Ed.), M.U.V., *Society for gynecological oncology,colposcopy and cervical pathology,Slovenian guidelines for management of pathological cercical smears*, http: and z.o.-i.s.f.u.u.d.s.p.S. web.pdf, Editors. 2011.

208. Tang, N.S., M.L. Tang, and I.S. Chan, *On tests of equivalence via non-unity relative risk for matched-pair design.* Stat. Med, 2003. **22**(8): p. 1217-1233.

209. McNemar, Q., *Note on the sampling error of the difference between correlated proportions or percentages.* Psychometrika, 1947. **12**(2): p. 153-157.

210. Fleiss, J.L., *Measuring agreement between two judges on the presence or absence of a trait.* Biometrics, 1975. **31**: p. 651-659.

211. De Francesco, M.A., et al., *Prevaccination distribution of human papillomavirus types in Italian women with high-risk lesions and cervical neoplasia.* Intervirology, 2010. **53**(6): p. 417-425.

212. Stoler, M.H., et al., *High-Risk Human Papillomavirus Testing in Women With ASC-US Cytology: Results From the ATHENA HPV Study.* Am J Clin Pathol, 2011. **135**(3): p. 468-475.

213. Levi, A.W., et al., *A Comparison of the Roche Cobas HPV Test With the Hybrid Capture 2 Test for the Detection of High-Risk Human Papillomavirus Genotypes.* Arch. Pathol. Lab Med, 2016. **140**(2): p. 153-157.
214. Arbyn, M., et al., *Cervical cytology biobanks as a resource for molecular epidemiology.* Methods Mol. Biol, 2011. **675**: p. 279-298.
215. Larsson, G.L., M.G. Karlsson, and G. Helenius, *HPV testing of biobanked liquid-based cytology - a validation study.* Int. J Biol. Markers, 2016. **31**(2): p. e218-e223.
216. Castle, P.E., et al., *Performance of carcinogenic human papillomavirus (HPV) testing and HPV16 or HPV18 genotyping for cervical cancer screening of women aged 25 years and older: a subanalysis of the ATHENA study.* Lancet Oncol, 2011. **12**(9): p. 880-890.
217. Monsonego, J., et al., *Prevalence of high-risk human papilloma virus genotypes and associated risk of cervical precancerous lesions in a large U.S. screening population: data from the ATHENA trial.* Gynecol. Oncol, 2015. **137**(1): p. 47-54.
218. Cuzick, J. and C. Wheeler, *Need for expanded HPV genotyping for cervical screening.* Papillomavir. Res, 2016. **2**: p. 112-115.
219. Eklund, C., T. Zhou, and J. Dillner, *A global proficiency study of Human Papillomavirus genotyping.* J. Clin. Microbiol, 2010. **2010**(48): p. 4147-4155.
220. Eklund, C., et al., *Global improvement in genotyping of Human Papillomavirus DNA: The 2011 HPV LabNet International Proficiency Study.* J. Clin. Microbiol, 2014. **52**(2): p. 449-459.
221. Bouvard, V., et al., *A review of human carcinogens--Part B: biological agents.* Lancet Oncol, 2009. **10**(4): p. 321-322.
222. Wentzensen, N., et al., *Eurogin 2016 Roadmap: How HPV knowledge is changing screening practice.* Int. J. Cancer, 2017. **140**(10): p. 2192-2200.
223. Melchers, W.J.G., et al., *Technical advance. Short fragment polymerase chain reaction reverse hybridization line probe assay to detect and genotype a broad spectrum of human papillomavirus types.* Am. J. Pathol, 1999. **155**: p. 1473-1478.
224. Kleter, B., et al., *Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus.* J. Clin. Microbiol, 1999. **37**: p. 2508-2517.
225. *Fujirebio Europe upgrades its HPV (Human Papillomamvirus) Genotyping panel with the launch of the INNO-LiPA HPV Genotyping Extra II Assay*. 2015 5/3/2015; Available from: http://www.webcitation.org/70dExIEuQ.
226. Xu, L., et al., *Assessment of the Roche Linear Array HPV genotyping test within the VALGENT framework.* J. Clin. Virol, 2018. **98**: p. 37-42.
227. Landis, J.R. and G.G. Koch, *The measurement of observer agreement for categorical data.* Biometrics, 1977. **33**: p. 159-174.

