A MULTIDIMENSIONAL APPROACH TO IMPROVE CERVICAL CANCER PREVENTION

Renée Ebisch

A multidimensional approach to improve cervical cancer prevention

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For reasons of consistency within this thesis, some terms and abbreviations have been standardized troughout the text, and might therefore slightly differ from the original publications.

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CHAPTER 1

Introduction, aim and outline of this thesis

INTRODUCTION

Cervical cancer is the fourth most common cancer in women, with an estimated 527,600 new cancer cases and 265,700 deaths worldwide in 2012.[1] Cervical cancer is preceded by a premalignant stage which is estimated to progress to cervical cancer in 10-15 years. Premalignant lesions can therefore be detected by screening, to be treated before malignant potential occurs.[2] Since the introduction of cytology-based cervical cancer screening, the incidence of cervical cancer in developed countries has substantially diminished.[3] Nowadays, cervical cytology is used as primary screening test in the majority of programs.[4] However, many Western countries are on the verge of replacing cytology as primary screening by testing for the presence of high-risk human papillomavirus (hrHPV). The Netherlands is among the first countries with a full hrHPV-based organized cervical cancer screening program in 2017.

Triage of high-risk HPV positive women

A persistent infection with an hrHPV is causally related to the development of cervical cancer.[5] This results in a high sensitivity of hrHPV-based cervical cancer screening. Other advantages of primary hrHPV testing over cytology-based screening are the objectivity of the assay, high-throughput testing, and the possibility of analyzing self-sampled material, which may improve the efficacy of cervical cancer screening by increasing participation of non-responders.[6-9] A limitation of hrHPV testing is its inability to distinguish transient infections from clinically relevant infections. This results in limited specificity, with higher numbers of unnecessary colposcopy referrals compared with cytology screening. Effective triage of hrHPV-positive women is therefore essential. Different triage options are available, which can be divided into: morphological triage techniques, like Pap cytology and other immunochemistry markers; and molecular triage techniques, like HPV genotyping and methylation markers. These markers all have their own advantages and disadvantages, and knowledge on clinical value of most of these markers should be increased before they can be implemented in a screening program.

Opportunities in improving colposcopic examination

When an abnormal result is detected during cervical screening and triage, women are generally referred to their gynecologist for a diagnostic colposcopic examination. Colposcopy is the visualization of the cervix using a stereoscopic binocular microscope of low magnification. Colposcopic examination combined with a biopsy and histologic evaluation is the gold standard for identifying premalignant cervical intraepithelial neoplasia (CIN). Low-grade CIN are usually monitored by watchful waiting, and regress to normal without treatment in the majority of cases. When a high-grade CIN is suspected, treatment is recommended in most cases. A limitation of colposcopy is the high degree

of inter- and intraobserver variability, with a low-to-average sensitivity of 61% with a specificity of 85% for CIN2 or worse. This results in missed lesions, unnecessary biopsies or overtreatment.[10-12] Improving diagnostics and management of women referred for colposcopy is therefore desirable.

Future risks of high-risk HPV infections

Because of the low specificity of hrHPV screening for young women, the Dutch screening program starts at 30 years of age. Younger women with cervical cancer or high-grade CIN lesions are missed with this screening strategy. The risk of sexual transmission of HPV generally peaks early in sexual life and declines with higher age.[13] However, only a minority of these infections in young women eventually develop into cervical cancer. Most ot these hrHPV infections are transient and clear spontaneously; two thirds of all infections can be cleared by the host immune system within 12 months, and over 90% can be cleared within 24 months.[14,15] It is yet unknown to which extent a transient or persistent hrHPV infection in young women is a risk factor for developing CIN or cervical cancer later in life. This knowledge may improve recommendations for women who test positive for hrHPV early in life.

When an hrHPV infection is not cleared by the host immune system, it becomes a persisting infection. A persisting infection may lead to the development of a high-grade CIN lesion. It is known that women diagnosed with high-grade CIN show an increased risk of developing malignancies of the anogenital region and head and neck region, probably as the result of an infection with an hrHPV. The extent of this risk has never been studied with data of Dutch women, and the extent of this risk for high-grade premalignancies after a diagnosis of CIN3 is also unknown. Knowledge on this risk is important to consider preventing future risks of hrHPV infections by prophylactic HPV vaccination and/or intensified screening for other HPV-related carcinomas and premalignancies when a high-grade CIN lesion is identified.

AIM AND OUTLINE OF THIS THESIS

In this thesis, we aim to improve cervical cancer prevention programs with studies on multiple dimensions of cervical cancer prevention.

Part I focuses on improving triage of hrHPV-positive women in cervical cancer screening. Triage is necessary because of the limited specificity of hrHPV-based screening. In **chapter 2** an overview of triage strategies for hrHPV-positive women is provided, differentiating between morphological and molecular triage techniques. Furthermore, future perspectives of promising triage-strategies are discussed. In **chapter 3** the clinical value of HPV genotyping in triage of women with hrHPV-positive self-samples was evaluated.

HPV genotyping is studied as individual triage method, and combined with Pap cytology at different thresholds. The clinical value of different combinations of triage-strategies with HPV genotyping is studied. Findings on p16/Ki-67 dual-stain as triage-technique for hrHPV-positive women are presented in **chapter 4**. This chapter evaluates the clinical utility of this dual-stain, either or not combined with other triage techniques, and results are put into perspective by comparing them with previously published studies.

Part II focuses on opportunities in improving colposcopic assessment after an abnormal cervical screening and/or triage result. If a high-grade intraepithelial lesion is suspected, treatment is recommended in most cases. Treatment can be performed after histological confirmation of the lesion, which is called a two-step approach. However, treatment can also be performed immediately if a high-grade lesion is suspected, without the need of an additional visit, which is called the see-and-treat strategy. It is important to decide under which circumstances see-and-treat management may be used with limited overtreatment-rates. **Chapter 5** systematically reviews overtreatment-rates in see-and-treat management in regard to cytology results and the colposcopic assessment. To further improve colposcopy, digital colposcopy techniques are upcoming and increasingly studied. In **chapter 6** we performed a pilot-study on fluorescence and reflectance spectroscopy, a digital colposcopy technique that combines two types of spectroscopy. In this study, we assessed performance and patient acceptance of this technique. As a large variety of digital colposcopy techniques are showing promising results, all with all different perspectives in screening, an overview of this novel technique is needed. We therefore performed a meta-analysis on results of three different colposcopy techniques which could potentially lower colposcopy referral rates. Result of this systematic review and meta-analysis are shown in **chapter 7**.

Part III focuses on future risks of hrHPV infections. The risk of future CIN lesions in women who tested HPV positive before the age of 30 was assessed in **chapter 8**. These women either cleared their infection or showed a persistent infection. Data on development of high-grade CIN lesions or cervical cancer within eight years of follow-up was obtained from the Dutch nationwide database of pathology. In **chapter 9** we take it one step further and look at the future risk of developing other HPV-related premalignancies and carcinomas when a high-grade CIN has been diagnosed. We compare this risk with the 'baseline risk' in a population without a previously diagnosed high-grade CIN of the cervix. An increased risk of other HPV-related premalignancies and carcinomas could be a reason for considering intensified screening or prophylactic HPV vaccination in women treated for a high-grade CIN lesion.

The results of these previous chapters are put into perspective in a general discussion in **chapter 10**, and a summary is given in **chapter 11**.

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CHAPTER 2

Triage of high-risk HPV positive women in cervical cancer screening

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ABSTRACT

Introduction High-risk human papillomavirus (hrHPV) testing is expected to replace cytology as primary screening method for cervical cancer screening in an increasing number of countries. The high sensitivity of hrHPV testing is combined with a limited specificity, which makes triaging of hrHPV-positive women necessary. As an ideal triage method does not yet exist, an optimal triage strategy for hrHPV-positive women, based on current knowledge, should be obtained. The aim of this article is to present an overview of available options for triage of hrHPV-positive women, with their strengths and limitations and possible future opportunities.

Areas covered Current knowledge on morphological biomarkers, molecular biomarkers and combined triage strategies will be discussed, to give an overview of the state-of-the-art on triaging hrHPV-positive women. The literature search was limited to studies on triage strategies for hrHPV-positive women.

Expert commentary Experience with morphology-based biomarkers makes these biomarkers a valuable triage method. However, they lack the ability of differentiating productive from transforming infections. Molecular biomarkers are objective, highly reproducible, can be used in high throughput testing, and show promising results. With more extensive knowledge on these molecular markers, cervical cancer screening may transform to a full molecular screening in the future.

1. INTRODUCTION

With an estimated 527,600 new cancer cases and 265,700 deaths in 2012, cervical cancer is the fourth most diagnosed cancer and fourth cause of cancer death in females worldwide. Developing countries account for almost 90% of all cervical cancer deaths, and in some countries in Melanesia, eastern, middle, and southern Africa, it even is the leading cause of cancer death amongst females. Cervical cancer incidence and mortality rates are lowest in Europe, eastern and western Asia, northern Africa, northern America, Australia and New Zealand.[1] The availability of screening and differences in human papillomavirus (HPV) prevalence are the cause of these major geographic variations.[2]

The role of HPV in development of cervical cancer was first described by zur Hausen in 1977, for which he received the Nobel Prize in Physiology or Medicine in 2008.[3] Approximately two decades later, Walboomers described the necessity of a persistent infection with high-risk human papillomavirus (hrHPV) for the development of precancerous and cancerous lesions of the cervix.[4] Since then, over 200 HPV genotypes have been identified and clinically relevant types are grouped by the innate risk of causing cervical cancer (Table 1).[5] Low-risk types generally only induce benign warts, whereas high-risk (hr)HPV types have the ability to induce cervical premalignancies and malignancies, of which approximately 70% is caused by types 16 and 18. The lifetime risk of an infection with hrHPV is high, however, only a minority of infections develop into cervical cancer. After 12 months, two-thirds of all infections are already cleared by the host immune system, and after 24 months over 90% are cleared.[6,7] Infections that are not cleared may develop into 'productive' infections, cytologically and histologically known as low-grade squamous intraepithelial lesion (LSIL), or histologically known as low-grade cervical intraepithelial neoplasia (CIN; CIN grade 1). These infections morphologically show dysplastic features overlapping with those in progressive precancers. However, such infections show no signs of cellular transformation and the majority of productive infections still clear quickly. Only a minority of all persistent hrHPV infections results in altered E6 and E7 viral gene expression, thereby becoming a 'transforming' infection. In transforming infections, the normal viral life cycle is aborted and the viral early genes *E6* and *E7* are overexpressed in proliferating cells, leading to altered expression of cell cycle and DNA repair regulators (Figure 1).[8-10] Transforming infections are cytologically

Table 1. Human papillomavirus types grouped by the innate risk of causing cervical cancer.

IARC group[6]	Risk estimate	HPV types
1	High-risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59
2A	Probable high-risk	68
2B	Possible high-risk	26, 53, 66, 67, 70, 73, 82
3	Low-risk	6, 11

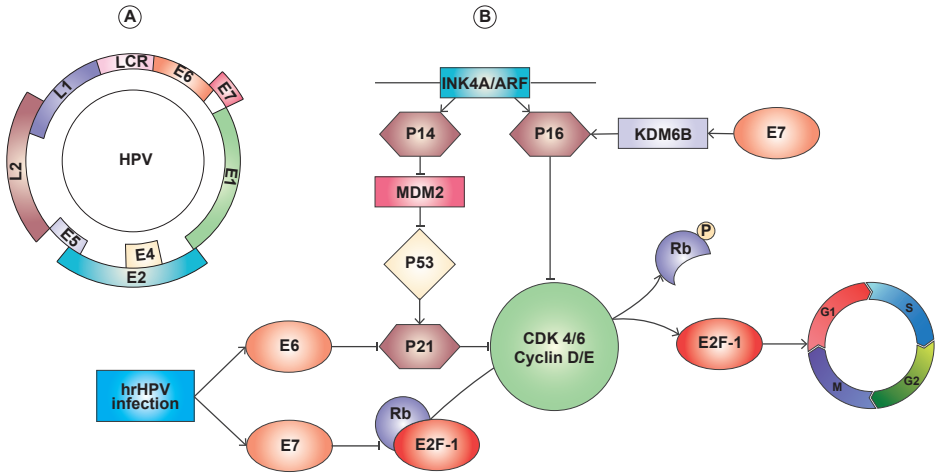

Figure 1. Molecular mechanisms by which the human papillomavirus induces cervical carcinogenesis. **(A)** Genome organization of the human papillomavirus (HPV). HPV are DNA viruses coding for a long control region (LCR), several early functional genes (E1-E7), and two late structural genes (L1-L2). **(B)** The INK4A/ARF locus at chromosome 9p21 encodes proteins $p16^{INK4a}$ (p16) and $p14^{ARF}$ (p14) that ultimately link the Retinoblastoma (Rb) and p53 tumor suppressor pathways. p16 is a cyclin-dependent kinase (CDK) inhibitor that prohibits progression form G1 phase to S phase and slowing down the cell cycle. In a normal cell, p16 acts as a tumor suppressor by binding to CDK4/6 and preventing its interaction with cyclin D resulting in arrest of cell proliferation. p14 inhibits mdm2, therefore promotes p53, which promotes p21 activation. p21 binds and inactivates certain cyclin-CDK complexes which otherwise would promote transcription of genes that would carry the cell trough G1/S checkpoint of the cell cycle resulting in S-phase induced p16 stimulation. When hrHPV types integrate into the host genome, loss of negative feedback control will result in increased expression of viral E6 and E7 oncogenes. HPV protein E6 binds tumor suppressor gene p53 and promotes its destruction, resulting in inhibition of apoptosis and loss of inhibition of cyclin-CDK complexes via loss of p21 stimulation. CDK4/6 binds cyclin D and forms an active protein complex that phosphorylates Rb which disassociates from transcription factor E2F1. Liberated E2F1 enters the nucleus and promotes transcription of target genes essential for transition from G1 to S phase. HPV protein E7 dissociates the E2F-Rb complex and binds and inactivates Rb. This also causes release of transcriptionally E2F1 dependent genes necessary for DNA replication, resulting in stop of growth arrest and therefore progression the cell cycle. HPV E7 oncoprotein expression also induces KDM6B histone demethylase expression, which triggers the p16 promoter, also resulting in upregulation of p16 expression. Stimulation of progression of the cell cycle, combined with loss of apoptosis results in immortalization, genomic instability and finally in increased risk of transformation and malignant progression.[8-11]

and histologically known as high-grade squamous intraepithelial lesion (HSIL) or histologically known as high-grade CIN (CIN grade 3), which may finally result in cancer if left untreated (Figure 2). These premalignant stages preceding cervical cancer allow for detection and treatment of these lesions before they progress to cervical cancer.

Screening has been very successful in decreasing cervical cancer incidence and mortality in Western countries.[12] Screening programs worldwide differ regarding age, frequency, participation rate and screening modality.[13] Nowadays, cervical cytology is used as primary screening test in the majority of programs.[14] However, many Western

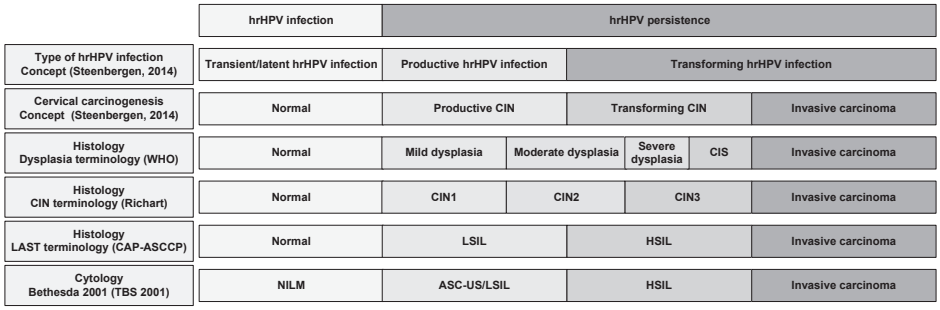

Figure 2. Cervical carcinogenesis and morphological abnormality.
Viral persistence of hrHPV can result in productive infections with a productive CIN lesion, and a transforming infection with a transforming CIN. Morphologically no clear distinction between productive and transforming CIN lesions can be made so histological and cytological terminology and classification systems are not one-on-one linkable to this concept of productive and transforming infections. In the 3-tiered CIN terminology productive CIN are visible as CIN1 and transforming CIN lesions are morphologically known as CIN3. CIN2 is a mixture and is not comparable with a biological state. Therefore, this classification system is arbitrary and does not correspond to our current understanding of HPV infection and precancer. The 4-tiered dysplasia spectrum includes mild dysplasia, moderate dysplasia, severe dysplasia and CIS. The most recent LAST terminology only distinguishes LSIL from HSIL histology and therefore corresponds most accurately with the concept of productive and transforming infections. Cytology is generally classified according to the Bethesda system. Low-grade cytology results as ASC-US and LSIL generally represent productive CIN lesions, and high-grade cytology as HSIL represents transforming CIN lesions. To simplify the figure, atypical glandular cells (AGC), and atypical squamous cell-cannot exclude high-grade squamous intraepithelial lesions (ASC-H) were not included. ASCCP = American Society for Colposcopy and Cervical Pathology; ASC-US = Atypical squamous cells of undetermined significance; CAP = College of American Pathologists; CIN = Cervical intraepithelial neoplasia; CIS = Carcinoma in situ; hrHPV = High-risk human papillomavirus; HSIL = High-grade squamous intraepithelial lesion; LAST = Lower Anogenital Squamous Terminology; LSIL = Low-grade squamous intraepithelial lesion; NILM = Negative for intraepithelial lesion or malignancy; TBS = The Bethesda System; WHO = World Health Organization.

countries are on the verge of replacing cytology as primary screening by testing for the presence of hrHPV. The Netherlands and Australia will be among the first countries to initiate full hrHPV-based organized screening in 2017.

Advantages of primary hrHPV testing are the objectivity of the assay and high-throughput testing, and its high sensitivity of 90% for CIN2 or worse (≥CIN2) and 95% for CIN3 or worse (≥CIN3), compared with moderate sensitivity of 30-87% for cytology.[15,16] Additionally, HPV screening holds the possibility to analyze self-sampled material of brush- or lavage based samples, which may improve the efficacy of cervical cancer screening by increasing participation of non-responders.[17-20] The major limitation of hrHPV testing is its inability to distinguish transient infections from clinically relevant infections, resulting in limited specificity compared with cytology. This limited specificity would result in higher numbers of unnecessary colposcopy referrals compared with cytology screening.[21,22] Effective triage of hrHPV-positive women is therefore essential.

An ideal triage strategy meets two important requirements: 1) The strategy gives a highly sensitive and specific result, differentiating between cervical cancer and CIN lesions that need treatment, and abnormalities with a risk that is low enough for a woman to safely return to the next screening round. Ideally, low risk groups would need no follow-up. 2) No additional sample or additional visit is needed to perform the triage, thus minimizing the efforts for the screened women, and limit loss to follow-up. The ideal triage method for hrHPV-positive women is not yet available; therefore, the most optimal triage strategy using current knowledge should be obtained. Current triage methods all have advantages and disadvantages and some may possibly be improved or combined to measure up to an ideal strategy as far as possible.

This review gives an outline of current knowledge and future opportunities for triage of hrHPV-positive women, which is especially important in the transition from cytology-based cervical cancer screening to primary hrHPV screening. A variety of triage options are discussed: morphological biomarkers such as Papanicolaou (Pap)-stained cytology or cytology with different types of immunochemistry, and molecular biomarkers such as HPV genotyping, RNA-based biomarkers, and methylation. We focus on the advantages and disadvantages of these strategies and their value as triage method of hrHPV-positive women. Finally, a five-year view on cervical cancer screening in a post-vaccination era will be discussed.

2. MORPHOLOGICAL BIOMARKERS

2.1 Cytology

Cytological examination of exfoliated cervical cells stained with the multichromatic Pap staining was introduced by Papanicolaou in the 1940s.[14] Triage of hrHPV-positive women with cytology is a common choice because of the worldwide experience with this technique. This widely evaluated triage method has shown to improve the initially limited specificity of hrHPV testing, and is known to reduce colposcopy referrals and follow-up testing.[23-28] However, cytological assessment in these studies is generally performed without knowledge on hrHPV-positive status, which is different to primary hrHPV-based screening. The knowledge on positive hrHPV status is known to affect the interpretation of cytology. In hrHPV-based screening with knowledge on hrHPV-positive status, this might result in a slightly higher sensitivity and a lower specificity compared with these previously published results.[29-31] The inability of high throughput testing and subjectivity of the examination are limitations of this technique as triage method for hrHPV-positive women. The performance of cytological examination highly depends on training and experience of cytotechnologists, resulting in variations in performance and quality. Quality management and benchmarking are therefore needed to obtain

and maintain high quality of cytological examination. With the introduction of primary hrHPV-based screening, the number of samples for cytological examination will decrease and maintaining highly trained cytotechnologists might become more difficult.

For cytological examination of Pap stained cells, a slide can be obtained from a primary hrHPV-positive clinician-taken cervical sample. In case of an hrHPV-positive self-sample, an additional clinician-taken sample will be necessary as cytology cannot be reliably performed on self-sampled materials.[32] Cytological triage has limited sensitivity, and therefore still needs follow-up for cases with an hrHPV-positive result combined with normal cytology, also additional follow-up is warranted for cytology positive cases which show no abnormalities during colposcopy or in a biopsy. The worldwide experience with cytological assessment of cervical samples makes cytology an interesting triage method for hrHPV-positive women; however, limitations as average sensitivity, subjectivity of the analysis, and inability to perform on self-sampled material are major disadvantages.

2.2 p16 staining

Expression of the HPV E7 oncoprotein induces KDM6B histone demethylase expression and causes epigenetic reprogramming. Through KDM6B, demethylation of the $p16^{INK4a}$ (p16) promoter is triggered, with upregulation of p16 expression as a result.[11] The upregulation of p16 in transforming infections might therefore be used as biomarker for differentiating between productive and transforming infections. HrHPV testing with p16-staining triage produces a significant increase in sensitivity compared with conventional cytology, with no substantial increase in referral to colposcopy.[33] The longitudinal sensitivity of p16-staining as triage method for hrHPV-positive women is 77.8% for ≥CIN3, with a 95% confidence interval (CI) ranging from 63.9-91.6%. The relative sensitivity of p16 triage of hrHPV-positive women between 35 and 60 years of age, compared with primary conventional cytology is 2.08 (95% CI 1.13-3.56).[34] Positive p16 staining distinguishes hrHPV-positive women in need of immediate colposcopy referral, from hrHPV-positive and p16 staining negative women who can safely be managed with repeat screening after a 2-3 year interval.[34] Expression of p16 is not limited to dysplastic cells but can also been found in normal cervical cells such as squamous metaplastic and endocervical cylindrical cells. This is not problematic in histological samples, but makes morphological assessment necessary in cytological samples. Assessment is therefore more subjective and limits reproducibility.[35] This can be partially overcome by combining p16 with the proliferation marker Ki-67.

2.3 p16 / Ki-67 dual staining

In normal cells expression of proliferation marker Ki-67 can be found in the nucleus during all proliferative cell cycle phases, and its expression is limited to basal or parabasal layers. In CIN lesions its expression extends above the first one-third of the epithelium.