228. Kleter, B., et al., *Novel short-fragment PCR assey for highly sensitive broad-spectrum detection of anogenital human papillomaviruses.* Am. J. Pathol, 1998. **153**(6): p. 1731-1739.
229. Iftner, T. and L.L. Villa, *Chapter 12: Human papillomavirus technologies.* J. Natl. Cancer Inst. Monogr, 2003(31): p. 80-88.
230. Fleiss, J.L., *Statistical Methods for Rates and Proportions.* 2nd ed. Wiley Series Probability and Mathematical Statistics., ed. R.A. Bradly, et al. 1981, New York: John Wiley & Sons.
231. Castle, P.E., M.A. Schiffman, and C.M. Wheeler, *Hybrid capture 2 viral load and the 2-year cumulative risk of cervical intraepithelial neoplasia grade 3 or cancer.* Am. J. Obstet. Gynecol, 2004. **191**(5): p. 1590-1597.
232. Franco, E.L. and F. Coutlee, *Prognostic value of measuring load of human papillomavirus DNA in cervical samples: an elusive target.* J Natl. Cancer Inst, 2009. **101**(3): p. 131-133.
233. Constandinou-Williams, C., et al., *Is human papillomavirus viral load a clinically useful predictive marker? A longitudinal study.* Cancer Epidemiol. Biomarkers Prev, 2010. **19**(3): p. 832-837.
234. Xi, L.F., et al., *Viral load in the natural history of human papillomavirus type 16 infection: a nested case-control study.* J. Infect. Dis, 2011. **203**(10): p. 1425-1433.
235. Fu, X.L., et al., *Type-dependent association between risk of cervical intraepithelial neoplasia and viral load of oncogenic human papillomavirus types other than types 16 and 18.* Int. J. Cancer, 2017. **140**(8): p. 1747-1756.
236. Depuydt, C.E., et al., *Changes in type-specific human papillomavirus load predict progression to cervical cancer.* J. Cell Mol. Med, 2012. **16**(12): p. 3096-3104.
237. von Karsa, L., et al., *European guidelines for quality assurance in cervical cancer screening. Summary of the supplements on HPV screening and vaccination.* Papillomavir. Res, 2015. **1**: p. 22-31.
238. Micalessi, I.M., et al., *High-throughput detection, genotyping and quantification of the human papillomavirus using real-time PCR.* Clin. Chem. Lab. Med, 2012. **50**(4): p. 655-661.
239. Xu, L., et al., *Clinical Evaluation of INNO-LiPA HPV Genotyping EXTRA II Assay Using the VALGENT Framework.* Int. J. Mol. Sci, 2018. **19**(9).
240. Viti, J., et al., *Validation of EUROArray HPV test using the VALGENT framework.* J. Clin. Virol, 2018. **108**: p. 38-42.
241. Ostrbenk, A., et al., *Clinical and analytical evaluation of the Anyplex II HPV HR Detection assay within the VALGENT-3 framework.* J. Clin. Microbiol, 2018. **56**(11): p. e01176-18.
242. Arbyn, M., et al., *European guidelines for quality assurance in cervical cancer screening: recommendations for collecting samples for conventional and liquid-based cytology.* Cytopathology, 2007. **18**(3): p. 133-139.
243. Depuydt, C.E., et al., *Clinical validation of a type-specific real time quantitative human papillomavirus PCR to the performance of Hybrid Capture 2 for the purpose of cervical cancer screening.* J. Clin. Microbiol, 2012. **50**(12): p. 4073-4077.

244. Ghittoni, R., et al., *Role of human papillomaviruses in carcinogenesis.* Ecancermedicalscience, 2015. **9**: p. 526.
245. Benoy, I., et al., *Using the VALGENT-3 framework to assess the clinical and analytical performance of the RIATOL qPCR HPV genotyping assay.* J. Clin. Virol, 2019. **120**: p. 57-62.
246. Bonde, J.H., et al., *Clinical and analytical performance of the BD Onclarity HPV Assay with SurePath screening samples from the Danish cervical screening program using the VALGENT framework.* J. Clin. Microbiol, 2020. **58**(2).
247. Bonde, J., et al., *The Valgent4 protocol: Robust analytical and clinical validation of 11 HPV assays with genotyping on cervical samples collected in SurePath medium.* J. Clin. Virol, 2018. **108**: p. 64-71.
248. Bian, M.L., et al., *Evaluation of the detection of 14 high-risk human papillomaviruses with HPV 16 and HPV 18 genotyping for cervical cancer screening.* Exp. Ther. Med, 2013. **6**(5): p. 1332-1336.
249. Xue, P., et al., *A direct comparison of four high-risk human papillomavirus tests versus the cobas test: Detecting CIN2+ in low-resource settings.* J. Med. Virol, 2019. **91**(7): p. 1342-1350.
250. Arbyn, M., et al., *Cervical Cytology Biobanking in Europe.* Int. J. Biol. Markers, 2010. **25**(3): p. 117-125.
251. Eklund, C., et al., *Continuing global improvement in human papillomavirus DNA genotyping services: The 2013 and 2014 HPV LabNet international proficiency studies.* J. Clin. Virol, 2018. **101**: p. 74-85.
252. Zuna, R.E., et al., *Determinants of human papillomavirus-negative, low-grade squamous intraepithelial lesions in the atypical squamous cells of undetermined significance/low-grade squamous intraepithelial lesions triage study (ALTS).* Cancer, 2005. **105**(5): p. 253-262.
253. Rebolj, M., E. Lynge, and J. Bonde, *Human papillomavirus testing and genotyping in cervical screening.* Expert. Rev. Anticancer Ther, 2011. **11**(7): p. 1025-1033.
254. Geraets, D.T., et al., *Correction for Geraets et al., Clinical Evaluation of a GP5+/6+-Based Luminex Assay Having Full High-Risk Human Papillomavirus Genotyping Capability and an Internal Control.* J Clin. Microbiol, 2016. **54**(7): p. 1927.
255. Harbord, R.M., et al., *A unification of models for meta-analysis of diagnostic accuracy studies.* Biostatistics, 2007. **8**: p. 239-251.
256. Harbord, R.M. and P. Whiting, *metandi: Meta-analysis of diagnostic accuracy using hierarchical logistic regression.* The Stata Journal, 2009. **9**(2): p. 211-229.
257. Takwoingi, Y., M.M. Leeflang, and J.J. Deeks, *Empirical evidence of the importance of comparative studies of diagnostic test accuracy.* Ann. Intern. Med, 2013. **158**(7): p. 544-554.
258. Castle, P.E., et al., *Risk assessment to guide the prevention of cervical cancer.* Am. J. Obstet. Gynecol, 2007. **197**(4): p. 356-356.

259. Arbyn, M., P. Martin-Hirsch, and N. Wentzensen, *Human papillomavirus-based triage of women showing a cervical cytology result of borderline or mild dyskaryosis.* BJOG, 2010. **117**(6): p. 641-644.
260. Del Mistro, A., et al., *Human papilloma virus genotyping for the cross-sectional and longitudinal probability of developing cervical intraepithelial neoplasia grade 2 or more.* Int. J. Cancer, 2018. **143**(2): p. 333-342.
261. Wentzensen, N., et al., *p16/Ki-67 Dual Stain Cytology for Detection of Cervical Precancer in HPV-Positive Women.* J. Natl. Cancer Inst, 2015. **107**(12): p. djv257-?
262. Davey, D.D., et al., *Atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion: review of ancillary testing modalities and implications for follow-up.* J. Low Genit. Tract. Dis, 2010. **14**(3): p. 206-214.
263. Zhao, C., et al., *Human papillomavirus testing and reporting rates in 2012: results of a College of American Pathologists national survey.* Arch. Pathol. Lab Med, 2015. **139**(6): p. 757-761.
264. Pretorius, R.G., et al., *Comparison of two signal-amplification DNA tests for high-risk HPV as an aid to colposcopy.* J. Reprod. Med, 2002. **47**: p. 290-296.
265. Galliano, G.E., et al., *Reflex high risk HPV testing in atypical squamous cells, cannot exclude high grade intraepithelial lesion: a large institution's experience with the significance of this often ordered test.* Acta Cytol, 2011. **55**(2): p. 167-172.
266. Wang, Z.M., *PAX1 methylation analysis by MS-HRM is useful in triage of high-grade squamous intraepithelial lesions.* Asian Pac. J. Cancer Prev, 2014. **15**(2): p. 891-894.
267. Monsonego, J., et al., *Detection of Human Papillomavirus Genotypes Among High-Risk Women: A Comparison of Hybrid Capture and Linear Array Tests.* Sex Transm. Dis, 2008. **35**(5): p. 521-527.
268. Saslow, D., et al., *American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology screening guidelines for the prevention and early detection of cervical cancer.* Am J Clin Pathol, 2012. **137**(4): p. 516-542.
269. Sherman, M.E., P.E. Castle, and D. Solomon, *Cervical cytology of atypical squamous cells-cannot exclude high-grade squamous intraepithelial lesion (ASC-H): characteristics and histologic outcomes.* Cancer, 2006: p. 298-305.
270. Katki, H.A., et al., *Five-Year Risks of CIN 3+ and Cervical Cancer Among WomenWho Test Pap-Negative But Are HPV-Positive.* J. Low Genit. Tract Dis, 2013. **17**(5 Suppl 1): p. S56-S63.
271. Cohen, D., et al., *Follow-up Outcomes in a Large Cohort of Patients With Human Papillomavirus–Negative ASC-H Cervical Screening Test Results.* Am. J. Clin. Pathol, 2012. **138**(4): p. 517-523.