Expression of both p16 and Ki-67 within the same cell is a sign of neoplastic transformation, independent from morphological criteria.[36] The clinical performance of p16/Ki-67 has been studied extensively as triage method for low-grade cytology; however, also studies in hrHPV-positive women were performed. In triage of hrHPV-positive women with a negative cytology result, sensitivity for ≥CIN2 varies between 67-92%, with a specificity of 73-82%.[36,37] In triage of women with hrHPV-positive low-grade cytology, sensitivity and specificity of ≥CIN2 vary from 75-94% and 51-88% respectively.[38-43] Limited studies have been performed with immediate p16/Ki-67 triage of hrHPV-positive women, without additional cytological interference. In a study with 1,509 hrHPV-positive women >30 years of age, dual-stain cytology shows similar sensitivity but a higher specificity for ≥CIN2 detection, when compared with Pap cytology with a threshold of atypical squamous cells of undetermined significance (ASC-US). For women with a positive dual-stain, immediate referral to colposcopy was justified. Dual-stain negative women had a risk of a high-grade lesion that was lower than the risk for hrHPV-positive women with normal cytology, which is considered low enough to re-examine after one year according to US management guidelines.[44,45] In another study that performed p16/Ki-67 dual-stained triage of 446 hrHPV-positive women, a sensitivity similar to cytology and cytology combined with HPV genotyping has been achieved. Specificity for ≥CIN3 was significantly higher than with cytology, either or not combined with HPV genotyping.[46] In a prospective population-based study in over 6,000 women, 396 women between 35 and 64 years of age were hrHPV-positive. Triage of these hrHPV-positive women by p16/Ki-67 dual-stain yielded a sensitivity of 87.6%, compared with 77.6% for cytology with an ASC-US threshold. Specificity and colposcopy referral rate were similar in both groups. Combined triage with cytology (threshold ASC-US or worse) and p16/Ki67 yielded an increased sensitivity but at the expense of a decreased specificity. With an adjusted threshold of HSIL for cytology, the sensitivity and specificity were similar to triage with p16/Ki-67 alone.[47]

Combined p16/Ki-67 dual staining shows promising results in triaging hrHPV-positive women, as well as in triaging hrHPV-positive women with negative or low-grade cytology. However, the subjectivity of the examination, the inability for high-throughput testing and its inability to be used on self-sampled material are shortcomings. Also, a negative dual-stain result still needs follow-up, as well as a positive dual-stain result without colposcopic or histologic abnormalities.

2.4 MCM2/TOP2A dual-stain

Minichromosome maintenance protein 2 (MCM2) and topoisomerase II-a (TOP2A) are both indicative for the formation of the origin recognition, and indicate an aberrant S phase induction when detected in suprabasal cells of the epidermis. Aberrant S phase induction is the premature and prolonged entry in the S-phase of the cell cycle, which

is induced by HPV E6 and E7 oncoproteins, resulting in G1/S cell-cycle checkpoint malfunction. Both MCM2 and TOP2A have shown to be overexpressed in high-grade CIN and cancer. The performance of the MCM2/TOP2A dual-stain has been examined as triage method of low-grade cytology showing varying sensitivity for the detection of ≥CIN2, when compared with cytology. In triage of hrHPV-positive women in one study, this dual-stain yielded a relative sensitivity of 1.30 (95% CI 1.20-1.41) and relative positive predictive value (PPV) of 2.89 (95% CI 2.58-3.15), when compared with cytology alone.[48] Knowledge on clinical utility of the assay in triage of hrHPV-positive women is limited and further studies on this morphology-dependent assay are needed to determine and validate its clinical value.

3. MOLECULAR BIOMARKERS

3.1 HPV genotyping

Cervical infections with HPV16 and HPV18 have demonstrated the highest risk of developing precancer and cancer. HPV16 is found in 50-60% of all cervical cancers, and HPV18 in 10-15% of cervical cancer cases.[49] HPV18 and HPV45 are especially known for their association with the less prevalent adenocarcinoma of the cervix.[50,51] Risk estimates for individual genotypes or the combination of different genotypes can be used for triage of hrHPV-positive women.

In the guideline of the American Society for Colposcopy and Cervical Pathology (ASCCP), HPV16/18 genotyping is already recommended for hrHPV-positive women with normal cytology results; all HPV16 or HPV18 positive women are immediately referred for colposcopy, women positive for other hrHPV types without morphological changes are advised follow-up after one year with hrHPV and cytology co-testing.[52] The additional value of genotyping with immediate referral of HPV16/18 positives was confirmed in the HPV FOCAL trial which included over 6,000 women screened by hybrid capture 2 (HC2) and Cobas hrHPV testing at baseline and liquid-based cytology (LBC) 24 months later. HrHPV-positive women were tested with HPV genotyping and reflex LBC for colposcopy triage. Of the ≥CIN2 lesions identified in the first round of screening, which includes baseline and 12 month follow-up, 55% had abnormal cytology and an additional 17% with normal cytology was HPV16/18 positive. Concluding that immediate referral of HPV16/18 positive women with normal cytology may allow for earlier detection of ≥CIN2 lesions.[53]

Multiple studies have been performed on HPV16/18 genotyping triage of hrHPV-positive women, of which the ATHENA trial is one of the largest. HrHPV testing with HPV genotyping was compared with LBC for cervical cancer screening in women over 21 years old. In a subgroup analysis of women >25 years of age, the sensitivity and PPV

for ≥CIN3 in triaging hrHPV-positive women with HPV16 and/ or HPV18 was equivalent to detecting ASC-US or worse. Detection of HPV16 and/or HPV18 or cytology with LSIL or HSIL threshold showed increased sensitivity and similar or increased PPV for ≥CIN3 detection than single cytology triage with an ASC-US threshold.[54] Other studies have also shown promising results with high negative predictive values (NPV) by combining an ASC-US threshold with HPV16/18 or HPV16/18/31/33/45 genotyping. However, these strategies generally showed high colposcopy referral rates.[23,24] In a recent European study, HPV16/18 genotyping performance was explored in a setting with prior knowledge on hrHPV-positive status during cytology assessment, similar to hrHPV-based screening. This study concludes that by adjusting the threshold of cytology and combining it with HPV16/18 genotyping, specificity for ≥CIN3 increases with similar sensitivity and no increase in colposcopy referrals. However, follow-up is still needed for hrHPV-positive women with low-grade cervical cytology results.[31] A cost-effectiveness study on triage strategies of primary hrHPV screening in women over 30 years of age, with screening sensitivity and specificity based on the ATHENA trial, indicates that incorporating HPV16/18 genotyping in primary hrHPV screening with reflex cytology with an ASC-US threshold is cost-effective.[55]

Compared with partial genotyping, extended genotyping methods are also available. The Onclarity HPV test offers extended genotyping of types 16, 19, 31, 45, 51 and 52, and identifies an additional three groups of 33/58, 56/59/66 and 35/39/68. The clinical and analytical performance of this test was recently assessed according to the VALGENT framework concluding that this assay offers applications for clinical workstreams.[56] Also other techniques as the GP5+/6+-LMNX, MALDI-TOF, qPCR test, and PapilloCheck offer extended genotyping. Extended genotyping methods for immediate triage of hrHPV-positive women have however not been studied as extensively as the previously mentioned partial genotyping tests. These studies might be performed in the near future.

A variety of hrHPV tests have the ability of immediate and combined genotyping, which makes this triage strategy easy to use. The promising results of previous studies, combined with the ability of immediate triage of both clinician-taken and self-sampled specimens, makes hrHPV genotyping an interesting triage option for hrHPV-positive women. However, adding Pap cytology to HPV16/18 genotyping increases clinical value of the triage, with the disadvantage of the need of a clinician-taken sample for cytology. Current knowledge indicates that HPV16/18 genotyping may improve triage of hrHPV-positive women; however, for management of hrHPV-positive women with infections other than HPV16 and HPV18, other techniques might still be necessary.

3.2 RNA-based biomarkers

RNAs can play an active role within cells by communicating responses to cellular signals, catalyzing biological reactions, and controlling gene expression with of small interfer-

ing RNA (siRNA) or micro RNA (miRNA). Cellular organisms use messenger RNA (mRNA) to direct synthesis of specific proteins, and miRNA to regulate the function of mRNA. RNA-based tests are able to detect differences or changes in gene expression related to cancer development, while DNA-based tests detect the presence or absence of the HPV genome.

3.2.1 E6/E7 messenger RNA-based biomarker

A limitation of the DNA-based hrHPV test is its inability to distinguish transient from persistent hrHPV infections resulting in low specificity. Cellular transformation of hrHPV infected cells begins with upregulation of E6 and E7 mRNA, and progression from hrHPV infection to cancer is dependable of E6/E7 integration. Overexpression of E6/E7 mRNA could therefore be used to detect only active infections which could result in high-grade lesions. E6/E7 mRNA-based test are widely studied as substitute for DNA HPV-based tests, indicating significance as diagnostic tool. Already three commercial E6/E7 mRNA-based tests are known.[57] A recent review of the clinical performance of the E6/E7 mRNA-based Aptima HPV assay indicates stable similar sensitivities of the E6/E7mRNA hrHPV assay for detection of CIN2/3 or worse (≥CIN2/3), independent from study design, compared with the HC2 and GP5+/6+DNA tests. This stable sensitivity was combined with a higher specificity of the mRNA-based HPV test.[58] In a second review the Aptima assay also showed consistently similar study-specific and pooled sensitivity and superior specificity for ≥CIN2 compared with HC2. The pooled relative sensitivity for the Aptima assay was 0.98 (90% CI 0.95-1.01) with a pooled relative specificity of 1.04 (90% CI 1.02-1.07).[59] In a study comparing multiple triage algorithms for hrHPV-positive women, an E7 mRNA test with HPV16/18/31/33/45/52/58 genotyping achieved similar performance to HPV16/18 genotyping and cytology in ≥CIN2 detection.[60] Triage of hrHPV-positive women with ASC-US cytology in the CLEAR HPV study was performed with the Aptima HPV 16 18/45 genotype assay. This study demonstrated that the assay has utility in stratifying low and high risk of ≥CIN2 and CIN3 among women with hrHPV-positive ASC-US.[61] These E6/E7 messenger RNA-based tests could possibly combine primary mRNA-based hrHPV testing with HPV genotyping. However, follow-up of hrHPV-positive women with genotypes other then 16, 18 or 45 would still be needed, and additional longitudinal studies and economic evaluations must be conducted before more solid conclusions regarding clinical applicability can be made.

3.2.2 Other messenger RNA-based biomarkers

It has been previously demonstrated that HPV infection alone is insufficient for development of cervical cancer; abnormal host genes play an important role in carcinogenesis.[62] The mRNA-based expression profile of normal cervical tissue is known to change during carcinogenesis, and expression of single genes or gene profiles might be used as mo-

lecular biomarker to distinguish between different stages of carcinogenesis or indicate response to particular treatment. The expression status of thousands of genes can be studied at once to create a profile of cellular function. DNA microarray, which measures expression of previously identified target genes, and the newer sequence-based techniques can be used to obtain gene expression profiles.[62] Also bioinformatics tools can be used to identify key genes and potential biomarkers, by analyzing gene expression profiles and differentially expressed genes between cervical samples of different stages in carcinogenesis.[63]

Previous studies compared expression profiles of cervical cancer with normal cervical tissue, early-stage with late-stage cervical cancer, and squamous cell carcinoma with adenocarcinoma of the cervix.[64-66] Also studies on therapy response and resistance were performed.[67,68] Differences between CIN lesions and cervical cancer or normal tissue have also been studied in a small number of studies, finally resulting in proposed genes for further research, with special attention to HOXC10, BPA1, HIF-1a, PTP, HME1, HNTH1 and PHGDH.[69-72] In a recent feasibility study the detection of mRNA-based biomarkers in cervical samples obtained by LBC was studied, showing promising results. Single markers and combinations of markers CDKN2A/p16, BIRC5, MMP9, TOP2A, MCM5 and MKI67 were studied. TOP2a was most sensitive, with a sensitivity of 97% for detection of HSIL, and CDKN2A/p16 was most specific with a specificity of 78%. The combination of TOP2A and CDKN2A/p16 was highly sensitive 96% (95% CI 88-99) with a specificity of 71% (95% CI 55-82).[73]

Identified differences in these genomic expression profiles may in the future be used to distinguish productive from transforming hrHPV infections, and may be able to identify prognostic markers and targets for molecular therapy. Knowledge on these biomarkers is however still at an early stage, and at current state knowledge on these markers is far too limited for implementing these in clinical practice.

3.2.3 miRNA-based biomarker

Micro RNA (miRNA) are more recently discovered and are noncoding parts of RNA that regulate messenger RNA (mRNA) function by modulating mRNA stability and the translation of mRNA into proteins. MiRNAs can be upregulated or downregulated and are thought to play an important role in processes as cell proliferation, metabolism, and apoptosis, with a possible role in onset or progression of cervical cancer. An important feature of miRNAs is that one miRNA often interacts with more than one mRNA and one transcript can be targeted by multiple miRNAs, indicating a variety of interactions for one miRNA.

In a meta-analysis comparing 27 studies, including 1,132 cancer samples and 943 normal samples, frequency of upregulation and downregulation of miRNAs was scored. Upregulation of miRNAs was most consistently reported for miR-20a and miR-21. Down-

regulation was shown most frequently for miR-143, miR-03 and miR-145.[74] In a large systematic study on deregulated miRNAs in cervical cancer development, 85 published reports with 3,922 cases and 2,099 noncancerous control tissue samples were analyzed. Expression of miRNAs in cervical cancer, as well as in different CIN lesions was reviewed. A meta signature of miRNAs reflecting the cervical carcinogenesis from CIN1, CIN2 and CIN3 to cervical cancer was made, reporting 42 upregulated and 21 downregulated miRNAs with a trend of increasing numbers of deregulated miRNAs during progression of CIN to carcinoma. The meta-analysis shows a selection of five upregulated and seven downregulated miRNAs in CIN1 compared with noncancerous tissue, which are also visible in more severe CIN lesions and cervical cancer, indicating a possible role in development of cervical cancer. CIN2 and CIN3 lesions showed an increased number of deregulated miRNAs with an additional 35 and 36 deregulated genes compared with CIN1. In cervical cancer, another five downregulated and ten upregulated miRNAs genes were reported.[75] Knowledge on these miRNA expression profiles may be used for disease classification or monitoring. To our knowledge, this profile has however not yet been studied in large prospective studies or on cervical samples, therefore, its value for triage of hrHPV-positive women is yet unknown.

3.3 HPV E6 protein biomarker

Expression of E6 and E7 genes is integral to hrHPV-induced malignant transformation, indicating that HPV E6/E7 protein markers could potentially serve as markers for identifying high-grade CIN. Most diagnostic E6/E7 protein markers are, as previously described, based on mRNA testing and therefore susceptible to degradation. Development of the whole-cell enzyme-linked immunoabsorbent assay (ELISA) using an HPV16, 18 and 45 anti E6 monoclonal antibody to detect HPV E6 protein in cervical samples could tackle this problem. In the first pilot-study as well as the first clinical trial and follow-up after one year, increased specificity with a considerable lower sensitivity for ≥CIN3 was found, compared with an hrHPV DNA test.[76-78] Clinical evaluation of the assay in triage of HC2 hrHPV-positive clinician-taken samples showed a high specificity for ≥CIN3 of 93.8% (95% CI 92.1-95.2), with again limited sensitivity of 54.2% (95% CI 43.7-64.4).[79] An explanation for this limited sensitivity could be the fact that this E6 test only covers HPV types 16, 18 and 45. Future research to possibly increase the number of covered hrHPV types in this ELISA-based test should reveal if sensitivity can be improved to increase its value as triage marker for hrHPV-positive women. Also, this marker has not yet been tested on self-sampled specimens, so its value in triaging hrHPV-positive self-sampled specimens is yet unknown.

3.4 Methylation markers

Methylation of CpG islands is a normal epigenetic event where functionally relevant changes to the genome are made without changing the nucleotide sequence. Abnormal DNA methylation in host or viral DNA promoter regions during carcinogenesis may result in inactivation of tumor suppressor genes and silencing of gene expression. These changes in the DNA sequence can be detected, and may possibly be used as biomarker to distinguish productive from transforming CIN lesions and cervical cancer. DNA methylation is easily detectable in clinician-taken, self-sampled and histological cervical specimens.[12] Understanding the role of epigenetic events in host and viral genes is an important and promising area of investigation and is expected to result in novel risk stratifying strategies for triage of hrHPV-positive women.

3.4.1 Viral gene methylation markers

Methylation of the HPV DNA genome shows type-specific variation within the viral life cycle and differs during carcinogenesis. Methylation of HPV DNA may be a host response to foreign intracellular agents, a method of evading immune recognition, or a signaling event indicating viral integration into the host genome. From studies using different HPV-positive cell lines it is known that methylation of the late region is indicative for integration of the viral genome.[80,81] Viral gene methylation may also be indicative of the likelihood of persistence or clearance of the infection, therefore possibly holding a strong diagnostic or prognostic value in triaging hrHPV-positive women.[82]

HPV genome sequence methylation is most widely studied in HPV16; hypermethylation of the HPV16 L1, L2, E2 and E4 regions is associated with an increased risk of CIN3 and HPV persistence, and hypermethylation of the E6 gene is associated with a lower likelihood of ≥CIN2. Some of the hypermethylated CpG sites also showed significant higher methylation levels in pre-diagnostic ≥CIN2 specimens with a median time of three years before diagnosis, compared with a control group, indicating a positive predictive property for high-grade lesions of these markers.[83-86] Conclusion of these studies is that elevated levels of methylation in the HPV16 genome may be useful in predicting concurrent or even future development of ≥CIN2. Methylation of other hrHPV types shows similar results to HPV16; hypermethylation of the L1, L2 regions of HPV18, 31, 33 and 45 was associated with high-grade CIN lesions, and increased methylation of the E2 region was found in HPV18, 31 and 45 induced high-grade CIN lesions.[87,88] HPV DNA methylation of HPV16, 18, 31, 33 and 45 may be useful in differentiating transforming from productive hrHPV infections. However, validation studies in large cohorts are necessary before these biomarkers could be used in clinical practice.

3.4.2 Host gene methylation markers

Methylation markers based on host DNA methylation have been studied extensively and have been summarized in reviews indicating a promising role in triage of hrHPV-positive women.[8,89] Studies in this field still continue, as for most genes no highly consistent result has yet been found. Combinations of markers most widely studied in hrHPV-positive women include the marker panels JAM3/EPB41L3/ TERT/C13ORF18 and JAM3/C13ORF18/ANKRD18CP, and various combinations of the markers CADM1, MAL, miR-124-2 and FAM19A4. Many other individual markers and marker panels have been studied less extensively and most of these studies did not include hrHPV status. Some small studies did include the hrHPV status when testing markers. Bi-marker panels, DLX4/SIM1,[90] tri-marker panel DAPK1/RARB/MGMT,[91] and single markers JAM3-M4,[92] EPB41L3,[93] hsa-miR-203,[94] PAX1,[95] and POU4F3,[96] show promising results; however, to our knowledge these are not yet confirmed by large prospective follow-up studies.

Triage of hrHPV-positive clinician-taken samples by methylation panel JAM3/ EPB41L3/ TERT/C13ORF18 yielded a ≥CIN3 detection rate of 65%. The panel is also shown feasible to use on self-sampled lavage and brush samples.[97,98] An adjusted panel of JAM3/C13ORF18/ ANKRD18CP has only been studied in women with positive cervical cytology.[99] Further validation in population-based cohorts and large prospective studies is the next step for these panels. Methylation panels CADM1/MAL and MAL/miR-124-2 were the first panels validated in a population-based screening setting. CADM1/MAL methylation levels are related to the degree of the cervical disease and the duration of preceding hrHPV infection and the methylation status in cervical scrapes appears to be representative for the worst underlying lesion.[110,101] The CADM1/MAL bi-marker panel was equally discriminatory for ≥CIN3 as cytology at similar specificity in the triage of hrHPV-positive women.[102,103] When combined with cytology, this panel showed a high sensitivity with a slight drop in specificity in triage of hrHPV-positive women.[103,104] In triaging 79 hrHPV-positive women, the marker panel showed a sensitivity of 70% and specificity of 78% for ≥CIN3.[105] In triaging women for colposcopy, the bi-marker panel MAL/miR-124-2 yielded a sensitivity of 64.9-71.6% at a specificity of 70% for ≥CIN3, which was significantly higher then the sensitivity for HPV16/18 genotyping in this specific study cohort.[106] In a large prospective study with 1,038 hrHPV-positive non-responders who were randomized between MAL/miR-124-2 and cytology triage, the DNA methylation panel was at least as sensitive as cytology at a threshold of borderline or mild dyskaryosis or worse for ≥CIN2 detection. The methylation panel showed a decreased mean time to diagnosis; however, at the cost of more colposcopy referrals.[107] In a recent pilot-study, FAM19A4 methylation in clinician-taken samples showed to be an attractive triage marker for hrHPV-positive women.[108] Validation of bi-marker FAM19A4/miR124-2 in lavage- and brush-based self-samples resulted in a ≥CIN3 sensitivity of 69.4-70.5% with a specificity of 67.8-76.4%.[109]

By adding HPV16/18 genotyping to the MAL/miR-124-2 methylation panel with an adjusted threshold, similar sensitivity with increased specificity for ≥CIN3 can be achieved, when compared with the methylation panel alone.[110] By adding HPV16/18 genotyping tot FAM19A4 methylation, sensitivity increased, with decreased specificity, when compared with cytology alone, FAM19A4 methylation alone and HPV16/18 genotyping alone.[111] Combined FAM19A/mir124-2 and HPV16/18 genotyping showed a ≥CIN3 sensitivity of 84.7% and specificity of 54.9%.[109] This indicates that combining host methylation markers and HPV16/18 genotyping may increase the clinical value of both techniques separately in triaging hrHPV-positive women.

3.4.3 Combined methylation marker panels

Recently, also studies combining HPV viral DNA genome methylation and host DNA methylation have been performed. A study with methylation of DAPK, L1 and L2 of HPV16, 18, and 54 shows lowest methylation in asymptomatic infections and increased methylation in progression to cancer.[112] Another combined methylation panel of L1 and L2 regions of HPV16, 18 and 31, and human gene EPB41L3, was tested in 1,493 hrHPV-positive exfoliated cervical specimens from a colposcopy referral cohort, and showed a sensitivity of 90%, combined with specificity of 36% and a PPV of 46%.[113] By adding HPV33 to the panel, specificity increased to 49% with a stable 90% sensitivity.[114] Validation of this assay in exfoliated cervical specimens of 710 women attending routine screening, of which 341 hrHPV-positive, yielded a similar specificity of 65% with better sensitivity than HPV16/18 genotyping (47% vs. 54%) in identifying ≥CIN2.[115]

In summary, currently studied methylation markers show great potential as triage marker for hrHPV-positive women and could be the key to full molecular screening, possibly even with predictive value. Marker panels CADM1/MAL, MAL/mir124-2, and FAM19A/mir124-2, and combined HPV methylation and host methylation panels currently show the most potential. However, previously studied markers generally do not detect all ≥CIN3 lesions and detect less CIN2 lesions than cytology.[8] Also high referral rates have been described with host methylation triage, resulting in an increase of unnecessary colposcopies. Therefore, at the moment, we do not have methylation markers that merit clinical use yet. Future research with large population-based studies will prove whether methylation marker panels, either or not combined with other triage strategies, will eventually result in a triage strategy with high sensitivity and specificity and limited referral rates, to play a role in the triage of hrHPV-positive women.

3.5 Chromosomal biomarkers

Cellular genomic alterations are needed for progression of HPV-induced premalignant lesions, which could make chromosomal biomarkers a valuable triage method. The chromosomal regions most frequently altered in cervical squamous cell carcinoma

are a loss at 3p and 11q, and gains at 3q, especially in HPV16-positive carcinomas.[116] The human telomerase RNA (hTERC) gene plays a role in maintenance of chromosome length and stability, and is located in chromosome 3q26 region. Gain of 3q26 shows a strong association with severity of dysplasia,[117] and several small studies triaging LSIL and ASC-US report a high NPV, with a possible role in triage of women with low-grade CIN.[118-120] Most of the studies on genomic alterations are small and retrospective. Before these markers could be considered as triage method further research with prospective large studies with long-term follow-up is needed.