272. Arbyn, M., et al., *Peri-natal mortality and other severe adverse pregnancy outcomes associated with treatment of cervical intraepithelial neoplasia: a meta-analysis.* BMJ, 2008. **337**: p. a1284.
273. Whiting, P.F., et al., *QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies.* Ann Intern Med, 2011. **155**(8): p. 529-536.
274. Reitsma, J.B., et al., *Bivariate analysis of sensitivity and specificity produces informative summary measures in diagnostic reviews.* J. Clin. Epidemiol, 2005. **58**(10): p. 982-990.
275. Nyaga, V.N., M. Arbyn, and A. M, *Metaprop: a Stata command to perform meta-analysis of binomial data.* Arch. Public Health, 2014. **72**(1): p. 39.
276. Harris, R., et al., *metan: fixed- and random-effects meta-analysis.* The Stata Journal, 2008. **8**(1): p. 3-26.
277. Takwoingi, Y. and J. Deeks *METADAS: A SAS macro for meta-analysis of diagnostic accuracy studies. Quick reference and worked example.* https://methods. cochrane. org/sites/methods. cochrane. org. sdt/files/public/uploads/MetaDAS%20Quick%20Reference%20v1. 3%20May%202012. pdf, 2010. Version 1.3.
278. Passamonti, B., et al., *Expression of p16 in abnormal pap-tests as an indicator of CIN2+ lesions: a possible role in the low grade ASC/US and L/Sil (lg) cytologic lesions for screening prevention of uterine cervical tumours.* Pathologica, 2010. **102**: p. 6-11.
279. Alaghehbandan, R., et al., *Performance of ProEx C and PreTect HPV-proofer E6/E7 mRNA tests in comparison with the hybrid capture 2 HPV DNA test for triaging ASCUS and LSIL cytology.* Diagn. Cytopathol, 2013. **41**: p. 767-775.
280. Katki, H.A., et al., *Five-year risks of CIN 3+ and cervical cancer among women with HPV-positive and HPV-negative high-grade Pap results.* J Low Genit. Tract. Dis, 2013. **17**(5 Suppl 1): p. S50-S55.
281. Castle, P.E., et al., *The age-specific relationships of abnormal cytology and human papillomavirus DNA results to the risk of cervical precancer and cancer.* Obstet. Gynecol, 2010. **116**(1): p. 76-84.
282. Owens, C.L., et al., *"Low-grade squamous intraepithelial lesion, cannot exclude high-grade squamous intraepithelial lesion" is a distinct cytologic category: histologic outcomes and HPV prevalence.* Am J Clin Pathol, 2007. **128**(3): p. 398-403.
283. Srodon, M., D.H. Parry, and B.M. Ronnett, *Atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion.* Cancer, 2006. **108**(1): p. 32-38.
284. Lonky, N.M., et al., *Triage of atypical squamous cells of undetermined significance with Hybrid Capture II: colposcopy and histologic human papillomavirus correlation.* Obstet. Gynecol, 2003. **101**(3): p. 481-489.

285. Wentzensen, N., et al., *Performance of p16/Ki-67 immunostaining to detect cervical cancer precursors in a colposcopy referral population.* Clin. Cancer Res, 2012. **18**(15): p. 4154-4162.

286. Lorenzato, M., et al., *Contribution of DNA ploidy image cytometry to the management of ASC cervical lesions.* Cancer, 2008. **114**(4): p. 263-269.

287. You, K., et al., *The use of HPV test in patients with cervical cytology interpreted as ASC-H.* Acta Cytol, 2013. **57**: p. 85-85.

288. Wu, H.H., et al., *Reflex high-risk human papilloma virus DNA test is useful in the triage of women with atypical squamous cells cannot exclude high-grade squamous intraepithelial lesion.* Diagn. Cytopathol, 2006. **34**(10): p. 707-710.

289. Reid-Nicholson, M., et al., *Atypical squamous cells, cannot exclude high grade intraepithelial lesion (ASC-H): Does HPV matter?* Diagn. Cytopathol, 2007. **35**(1): p. 1-5.

290. You, K., et al., *High-risk human papillomavirus DNA testing and high-grade cervical intraepithelial lesions.* Aust N Z J Obstet Gynaecol, 2007. **47**(2): p. 141-144.

291. Bandyopadhyay, S., et al., *Adjunctive human papillomavirus DNA testing is a useful option in some clinical settings for disease risk assessment and triage of females with ASC-H Papanicolaou test results.* Arch Pathol Lab Med, 2008. **132**(12): p. 1874-1881.

292. Rao, A., et al., *The role of HPV testing in patients with possible high-grade cervical cytology.* J. Obstet. Gynaecol. Res, 2009. **35**(3): p. 503-506.

293. Samarawardana, P., et al., *p16(INK4a) is superior to high-risk human papillomavirus testing in cervical cytology for the prediction of underlying high-grade dysplasia.* Cancer Cytopathol, 2010. **118**(3): p. 146-156.

294. Sung, C.O., et al., *The use of p16(INK4A) immunocytochemistry in "Atypical squamous cells which cannot exclude HSIL" compared with "Atypical squamous cells of undetermined significance" in liquid-based cervical smears.* Diagn. Cytopathol, 2010. **38**(3): p. 168-171.

295. Gilani, S.M., R. Tashjian, and L. Fathallah, *Cervical cytology with a diagnosis of atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion (ASC-H): a follow-up study with corresponding histology and significance of predicting dysplasia by human papillomavirus (HPV) DNA testing.* Arch. Gynecol. Obstet, 2014. **289**(3): p. 645-648.

296. Koo, Y.J., et al., *Dual immunostaining of cervical cytology specimens with atypical squamous cells for p16/Ki-67 does not exclude the existence of a high-grade squamous intraepithelial lesion.* Virchows Arch, 2013. **463**(5): p. 689-696.

297. Lee, S., et al., *Clinical significance of HPV DNA cotesting in Korean women with ASCUS or ASC-H.* Diagn. Cytopathol, 2014.

298. Killeen, J.L., et al., *Improved Abnormal Pap Smear Triage Using Cervical Cancer Biomarkers.* J. Low Genit. Tract. Dis, 2014. **18**(1): p. 1-7.

299. Pabuccu, E.G., et al., *Diagnostic performance of p16 staining in atypical squamous cells 'cannot exclude high-grade squamous epithelial lesion' in predicting high-grade cervical pathology.* J. Obstet. Gynaecol, 2014(34): p. 1-5.
300. Chen, L., et al., *HPV testing results and histologic follow-up in women with ASC-H cytology in different age groups.* J Am Soc Cytopathology, 2015. **4**(4): p. 225-231.
301. Monsonego, J., et al., *Linear array genotyping and hybrid capture II assay in detecting human papillomavirus genotypes in women referred for colposcopy due to abnormal Papanicolaou smear.* Int. J. STD AIDS, 2008. **19**(6): p. 385-392.
302. Arbyn, M., et al., *Use of HC2 to triage women with borderline and mild dyskaryosis in the UK.* Br J Cancer, 2011. **105**(7): p. 877-880.
303. Katki, H.A., et al., *Five-Year Risks of CIN 3+ and Cervical Cancer Among Women With HPV Testing of ASC-US Pap Results.* J. Low Genit. Tract. Dis, 2013. **17**(5 Suppl 1): p. S36-S42.
304. Gupta, S., et al., *Outcome of "Atypical squamous cells" in a cervical cytology screening program: implications for follow up in resource limited settings.* Diagn. Cytopathol, 2007. **35**(11): p. 677-680.
305. Liman, A.K., E.J. Giampoli, and T.A. Bonfiglio, *Should women with atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion, receive reflex human papillomavirus-DNA testing?* Cancer, 2005. **105**: p. 457-460.
306. Arbyn, M., et al., *Triage of women with equivocal or low-grade cervical cytology results. A meta-analysis of the HPV test positivity rate.* J. Cell. Mol. Med, 2009. **13**(4): p. 648-659.
307. Guyatt, G.H., et al., *What is "quality of evidence" and why is it important to clinicians?* BMJ, 2008. **336**(7651): p. 995-998.
308. Einstein, M.H., et al., *Comparative immunogenicity and safety of human papillomavirus (HPV)-16/18 vaccine and HPV-6/11/16/18 vaccine: Follow-up from Months 12-24 in a Phase III randomized study of healthy women aged 18-45 years.* Hum. Vaccin, 2011. **7**(12): p. 1343-1358.
309. Bosch, F.X., et al., *Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia.* Vaccine, 2008. **26 Suppl 10**: p. K1-K16.
310. Bosch, F.X., *HPV vaccines and cervical cancer.* Ann. Oncol, 2008. **19 Suppl 5**: p. v48-v51.
311. Lehtinen, M., et al., *Population-based enrolment of adolescents in a long-term follow-up trial of human papillomavirus vaccine efficacy.* Int. J. STD AIDS, 2006. **17**(4): p. 237-246.
312. Baldur-Felskov, B., et al., *Incidence of cervical lesions in Danish women before and after implementation of a national HPV vaccination program.* Cancer Causes Control, 2014. **25**(7): p. 915-922.
313. Baldur-Felskov, B., et al., *Early Impact of Human Papillomavirus Vaccination on Cervical Neoplasia--Nationwide Follow-up of Young Danish Women.* J. Natl. Cancer Inst, 2014.