3.6 Other molecular biomarkers

Several other potential triage methods for hrHPV-positive women have been proposed; cellular proliferation-associated proteins, viral markers as HPV L1 capsid protein and E4 markers, the cervical microbiome and its cytokine profile, and proteomics based on differences in expressed proteins, all in a limited number of small studies.[121-125] Further prospective research is needed to determine the utility of these molecular biomarkers in triage of hrHPV-positive women.

4. EXPERT COMMENTARY

With the introduction of hrHPV-based screening with high sensitivity but limited specificity, effective triaging of hrHPV-positive women is essential. None of the triage strategies discussed in this review currently meets the criteria for an ideal triage method; however, several strategies show great potential each with their own advantages and limitations.

The worldwide experience with morphology-based Pap cytology makes this a common triage method for hrHPV-positive women. To improve sensitivity, different immunochemistry stains, as p16 staining, or p16/Ki-67 dual-stain can be used as biomarker. However, these triage strategies are still more or less based on morphological criteria and cannot differentiate between productive and transforming infections or predict the development of high-grade lesions. Besides they are not applicable on self-sampled specimens, which may play an important future role in hrHPV-based screening. An additional clinician-taken sample would therefore still be needed for further triage of hrHPV-positive women.

Molecular biomarkers have been extensively studied with a consistent increase of knowledge in this area. Molecular triage of hrHPV-positive women could in theory differentiate productive from transforming infections, and some studies have already shown to be able to predict development of high-grade lesions. Molecular biomarkers are objective and highly reproducible, and can be used in high throughput testing. They do not need high cellularity, and some have already been shown applicable on lavage- and

brush based self-samples. Studies on these molecular techniques as single biomarker or as a combination of biomarkers show promising results. Yet, no molecular triage method can differentiate all women with a high risk from women with a low risk for high-grade CIN. However, with further increasing knowledge on these molecular markers, cervical cancer screening may transform to full molecular screening in the future.

5. FIVE-YEAR VIEW

It is expected that in the next five years primary hrHPV testing, due to its high sensitivity, is increasingly incorporated in programs for cervical cancer screening in many Western countries. Results from the first years of primary hrHPV screening in some countries will then already be available. With the increased number of countries incorporating hrHPV screening, improving triage of hrHPV-positive women becomes more and more important. As knowledge on the molecular genesis of cervical precancer and cancer is expanding; triage tests other than cytology could fulfil the role of an additional triage test for HPV positive women. In the next five years, p16/Ki-67 dual-stain may replace or be added to morphology dependent Pap cytology as triage method for hrHPV-positive women. However, it is expected that triage of hrHPV-positive women by morphological biomarkers will finally be taken over by molecular triage techniques with advantages as objective evaluation and high-throughput triage. HPV genotyping has been widely studied and could be used as triage method for hrHPV-positive women on short notice. Methylation of host DNA is being widely studied and may also be used as triage method for hrHPV-positive women in the near future. The number of studies on predictive value of biomarkers is expected to increase and may finally result in biomarkers with predictive characteristics to detect high-grade abnormalities even earlier in the process of carcinogenesis. To improve attendance to the screening program in the Netherlands, self-sampling will be offered to non-responders of the new hrHPV-based screening program. If this approach turns out successful, other countries may also consider implementing self-sampling in their screening programs in the future. Cervical cancer screening is expected to gradually transform into a more woman-friendly program with more objective screening and triage methods with higher clinical accuracy.

Approximately ten years ago, vaccines for HPV types 16 and 18 first became available, and were followed-up by the quadrivalent and nonavalent vaccines. Vaccination programs based on vaccinating girls in their pre-sexarche for high-risk types 16 and 18, and possibly also other types, are introduced. Recently published large studies show great promise of these vaccines with strong herd protection and no sign of type-replacement yet. It will however take many years before the vaccination program will affect the incidence of cervical cancer, and it is not yet fully known how this will affect

the effectiveness of screening programs. Screening will therefore remain necessary for the next decades. Vaccination will however decrease the prevalence of high-grade CIN and therefore test characteristics of screening must show high sensitivity and specificity. In time screening programs might need to be re-evaluated and adjusted again.

6. KEY ISSUES

- hrHPV testing is expected to replace cytology as primary screening method for cervical cancer screening in an increasing number of countries. The high sensitivity of hrHPV testing combined with a limited specificity makes triaging of hrHPV-positive women necessary.
- An ideal triage strategy consists of a biomarker that can be used on the primary screening sample, resulting in a highly sensitive and specific result that differentiates women with cervical cancer or high-grade CIN lesions from women with a low risk for these lesions who can safely return to the next screening round. An ideal triage strategy does not yet exist. Therefore, the most optimal triage strategy should be obtained based on current knowledge.
- Multiple options for triaging hrHPV-positive women are available. Previous experience with morphologically-based methods makes them a logical first choice as triage method for hrHPV-positive women. However, these morphological markers lack properties that make molecular biomarkers more attractive as triage method such as: objectivity, option for using high-throughput systems, the capacity to distinguish productive from transforming infections, predict developing high-grade CIN lesions, and the option to be performed on self-sampled material.
- At the moment, most biomarkers lack sufficient evidence to introduce them as triage method for hrHPV-positive women in screening programs. Improved sensitivity and specificity, and more evidence from large prospective studies is needed before introducing these biomarkers as triage test into standard of care in cervical cancer screening programs. Different triage techniques may also be combined to improve diagnostic value as triage method for hrHPV-positive women.
- In the near future, cervical cancer screening programs are expected to be based on full molecular screening with primary hrHPV testing and molecular triage of hrHPV-positive women.
- The number of studies on predictive value of biomarkers is expected to increase and may finally result in biomarkers with predictive characteristics to detect high-grade abnormalities even earlier in the process of carcinogenesis.
- Self-sampling may attain a role in hrHPV-based screening programs, to finally result in a more women-friendly screening program with less loss to follow-up.

- It will take many years before vaccination programs will affect the incidence of cervical cancer, and it is not yet known how this will affect the effectiveness of screening programs. In time screening programs might need to be re-evaluated and adjusted again.

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CHAPTER 3

The clinical value of HPV genotyping in triage of women with high-risk-HPV-positive self-samples

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ABSTRACT

Objective Cytology alone, or combined with HPV16/18 genotyping, might be an acceptable method for triage in high-risk human papillomavirus (hrHPV)-cervical cancer screening. Previously studied HPV-genotype based triage algorithms are based on cytology performed without knowledge of hrHPV status. The aim of this study was to explore the value of hrHPV genotyping combined with cytology as triage tool for hrHPV-positive women.

Methods 520 hrHPV-positive women were included from a randomized controlled self-sampling trial on screening non-attendees (PROHTECT-3B). Eighteen baseline triage strategies were evaluated for cytology and hrHPV genotyping (Cobas 4800, Roche) on physician-sampled triage material. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), referral rate, and number of referrals needed to diagnose (NRND) were calculated for ≥CIN2 and ≥CIN3. A triage strategy was considered acceptable if the NPV for ≥CIN3 was ≥98%, combined with maintenance or improvement of sensitivity and an increase in specificity in reference to the comparator, being cytology with a threshold of atypical cells of undetermined significance (ASC-US).

Results Three triage strategies met the criteria: HPV16+ and/or ≥LSIL; HPV16+ and/or ≥HSIL; (HPV16+ and/or HPV18+) and/or ≥HSIL. Combining HPV16+ and/or ≥HSIL yielded the highest specificity (74.9%, 95% CI 70.5–78.9), with a sensitivity (94.4%, 95% CI 89.0–97.7) similar to the comparator (93.5%, 95% CI 87.7–97.1), and a decrease in referral rate from 52.2% to 39.5%.

Conclusion In case of prior knowledge of hrHPV presence, triage by cytology testing can be improved by adjusting its threshold, and combining it with HPV16 /18 genotyping. These strategies improve the referral rate and specificity for detecting ≥CIN3 lesions, while maintaining adequate sensitivity.

INTRODUCTION

A persistent cervical infection with high-risk human papillomavirus (hrHPV) is a necessary cause for the development of cervical neoplasia.[1] Western countries therefore increasingly consider implementation of primary hrHPV testing for cervical cancer screening, and replace primary cytology screening. The main concern surrounding the use of hrHPV testing for primary screening is its relatively low specificity, the direct result of the assay's inability to distinguish transient from persistent HPV infection. Therefore, additional triage is required to identify women with the highest risk of cervical precancer or cancer, in need of treatment.[2]

In the Netherlands, primary hrHPV testing will soon be implemented in the organized screening program. The proposed strategy for hrHPV-positive women is cytology triage at baseline, with repeat cytology after six months for hrHPV-positive and cytology-negative cases. However, this follow-up algorithm demonstrates limited specificity and requires repeat testing which in turn increases the risk of loss to follow-up. A suitable supplemental or alternative triage method may further reduce unnecessary colposcopies, invasive diagnostics such as biopsies, overtreatment, patient anxiety, and waste of financial sources.[3-6] HrHPV genotyping, supplemental to cytology, may be a potential solution to this clinical dilemma in the management of hrHPV-positive women.

Previous studies on triage strategies evaluating cytology with hrHPV genotyping show promising results with equal or increased sensitivity,[7,8] combined with an increased positive predictive value (PPV) and specificity,[9,10] resulting in higher detection of moderate dysplasia (cervical intraepithelial neoplasia grade 2) or worse (≥CIN2) at baseline. The latter studies have, however, been performed with cytology scoring without knowledge of hrHPV presence. Understanding the extent to which prior knowledge of hrHPV status affects cytology-based triage algorithm outcomes is critical because primary hrHPV testing becomes increasingly used for cervical cancer screening.

The aim of this study was to assess the clinical value of hrHPV genotyping as a supplemental or alternative triage strategy for hrHPV-positive women, compared with Pap cytology testing with a threshold of atypical squamous cells of undetermined significance (ASC-US), in a setting comparable to the future cervical cancer screening program in the Netherlands, based on primary hrHPV screening.

METHODS

We conducted a post-hoc analysis on 520 physician-taken triage cervical scrape samples of former non-responders who were recruited into the screening program by offering self-sampling for hrHPV testing in the PROHTECT-3B study (PRotection by Offering HPV

TEsting on self-sampled Cervicovaginal specimens Trial-3B). The PROHTECT-3B study is a randomized controlled trial designed to determine whether the participation rate by offering a brush-based cervicovaginal self-sampling device (Evalyn Brush, Rovers Medical Devices) is non-inferior to that of a lavage-based device (Delphi screener, Rovers Medical devices) for self-collection of material for hrHPV testing.[11] The Ministry of Health gave ethical approval for this study (No. 2010/WBO04). In short, 35,477 non-responders to the regular cervical screening program, 33 to 63 years of age, were invited to participate between October 2011 and September 2012. 10,027 women participated by returning self-sampled material to the laboratory for hrHPV testing (GP5+/6+ PCR; EIA HPV GP HR kit; Diassay, Rijswijk, The Netherlands). Women who tested hrHPV negative were advised to participate in the next screening round. HrHPV-positive women were advised to have an additional cervical smear taken by a physician for liquid based cytology (LBC) testing (ThinPrep). The latter population is the focus of this post-hoc analysis (Figure 1). The regional institutional review board approved the protocol for this post-hoc analysis, since all women provided written informed consent.

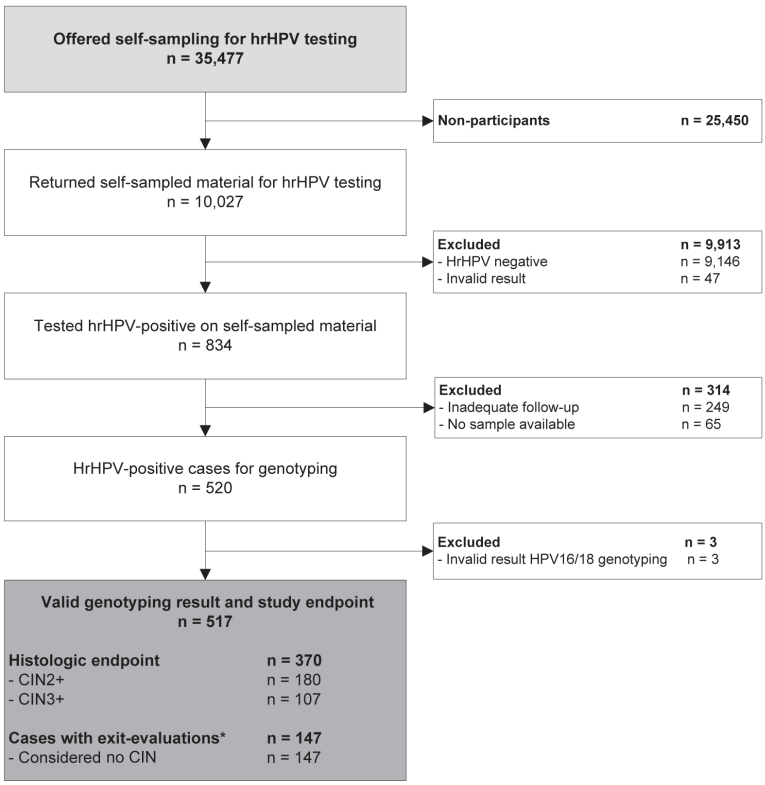

Figure 1. Trial Profile.
* At exit evaluation, women with an initial cytology test result negative for intraepithelial lesion or malignancy (NILM), and after six months a NILM result and an hrHPV-negative test are considered to have no CIN.

All cervical cytology samples were tested in the laboratory of the Department of Pathology, Radboud university medical center, Nijmegen, the Netherlands. The Dutch CISOE-A (composition, inflammation, squamous cells, other and endometrium, endocervical cylindrical epithelium, adequacy) classification system was used to report the test results for the cervical smears, which can easily be translated into the Bethesda nomenclature.[12] Cytologic results were reported as negative for intraepithelial lesion or malignancy (NILM), ASC-US, low-grade squamous intraepithelial lesions (LSIL), or high-grade squamous intraepithelial lesions (HSIL). Atypical squamous cells cannot exclude high-grade squamous intraepithelial lesion (ASC-H) results are not scored as a separate group in the Dutch classification system, thus spread amongst ASC-US or LSIL. Cervical cytology samples were listed as ASC-US when they showed atypical squamous cells or squamous metaplasia, atypical repair, or atypical glandular cells (scored as S2-3, O3, or E3 in the CISOE-A classification), and listed as LSIL, when they showed mild dyskaryosis of the squamous epithelium, mildly atypical endometrium, or mildly-moderately atypical endocervical epithelium (scored as S4, O4, or E4-5). Cervical smears that showed moderate dyskaryosis of squamous epithelium or worse, moderately atypical endometrium or worse, or severe atypical endocervical epithelium or worse (scored as S5-9, O5-9, or E6-9), were registered as HSIL.[12]

Women with abnormal cytology results (determined by the ASC-US threshold) were referred for a colposcopy-directed biopsy, while women with NILM cytologic results were re-invited for an exit test (cytology and hrHPV co-testing) six months later. Women with a positive exit test (ASC-US or worse (≥ASC-US) cytology and/or hrHPV-positive test results) were referred for a colposcopy-directed biopsy. Colposcopists were aware of the hrHPV-positive status and colposcopy was performed according to the Dutch national guidelines. Women with an abnormal biopsy result, ≥CIN2, were treated according to the Dutch national guidelines.[13,14] If no abnormalities were seen at colposcopy, the gynecologist was advised to take two random biopsies according to the study protocol. Histological results were obtained from gynecologists, and missing data were retrieved from the Dutch nationwide registry of histopathology and cytopathology (PALGA).[15] The outcomes were classified as ≥CIN2 and/or severe dysplasia (CIN3) or worse (≥CIN3), mild dysplasia (CIN1), or no dysplasia (no CIN) based on the histological test results. When multiple results were registered, the most severe histological diagnosis was used for analysis. Women with a double-negative exit test (NILM cytology and negative hrHPV results) after six months were considered to have a minimal risk of ≥CIN2 lesions and were not referred for colposcopy; these women were classified as having no CIN. We included all results recorded before June 2013. At this point, the database was closed with a mean follow-up of 15 months (range 6–18 months).

All physician-taken LBC samples, from women with ≥ASC-US cytology who were referred for colposcopy, and from women with NILM cytology who were invited for an exit

test six months later, were analyzed for the presence of different hrHPV genotypes using the clinically validated Cobas 4800 test (Roche) according to the manufacturer's recommendations in the laboratory of the Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, the Netherlands.[16] This test provides separate results for HPV16, 18, and a pool of 12 other hrHPV types (i.e., 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68).[17]

The hrHPV genotyping results were categorized: hrHPV-positive (positive for any of 14 hrHPV types) and negative (negative for all 14 hrHPV types). The hrHPV-positive group was subcategorized in two groups; HPV16 and/or 18 positive (positive for HPV16 and/or 18, regardless of the presence or absence of 12 other hrHPV types) and, positive for 12 other hrHPV types (positive for one or more of the 12 other hrHPV types and negative for HPV16 and 18).

Eighteen different baseline triage strategies were evaluated, combining genotyping for HPV16, HPV16 and/or HPV18, and different thresholds for cytologic interpretations: ≥ASC-US, LSIL or worse (≥LSIL), or HSIL or worse (≥HSIL). These strategies were assessed to identify potentially better algorithms for triage to immediate colposcopy.

The performance of each strategy in detecting ≥CIN3, or ≥CIN2, was explored. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and referral rate (REF) were estimated for each screening strategy. To analyze the extent of overdiagnosis, we estimated the number of referrals needed to diagnose (NRND) one lesion with endpoint ≥CIN2 or ≥CIN3, by dividing one by the PPV. In evaluating the strategies, we considered a NPV for ≥CIN3 of at least 98%, combined with maintenance or improvement of sensitivity and an increase in specificity relative to the comparator, being cytology with an ASC-US threshold, to be a minimal requirement. All strategies were analyzed in reference to this comparator. The threshold of 98% is based on currently used management strategies, in which a scientific risk estimation of <2% risk of cancer or precancer in the subsequent 2–3 years (i.e., NPV for ≥CIN3 for at least 98%) is considered acceptably low to dismiss further testing until the next screening round.[7,18] 95% confidence intervals (CI) were calculated for sensitivity, specificity, PPV, and NPV. Differences between the comparator and the other strategies in sensitivity and specificity were evaluated with McNemar's χ^2 test, and differences in PPV and NPV, with Kosinski and colleagues' method.[19]

We used SPSS version 20.0.1 to analyze the data. Differences in PPV and NPV were estimated with R, version 3.0.1.[20,21]

RESULTS

In the PROHTECT-3B study, 35,477 women who had not responded to an invitation for regular screening were invited to submit a self-collected sample for hrHPV testing. From the 10,027 women who participated in the PROHTECT-3B study, 834 had a positive hrHPV test. Exclusion of women with inadequate follow-up (n=249) or no available sample for genotyping (n=65), resulted in a study population of 520 women (Figure 1). The mean age of the study group was 42 years (range 33–63). There was no difference in mean age between the included and excluded group of women.

A total of 271 women (52.1%) had an ≥ASC-US result, and the remaining 249 women had a NILM cytologic test result. In this group of 249 women with a NILM result, 36 women (14.5%) had an ≥ASC-US result at cytologic testing at exit exam at six months and were referred for colposcopy. Sixtysix women from the NILM group at exit exam, remained HPV positive and were therefore also referred for colposcopic examination. A histological diagnosis was available for 373 of the 520 women. Women who were referred for colposcopy after their first smear result (n=269), and women who underwent colposcopy after their exit exam at six months were combined: 122 had no CIN;CIN1 (n=71); CIN2 (n=73); CIN3 (n=92); and invasive cervical carcinoma (n=15). The remaining 147 women were considered to have no CIN, based on both an hrHPV-negative and NILM cytologic result at exit exam at six months. Seven ≥CIN3 lesions, and eight CIN2 lesions were diagnosed during follow-up of hrHPV-positive women with an initial NILM cytologic result, of which one CIN3 and four CIN2 in random biopsies in hrHPV-positive women with an NILM cytology at exit exam.

Of the 520 physician-taken triage samples, three were invalid for genotype testing and were therefore excluded. All 107 women with ≥CIN3 lesions were hrHPV-positive, and samples were frequently positive for more than one type of hrHPV; HPV16 was detected in 62.6% (67 of 107), HPV18 was detected in 16.8% (18 of 107), and the other 12 types were detected in 58.9% (63 of 107) of women with ≥CIN3. In samples of women with ≥CIN2 lesions, HPV16 was positive in 55.0% (99 of 180), HPV18 in 16.7% (30 of 180), and other, non-HPV16/18 HPV was positive in 67.2% (121 of 180). A total of 2.2% (4 of 180) ≥CIN2 lesions were hrHPV negative. Table 1 shows the hrHPV genotype distribution per outcome category.

In this post-hoc analysis of the PROHTECT-3B study, the performance of baseline triage strategies by hrHPV genotyping, supplemental or alternative to baseline cytology triage were explored. Triage with cytology testing with an ASC-US threshold (strategy 2), was used as the comparator, yielding a sensitivity of 93.5% (95% CI 87.7–97.1), a specificity of 58.5% (95% CI 53.7–63.2), a PPV of 37.0% (95% CI 31.4–42.9), and a NPV of 97.2% (95% CI 94.6–98.8) for ≥CIN3. With this strategy, referral rate was 52.2%, and NRND was 2.70. The characteristics of all 18 strategies for detecting ≥CIN3 lesions are detailed in Table 2.

Table 1. Distribution of hrHPV types in relation to histology lesions.

	Considered no CIN*	No CIN	CIN1	CIN2	CIN3	Carcinoma
hrHPV negative**	78/147 (53.1%)	18/119 (15.1%)	6/71 (8.5%)	4/73 (5.5%)	0/92 (0.0%)	0/15 (0.0%)
hrHPV-positive†	69/147 (46.9%)	101/119 (84.9%)	65/71 (91.5%)	69/73 (94.5%)	92/92 (100.0%)	15/15 (100.0%)
HPV16/18‡	14/147 (9.5%)	36/119 (30.3%)	21/71 (29.6%)	29/73 (39.7%)	68/92 (73.9%)	12/15 (80.0%)
Other 12 hrHPV types¥	55/147 (37.4%)	65/119 (54.6%)	44/71 (62.0%)	40/73 (54.8%)	24/92 (26.1%)	3/15 (20.0%)

CIN = cervical intraepithelial neoplasia; hrHPV = high-risk human papillomavirus. * Considered to be no CIN based on an HPV-negative result and a cytologic result within normal limits at six months of follow-up. ** Negative for all 14 hrHPV types. † Positive for any of 14 hrHPV types. ‡ Positive for HPV16 and/or 18, regardless of the presence or absence of 12 other HPV types (types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). ¥ Positive for one or more of the 12 other HPV types and negative for HPV16 and 18.

Six triage strategies met the test criterion of a NPV of at least 98% for ≥CIN3, although some with limited power. The PPVs of these strategies ranged from 33.1% to 49.5%. Three triage strategies with a combination of cytologic testing and genotyping showed a higher specificity for ≥CIN3 than the comparator, without decreasing the sensitivity. Of these strategies, the one with positivity for HPV16 and/or ≥HSIL cytology (strategy 11 in Table 2) revealed the highest specificity (74.9%, 95% CI 70.5–78.9; p<0.001) with a referral rate of 39.5%, and a NRND of 2.02. The sensitivity of this strategy was similar to the comparator. A specificity of 70.0% (95% CI 65.4–74.3) was achieved with HPV16 and/or 18 positivity and/or ≥HSIL cytology (strategy 17), with a sensitivity of 97.2% (95% CI 92.9–99.3), a referral rate of 43.9% and NRND of 2.18. The sensitivity of combining HPV16 positivity and/or ≥LSIL cytology (strategy 9) was also similar the comparator, with a specificity of 65.6% (95% CI 60.9–70.1; p=0.003), a referral rate of 47.2%, and a NRND of 2.37. Genotyping for HPV16 alone or combined with HPV18, yielded a lower ≥CIN3 sensitivity (62.6%, 95% CI 53.2–71.4, and 74.8%, 95% CI 66.0–82.3 respectively) than the comparator.

The test characteristics of all 18 strategies for detecting ≥CIN2 lesions are given in Table 3. Only the strategy HPV16 positivity and/or ≥LSIL cytology (strategy 9, Table 3) showed an improvement in specificity to 75.1% (95% CI 70.3–79.5) relative to the comparator (68.8% (95% CI 63.8–73.6; p=0.024) at similar sensitivity. None of the other strategies showed improvement in specificity without a decreased sensitivity for the detection of ≥CIN2 lesions as compared with baseline cytology.

Table 2. Strategies for triage of HPV-positive women for identifying women with CIN3 or worse.

No.	Strategy	Sensitivity (*n*=107)		Specificity (*n*=410)		PPV		NPV		REF (*N*=517)	NRND
		% (95% CI)	*p*-value	% (95% CI)	*p*-value	% (95% CI)	*p*-value	% (95% CI)	*p*-value	%	Ratio
1	**None***	100	...	0.0	...	20.7 (17.4–24.3)	...	-	...	100	4.83
2	**≥ASC-US**	93.5 (87.7–97.1)	*COM*	58.5 (53.7–63.2)	*COM*	37.0 (31.4–42.9)	*COM*	97.2 (94.6–98.8)	*COM*	52.2	2.70
3	**≥LSIL**	86.9 (79.7–92.4)	*0.016*	78.0 (73.9–81.9)	*<0.001*	50.8 (43.6–58.0)	*<0.001*	95.8 (93.3–97.6)	*0.129*	35.4	1.97
4	**≥HSIL**	82.2 (74.3–88.7)	*<0.001*	90.2 (87.1–92.9)	*<0.001*	68.8 (60.4–76.4)	*<0.001*	95.1 (92.7–97.0)	*0.058*	24.8	1.45
5	**HPV16+**	62.6 (53.2–71.4)	*<0.001*	81.7 (77.8–85.2)	*<0.001*	47.2 (39.1–55.4)	*0.002*	89.3 (85.9–92.2)	*<0.001*	27.5	2.12
6	**HPV16+ and/or 18+**	74.8 (66.0–82.3)	*<0.001*	75.6 (71.3–79.6)	*<0.001*	44.4 (37.3–51.7)	*0.008*	92.0 (88.8–94.6)	*0.007*	34.8	2.25
7	**HPV16+ and/or ≥ASC-US**	99.1 (95.9–99.9)	*0.031*	50.7 (45.9–55.6)	*<0.001*	34.4 (29.3–39.8)	*0.003*	99.5 (97.9–100)	*0.026*	59.6	2.91
8	**HPV16+ and ≥ASC-US**	57.0 (47.5–66.1)	*<0.001*	89.5 (86.3–92.2)	*<0.001*	58.7 (49.1–67.8)	*<0.001*	88.9 (85.6–91.7)	*<0.001*	20.1	1.70
9	**HPV16+ and/or ≥LSIL**	96.3 (91.5–98.8)	*0.508*	65.6 (60.9–70.1)	*0.003*	42.2 (36.1–48.5)	*0.002*	98.5 (96.6–99.5)	*0.227*	47.2	2.37
10	**HPV16+ and ≥LSIL**	53.3 (43.8–62.6)	*<0.001*	94.1 (91.6–96.1)	*<0.001*	70.4 (59.9–79.6)	*<0.001*	88.5 (85.3–91.3)	*<0.001*	15.7	1.42
11	**HPV16+ and/or ≥HSIL**	94.4 (89.0–97.7)	*1.000*	74.9 (70.5–78.9)	*<0.001*	49.5 (42.7–56.3)	*<0.001*	98.1 (96.2–99.2)	*0.433*	39.5	2.02
12	**HPV16+ and ≥HSIL**	50.5 (41.1–59.8)	*<0.001*	97.1 (95.1–98.4)	*<0.001*	81.8 (71.4–89.8)	*<0.001*	88.2 (85.1–91.0)	*<0.001*	12.8	1.22
13	**(HPV16+ and/or 18+) and/or ≥ASC-US**	100 (96.6–100)	*0.014*	47.3 (42.5–52.2)	*<0.001*	33.1 (28.1–38.4)	*<0.001*	100.0 (96.6–100)	*0.017*	62.5	3.02
14	**(HPV16+ and/or 18+) and ≥ASC-US**	68.2 (59.0–76.5)	*<0.001*	86.8 (83.3–89.9)	*<0.001*	57.5 (48.8–65.9)	*<0.001*	91.3 (88.2–93.8)	*<0.001*	24.6	1.74
15	**(HPV16+ and/or 18+) and/or ≥LSIL**	98.1 (94.3–99.7)	*0.180*	61.5 (56.7–66.1)	*0.281*	39.9 (34.1–45.9)	*0.076*	99.2 (97.6–99.9)	*0.082*	50.9	2.50
16	**(HPV16+ and/or 18+) and ≥LSIL**	63.6 (54.2–72.3)	*<0.001*	92.2 (89.3–94.5)	*<0.001*	68.0 (58.5–76.6)	*<0.001*	90.6 (87.6–93.2)	*<0.001*	19.3	1.47
17	**(HPV16+ and/or 18+) and/or ≥HSIL**	97.2 (92.9–99.3)	*0.344*	70.0 (65.4–74.3)	*<0.001*	45.8 (39.4–53.3)	*<0.001*	99.0 (97.3–99.7)	*0.122*	43.9	2.18
18	**(HPV16+ and/or 18+) and ≥HSIL**	59.8 (50.4–68.8)	*<0.001*	95.9 (93.6–97.5)	*<0.001*	79.0 (69.3–86.9)	*<0.001*	90.1 (87.1–97.2)	*<0.001*	15.7	1.27

ASC-US = atypical squamous cells of undetermined significance or worse; CIN3 = cervical intraepithelial neoplasia, grade 3; COM = comparator; HPV = human papillomavirus; ≥HSIL = high-grade squamous intraepithelial lesions or worse; ≥LSIL = low-grade squamous intraepithelial lesions or worse; NPV = negative predictive value; NRND = number of referrals needed to diagnose one ≥CIN3 lesion; PPV = positive predictive value; REF = referral rate. * All women with HPV-positive self-samples were referred for colposcopy. All data are crude estimates. The *p*-values represent comparisons of various triage strategies to ASC-US or worse. Differences in sensitivity and specificity between the reference strategy and the other strategies were evaluated with McNemar's χ^2 test, and differences in PPV and NPV were evaluated with Kosinski and colleagues' method.[19] ASC-US or worse includes cytologic interpretations of ASC-US, AGC, LSIL, and HSIL. The strategies meeting the criterion for a NPV of at least 98%, with similar sensitivity, and significantly better specificity and PPV for ≥CIN3 than the reference triage strategy at a ≥CIN3 threshold, are marked in grey.

Table 3. Strategies for triage of HPV-positive women for identifying women with CIN2 or worse.

No.	Strategy	Sensitivity (*n*=180)		Specificity (*n*=337)		PPV		NPV		REF (*N*=517)	NRND
		% (95% CI)	*p-value*	% (95% CI)	*p-value*	% (95% CI)	*p-value*	% (95% CI)	*p-value*	%	Ratio
1	**None***	100	...	0	...	34.8 (30.8–39.0)	...	-	...	100	2.87
2	**≥ASC-US**	91.7 (87.0–95.1)	*COM*	68.8 (63.8–73.6)	*COM*	61.1 (55.2–66.8)	*COM*	93.9 (90.5–96.5)	*COM*	52.2	1.64
3	**≥LSIL**	77.8 (71.3–83.4)	*<0.001*	87.2 (83.4–90.5)	*<0.001*	76.5 (70.0–82.3)	*<0.001*	88.0 (84.3–91.2)	*<0.001*	35.4	1.31
4	**≥HSIL**	62.2 (55.0–69.1)	*<0.001*	95.3 (92.6–97.2)	*<0.001*	87.5 (81.0–92.5)	*<0.001*	82.5 (78.5–86.1)	*<0.001*	24.8	1.14
5	**HPV16+**	49.4 (42.2–56.7)	*<0.001*	84.3 (80.1–87.9)	*<0.001*	62.7 (54.5–70.4)	*0.681*	75.7 (71.2–79.9)	*<0.001*	27.5	1.60
6	**HPV16+ and/or 18+**	60.6 (53.3–67.5)	*<0.001*	78.9 (74.4–83.1)	*<0.001*	60.6 (53.3–67.5)	*0.870*	78.9 (74.4–83.1)	*<0.001*	34.8	1.65
7	**HPV16+ and/or ≥ASC-US**	96.7 (93.4–98.7)	*0.004*	60.2 (54.9–65.4)	*<0.001*	56.5 (50.9–62.0)	*<0.001*	97.1 (94.3–98.8)	*0.011*	59.6	1.77
8	**HPV16+ and ≥ASC-US**	44.4 (37.3–51.7)	*<0.001*	92.9 (89.8–95.3)	*<0.001*	76.9 (68.2–84.3)	*<0.001*	75.8 (71.5–79.8)	*<0.001*	20.1	1.30
9	**HPV16+ and/or ≥LSIL**	88.9 (83.8–92.9)	*0.405*	75.1 (70.3–79.5)	*0.024*	65.6 (59.5–71.4)	*0.052*	92.7 (89.2–95.4)	*0.471*	47.2	1.53
10	**HPV16+ and ≥LSIL**	38.3 (31.4–45.6)	*<0.001*	96.4 (94.1–98.1)	*<0.001*	85.2 (76.4–91.8)	*<0.001*	74.5 (70.3–78.5)	*<0.001*	15.7	1.17
11	**HPV16+ and/or ≥HSIL**	78.3 (71.9–83.9)	*<0.001*	81.3 (76.9–85.2)	*<0.001*	69.1 (62.6–75.2)	*0.006*	87.5 (83.6–90.9)	*0.003*	39.5	1.45
12	**HPV16+ and ≥HSIL**	33.3 (26.7–40.4)	*<0.001*	98.2 (96.4–99.3)	*<0.001*	90.9 (82.4–96.3)	*<0.001*	73.4 (69.2–77.3)	*<0.001*	12.8	1.00
13	**(HPV16+ and/or 18+) and/or ≥ASC-US**	97.2 (94.1–99.0)	*0.002*	56.1 (50.8–61.3)	*<0.001*	54.2 (48.7–59.6)	*<0.001*	97.4 (94.5–99.1)	*0.012*	62.5	1.85
14	**(HPV16+ and/or 18+) and ≥ASC-US**	55.0 (47.7–62.2)	*<0.001*	91.7 (88.4–94.3)	*<0.001*	78.0 (70.2–84.6)	*<0.001*	79.2 (75.0–83.1)	*<0.001*	24.6	1.28
15	**(HPV16+ and/or 18+) and/or ≥LSIL**	90.6 (85.7–94.3)	*0.832*	70.3 (65.3–75.0)	*0.675*	62.0 (56.0–67.7)	*0.707*	93.3 (89.8–96.0)	*0.726*	50.9	1.61
16	**(HPV16+ and/or 18+) and ≥LSIL**	47.8 (40.6–55.1)	*<0.001*	95.8 (93.4–97.6)	*<0.001*	86.0 (78.3–91.9)	*<0.001*	77.5 (73.3–81.3)	*<0.001*	19.3	1.16
17	**(HPV16+ and/or 18+) and/or ≥HSIL**	81.1 (75.0–86.4)	*0.003*	76.0 (71.2–80.3)	*0.028*	64.3 (57.9–70.4)	*0.255*	88.3 (84.2–91.6)	*0.009*	43.9	1.55
18	**(HPV16+ and/or 18+) and ≥HSIL**	41.7 (34.6–48.9)	*<0.001*	98.2 (96.4–99.3)	*<0.001*	92.6 (85.6–97.0)	*<0.001*	75.9 (71.8–79.8)	*<0.001*	15.7	1.08

≥ASC-US = atypical squamous cells of undetermined significance or worse; CIN3 = cervical intraepithelial neoplasia grade 3; COM = comparator; HPV = human papillomavirus; ≥HSIL = high-grade squamous intraepithelial lesions or worse; ≥LSIL = low-grade squamous intraepithelial lesions or worse; NPV = negative predictive value; NRND = number of referrals needed to diagnose one ≥CIN2 lesion; PPV = positive predictive value; REF = referral rate. * All women with HPV-positive self-samples were referred for colposcopy. All data are crude estimates. The *p*-values represent comparisons of various triage strategies to ASC-US or worse. Differences between the reference strategy and the other strategies in sensitivity and specificity were evaluated with McNemar's χ^2 test. Differences in PPV and NPV were evaluated with Kosinski and colleagues' method.[19] ASC-US or worse includes cytologic interpretations of ASC-US, AGC, LSIL, and HSL.

DISCUSSION

Three of the 18 evaluated strategies for the triage of hrHPV-positive women met the NPV criterion of at least 98% for ≥CIN3, and showed a higher specificity for ≥CIN3 than the comparator being cytology with an ASC-US threshold. These three strategies were 1) HPV16 genotyping and/or cytology at ≥LSIL threshold, 2) HPV16 genotyping and/ or cytology at ≥HSIL threshold, and 3) HPV16/18 genotyping and/or ≥HSIL cytology. The three strategies all showed high sensitivities and a significantly better PPV for ≥CIN3, the comparator (i.e. cytology with prior knowledge on hrHPV status), and in addition, the referral rates decreased by 5.0–12.7%. With a ≥CIN2 threshold only the strategy that combines HPV16 genotyping and/or cytology at ≥LSIL threshold showed improved specificity with similar sensitivity, relative to the comparator. However, in case of a CIN2 lesion a watchful waiting policy could be considered, especially in young and fertile women.

Our findings are consistent with those from the ATHENA study[9], which is a large clinical trial that assessed hrHPV genotyping and cytology co-testing for cervical cancer screening. Strategies that included integrated HPV16/18 testing provided efficient referral to colposcopy. When the cytologic threshold is raised to HSIL as well as hrHPV genotyping added to cytologic triage, our results confirm the improvement of diagnostic accuracy. Our study differed from the Athena trial as we assessed hrHPV genotyping strategies in the physician-taken triage material of women 33 to 63 years of age after hrHPV-positive self-sampling, in contrast to the ATHENA trial where hrHPV genotyping was added in an opportunistic screening population that also included women between 29 and 32 years of age. Rijkaart et al.[7] showed a NPV of >98% in a triage strategy combining cytology at ASC-US threshold and HPV16/18/31/33/45 genotyping in an organized cervical cancer screening setting. Dijkstra et al.[8] showed a NPV of >98% by combining cytology at ASC-US threshold and HPV16/18 genotyping. However, both studies showed higher colposcopy referral rates. Our study adds to previous findings because genotyping performance was explored in a setting with prior knowledge on hrHPV-positive status, rather than cytology positivity, which will become the new practice in hrHPV-based population-screening.

The choice for one of the three favorable strategies will depend on clinical needs. When the proposed cytologic triage is used in clinical practice, there may be a need to reduce the colposcopy rate because of risk of overdiagnosis, high costs, and high levels of anxiety for the women involved. In these cases, referring women with HPV16 and/or ≥HSIL cytology test results (strategy 11) may be a good alternative since the specificity is significantly improved, and the sensitivity is similar to that of the comparator. As a result, the referral rate decreases from 52.2% to 39.5%, and instead of 2.70 women, only 2.02 women need to be referred to diagnose one ≥CIN3. This may be considerable for

young women for whom overtreatment may be harmful in future pregnancies.[22] However, women with low-grade abnormal cervical smear results will not be referred in this strategy. Lowering the cytology threshold to LSIL, combined with HPV16 genotyping (strategy 9) might partially resolve this issue. In women with an ASC-US cervical smear result, follow-up could be considered. When there is a clinical need to use a test with the highest possible sensitivity, strategy 13 (HPV16 and/or HPV18 positive or ≥ASC-US cytologic testing) with 100% sensitivity for ≥CIN3 (95% CI 96.6–100) may be an alternative. The referral rate increased to 62.5%, and the NRND to 3.02, while the specificity decreased to 47.3%. However, the length of follow-up in this study is too short to determine the actual consequences of their return to the next screening round. Furthermore, this strategy did not yield such high sensitivity in previous trials.[7,9]

Previous studies have shown a 10-year cumulative incidence rate of ≥CIN3 lesions of about 17–20% among HPV16-positive women, 13–17% among HPV18-positive women and only 3% among hrHPV-positive, HPV16/18-negative women.[18,23] An advantage of hrHPV genotyping is the possibility to objectively select women who have an increased risk (e.g. HPV16 positive), or a relatively low risk (e.g. positive for types other than HPV16/18) of developing ≥CIN3 lesions. Adequate follow-up based on individual risk stratification can therefore be provided.[23,24] However, in the use of either of these triage methods there is still a group of hrHPV-positive women that will not be referred for additional diagnostics. This may result in anxiety amongst women, even if their ≥CIN3 risk is statistically low enough for them to return to the next screening round. It is therefore important for the last triage test to be negative; a repeat visit for cytologic testing at six to 12 months could be used to accomplish this.[7,25] A disadvantage of repeat testing is loss to follow-up, while existing ≥CIN3 lesions may be missed.[26]

An important strength of this study is the study design, comparable with the future Dutch cervical cancer screening program with hrHPV testing and cytology triage. It is known that the performance of cytology is influenced by the cytotechnicians' knowledge of the positive hrHPV status.[27] In this setting with knowledge of prior hrHPV status, we observed high sensitivity for cytology.[2] A second strength of this study is that for some HPV tests it is possible to combine hrHPV screening with immediate HPV16/18 genotyping, (e.g. the Cobas 4800 test used in this study). By combining hrHPV screening with HPV16/18 genotyping, the proposed strategies in this study will be easily available, without any additional costs. A limitation of this study is the lack of histology in the group of women who had a NILM cytologic triage result and, after six months of follow-up, an hrHPV-negative result and a NILM cytologic result. However, the Ministry of Health did not approve colposcopy with random biopsies in these women, because the risk of ≥CIN2 in his group of women is low.[25] Furthermore, women with inadequate follow-up were excluded from this study, which might over-represent women with NILM cytology at baseline, and the current study also has a relative short duration of follow-

up. When ruling out the need for intensified surveillance, we should wait for long-term follow-up for a more solid result, especially when strategies without referring HPV18 positive women will be considered. Next, it might be difficult to distinguish between ASC-US and LSIL in cytology. When a LSIL threshold is used to distinguish women who need to visit a gynecologist for colposcopy, from women who do not, this might hold a risk for differences in scoring of these low-risk cervical smears. Therefore, the reproducibility of this difference still has to be confirmed. Furthermore, this study was performed in a non-responder population, which involves substantial numbers of underscreened women and women who were never screened before.[28] For a responder population, these algorithms might show slightly different results. These factors altogether may lead to an under- or overestimation of the screening results.

As knowledge about the molecular oncogenesis of cervical precancer and cancer is expanding, the clinical utility of a wide variety of novel biomarkers in supplementary or stand-alone triage tests is being studied extensively and could also fulfil a role in triage of hrHPV-positive women. Triage strategies, such as mRNA HPV testing, p16 staining, p16/Ki-67 dual staining, MSM2/TOP2A dual staining, E6/E7 protein markers, and DNA methylation markers, may help to reduce the number of unnecessary referrals for colposcopy.[29-31] The clinical performance of these various triage strategies still needs further assessment in regular screening programs; also, these strategies are not yet combined in one test with HPV testing, as is hrHPV genotyping.

In regular European screening programs, hrHPV testing is expected to replace cytology testing as a primary screening tool in the coming years. In the Netherlands, primary hrHPV screening is scheduled to start in 2017, and non-responders will be able to opt in for HPV self-sampling. For the time being, if an HPV self-sampling result is positive, triage will consist of cytology on an extra physician-taken cervical smear. In such a screening program, it would be possible to combine hrHPV testing with HPV16 and HPV18 genotyping, and have knowledge on the presence of cellular abnormalities, which offers opportunities for personalized risk management.

We conclude that HPV16 and/or 18 genotyping combined with raising the threshold of cervical cytological abnormalities from ≥ASC-US to ≥LSIL or ≥HSIL improves the referral rate and the specificity for detecting ≥CIN3 lesions while maintaining adequate sensitivity.

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CHAPTER 4

Evaluation of p16/Ki-67 dual-stained cytology as triage test for high-risk human papillomavirus-positive women

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ABSTRACT

Objective The aim of this study was to evaluate the clinical utility of p16/Ki-67 dual staining, for the identification of cervical intraepithelial neoplasia (CIN) in high-risk human papillomavirus (hrHPV)-positive women from a non-responder screening cohort.

Methods P16/Ki-67 dual staining, Pap cytology and HPV16/18 genotyping were performed on physician-taken liquid-based cytology samples from 495 women who tested hrHPV-positive on self-sampled material (PROHTECT3B study). Different triage strategies involving p16/Ki-67 dual staining were evaluated for sensitivity, specificity, and predictive value for ≥CIN2 and ≥CIN3, and compared with Pap cytology with a threshold of atypical cells of undetermined significance (ASC-US). Centrally revised histology or an adjusted endpoint with combined hrHPV negative and cytology negative follow-up at six months was used as gold standard.

Results Triage of hrHPV-positive samples by Pap cytology with an ASC-US-threshold showed a sensitivity of 93% (95% CI 85-98) with a specificity of 49% (95% CI 41-56) for ≥CIN3. Three triage strategies with p16/Ki-67 showed a significantly increased specificity with similar sensitivity. P16/Ki-67 triage of all hrHPV-positive samples had a sensitivity of 92% (95% CI 84-97) and a specificity of 61% (95% CI 54-69) for ≥CIN3. Applying p16/Ki-67 triage to only hrHPV-positive women with low-grade Pap cytology showed a similar sensitivity of 92% (95% CI 84-97), with a specificity for ≥CIN3 of 64% (95% CI 56-71). For hrHPV-positive women with low-grade and normal Pap cytology, triage with p16/Ki-67 showed a sensitivity of 96% (95% CI 89-99), and a specificity of 58% (95% CI 50-65). HPV16/18 genotyping combined with Pap cytology showed a sensitivity and specificity for ≥CIN3 similar to Pap cytology with an ASC-US threshold.

Conclusion Because the quality of Pap cytology worldwide varies, and differences in sensitivity and specificity are limited between the three selected strategies, p16/Ki-67 triage of all hrHPV-positive samples would be the most reliable strategy in triage of hrHPV-positive women with an increased specificity and similar sensitivity compared with Pap cytology triage.