314. Brotherton, J.M., et al., *Early effect of the HPV vaccination programme on cervical abnormalities in Victoria, Australia: an ecological study.* Lancet, 2011. **377**(9783): p. 2085-2092.
315. Kavanagh, K., et al., *Introduction and sustained high coverage of the HPV bivalent vaccine leads to a reduction in prevalence of HPV 16/18 and closely related HPV types.* Br. J. Cancer, 2014. **110**(11): p. 2804-2811.
316. Leval, A., et al., *Quadrivalent human papillomavirus vaccine effectiveness: a Swedish national cohort study.* J. Natl. Cancer Inst, 2013. **105**(7): p. 469-474.
317. Markowitz, L.E., et al., *Reduction in Human Papillomavirus (HPV) Prevalence Among Young Women Following HPV Vaccine Introduction in the United States, National Health and Nutrition Examination Surveys, 2003-2010.* J. Infect. Dis, 2013. **208**(3): p. 385-393.
318. Markowitz, L.E., et al., *Human papillomavirus vaccine effectiveness by number of doses: Systematic review of data from national immunization programs.* Vaccine, 2018. **36**(32 Pt A): p. 4806-4815.
319. Merckx, M., et al., *Early effects of HPV vaccination in Belgium.* Eur. J. Cancer Prev, 2015. **24**(4): p. 340-342.
320. Gee, J., et al., *Monitoring the safety of quadrivalent human papillomavirus vaccine: findings from the Vaccine Safety Datalink.* Vaccine, 2011. **29**(46): p. 8279-8284.
321. Moro, P.L., et al., *Safety of influenza A (H1N1) 2009 live attenuated monovalent vaccine in pregnant women.* Obstet Gynecol, 2013. **122**(6): p. 1271-8.
322. Suragh, T.A., et al., *Safety of bivalent human papillomavirus vaccine in the US vaccine adverse event reporting system (VAERS), 2009-2017.* Br J Clin Pharmacol, 2018. **84**(12): p. 2928-2932.
323. Shimabukuro, T.T., et al., *Safety of the 9-Valent Human Papillomavirus Vaccine.* Pediatrics, 2019. **144**(6).
324. Arana, J.E., et al., *Post-licensure safety monitoring of quadrivalent human papillomavirus vaccine in the Vaccine Adverse Event Reporting System (VAERS), 2009-2015.* Vaccine, 2018. **36**(13): p. 1781-1788.
325. Donahue, J.G., et al., *Near Real-Time Surveillance to Assess the Safety of the 9-Valent Human Papillomavirus Vaccine.* Pediatrics, 2019. **144**(6).
326. Kreimer, A.R., et al., *Proof-of-principle evaluation of the efficacy of fewer than three doses of a bivalent HPV16/18 vaccine.* J. Natl. Cancer Inst, 2011. **103**(19): p. 1444-1451.
327. Arbyn, M., et al., *The European response to the WHO call to eliminate cervical cancer as a public health problem.* Int. J. Cancer, 2020.
328. Ejegod, D.M., et al., *Clinical validation of the Cobas 4800 HPV assay using cervical samples in SurePath medium under the VALGENT4 framework.* J. Clin. Virol, 2020. **128**: p. 104336.
329. Heideman, D.A.M., et al., *Clinical performance of the HPV-Risk assay on cervical samples in SurePath medium using the VALGENT-4 panel.* J. Clin. Virol, 2019. **121**: p. 104201-1- 5.