INTRODUCTION

The introduction of cytology-based organized cervical cancer screening programs has contributed to decreased cervical cancer incidence and mortality in developed countries.[1-3] Compared with cytology, human papillomavirus (HPV) DNA testing has a higher sensitivity with a higher negative predictive value (NPV) for detection of cervical intraepithelial neoplasia (CIN) and cancer. The high reassurance of a low risk of cervical cancer for high-risk HPV (hrHPV) negative women is one of the advantages of the HPV DNA test, which has resulted in a shift from cytology-based screening towards HPV DNA detection as primary screening method.[4,5]

However, the high sensitivity of HPV DNA testing is combined with a lower positive predictive value (PPV), due to the fact that most hrHPV infections clear spontaneously and do not result in cancer.[6] Additional triage of hrHPV-positive women is therefore required to limit the number of unnecessary referrals for follow-up procedures in women without clinically meaningful hrHPV infections.[7] Currently proposed triage strategies for hrHPV-positive women in HPV DNA-based screening programs are repeated Pap cytology and/or HPV16 and HPV18 genotyping. Pap cytology is a relatively subjective test for which high expertise is required. Owing to its limited sensitivity repeat cytology testing within 6-12 months is needed before returning hrHPV-positive women with normal cytology back to regular screening. This bears the risk of losing them during follow-up.[8] HPV16/18 genotyping is an objective test to triage hrHPV-positive women, however, this strategy alone yields limited sensitivity as it only identifies cervical lesions associated with these two hrHPV types. Sensitivity of HPV16/18 genotyping can be improved by combining it with Pap cytology at the cost of a lower specificity.[9-11]

Another biomarker widely studied for triage is the p16/Ki-67 dual staining. $P16^{INK4a}$ (p16) is a cell-cycle regulatory protein that induces cell-cycle arrest under normal physiological conditions, and Ki-67 is a marker expressed during cell proliferation.[12,13] The simultaneous detection of p16 and Ki-67 within the same cervical epithelial cell will not occur under physiological conditions and may be used as a surrogate marker of cell-cycle deregulation mediated by transforming hrHPV infections. P16/Ki-67 dual-stain has been previously studied as a potential primary screening test, as triage test for women with atypical cells of undetermined significance (ASC-US), for surveillance of women treated for high-grade CIN, and also in a limited number of studies as triage test in women with a positive hrHPV test.[14-22]

The aim of the current study was to evaluate the overall clinical performance of the p16/Ki-67 dual-stain test as triage method for hrHPV-positive women from a non-responder screening cohort.

METHODS

This study is a post-hoc analysis on physician-taken triage cervical scrapes of former non-responder women who were recruited into the screening program by offering self-sampling for hrHPV testing in the PROHTECT-3B study in 2011 and 2012. (PRotection by Offering HPV TEsting on self-sampled Cervicovaginal specimens Trial-3B).[23] In this trial, non-responders to the regular cervical screening program, between 30 and 60 years of age, were invited to participate by returning self-sampled material (Evalyn Brush, or Delphi Screener, Rovers Medical Devices) to the laboratory for hrHPV testing (GP5+/6+ polymerase chain reaction; EIA HPV GP HR kit; Diassay, Rijswijk, The Netherlands). Women who tested hrHPV-positive on their self-sample were advised to have an additional cervical smear taken by a physician for Pap cytology testing. Women with an abnormal cervical smear result were referred to a gynecologist for colposcopic examination, and for women with a normal cervical smear result a 6-month follow-up smear was performed for both hrHPV testing and Pap cytology. Further details of the PROHTECT-3B study design are reported elsewhere.[23] All women provided written informed consent. The Ministry of Health gave ethical approval for this study (No.2010/WBO04), and the regional institutional review board approved the protocol for this post-hoc analysis.

From 495 of the total of 834 hrHPV-positive women in the PROHTECT-3B study, a study-endpoint was known, and a physician-taken triage liquid-based cytology cervical scrape was available. Women with abnormal cytology results (defined as ≥ASC-US) were referred for a colposcopy-directed biopsy, whereas women with normal cytology results (defined by negative for intraepithelial lesion or malignancy (NILM) cytology result) were re-invited for an exit test with Pap cytology and hrHPV co-testing six months later. Women with a positive exit test, defined as ASC-US or worse (≥ ASC-US) cytology and/or hrHPV-positive test results, were referred for a colposcopy-directed biopsy. Colposcopists were aware of the hrHPV-positive status, and colposcopy was performed according to the Dutch national guidelines. If no abnormalities were seen at colposcopy, it was advised to take two random biopsies according to the study protocol. Women with a double-negative exit test (NILM cytology and negative hrHPV results) after six months were considered to have a minimal risk of ≥CIN2 lesions and were not referred for colposcopy; these women were classified as not having CIN2 (<CIN2). We included all results recorded before June 2013. At this point, the database was closed with a mean follow-up of 15 months (range 6–18 months).

All liquid-based cytology samples were processed and reported in the laboratory of the department of Pathology, Radboud university medical center, Nijmegen, the Netherlands. The ThinPrep 3000 was used for processing, and cytological classification was performed according to the primarily used Dutch CISOE-A (composition, inflammation, squamous cells, other and endometrium, endocervical cylindrical epithelium, ad-

equacy) classification. For analysis of cervical smears, the CISOE-A classification system was translated into the Bethesda nomenclature; in which borderline or mild dyskaryosis equals ASC-US and low-grade squamous intraepithelial lesion (LSIL), and worse than borderline or mild dyskaryosis equals high-grade squamous intraepithelial lesion (HSIL).[24] All abnormal cytology was analyzed independently by two cytotechnicians who were aware of the hrHPV-positive status, but unaware of the p16/Ki-67 and HPV16/18 genotype results.

Histological results were obtained from records of the pathologists, and missing data were retrieved from the Dutch nationwide registry of histopathology and cytopathology (PALGA).[25] The histology outcomes were classified as CIN2 or worse (≥CIN2) or CIN3 or worse (≥CIN3), CIN1, or no CIN. AIS was included in the CIN3 group. When multiple results were registered per woman, the most severe histological diagnosis was used for analysis. Histology samples collected during colposcopy procedures were subjected to central pathologist review. If the first pathologist disagreed with the initial diagnosis, a pathologist specialized in gynecologic oncology independently reviewed the case resulting in a final diagnosis. Majority consensus diagnoses were established on all available cervical tissue specimens. Pathologists were blinded to all other study results.

Partial HPV genotyping was done by the Cobas 4800 test (Roche), according to the manufacturer's recommendations in the laboratory of the Department of Medical Microbiology, Radboud university medical center, Nijmegen, the Netherlands.[26] This test provides separate result for HPV16 and 18, and a pool of 12 other hrHPV types (i.e., 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). The HPV genotyping results were categorized as HPV16/18 positive when HPV16 and/or HPV18 were present, regardless of the presence or absence of 12 other HPV types. Other results were scored as non-16/18 HPV positive.

After hrHPV testing and Pap cytology testing the residual liquid-based cytology (LBC) material was stored for approximately two years in the original ThinPrep vials (Hologic UK Ltd, Crawley, West Sussex, UK) at room temperature (storage between 4 and 30° Celsius is advised by the manufacturer). A slide for p16/Ki-67 was prepared from the residual PreservCyt material using the ThinPrep 2000 Processor (Hologic). The CINtec PLUS Cytology kit (Roche mtm Laboratories AG, Mannheim, Germany) was used according to the instructions of the manufacturer. Staining was performed on a Ventana benchmark ultra. (Roche mtm Laboratories AG, Mannheim, Germany), and each run included one control specimen.

Six trained observers each independently reviewed one-third of the cases for the presence of dual-stained cells, resulting in two independent results for each case. A case was considered positive if one or more cervical epithelial cells were stained both with a red nuclear stain for Ki-67 and a brown cytoplasmic stain for p16, independent of cellularity criteria (Figure 1). Cases were considered negative when no staining or only single staining of p16 or Ki-67 was observed in a single cell. A case was scored as

inadequate if background staining prohibited adequate evaluation, no p16 and/or Ki-67 staining was visible as internal control, or if slides did not meet the squamous cellularity criteria as specified in the Hologic criteria (≥5,000 cells per slide). Inadequate cases were excluded from evaluation. In case of disagreement between two observers, a consensus score using a multi-headed microscope was obtained. The observers were unaware of the Pap cytology result, HPV16/18 genotyping result, or follow-up data. Our data on p16/ki-67 triage of hrHPV-positive women was compared with previous studies found in a systematic search combining synonyms for p16/Ki-67 dual-stain and HPV. Information on sensitivity, specificity, number of participants, hrHPV status, cytology result, and p16/Ki-67 stain was obtained from relevant studies.

Descriptive statistics were used to calculate sensitivities, specificities, predictive values with corresponding 95% confidence intervals (CI), and referral rate (REF). The extent of overdiagnosis was estimated by using the number of referrals needed to diagnose (NRND) one lesion with endpoint ≥CIN2 or ≥CIN3, this equals by dividing one by the PPV. Differences in sensitivity and specificity between the comparator strategy and the other strategies were evaluated with McNemar's χ^2 test with continuity correction. Statistical analyses were conducted using SPSS version 20.0.1. Differences in sensitivity and specificity were estimated with R, version 3.0.1.[27]

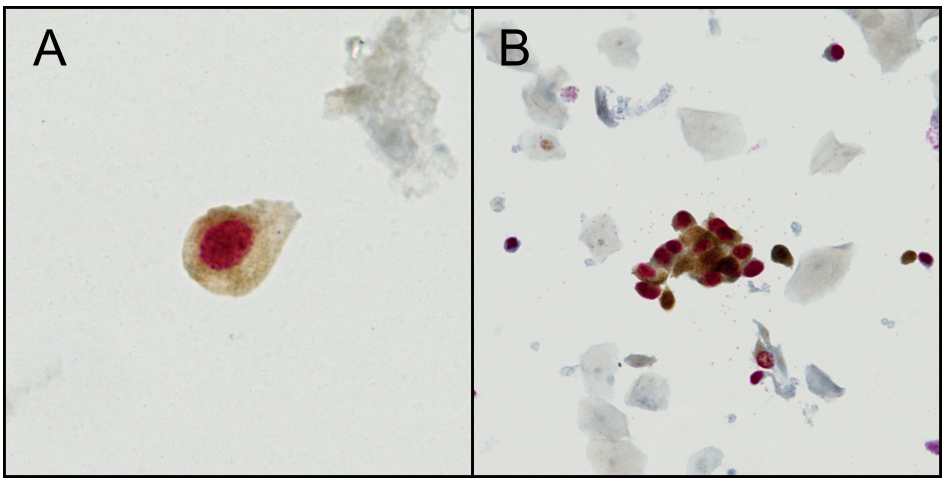

Figure 1. Example of p16/Ki-67 dual staining in cervical cytology.
A) A p16/Ki-67 dual-stain positive single-cell. B) A p16/Ki-67 dual-stained cluster of cells. A case is considered dual-stain positive if one or more cervical epithelial cells are stained with a red nuclear stain for Ki-67 and a brown cytoplasmic stain for p16.

RESULTS

In the PROHTECT-3B study, 35,477 women were offered self-sampling for hrHPV testing. Out of 10,027 women who participated in the study, 834 (8.3%) were hrHPV-positive on a self-sample. Of the 834 hrHPV-positive women, 287 were excluded because of inadequate follow-up, 47 because no LBC left-over sample was available, and 38 women because no histology sample was available for revision, leaving 462 cases available for p16/Ki-67 dual-stain cytology and hrHPV genotyping (Figure 2). The mean age of the study group was 42 years, with a median age of 38 years (range, 33-63 years).

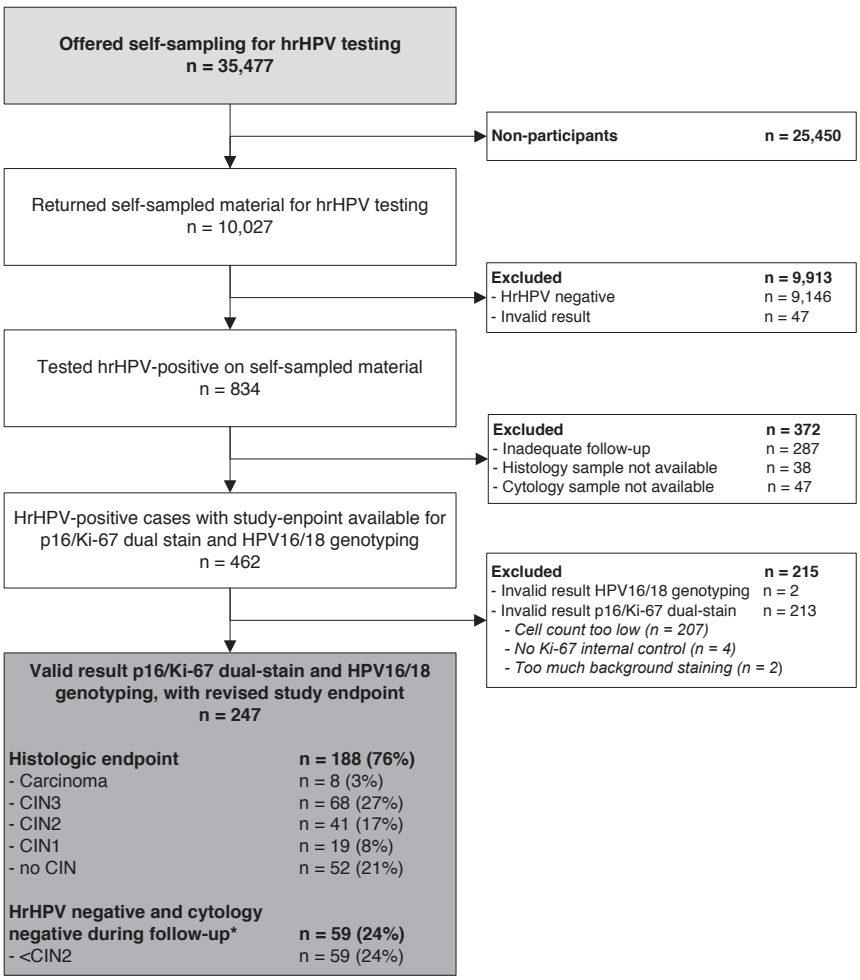

Figure 2. Trial Profile.

* HrHPV negative and cytology negative during follow-up; women with an initial cytology test result negative for intraepithelial lesion or malignancy, and after six months a negative for intraepithelial lesion or malignancy result and an hrHPV-negative test are considered to have less than CIN2 (<CIN2).

From the total group of 462 cases available for p16/Ki-67 dual staining, 136 were positive, 113 negative, and 213 were inadequate. In 207 of the latter, the cell count was too low, in four cases no Ki-67 internal control was visible, and in two cases the background staining made adequate evaluation impossible. The low cell count in the majority of cases was probably due to the fact that too little of the sample material was left after previous tests. Of the remaining 249 women, two had an inadequate genotyping result; leaving 247 cases available for analysis. There was no difference in age between the included and excluded women with a median age of 38 years in both groups and a mean age of 42 years and 41 years respectively. The excluded group contained more low-grade and normal histology results. Resulting in 47% ≥CIN2 and 71% CIN1 or less (≤CIN1) in the included group, and 29% ≥CIN2 and 53% ≤CIN1 in the excluded group.

From the total of 247 women with a valid result for p16/Ki-67 dual-stain and HPV16/18 genotyping, 159 (64%) women had an abnormal cytology result of ASC-US or worse, 136 women (55%) had a p16/Ki-67 positive score, and 99 women (40%) were positive for HPV16/18 (Table 1). Of the total group of 247 women, 188 women had a revised histological endpoint: eight women had a cervical carcinoma, 68 were diagnosed with CIN3, 41 with CIN2, 19 with CIN1, and 52 with no CIN. The remaining 59 women were considered to have no CIN, based on both an hrHPV negative and NILM cytology result after six months (Figure 2). Revision of the 188 histology results yielded a similar result in 153 (81%), cases and a different result in 35 cases (19%) Revision leaded to shift of three cases scored as ≥CIN2 towards <CIN2, and 12 cases scored as <CIN2, towards ≥CIN2, resulting in a 3.6% higher ≥CIN2 prevalence after revision.

Table 1. Test results of p16/Ki-67 dual-stain in regard to Pap cytology and HPV16/18 genotyping.

		Pap cytology			
		NILM	**ASC-US and LSIL**	**HSIL**	**Total**
p16/Ki-67 positive	HPV16/18 positive	4	18	57	79 (32%)
	HPV16/18 negative	9	23	25	57 (23%)
	Total	13	41	82	136 (55%)
p16/Ki-67 negative	HPV16/18 positive	10	7	3	20 (8%)
	HPV16/18 negative	65	20	6	91 (37%)
	Total	75	27	9	111 (45%)
Total		88 (36%)	68 (27%)	91 (37%)	247 (100%)

≥ASC-US = atypical squamous cells of undetermined significance or worse; HPV = human papillomavirus; HSIL = high-grade squamous intraepithelial lesion; LSIL = low-grade squamous intraepithelial lesion; NILM = negative for intraepithelial lesion or malignancy.

In this post-hoc analysis of the PROHTECT-3B study, first the performance of six baseline triage strategies by p16/Ki-67 dual stain, Pap cytology and HPV16/18 genotyping was explored with endpoints ≥CIN2 and ≥CIN3. Baseline triage with cytology testing

with an ASC-US threshold was used as comparator strategy. This strategy yielded a sensitivity of 93% (95% CI 85-98), with a specificity of 49% (95% CI 41-56), PPV of 45% (95% CI 37-53) and NPV of 94% (95% CI 87-98) for ≥CIN3. Five women with CIN3 were missed with this strategy. P16/Ki-67 dual staining showed a similar sensitivity of 92% (95% CI 84 -97), with an increased specificity of 61% (95% CI 54-69) for ≥CIN3. Six women with CIN3 were missed with this strategy. HPV16/18 genotyping alone showed a significantly lower sensitivity of 75% (95% CI 64-97), with 19 missed CIN3 cases, and a significant improvement in specificity of 75% (95% CI 68-82) for ≥CIN3 with a referral rate of only 40%.

Three strategies showed similar sensitivity with improved specificity, in regard to the comparator strategy for ≥CIN3. Similar was defined by a non-significant difference from Pap cytology triage. The first strategy was p16/Ki-67 triage of all hrHPV-positive women. The second strategy was baseline cytology with p16/Ki-67 triage restricted to hrHPV-positive women with ASC-US or LSIL cytology, which showed a sensitivity of 92% (95% CI 84-97), with the highest specificity of 64% (95% CI 56-71), and a false negative result for six women with CIN3. The third strategy was 16/Ki-67 triage of hrHPV-positive women with NILM, ASC-US or LSIL cytology, resulted in an equal sensitivity of 96% (95% CI 89-99), with a specificity of 58% (95% CI 50-65), and three false negative cases for CIN3. Combined p16/Ki-67 dual staining with HPV16/18 genotyping showed a similar sensitivity, with only two missed CIN3 lesions, and a similar specificity, compared with Pap cytology triage. Adding p16/Ki-67 triage to hrHPV-positive women with normal cytology detected four women with CIN3, and another eight with CIN2, however, at the cost of 32 and 24 unnecessary referrals, respectively (Table 2).

For ≥CIN2 the comparator cytology strategy showed a sensitivity of 94% (95% CI 88-98) with a specificity of 62% (95% CI 53-71), PPV of 69% (95% CI 61-76) and NPV of 92% (95% CI 84-97). The strategy combining p16/Ki-67 triage of ASC-US and LSIL cytology also yielded a similar sensitivity but with a statistically significant increase in specificity for ≥CIN2, compared with Pap cytology triage. The sensitivity of this strategy was 89% (95% CI 82-94) with a specificity of 79% (95% CI 70-85) (Table 3).

Our systematic search yielded five studies that previously reported on triage of hrHPV-positive women with all cytology categories, representing 3,270 women.[22,28-31] Results are summarized in Table 4. Results of these studies show sensitivities ranging from 83% to 93% for ≥CIN2 and 87% to 95% for ≥CIN3. Specificities range from 53% to 75% for ≥CIN2 and 48% to 57% for ≥CIN3. Only the specificity for ≥CIN3 found in this study falls outside the range, but is not significantly higher compared with previously published numbers.

The overall κ-value of dual-stain cytology for comparing scores of two independent evaluators was 0.67 (95% CI 0.59-0.76), which is considered substantial according to the classification of Landis and Koch.[32]

Table 2. Clinical performance of p16/Ki-67 dual-stain and HPV16/18 genotyping in triage or hrHPV-positive women to detect ≥CIN3.

	TP	FP	TN	FN	Sensitivity		Specificity		PPV	NPV	REF	NRND
					% (95% CI)	P-value	% (95% CI)	P-value	% (95% CI)	% (95% CI)	%	ratio
Pap cytology ≥ ASC-US	71	88	83	5	93 (85-98)	COM	49 (41-56)	COM	45 (37-53)	94 (87-98)	64	2.2
p16/Ki-67 dual stain	70	66	105	6	92 (84-97)	1.00	61 (54-69)	<0.01	52 (43-60)	95 (89-98)	55	1.9
HPV16/18 genotyping	57	42	129	19	75 (64-84)	<0.01	75 (68-82)	<0.01	58 (47-68)	87 (81-92)	40	1.7
p16/Ki-67 and/or HPV16/18 genotyping	74	82	89	2	97 (91-100)	0.37	52 (44-60)	0.45	47 (39-56)	98 (92-100)	63	2.1
Pap cytology, with p16/Ki-67 triage of ASC-US and LSIL*	70	62	109	6	92 (84-97)	1.00	64 (56-71)	<0.01	53 (44-62)	95 (89-98)	53	1.9
Pap cytology, with p16/Ki-67 triage of NILM**	74	98	73	2	97 (91-100)	0.25	43 (35-51)	<0.01	43 (36-51)	97 (91-100)	70	2.3
Pap cytology, with p16/Ki-67 triage of NILM, ASC-US and LSIL†	73	72	99	3	96 (89-99)	0.62	58 (50-65)	0.01	50 (42-59)	97 (92-99)	59	2.0

≥ASC-US = atypical squamous cells of undetermined significance or worse; CI = confidence interval; ≥CIN3 = cervical intraepithelial neoplasia grade 3 or worse; COM = comparator; FN = false negatives; FP = false positives; hrHPV = high-risk human papillomavirus; LSIL = low-grade squamous intraepithelial lesion; NILM = negative for intraepithelial lesion or malignancy; NPV = negative predictive value; NRND = number of referrals needed to diagnose one ≥CIN3 lesion; PPV = positive predictive value; REF = referral rate; TN = true negatives; TP = true positives. *Pap cytology as triage method for hrHPV-positive women, with p16/Ki-67 triage of ASC-US and LSIL Pap cytology results. ASC-US and LSIL Pap cytology, p16/Ki-67 positive samples were scored as positive, and ASC-US and LSIL Pap cytology, p16/Ki-67 negative were scored as negative. ** Pap cytology as triage method for hrHPV-positive women, with p16/Ki-67 triage of NILM Pap cytology results. NILM Pap cytology, p16/Ki-67 positive samples were scored as positive, and NILM Pap cytology, p16/Ki-67 negative were scored as negative. † Pap cytology as triage method for hrHPV-positive women, with p16/Ki-67 triage of NILM, ASC-US and LSIL Pap cytology results. NILM, ASC-US and LSIL Pap cytology, p16/Ki-67 positive samples were scored as positive, and NILM, ASC-US and LSIL Pap cytology, p16/Ki-67 negative were scored as negative.

Table 3. Clinical performance of p16/Ki-67 dual-stain and HPV16/18 genotyping in triage of hrHPV-positive women to detect ≥CIN2.

	TP	FP	TN	FN	Sensitivity		Specificity		PPV	NPV	REF	NRND
					% (95% CI)	P-value	% (95% CI)	P-value	% (95% CI)	% (95% CI)	%	ratio
Pap cytology ≥ ASC-US	110	49	81	7	94 (88-98)	COM	62 (53-71)	COM	69 (61-76)	92 (84-97)	64	1.5
p16/Ki-67 dual stain	101	35	95	16	86 (79-92)	0.04	73 (65-81)	0.03	74 (66-81)	86 (78-92)	55	1.4
HPV16/18 genotyping	77	22	108	40	66 (57-74)	<0.01	83 (76-89)	<0.01	78 (68-86)	73 (65-80)	40	1.3
p16/Ki-67 and/or HPV16/18 genotyping	110	46	84	7	94 (88-98)	1.00	65 (56-73)	0.75	71 (63-78)	92 (85-97)	63	1.4
Pap cytology, with p16/Ki-67 triage of ASC-US and LSIL*	104	28	102	13	89 (82-94)	0.41	79 (70-85)	<0.01	79 (71-85)	89 (82-94)	53	1.3
Pap cytology, with p16/Ki-67 triage of NILM**	113	59	71	4	97 (92-99)	0.25	55 (46-63)	<0.01	66 (58-73)	95 (87-99)	70	1.5
Pap cytology, with p16/Ki-67 triage of NILM, ASC-US and LSIL†	107	38	92	10	92 (85- 96)	0.51	71 (62-78)	0.07	74 (66-81)	90 (83-95)	59	1.4

≥ASC-US = atypical squamous cells of undetermined significance or worse; CI = confidence interval; ≥CIN2 = cervical intraepithelial neoplasia grade 2 or worse; COM = comparator; FN = false negatives; FP = false positives; hrHPV = high-risk human papillomavirus; LSIL = low-grade squamous intraepithelial lesion; NILM = negative for intraepithelial lesion or malignancy; NPV = negative predictive value; NRND = number of referrals needed to diagnose one ≥CIN2; PPV = positive predictive value; REF = referral rate; TN = true negatives; TP = true positives. *Pap cytology as triage method for hrHPV-positive women, with p16/Ki-67 triage of ASC-US and LSIL Pap cytology results. ASC-US and LSIL Pap cytology, p16/Ki-67 positive samples were scored as positive, and ASC-US and LSIL Pap cytology, p16/Ki-67 negative were scored as negative. ** Pap cytology as triage method for hrHPV-positive women, with p16/Ki-67 triage of NILM Pap cytology results. NILM Pap cytology, p16/Ki-67 positive samples were scored as positive, and NILM Pap cytology, p16/Ki-67 negative were scored as negative. † Pap cytology as triage method for hrHPV-positive women, with p16/Ki-67 triage of NILM, ASC-US and LSIL Pap cytology results. NILM, ASC-US and LSIL Pap cytology, p16/Ki-67 positive samples were scored as positive, and NILM, ASC-US and LSIL Pap cytology, p16/Ki-67 negative were scored as negative.

Table 4. Characteristics of the included studies.

Study	Included patients	Cytology technique	HPV test	p16/Ki-67 (≥CIN2)		Pap cytology (≥CIN2)		p16/Ki-67 (≥CIN3)		Pap cytology (≥CIN3)	
				Sensitivity % (95% CI)	Specificity % (95% CI)	Sensitivity % (95% CI)	Specificity % (95% CI)	Sensitivity % (95% CI)	Specificity % (95% CI)	Sensitivity % (95% CI)	Specificity % (95% CI)
Allia, 2015[30]	477	LBC ThinPrep	HC2	NA	64 (57-70)	NA	NA	NA	NA	NA	NA
Gustinucci, 2016[28]	375	Cobas PCR medium	Cobas	88 (76-94)	75 (69-79)	78 (65-87)	73 (67-77)	92 (75-99)	NA	96 (81-100)	NA
Luttmer, 2016[22]	446	LBC ThinPrep	GP5+/6+	86 (80-91)	60 (54-66)	87 (82-92)	54 (49-60)	94 (89-99)	51 (46-56)	88 (80-95)	45 (40-50)
Wentzensen 2015[29]	1,509	LBC Surepath	HC2	83 (77-89)	59 (56-62)	77 (70-83)	50 (47-52)	87 (79-93)	57 (54-60)	84 (75-91)	49 (46-51)
Yu, 2016[31]	463	LBC ThinPrep	Cobas	93 (88-95)	53 (46-59)	95 (91-97)	54 (47-60)	95 (91-97)	48 (69-80)	98 (95-99)	49 (43-55)
Ebisch	247	LBC ThinPrep	GP5+/6+	86 (79-92)	73 (65-81)	94 (88-98)	62 (53-71)	92 (84-97)	61 (54-69)	93 (85-98)	49 (41-56)

CI = confidence interval; CIN = cervical intraepithelial neoplasia; HC = Hybrid Capture 2; HPV = human papillomavirus; LBC = liquid-based cytology; NA = not available; PCR = polymerase chain reaction.

DISCUSSION

In this study we evaluated the clinical utility of p16/Ki-67 dual staining, either or not combined with Pap cytology and/or HPV16/18 genotyping, for the identification of ≥CIN2 and ≥CIN3 in hrHPV-positive women from a non-responder screening cohort. Three of the proposed strategies for triaging hrHPV-positive women showed increased specificity with similar sensitivity for ≥CIN3, compared with Pap cytology. These strategies were: p16/Ki-67 triage of all hrHPV-positive women, p16/Ki-67 triage of hrHPV-positive women with ASC-US or LSIL cytology, and p16/Ki-67 triage of hrHPV-positive women with NILM, ASC-US or LSIL cytology. With a ≥CIN2 threshold only the strategy with p16/Ki-67 triage of hrHPV-positive women with ASC-US or LSIL cytology showed increased specificity with similar sensitivity compared with Pap cytology triage of hrHPV-positive women.

Our findings on sensitivity and specificity of p16/Ki-67 triage of hrHPV-positive women independent of cytology result are comparable with previously published studies, with only small differences in sensitivity and specificity between Pap cytology and p16/Ki-67 dual-stain. These studies have been performed with different hrHPV tests and different cell collection medium. Also, the populations in these studies are different, some are performed in a general population, others in an outpatient population, and none were previously performed in a non-responder population, which could explain slightly different results. A recent large study on p16/Ki-67 performance in hrHPV-positive women by Wentzensen et al.[29] shows an increased specificity with a maintained sensitivity for ≥CIN3 detection, compared with Pap cytology in triage of hrHPV-positive women. This was also confirmed by Luttmer et al. [22] who show a good clinical performance of p16/Ki-67 dual-stained cytology as triage method for hrHPV-positive women with an increase in sensitivity and specificity for ≥CIN3. A previously performed study on p16/Ki-67 triage of hrHPV-positive women with normal cytology showed that p16/Ki-67 dual-stained cytology detects more than 70% of underlying ≥CIN3 lesions in hrHPV-positive women with normal cytology at baseline. They conclude to state that this strategy is suitable for triaging these women to colposcopy.[33] Our study also confirms the additional detection of high-grade lesions in hrHPV-positive women with a normal cytology result, however, at the cost of additional colposcopy referrals.

Previous studies have also analyzed the clinical value of p16/Ki-67 triage of women with low-grade or normal cytology in hrHPV-positive cohorts. To our knowledge, none of them show results on sensitivity, specificity and predictive values of overall triage strategies with p16/Ki-67 triage of certain Pap cytology subgroups in hrHPV-positive women. This approach gives an overview of the whole triage strategy, instead of only results on triage of a certain cytology subgroup. Our data show that adding p16/Ki-67 triage to either low-grade only, or alternatively, low-grade and normal Pap cytology in hrHPV-positive women would improve the specificity of the triage-step, while maintain-

ing similar sensitivity. With improved specificity by additional p16/Ki-67 triage of these low-grade and/or normal cytology results, unnecessary colposcopy referrals could be prevented. By adding p16/Ki-67 triage for women with ASC-US or LSIL cytology, the referral rate can be lowered from 64% to 53% with a decrease in NRND of ≥CIN3 lesion from 2.2 to 1.9. These strategies can easily be combined with Pap cytology triage of hrHPV-positive women which will be used in the new Dutch cervical cancer screening program which started in January 2017.

P16/Ki-67 dual-stain was also combined with HPV16/18 genotyping. An attractive feature of HPV16/18 genotyping is that this triage method could be implemented without additional costs, because most hrHPV tests also have the ability of immediate HPV16/18 genotyping. In this study, triage with HPV16/18 genotyping shows an increase in specificity, however, at the cost of a lower sensitivity.

An advantage of p16/Ki-67 dual-stain over Pap cytology is the reduced role of morphology. It has been previously shown that p16/Ki-67 dual-stain shows substantial to good reproducibility with almost identical performance by novice evaluators compared with reference evaluations, indicating that it can be implemented in clinical practice with limited training.[34,35] The Kappa found in this study was lower-than-expected, this might be caused by the overall low quality of the samples. A disadvantage of the technique is that it cannot be reliably used on self-sampled material, as cellularity of cervical indicator cells in these samples is limited, resulting in a low sensitivity.[36] Women testing hrHPV-positive on self-samples would therefore still need to visit their doctor for an additional clinician-taken cervical smear for triage. As such, in this setting direct triage strategies applicable to self-samples like HPV16/18 or other biomarkers such as DNA methylation analysis are preferred.[7,37] Dual staining neither rules-out the need for follow-up, and in case the technique is used as additional triage tool after Pap cytology triage, this would result in additional triage-costs. A wide variety of novel triage tests for hrHPV-positive women is currently being extensively studied for triage purposes. Molecular techniques based on host- and viral DNA methylation markers, and differences in gene-expression can be used as triage method in the future, possibly even with predictive characteristics.[38-40] Most of these markers have not yet been sufficiently validated to be ready for implementation in screening programs, but among them p16/Ki-67 dual-stained cytology or host cell DNA methylation analysis, with or without additional HPV16/18 genotyping, are attractive options for the near future.[41]

An important strength of the study is that the study design is comparable to future cervical screening program with primary hrHPV testing. The potential bias of HPV knowledge increases cytology sensitivity and decreases cytology specificity, which gives more reason for adding p16/Ki-67 to triage of hrHPV-positive women.[9,42] A limitation of this study is the large number of samples that had to be excluded because of a limited cell count, most likely due to insufficient left-over material, or because of the longer shelf-

life used in this study than advised by the manufacturer, which was within six weeks of collection.

As most of these samples are expected to be negative (because if a dual-stained cell was visible, the sample was scored as positive), the specificities found in this study might be an underestimation, with a slight overestimation of sensitivity. Another potential bias could be the fact that this study was performed in a non-responder population, this might result in slightly different results in a responder population with an expected lower hrHPV positivity rate.

The Netherlands and Australia will be among the first countries to initiate full hrHPV-based organized cervical cancer screening. It is expected that an increasing number of countries will also replace Pap cytology-based screening with hrHPV-based screening. The high sensitivity, reproducibility of the test, and possibility of high-throughput testing, are advantages of hrHPV-based screening. Triage with cytology is an obvious option because of the widespread knowledge on this technique. In line with others, our results indicate that the specificity of triage in hrHPV-based screening programs can be increased by replacing Pap cytology with p16/Ki-67 dual stain, or adding dual-stain cytology as an additional triage step for low-grade Pap cytology.

Because the quality of Pap cytology worldwide varies, and two of the selected strategies are based on quality of Pap cytology and differences in sensitivity and specificity are limited, it would be preferable to choose for primary p16/Ki-67 dual-stain triage of hrHPV-positive women. We therefore conclude that p16/Ki-67 dual-stained cytology of hrHPV-positive women shows increased specificity for ≥CIN3 with a maintained adequate sensitivity compared with Pap cytology in triage of hrHPV-positive women.

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CHAPTER 5

Evidence Supporting See-and-Treat Management of Cervical Intraepithelial Neoplasia: a Systematic Review and Meta-analysis

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ABSTRACT

Objective Studies of see-and-treat management of cervical intraepithelial neoplasia (CIN) vary in their inclusion criteria, resulting in a broad range of overtreatment rates. To determine overtreatment rates in see-and-treat management of women referred for colposcopy because of suspected CIN, in order to define circumstances supporting see-and-treat management.

Methods MEDLINE, EMBASE, and the Cochrane Library were searched from inception up to 12 May 2014. Studies of see-and-treat management in women with a reported cervical smear result, colposcopic impression, and histology result were included. Methodological quality was assessed with the Newcastle-Ottawa scale. We used the inverse variance method for pooling incidences, and a random effects model was used to account for heterogeneity between studies. Overtreatment was defined as treatment in patients with no CIN or CIN1.

Results Thirteen studies (n= 4,611) were included. The overall overtreatment rate in women with a high-grade cervical smear and a high-grade colposcopic impression was 11.6% (95% confidence interval (CI) 7.8-15.3). The overtreatment rate in women with a high-grade cervical smear and low-grade colposcopic impression was 29.3% (95% CI 16.7-41.9), and in the case of a low-grade smear and high-grade colposcopic impression it was 46.4% (95% CI 15.7-77.1). In women with a low-grade smear and low-grade colposcopic impression, the overtreatment rate was 72.9% (95% CI 68.1-77.7).

Conclusion The pooled overtreatment rate in women with a high-grade smear and high-grade colposcopic impression is at least comparable with the two-step procedure, which supports the use of see-and-treat management in this subgroup of women.

INTRODUCTION

Cervical cancer is the fourth most common cancer in women worldwide, with about half a million newly diagnosed cases per year.[1] Cervical cancer screening has decreased the incidence of cervical cancer in the last decades, especially in the developed world.[2,3] Women with abnormal cervical smears are generally referred for a diagnostic colposcopy. Low-grade cervical intraepithelial lesions ((CIN) grade 1), are usually monitored by watchful waiting, and regress to normal without treatment in the majority of cases.[4] In high-grade intraepithelial lesions (CIN grade 2 or worse), treatment is recommended in most cases. The two-step procedure is generally accepted and consists of initial colposcopy with biopsy, followed by treatment at a second visit if the biopsy shows CIN2 or worse. The overtreatment rate of this procedure varies between 11 and 35%.[5-13] Different definitions of overtreatment are described il the literature, but in this systematic review overtreatment is defined as the percentage of women undergoing treatment without the presence of a high-grade lesion. The main reason for overtreatment in a two-step procedure is probably the limited concordance between biopsy and histology of the excised lesion, varying between 71.4 and 85.8%,[5,6,9,14,15] mainly caused by high intra- and inter-observer variability.

A see-and-treat procedure of cervical abnormalities combines colposcopy and treatment of the lesion in one visit, with subsequently higher patient compliance, lower treatment costs, and less emotional stress and anxiety for the women.[16-21] A loop electrosurgical excision procedure (LEEP) is performed immediately under local anesthesia when the colposcopist is convinced that the cervical lesion is a high-grade CIN. The overtreatment rate for this procedure varies between 13.3 and 83.3%,[17] with a subsequent risk of morbidity (hemorrhage, infection and cervical stenosis),[16,22,23] and increased risk of future premature labor due to cervical insufficiency.[24-27]

Many studies of this see-and-treat strategy have been performed, including different patient groups, with wide ranges in age and referral cervical smear criteria, with different thresholds to perform a see-and-treat LEEP, and with a variety of excision techniques. These studies show a broad range of overtreatment rates for suspected high-grade intraepithelial lesions,[17] which makes it difficult to establish in which cases see-and-treat management is justified or should be avoided.

The objective of this systematic literature review is to estimate overtreatment rates of see-and-treat management of women requiring colposcopic evaluation for suspected CIN, in relation to the referral cervical smear and colposcopic impression, in order to define circumstances supporting see-and-treat management.

METHODS

We searched MEDLINE, EMBASE, and the Cochrane Library from inception up to 12 May 2014 for studies of see-and-treat management in women with suspected CIN (Supplemental material Table S1). The search query combined synonyms for see-and-treat management, CIN, colposcopy and LEEP. We also performed a reference, related article, and conference proceedings search. Duplicate articles were manually filtered using the bibliographic database of EndNote version X5.

Studies were eligible for inclusion if the following criteria were fulfilled. 1) A preceding cervical smear had to be reported. Atypical squamous cells of undetermined significance (ASC-US) and low-grade intraepithelial lesion (LSIL) cervical smear results were defined as low-grade, whereas high-grade squamous intraepithelial lesion (HSIL) and atypical squamous cells (ASC-H) smear results were defined as high-grade. 2) A colposcopic evaluation with LEEP was performed in one visit, with a colposcopic impression differentiating between high- and low-grade CIN. 3) A histological outcome was reported, classified as low-grade with a histology result of no CIN, or CIN1, and as high-grade when the histology result was CIN2 or worse. 4) At least ten or more see-and-treat patients were included. Language was restricted to English. Two reviewers (RMFE and RLMB) independently assessed the eligibility of the identified papers. Any disagreements were resolved by discussion with a third reviewer (MMR).

From the relevant articles, we extracted information on study design, characteristics, number of participants, and outcomes. The primary outcome of interest was overtreatment, which we defined as treatment in patients with a final histopathology result of no CIN or CIN1. Two reviewers independently determined the quality score of the included studies according to the Newcastle-Ottawa scale for cohort studies, with a maximum score of nine stars.[28] Seven stars or higher is considered high quality, between four and six stars is considered intermediate quality, and between one and three stars is considered low quality. We designed the review protocol in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines for reporting of meta-analyses, which was registered in PROSPERO (CRD42014010440).

Individual study results in different subgroups were plotted: 1) a high-grade referral cervical smear and a high-grade colposcopic impression; 2) a high-grade referral cervical smear and low-grade colposcopic impression; 3) a low-grade referral cervical smear and a high-grade colposcopic impression; and 4) a low-grade referral cervical smear and a low-grade colposcopic impression. The inverse variance method was used for pooling incidences and to calculate the corresponding 95% confidence intervals (CI). The I^2 test was used to measure heterogeneity. As heterogeneity between studies was expected, a random-effects meta-analysis was used for the primary analyses. A fixed value of 0.5 was added to all cells of a table in the groups where a zero-cell count was observed,

in order to calculate standard error.[29] Sensitivity analyses were performed to study the effect of small studies on the overtreatment rate by excluding studies with less than 100 included see-and-treat patients, and to study the effect of excluding the largest study by Bosgraaf et al. Review Manager version 5.0 was used for all analyses. We followed both the Meta-analysis of Observational Studies in Epidemiology (MOOSE) and the PRISMA guidelines in reporting the results.[30]

RESULTS

We retrieved 3,732 publications (Figure 1), of which 66 were considered potentially eligible. Studies were excluded because they appeared to be duplicates (n=1,221), not relevant (n=2,441), or written in a non-English language (n=4). After reading the full text, another 53 studies were excluded because they did not meet the inclusion criteria: three studies did not include see-and-treat patients; ten studies were unable to differentiate see-and-treat patients from other patient groups; nine studies did not report on cervical smear results; and two did not report on histology results. In another 18 studies, it was not possible to define the colposcopic impression, and in 11 studies it was not possible to link cervical smear, colposcopic impression and histology result. Finally, 13 studies were included, representing 4,611 see-and-treat patients.

Table 1 summarizes the characteristics of the different studies. Four studies included women with high- and low-grade cervical smear results, and nine studies only included women with high-grade cervical smear results. Seven studies included women with low- and high-grade colposcopic impression and six studies only included women with high-grade colposcopic impression. All 13 studies included women with high-grade cervical smear and high-grade colposcopic impression. The overall quality of the studies was moderate to high (Supplemental material Table S2).

In women with a high-grade cervical smear and a high-grade colposcopic impression (n=3,403), the pooled overtreatment rate is 11.6% (95% CI 7.8-15.3; I^2=90%) (Figure 2). In the seven studies that include only women with a high-grade smear and low-grade colposcopic impression (n=374), the pooled overtreatment rate is 29.3% (95% CI 16.7-41.9; I^2=80%) (Figure 3). In Figure 4, the subgroup of women with a low-grade cervical smear and high-grade colposcopic impression (n= 506) is shown. The pooled overtreatment rate is 46.4% (95% CI 15.7-77.1; I^2=98%). In the subgroup of women with a low-grade smear and low-grade colposcopic impression (n=328), shown in Figure 5, the pooled overtreatment rate is 72.9% (95% CI 68.1-77.7; I^2=0%). The sensitivity analysis excluding small studies, did not change the interpretation of our results. The sensitivity analysis, excluding the largest study by Bosgraaf et al., did not result in clinically significant changes in the data.

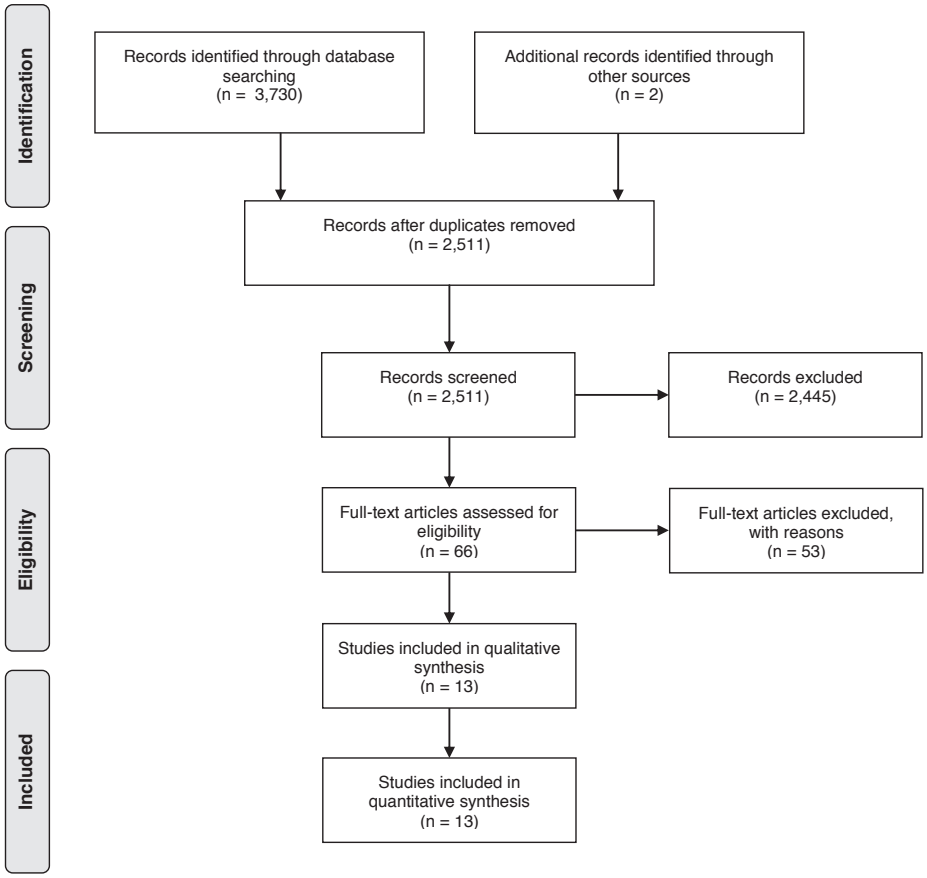

Figure 1. Flowchart of the systematic review process, per Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).

DISCUSSION

Our review shows that an overtreatment rate of 11.6% in see-and-treat management of women with a high-grade smear and high-grade colposcopic impression is at least comparable with the two-step overtreatment rates reported in literature, varying from 11 to 35%. This observation supports the see-and-treat strategy in this subgroup of women, who also benefit from the additional advantages of undergoing a slingle procedure.

In women with discrepancy between the cervical smear and colposcopic impression, we report an overtreatment rate that is higher than the two-step overtreatment rate. We therefore believe that in these two subgroups a biopsy prior to treatment is advisable in young and fertile women. In women with a completed family, or who are menopauzal or sterilized, a see-and-treat procedure may be considered after shared decision-making with the patient. In case of a low-grade smear and low-grade colposcopic impression, in

Table 1. Characteristics of the included studies.