330. Xu, L., et al., *Accuracy of genotyping for HPV16 and 18 to triage women with low-grade squamous intraepithelial lesions: a pooled analysis of VALGENT studies.* Expert. Rev. Mol. Diagn, 2019. **19**(6): p. 443-451.
331. Xu, L., et al., *Evaluation and optimization of the clinical accuracy of Hybribio's 14 High-risk HPV with 16/18 Genotyping assay within the VALGENT-3 framework.* J. Clin. Microbiol, 2020. **58**(6): p. ee00234-20.
332. Arbyn, M., et al., *Cervical cancer screening program and Human Papillomavirus (HPV) testing, part II: Update on HPV primary screening.* KCE reports, 2015. **238**: p. 1-282.
333. Dijkstra, M.G., et al., *Safety of extending screening intervals beyond five years in cervical screening programmes with testing for high risk human papillomavirus: 14 year follow-up of population based randomised cohort in the Netherlands.* BMJ, 2016. **355**: p. i4924.
334. Kitchener, C., et al., *The clinical effectiveness and cost-effectiveness of primary human papillomavirus cervical screening in England: extended follow-up of the ARTISTIC randomised trial cohort through three screening rounds.* Health Technol. Assess, 2014. **18**(23): p. 1-196.
335. Verdoodt, F., et al., *High-risk HPV testing in the management of atypical glandular cells: a systematic review and meta-analysis.* Int. J. Cancer, 2016. **138**(2): p. 303-310.
336. WHO *Global strategy towards the elimination of cervical cancer as a public health problem*. WHO, 2019.
337. Simms, K.T., et al., *Impact of scaled up human papillomavirus vaccination and cervical screening and the potential for global elimination of cervical cancer in 181 countries, 2020-99: a modelling study.* Lancet Oncol, 2019. **20**(3): p. 394-407.
338. WHO, *Global market study-HPV*. 2019.
339. El-Zein, M., L. Richardson, and E.L. Franco, *Cervical cancer screening of HPV vaccinated populations: Cytology, molecular testing, both or none.* J Clin. Virol, 2016. **76 Suppl 1**: p. S62-S68.
340. Simms, K.T., et al., *Optimal Management Strategies for Primary HPV Testing for Cervical Screening: Cost-Effectiveness Evaluation for the National Cervical Screening Program in Australia.* PLoS. ONE, 2017. **12**(1): p. e0163509.
341. Kim, J.J. and S.J. Goldie, *Cost effectiveness analysis of including boys in a human papillomavirus vaccination programme in the United States.* BMJ, 2009. **339**: p. b3884.
342. Ronco, G., et al., *Extension of organized cervical cancer screening programmes in Italy and their process indicators, 2011-2012 activity.* Epidemiol. Prev, 2015. **39**(3 Suppl 1): p. 61-76.
343. Verhoef, V.M., et al., *Triage by methylation-marker testing versus cytology in women who test HPV-positive on self-collected cervicovaginal specimens (PROHTECT-3): a randomised controlled non-inferiority trial.* Lancet Oncol, 2014. **15**(3): p. 315-22.

344. Malagon, T., C. Laurie, and E.L. Franco, *Human papillomavirus vaccination and the role of herd effects in future cancer control planning: A review.* Expert Rev. Vaccines, 2018. **17**(5): p. 395-409.

345. Arbyn, M., et al., *VALHUDES: a protocol for VALidation of HUman papillomavirus assays and collection DEvices for HPV testing on Self-samples and urine samples.* J. Clin. Virol, 2018. **117**: p. 52-56.

346. Blatt, A.J., et al., *Comparison of cervical cancer screening results among 256,648 women in multiple clinical practices.* Cancer Cytopathol, 2015. **123**(5): p. 282-288.