Author	Year	Country	Total number of patients	Included 'see-and-treat' patients	Referral cervical smear	Colposcopic impression
Aue-aungkul[31]	2011	Thailand	192	192	ASC-US, ASC-H, LSIL, HSIL, Carcinoma	Low-grade, high-grade
Bosgraaf[16]	2013	The Netherlands	4,808	2,335	ASC-US, ASC-H, LSIL, HSIL, Carcinoma	Low-grade, high-grade
Charoenkwan[32]	2004	Thailand	55	51	HSIL	Normal, low-grade, high-grade
Cho[5]	2009	South-Korea	1,011	432	Normal, ASC-US, LSIL, HSIL	High-grade
Errington[33]	2006	UK	607	378	HSIL	High-grade
Irvin[34]	2002	USA	61	50	HSIL	High-grade
Kietpeerakool[35]	2009	Thailand	108	58	ASC-H	Normal, low-grade, high-grade
Megevand[36]	1996	South-Africa	2,619	22	HSIL	High-grade
Monteiro[37]	2009	Brazil	900	294	HSIL	High-grade
Sadan[9]	2007	Israel	144	79	HSIL	High-grade
Smith[38]	2001	UK	870	461	LSIL, HSIL	Normal, low-grade, high-grade
Suntornlimsiri[39]	2004	Thailand	592	167	HSIL	Low-grade, high-grade
Szurkus[40]	2002	USA	104	92	HSIL	Normal, low-grade, high-grade
Total			12,071	4,611		

which we found a high pooled overtreatement rate, there is no indication for see-and-treat management, unless preferred for other reasons.

The major strengths of this study are the systematic approach, the inclusion of a large number of patients (n=4,611), and the criteria used for inclusion (i.e. reported cervical smear, colposcopic impression, and histology). We have provided a comprehensive assessment of the risk of overtreatment in four subgroups, which are relevant to both clinicians and patients. Some potential limitations should also be discussed. First, important information regarding age of the patients are not included in our analyses, as these were either not reported or reported in such a heterogeneous way that stratification of the analysis was not possible. Our quality assessment shows overall moderate to high quality, indicating that there is a low risk of methodological bias. Second, colposcopic impression is a subjective measure in the differentiation between high- and low-grade CIN, and the quality partially depends on the training and experience of the colposcopist, which we were unable to extract from most studies. Third, the study by Bosgraaf et

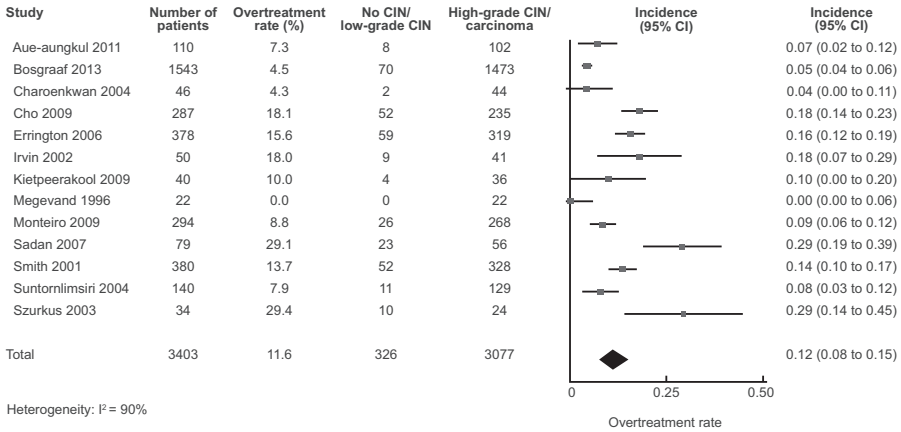

Figure 2. Forest plot of overtreatment rate in women with a high-grade cervical smear and high-grade colposcopic impression.

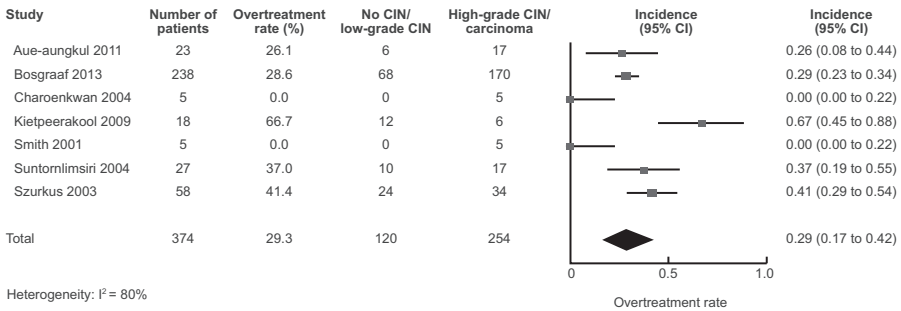

Figure 3. Forest plot of overtreatment rate in women with a high-grade cervical smear and low-grade colposcopic impression.

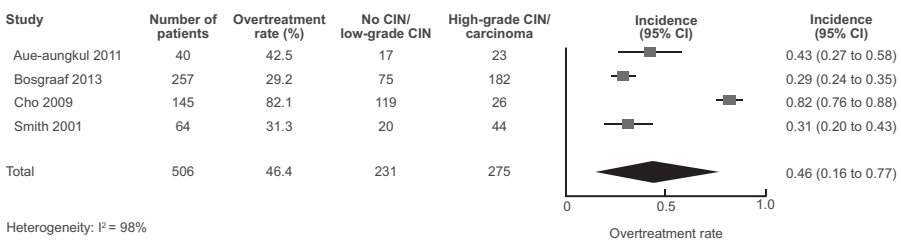

Figure 4. Forest plot of overtreatment rate in women with a low-grade cervical smear and high-grade colposcopic impression.

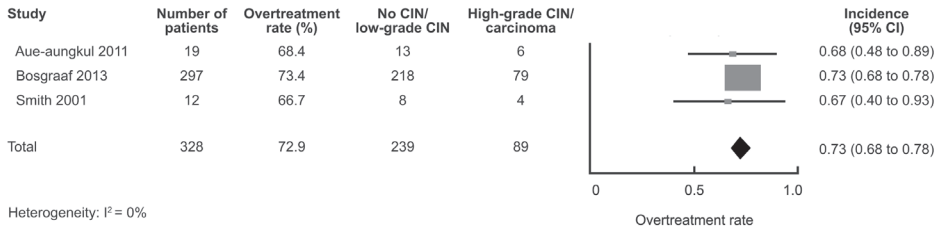

Figure 5. Forest plot of overtreatment rate in women with a low-grade cervical smear and low-grade colposcopic impression.

al.[16] includes a large proportion of the total of patients included in this meta-analysis. Sensitivity analysis excluding this study showed no clinically significant changes in the data. Fourth, the heterogeneity between studies was considerable, resuling in a wide confidence interval. Fifth, we found some asymmetry in the funnel plots, suggesting publication bias and, in particular, that smaller low-quality studies might not have been published. Our sensitivity analyses, however, showed that our results were robust to the removal of small studies, so we do not expect that any unpublished smaller studies would change our results.

Our results are largely in agreement with the guidelines of the American Society for Colposcopy and Cervical Pathology (ASCCP), although they do not include colposcopic impression as a criterion for see-and-treat management. The ASCCP guideline states that it is acceptable to treat women with a high-grade smear according to the see-and-treat strategy, except in case of pregnancy or age between 21-24 years old.[41] We believe it would be an addition to include the colposcopic impression as a prerequisite, because our results confirm an increased overtreatment rate in low-grade colposcopic impression as compared with high-grade colposcopic impression, in women with a high-grade smear. Our results are also in agreement with the guideline of the European Federation for Colposcopy and Pathology of the Lower Genital Tract (EFC), because they recommend targeting any CIN in ≥90% of the excised specimens in see-and-treat management.[42] We show that this percentage is already achievable by a threshold of CIN2 or worse, indicating that the EFC guideline may be adjusted accordingly. The EFC and the guidelines of the British National Health Services Cervical Screening Programme (NHSCSP) advise not to perform a see-and-treat strategy in borderline or mild dyskaryosis smears, and only use see-and-treat management in exceptional cases, which is in line with our results. Regarding quality control, we agree with the NHSCSP in using see-and-treat management in this group of women only when an audit has identified that CIN2/3 or high-grade cervical glandular intraepithelial neoplasia is present in ≥90% of the excised specimens.[43]

In conclusion, our findings suggest that the use of see-and-treat management is supported by evidence in women with a high-grade smear and high-grade colposcopic

impression. The pooled overtreatment rate in this group is at least comparable with the overtreatment rate in the two-step approach (11.6% versus 11-35%). In case of a discrepancy between cervical smear and colposcopic impression, a two-step procedure remains justifiable.

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SUPPLEMENTAL MATERIAL

Table S1. Search strategy for identification of studies in PubMed.

(((see and treat[tiab] OR single session[tiab] OR one session[tiab] OR one visit[tiab] OR single visit[tiab] OR single clinic visit[tiab] OR one procedure[tiab] OR three step[tiab] OR combined treatment[tiab] OR diagnostic treatment[tiab] OR one stop[tiab] OR single stop[tiab] OR first visit[tiab])
AND
(Uterine Cervical Dysplasia[mesh] OR Uterine Cervical Neoplasms[mesh] OR Cervical Intraepithelial Neoplasia[mesh] OR cervical intraepithelial[tiab] OR cervical intra-epithelial[tiab] OR CIN[tiab] OR Cervical abnormalit*[tiab] OR cervical neoplas*[tiab] OR cervical cancer*[tiab] OR cervical dysplasia[tiab] OR Cervix carcinoma*[tiab] OR Cervix cancer*[tiab] OR Cervix dysplasia[tiab]))
NOT
(radiation[tiab] OR radiotherap*[tiab] OR chemotherap*[tiab]))

OR

((“colposcopy”[mesh] OR Colposcop*[tiab])
AND
(“conization”[mesh] OR loop excision[tiab] OR LEEP[tiab] OR loop electrosurgical excision procedure[tiab] OR LLETZ[tiab] OR LETZ[tiab] OR large loop diathermy excision[tiab] OR LEC[tiab] OR conization*[tiab] OR conisation*[tiab]))

Terms used for search strategy in MEDLINE, EMBASE, and The Cochrane Library from inception to May 12th 2014. Terms with [tiab] reflect free text terms appearing in title or abstract.

Table S2. Risk of bias assessment.

Reference	Selection	Comparability	Outcomes	Final score
Aue-aungkul 2011	****		***	7 of 9
Bosgraaf 2013	***	*	***	7 of 9
Charoenkwan 2004	***	*	***	7 of 9
Cho 2009	****	**	***	9 of 9
Errington 2006	***		***	6 of 9
Irvin 2002	****	*	***	8 of 9
Kietpeerakool 2009	****		***	7 of 9
Megevand 1996	***	**	***	8 of 9
Monteiro 2009	***		***	6 of 9
Sadan 2007	****		***	7 of 9
Smith 2001	***	*	***	7 of 9
Suntornlimsiri 2004	***	*	***	7 of 9
Szurkus 2002	***	*	***	7 of 9

CHAPTER 6

Multimodal hyperspectroscopic imaging for detection of high-grade cervical intraepithelial neoplasia

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ABSTRACT

Objective Numerous new alternative digital colposcopy techniques have been developed, of which multimodal hyperspectroscopy (MHS) showed a high sensitivity in previous studies. The objective of this prospective single-center cohort study was to evaluate the clinical value of MHS for detecting high-grade cervical intraepithelial neoplasia in a colposcopy referral population and colposcopy follow-up population, to assess whether MHS could be safely used to improve care for women at risk for high-grade cervical intraepithelial neoplasia.

Methods A total of 125 women from a colposcopy referral population and colposcopy follow-up population were evaluated with MHS and tested for the presence of high-risk human papillomavirus (hrHPV) with HPV16/18 genotyping. Spectroscopic measurements of the cervix were taken and compared with an endpoint based on histology, hrHPV and cytology. Evaluable data for analysis were collected for 102 of the subjects. Sensitivity, specificity and predictive values were calculated for MHS and colposcopic impression based on conventional colposcopic examination.

Results From the total study population of 102 patients, 47 were enrolled in the colposcopy referral group and 55 in the colposcopy follow-up group. The MHS yielded a sensitivity of 93.6% (95% CI 78.6-99.2), with a corresponding specificity of 42.3% (95% CI 30.6-54.6) in the group with a composite endpoint. No adverse effects occurred, and patient acceptability was high.

Conclusion MHS is a digital colposcopy technique that offers an easy, rapid, well-tolerated point-of-care assessment with a high sensitivity for the presence of high-grade cervical intraepithelial lesions, however, with a low specificity, resulting in limited clinical value.

INTRODUCTION

Colposcopy involves the visualization of the cervix using a stereoscopic binocular microscope of low magnification to identify cervical lesions.[1] The reported performance of colposcopic examination varies because of interobserver and intraobserver variability,[2,3] with an average sensitivity ranging between 55% and 89% and a specificity of 52% to 85%.[4-7] This may result in missed lesions on one hand, and in unnecessary biopsies and overtreatment on the other hand.

To improve colposcopic examination, numerous new alternative digital colposcopy techniques have been developed. Previous studies have shown that a digital cervical scan based on fluorescence and reflectance spectroscopy, also referred to as multimodal hyperspectroscopy (MHS), is a potential method for triage of women at risk for moderate- and high-grade dysplasia.[8,9] The technology of multimodal spectroscopy is based on the combination of both reflectance characteristics and tissue fluorescence. Fluorescent spectra detect metabolic activity of epithelial cells, which change during carcinogenesis,[10] and reflectance spectroscopy detects morphologic changes that occur when normal epithelial cells develop into neoplastic cells.[11,12] By combining fluorescence and reflectance spectroscopy, a prediction of tissue type is obtained, based on morphologic and biochemical changes that occur during pathogenesis of neoplasia.

The goal of this study was to provide a prospective evaluation of the clinical value of MHS for detecting high-grade cervical intraepithelial neoplasia (CIN) in a Dutch colposcopy referral population and colposcopy follow-up population, in order to investigate whether MHS could be safely used to improve care for women at risk for high-grade CIN.

METHODS

We conducted a prospective single-center cohort study from November 2014 to December 2015. Women 18 years or older were consecutively evaluated for inclusion before their visit to the outpatient clinic of the department of Obstetrics and Gynecology of the Radboud university medical center, Nijmegen, the Netherlands. They visited the outpatient clinic for a scheduled colposcopic examination after an abnormal cervical smear (defined by the atypical squamous cells of undetermined significance threshold) or a scheduled follow-up cervical smear after previous colposcopic examination. Women were excluded if; they were pregnant at the time of the examination or if they were pregnant in the previous three months, were undergoing treatment for cervical cancer, or had received previous pelvic radiotherapy.

After obtaining informed consent, women were prepared for pelvic examination. Multimodal hyperspectroscopy was performed before the colposcopic examination or

before a cervical smear was taken. Excessive mucus or blood was removed from the cervix with a gauze. The MHS was calibrated, and the cervical tube was inserted through the speculum into the vagina using a live video feed, until the external ostium of the cervix was visible and focused in the field of view. Measurements were made automatically under software control for one minute. After completion of the scan, a second video image was displayed to ensure that the external ostium of the cervix was still in view and there was no significant movement. The cervical tube was then removed. The MHS results were scored as low risk, moderate risk, and high risk of high-grade CIN. Investigators and patients were blinded from the MHS output.

After finishing the MHS scan, colposcopic examination or a follow-up cervical smear was performed, according to the Dutch guidelines.[13] Colposcopic examination was conducted with the use of acetic acid and Lugol's solution. Either a biopsy was taken from suspicious areas of the cervix, an immediate loop electro-excision procedure (LEEP) was performed, or a watchful waiting strategy was conducted. The location of biopsies or a LEEP was marked on the MHS scan.

Expert pathologists from the Department of Pathology at the Radboud university medical center, Nijmegen, the Netherlands, assessed all LEEP and biopsy specimens, classifying them as follows: no CIN, CIN1, CIN2, CIN3 or cervical cancer. Follow-up cervical smears were assessed by cytotechnologists from the same department and were scored by the Dutch CISOE-A (composition, inflammation, squamous cells, other and endometrium, endocervical cylindrical epithelium, adequacy) classification system, which can be easily translated into the Bethesda nomenclature.[14] Pathologists and cytotechnicians were blinded from the output of the MHS scan.

Cervical smears were analyzed for the presence of high risk human papillomavirus (hrHPV), using the clinically validated Cobas 4800 test (Roche). This test detects separate results for HPV16 and HPV18 and a pooled result for 12 other hrHPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). In the colposcopy follow-up population, the diagnostic cervical smear was tested for the presence of hrHPV. In the colposcopy referral population, an additional cervical smear for hrHPV testing was taken.

Women were asked to fill out a patient acceptability questionnaire after the MHS. This questionnaire consisted of a pain scale from 1 to 5 (1 = no discomfort from the MHS scan, 2 = some discomfort, 3 = painful but tolerable, 4 = painful and the procedure had to be paused, and 5 = a setting too painful to continue without intervention, in which case the spectroscopic evaluation had to be stopped).

Up to the 6-month follow-up, data on cytology and histology results were collected. This study was approved by the local medical research ethics committee, region Arnhem Nijmegen with number 2014-1171, and informed consent from all women enrolled in the study was obtained.

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), with 95% confidence Intervals (CI) were calculated. First for the colposcopic impression based on conventional colposcopic examination and second for the results of the MHS scan as a total group, as well as for different subgroups. Two major subgroups are the colposcopy follow-up group and the colposcopy referral group, of which the second was subdivided according to the cytology referral result differentiating between low-grade and high-grade referral cervical smears. Low-grade referral cervical smears include atypical squamous cells of undetermined significance (ASC-US) and low-grade squamous intraepithelial lesions cytology (LSIL), and the high-grade group is represented by high-grade squamous intraepithelial lesions referral cervical smears (HSIL). The primary endpoint was based on histology, with either an immediate histology result or a 6-month follow-up histology result, or a double- negative cervical smear with a negative for intraepithelial lesion or malignancy (NILM) cytology result and a negative hrHPV test. Pap cytology alone was used as adjusted endpoint if no primary endpoint was available. A composite outcome was obtained using the adjusted endpoints combined with the primary endpoints. A positive (high-grade) endpoint was defined by CIN2 or worse (≥CIN2) histology or high-grade squamous intraepithelial lesion or worse (≥HSIL) cytology, and a negative endpoint was defined by CIN1 or less (≤CIN1) histology, or low-grade squamous intraepithelial lesion or less (≤LSIL) cytology. A colposcopic impression of ≥CIN2 was considered positive, and a colposcopic impression of ≤CIN1 was considered negative. High-risk and moderate-risk MHS results were classified as a positive scan result, and low-risk MHS results were classified as negative.

True positives were defined by all women with a positive scan result and a positive, high-grade endpoint. True negatives were women with a negative scan result, and a negative, low-grade endpoint. False positives were defined as women with a positive scan result and a negative endpoint, and false negatives were women with negative scan result and a positive endpoint. The McNemar test was used to compare sensitivity and specificity of the colposcopic impression with the sensitivity and specificity of MHS in women with a primary and composite endpoint.

RESULTS

In total, 152 women fulfilled the inclusion criteria, signed informed consent, and were enrolled in the study. In 27 cases (18%), the scan could not be performed; in 13 cases the speculum could not be opened wide enough because of pain, in nine cases the device gave a technical error, in three cases the calibration time was overdue and there was no time for a second calibration, in one case there was too much blood on the cervix, and in one case the external ostium could not be visualized. The MHS scan was therefore performed in

125 women. In 22 cases (14%), the scan result was invalid, and one woman was diagnosed with vaginal intraepithelial neoplasia, which caused the abnormal smear, and was therefore excluded from this study, resulting in 102 valid MHS scans (Figure 1). The median age of the included 102 women was 37 years, with a range from 20 to 57 years. The study group consisted of 55 women in the colposcopy follow-up group, who visited the outpatient clinic for a follow-up cervical smear after colposcopic examination, and the 47 women in the colposcopy referral group, who visited the clinic for a colposcopic examination. From the colposcopy referral group, 31 women visited the outpatient clinic with a low-grade cytology referral result and 16women visitied theoutpatient clinic with a high-grade cytology referral result. The composite endpoint with regard to the reason for visiting the outpatient clinic is shown in Table 1. An high-grade squamous intraepithelial lesion endpoint was found in 16% of the colposcopy follow-up group and in 47% of the colposcopy referral group, with the highest percentage of 87.5% in the subgroup of women with a high-grade cytology referral result. Table 2 shows the hrHPV prevalence for each subgroup of reason for visiting the outpatient clinic. The colposcopy referral group with high-grade referral cytology yields the highest HPV16/18 prevalence with 47%, and in women referred for colposcopic examination with low-grade cytology, the lowest hrHPV prevalence of 24% is shown.

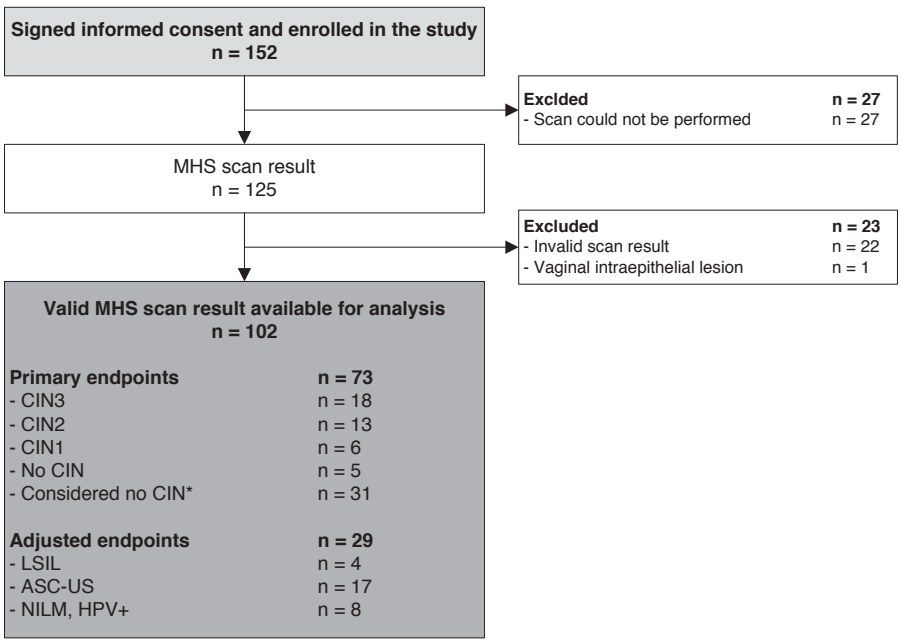

Figure 1. Trial Profile.
*Women with a cytology test result negative for intraepithelial lesion or malignancy (NILM) and an hrHPV-negative test are considered to have no CIN. ASC-US = atypical squamous cells of undetermined significance; CIN = cervical intraepithelial neoplasia; HPV = human papillomavirus; LSIL = low-grade squamous intraepithelial lesion; MHS = multimodal hyperspectroscopy.

Table 3 shows the sensitivity, specificity, PPV and NPV with 95% CI for the colposcopic impression based on conventional colposcopic examination and for the total MHS group as well as for the different subgroups with a composite endpoint. For all subgroups combined, the sensitivity of MHS for high-grade lesions was 93.6% (95% CI 78.6-99.2), with a specificity of 42.3% (95% CI 30.6-54.6). When MHS would be used as a triage method to lower colposcopy referrals, six (13%) of the 47 women would not have been referred for colposcopic examination, of which five without a high-grade lesion, and one with a HPV16/18 positive CIN2 lesion. Sensitivity of the colposcopic impression was 72.7% (95% CI 49.8-89.3), with a specificity of 88.0 (95% CI 68.8-97.5), with six missed high-grade lesions when no biopsy would have been taken. In the group of women visiting the outpatient clinic for a follow-up smear after previous colposcopic examination, eight women with high-grade lesions would have been detected with the MHS scan, and one HPV16/18 positive CIN3 would have been missed. Thus, the MHS in the colposcopy examination group had a significant (P<0.001) higher sensitivity but lower specificity than the colposcopic impression based on conventional colposcopic examination.

Table 1. Prevalence of composite endpoints in each group.

	Normal endpoint (%)	LSIL endpoint (%)	HSIL endpoint (%)	Total
Colposcopy referral group	6 (13%)	19 (40%)	22 (47%)	47
Low-grade cytology referral	4 (13%)	19 (61%)	8 (26%)	31
High-grade cytology referral	2 (12.5%)	0 (0%)	14 (87.5%)	16
Colposcopy follow-up group	38 (69%)	8 (15%)	9 (16%)	55
Total	**44 (43%)**	**27 (27%)**	**31 (30%)**	**102**

HSIL = high-grade squamous intraepithelial lesion; LSIL= low-grade squamous intraepithelial lesion.

Table 2. hrHPV prevalence in each group.

	HPV16/18 positive (%)	Other hrHPV-positive (%)	hrHPV negative (%)	Total
Colposcopy referral group	13 (33%)	16 (40%)	11 (27%)	40
Low-grade cytology referral	6 (24%)	13 (52%)	6 (24%)	25
High-grade cytology referral	7 (47%)	3 (20%)	5 (33%)	15
Colposcopy follow-up group	19 (37%)	9 (17%)	24 (46%)	52
Total	**32 (35%)**	**25 (27%)**	**35 (38%)**	**92**

Of the total of 102 cases, ten hrHPV results were missing.

Of the total group, 73 women had a primary endpoint. The sensitivity of MHS for this group with a primary endpoint is 93.6% (95% CI 78.6-99.2), with a specificity of 47.6% (95% CI 32.0-63.6). For the colposcopic impression the sensitivity is 72.7% (95% CI 49.8-89.3), with a specificity of 72.7% (95% CI 39.0-94.0) (Table 4). In addition, in this group, MHS had a significant (P<0.004) higher sensitivity with a lower specificity compared with the colposcopic impression based on conventional colposcopic examination.

Table 3. Sensitivity, specificity, PPV, and NPV for each group with a composite endpoint.

	TP	FP	TN	FN	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Colposcopic impression based on conventional colposcopy	16	3	22	6	72.7 (49.8-89.3)	88.0 (68.8-97.5)	84.2 (60.4-96.6)	78.6 (59.1-91.7)
MHS scan								
Colposcopy referral group	21	20	5	1	95.5 (77.2-99.9)	20.0 (6.8-40.7)	51.2 (35.1-67.1)	83.3 (35.9-99.6)
Low-grade cytology referral	7	18	5	1	87.5 (47.4-99.7)	21.7 (7.5-43.7)	28.0 (12.1-49.4)	83.3 (35.9-99.6)
High-grade cytology referral	14	2	0	0	100 (76.8-100)	0.0 (0.0-84.2)	87.5 (61.7-98.5)	NA
Colposcopy follow-up group	8	21	25	1	88.9 (51.8-99.7)	54.4 (39.0-69.1)	27.6 (12.7-47.2)	96.2 (80.4-99.9)
Total MHS	29	41	30	2	93.6 (78.6-99.2)	42.3 (30.6-54.6)	41.4 (29.8-53.8)	93.8 (79.2-99.2)

CI = confidence interval; FN = false negative; FP = false positive; MHS = multimodal hyperspectroscopy; NPV = negative predictive value; PPV = positive predictive value; TN = true negative; TP = true positive.

Table 4. Sensitivity, specificity, PPV, and NPV for each group with a primary endpoint.

	TP	FP	TN	FN	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Colposcopic impression based on conventional colposcopy	16	3	8	6	72.7 (49.8-89.3)	72.7 (39.0-94.0)	84.2 (60.4-96.6)	57.1 (28.9-82.3)
MHS scan								
Colposcopy referral group	21	9	2	1	95.5 (77.2-99.9)	18.2 (2.3-51.8)	70.0 (50.6-85.3)	66.7 (9.4-99.2)
Low-grade cytology referral	7	7	2	1	87.5 (47.4-99.7)	22.2 (2.8-60.0)	50.0 (23.0-77.0)	66.7 (9.4-99.2)
High-grade cytology referral	14	2	0	0	100 (76.8-100)	0.0 (0.0-84.2)	87.5 (61.7-98.5)	NA
Colposcopy follow-up group	8	13	18	1	88.9 (51.8-99.7)	58.1 (39.1-75.5)	38.1 (18.1-61.6)	94.7 (74.0-99.9)
Total MHS	29	22	20	2	93.6 (78.6-99.2)	47.6 (32.0-63.6)	56.9 (42.3-70.7)	90.9 (70.8-98.9)

CI = confidence interval; FN = false negative; FP = false positive; MHS = multimodal hyperspectroscopy; NPV = negative predictive value; PPV = positive predictive value; TN = true negative; TP = true positive.

No adverse effects were reported. Patient acceptability of the scan was high with an 84% score of no discomfort, one paused MHS examination, and an average score of 1.14 (95% CI 1.06-1.22) on the acceptability questionnaire (Table 5).

Table 5. Patient acceptability of the MHS digital colposcopy scan.

	Number (%)
No discomfort	86 (84%)
Some discomfort	9 (9%)
Painful but tolerable	2 (2%)
Painful and the procedure had to be paused	1 (1%)
Too painful to continue without intervention	0 (0%)
Missing	4 (4%)

DISCUSSION

The overall sensitivity of the MHS device for detecting high-grade cervical lesions is significantly higher compared with the colposcopic impression based on conventional colposcopic examination. The scan also shows good patient acceptability. The specificity of the MHS device is, however, significantly lower, resulting in a limited clinical value.

Our results are in line with previously published studies performed with the MHS device. Early studies have shown the value of this technique in identifying CIN lesions.[15-17] A more recent large study by Twiggs et al.[8] included 1,607 women from a colposcopy referral population. The MHS scan was well tolerated and resulted in a sensitivity of 91.3% (95% CI 87.3-94.3), and a specificity of 38.9% (95% CI 34.9-43.1) for ≥CIN2. A second study by DeSantis et al.[9] included 572 women from a colposcopy referral population and a colposcopy follow-up population, similar to our population. They also concluded that the test was simple to implement, well accepted by subjects and showed a high sensitivity with a limited specificity. In a recent systematic review, six studies on MHS were analyzed and a meta-analysis of 2,530 women included in these studies showed an overall sensitivity of 93% (95% CI 89-95) and specificity of 62% (95% CI 47-76). The MHS technique was compared with two other digital colposcopy techniques of which MHS showed the highest sensitivity, but not the highest specificity.[18] A difference between this study and the previously performed studies is that this study has been performed with a newer and improved device, which only needs 1 minute of scan time, compared with the older devices which needed at least 4.5 minutes of scan time.

The sensitivity of MHS is highest in women with a high-grade cervical smear who visited the outpatient clinic for colposcopic examination. It is, however, debatable whether this group of women needs triage before colposcopic examination after a high-grade cytology referral smear, because more than 90% of women with a high-grade referral smear indeed show a high-grade lesion.[19] In triage of women with a low-grade cytology referral smear, the MHS may be of more value. In this group of women with a histologic endpoint, the sensitivity of MHS was 87.5% (95% CI 47.4-99.7), compared with 72.7%

(95% CI 49.8-89.3) of conventional colposcopic examination. However, only six women (19.4%) with low-grade referral smears would not have been referred, of which one with a high-grade CIN lesion, and the specificity of MHS was much lower compared with the colposcopic impression. In the colposcopy follow-up population, the MHS scan might play a role in identifying women with a high risk on high-grade cervical lesions during follow-up. The MHS scan showed a positive result for 30 women, of which eight were true positives, and 26 women would not have been referred for colposcopic examination, of which one would be a false negative, resulting in one missed ≥CIN2 lesion. Follow-up with MHS may however be more costly compared with Pap cytology follow-up, and it does not rule out the need for visiting a doctor or use of a speculum.

Advantages of implementing MHS before colposcopic examination are the high sensitivity and increased NPV of MHS, compared with the colposcopic impression based on conventional colposcopic examination. This is important because it results in a low number of missed high-grade CIN lesions, and a negative MHS results gives a high assurance of the absence of a high-grade CIN lesion. Another advantage may be that the device can be used by a trained healthcare worker without colposcopic experience, it is less time-consuming compared with conventional colposcopy, and therefore possibly cheaper. However, the specificity and PPV of the MHS scan are low, resulting in a large proportion of women still being referred for conventional colposcopic examination. This large proportion of women will contain a high number of false positives which are still referred for conventional colposcopic examination. This may result in high costs of MHS per woman not referred for conventional colposcopic examination, making it less likely to be cost-effective to use MHS as an additional step before conventional colposcopic examination. Another limitation of the test is the absence of knowledge on clinical value of the MHS scan on detecting lesions in the endocervical canal. In our study, no women with endocervical high-grade lesions were included; it was therefore impossible to determine if the MHS scan detects lesions limited to the endocervix.

From the total group of 152 women, the scan could not be performed in 27 women (18%). The most frequent reason was because of too much pain by opening the speculum wide enough for the cervical guide to pass. The size of the cervical guide should, however, not be smaller because a certain size is needed for scanning the entire cervix at once. Other options would be a device that can be used without a speculum or a smaller device with a point-probe technique for multiple measurements; this might, however, be less easy to use. Furthermore, patient support might need improvement to obtain a better state of relaxation for the patient, resulting in less complaints of pain by opening the speculum. The second most frequent reason why the scan could not be performed was due to technical errors, which should be addressed by the manufacturer. Another 22 women (14%) were excluded because of an invalid scan result, which could possibly be avoided by better cleaning the cervix before scanning. In our study the device was on

study setting, and we could not see if the scan was valid; in a clinical setting this would indeed be possible and a new scan could be performed after cleaning the cervix again. Not all women from the colposcopy follow-up group and colposcopy referral group had a histologic endpoint. Results from the composite endpoint were therefore based on cytology results and histology results combined. This may have caused bias of the results. However, sensitivity, specificity, and predictive values did not differ much between the primary endpoint group and the group with a composite endpoint.

An increasing number of countries are on the verge of implementing hrHPV-based cervical cancer screening. This screening strategy has a high sensitivity, but a limited specificity, which is a result of the assays' inability to distinguish transient from persistent hrHPV infections. This limited specificity of hrHPV-based screening will result in an increased number of referrals for colposcopic evaluation, with the risk of unnecessary colposcopic examination and unnecessary treatment.[20] Referral rates for conventional colposcopic examination could possibly be reduced by implementing digital colposcopy techniques as triage method to separate women with a high risk of high-grade CIN in need of colposcopic examination, from women with a low risk of high-grade CIN, which could be managed with follow-up smears. Sensitivity of a technique needs to be high to safely limit missing high-grade lesions, and a decent specificity should limit the number of unnecessary referrals.

In summary, cervical multimodal hyperspectroscopy is a digital colposcopy technique that offers an easy, rapid, well tolerated point-of-care assessment with a high sensitivity for the presence of high-grade CIN lesions, but with low specificity, and a failure to produce a result in 32% in this study, resulting in a limited clinical value. A large study in an hrHPV-positive population should point out whether this MHS scan may be safely used to limit colposcopy referrals from a population-based screening program, especially in the group with low-grade cytology results.

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CHAPTER 7

Alternative Colposcopy Techniques: A Systematic Review and Meta-analysis

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ABSTRACT

Objective To assess the diagnostic value of alternative (digital) colposcopy techniques for detection of cervical intraepithelial neoplasia (CIN) 2 or worse in a colposcopy population.

Methods MEDLINE, EMBASE, ClinicalTrials.gov, and the Cochrane Library were searched from inception up to January 11, 2016, for studies that evaluated the diagnostic value of alternative (digital) colposcopy techniques. Inclusion criteria were: 1) an alternative (digital) colposcopy technique was used in a colposcopy population; 2) A histological outcome was reported, classified as CIN, differentiating between mild dysplasia or less (≤CIN1), and moderate dysplasia or worse (≥CIN2); 3) The entire cervix was scanned at once, or a per-woman analysis was performed; 4) No other topical application than acetic acid and Lugol's solution was used; 5) At least three eligible studies had to be available within a single technique; and 6) Studies obtained research ethics approval. Language was restricted to English.

Results Two reviewers assessed the eligibility of the identified articles. Disagreements were resolved by a third reviewer. Thirteen studies met the inclusion criteria. We found six studies on fluorescence and reflectance spectroscopy, including 2,530 women, with a pooled sensitivity of 93% (95% confidence interval (CI) 89-95), and specificity of 62% (95% CI 47–76). Four studies on dynamic spectral imaging were found including 1,173 women, with a pooled sensitivity of 69% (95% CI 48–85), and specificity of 83% (95% CI 76–88). We found three studies on optical coherence tomography including 693 women, with a pooled sensitivity of 48% (95% CI 32–64), and specificity of 77% (95% CI 52–91). Previously published conventional colposcopy results showed a sensitivity of 61% (95% CI 58–63) and a specificity of 85% (95% CI 83-86).

Conclusion Alternative (digital) colposcopy techniques may result in increased sensitivity and specificity, but no recommendation for introduction in clinical practice can be made yet.

INTRODUCTION

Colposcopy is the visualization of the cervix using a stereoscopic binocular microscope of low magnification (10x to 40x).[1] Colposcopic examination combined with a biopsy and histological evaluation is the gold standard for identifying cervical intraepithelial neoplasia (CIN). However, limitations of colposcopy include a low-to-average sensitivity of 61% (95% confidence interval (CI) 58–63) and a specificity of 85% (95% CI 83-86),[2] resulting in missed lesions, unnecessary biopsies or overtreatment, and a high degree of interobserver and intraobserver variability.[3,4] Improving diagnostics and management of women with CIN lesions is therefore desirable.

Alternative colposcopy techniques such as digital colposcopy might improve diagnosis and management of women with CIN lesions. Digital colposcopy is defined as every form of colposcopy using image enhancement by a computer.[5] The use of digital colposcopy techniques has been increasingly studied in the last decade. These studies all claim promising results on improving detection of CIN lesions when compared with colposcopy alone, although previously published sensitivity and specificity results highly vary.

The variety of digital colposcopy techniques can be categorized into groups based on their mechanism of detection of CIN lesions. Computerized colposcopy and telecolposcopy, both adjunct to regular colposcopy, may be used to improve regular colposcopic assessment. These techniques use computerized images of the cervix without taking any additional measurements.[6,7] Spectroscopy is a non-invasive technique that involves light or an electric current to detect CIN lesions based on morphologic and biochemical changes that occur during pathogenesis of neoplasia.[8,9] This technique includes optical spectroscopy (fluorescence, reflectance, and Raman spectroscopy),[10] electrical impedance spectroscopy,[10,11] Truscreen,[12,13] and dynamic spectral imaging.[10] Another technique, optical coherence tomography uses infrared light to distinguish normal cervical tissue from CIN.[14-16] Lastly, confocal (endo)microscopy is used to reconstruct a three-dimensional image, without removing tissue. This enables detection of intracellular details, although with limited field of view.[17,18]

Methods used for digital colposcopy can be divided into two groups; single point-probes that measure a small site (2-3mm) of the cervix, and widespread or multispectral imaging, which measures the entire cervix on multiple sites at once,[19] therefore allowing use of the device by healthcare workers with limited knowledge on cervical abnormalities.[20]

Various digital alternative colposcopy techniques have been developed lately, and it is difficult to get a proper overview of these techniques and their qualities. Therefore, the aim of this systematic review is to assess the diagnostic value of a variety of alternative

digital colposcopy techniques for detection of moderate dysplasia or worse (≥CIN2) in a colposcopy population.

We aimed to include all previously described techniques in this review. However, only three techniques finally met our inclusion criteria.

(1) Fluorescence and reflectance spectroscopy combines two methods of spectrum analysis: fluorescent spectra represent metabolic activity of epithelial cells, which changes during carcinogenesis,[21] and reflectance spectroscopy detects morphologic changes that occur when normal epithelial cells develop into neoplastic cells.[22,23] The device scans the entire cervix in 1 minute. By combining fluorescence and reflectance spectroscopy, a prediction of tissue type depending on morphologic and biochemical changes that occur during pathogenesis of neoplasia is obtained. A strength of this technique is the possibility of multimodal measuring, resulting in an easy-to-use single scan which can be used independently from colposcopy. However, this single scan measures a limited area of the cervix, which could possibly result in missed lesions.

(2) Dynamic spectral imaging is a colposcopy technique that creates a color-coded map of the entire cervix based on the intensity and time-evolution of the changes that develop when acetic acid is applied to both neoplastic and normal cells, with acetowhitening effect representing the severity of cervical neoplasia.[24-26] The location of the biopsy is objectively determined and biopsies or a loop electrosurgical excision procedure (LEEP) can be taken based on this color-coded map. This makes accurate colposcopic assessment possible for healthcare workers with limited colposcopy experience and might lower unnecessary biopsies. A possible restraint of dynamic spectral imaging is the long lasting acetowhitening effect, which lasts up to 45 minutes. If the first assessment fails, a second assessment may not be reliable because it can interfere with the time-evolution of acetowhitening effect of the first examination.

(3) Optical coherence tomography is a noninvasive imaging technique that uses near-infrared light to measure low-coherence interferometry of tissue.[14,15] It scans the entire cervix and is mainly used as an adjunct to colposcopy. Optical coherence tomography provides a higher resolution and better differentiation between diseased and normal tissue at superficial levels compared with established imaging techniques such as computed tomography, magnetic resonance imaging, positron emission tomography, ultrasonography, and nuclear imaging.[27] A possible restraint of optical coherence tomography is the possibility of misinterpretation caused by inflammatory changes of the cervix.[28] Furthermore, optical coherence tomography scans only one quadrant at a time; therefore four scans are needed to assess the entire cervix. Optical coherence tomography is used additionally to colposcopy in the reviewed studies and will therefore not lower colposcopy referrals.

METHODS

We systematically searched MEDLINE, EMBASE, ClinicalTrials.gov, and the Cochrane Library from inception up to September 21, 2015, for studies on digital colposcopy for the detection of cervical intraepithelial lesions. On January 11, an additional search was performed to include studies from September 21, 2015, up to January 11, 2016. The search query combined synonyms for cervical intraepithelial lesions, digital colposcopy and diagnostic accuracy (Supplemental material Table S1). We also performed a reference and related article search. Duplicate articles were manually filtered using the bibliographic database EndNote version X5.

Studies were eligible for inclusion if the following criteria were fulfilled: 1) a digital colposcopy technique was used to determine the diagnostic value in a colposcopy population, defined as women referred for colposcopy with an abnormal Pap cytology result or symptoms suggesting CIN or women referred for follow-up colposcopy; 2) A histological outcome was reported, classified as CIN, differentiating between mild dysplasia or less (≤CIN1), and moderate dysplasia or worse (≥CIN2); 3) The entire cervix was scanned at once or a per-woman analysis was performed; 4) No other than acetic acid and Lugol's solution, as topical application, was used; 5) At least three eligible studies were available within each individual technique. In case of less than three studies, this technique was excluded from this systematic review and meta-analysis; and 6) studies obtained research ethics approval. Language was restricted to English. Two reviewers (MH and RMFE) independently assessed the eligibility of the identified articles. Any disagreements were resolved by discussion with a third reviewer (RLMB).

From the relevant articles information on study design, year of publication, digital colposcopy technique, number of participants, age of participants, sensitivity and specificity of conventional colposcopy, and outcome of digital colposcopy technique was extracted. The primary outcome of interest was the detection of ≥CIN2 by the digital colposcopy technique.

Two reviewers individually assessed the risk of bias and the applicability of the studies, using the Quality Assessment of Diagnostic Accuracy studies scoring system,[29] which is a validated tool for assessment of the methodological quality and applicability of diagnostic test accuracy studies. The four domains scored are: patient selection, index test, reference standard and flow and timing. The system indicates the risk of bias and applicability of the study as low, high or unknown.

The review protocol was designed in accordance with Preferred Reporting Items for Systematci Reviews and Meta-Analyses (PRISMA) guidelines for the reporting of systematic reviews and meta-analyses,[30] which was registered in PROSPERO (CRD42015027895).

Data from each study were inserted in Review Manager version 5.0 to calculate sensitivity, specificity and the 95% CI. To determine pooled estimates of sensitivity and

specificity with 95% CI, the METADAS tool was used within the statistical software package SAS version 9.2. to perform a random-effects bivariate logistic regression analysis. Forest plots were drawn to visualize variation in sensitivity and specificity. A sensitivity analysis was performed to explore the effect of excluding the study by Park et al, which only includes women with high-grade Pap cytology results.

RESULTS

In the initial search we retrieved 8,282 publications (Figure 1) of which 153 were considered potentially eligible. Studies were excluded because they appeared to be duplicates (n = 3,009), not relevant (n = 5,105), or written in a non-English language (n = 15). After reading the full text, another 141 studies were excluded because they did not meet the inclusion criteria: 29 studies were conference abstracts; in 57 studies the objective was not to define diagnostic value of the digital colposcopy technique in a colposcopy population; 31 studies did not perform a per-women analysis; in nine studies the CIN2 threshold was not used and could not be determined; in eight studies it was not possible to calculate sensitivity and specificity; three studies used a form of topical application before optical imaging; and one study was a sub study of another included study. Two studies on electrical impedance and one study on reflectance spectroscopy were excluded because for these techniques, less than three eligible studies remained. Only one study from the second search met the inclusion criteria, including a total number of 13 studies. All studies were prospective cohorts. We found six studies on fluorescence and reflectance spectroscopy, representing 2,530 women;[31-36] four studies on dynamic spectral imaging, representing 1,173 women;[24-26,37] and three studies on optical coherence tomography, representing 693 women.[27,38,39] Data on age of the participants is not included in the results, because this was reported in such a heterogeneous way making it impossible to show mean age, median age, or age range for all studies. The overall quality of the studies was moderate to high (Supplemental material Figure S2). Five studies had low risk of bias and low risk of applicability concerns in all seven domains. Three studies had an unclear risk of bias in one domain, one study had an unclear risk of bias in two domains and one study had a high risk of bias in one domain. Two studies had a high risk of bias in one domain and an unclear risk of bias in another; one study of these two studies had an unclear risk of applicability concern.

Fluorescence and reflectance spectroscopy combines two methods of spectrum analysis, which results in a prediction of tissue type depending on morphologic and biochemical changes. Table 1 summarizes the characteristics of the studies on fluorescence and reflectance spectroscopy. Four studies included women from a colposcopy referral population, and two also included women from a colposcopy follow-up population. Park

et al. included only women with high-grade Pap cytology results referred for colposcopy. Study populations range from 29 to 1,447 women. The pooled data from these six studies on fluorescence and reflectance spectroscopy shows a sensitivity of 93% (95% CI 89–95) and a pooled specificity of 62% (95% CI 47–76) (Figure 2). The sensitivity analysis, excluding the study by Park et al.,[34] resulted in a sensitivity of 94% (95% CI 90-97) and a specificity of 60% (95% CI 44-74).

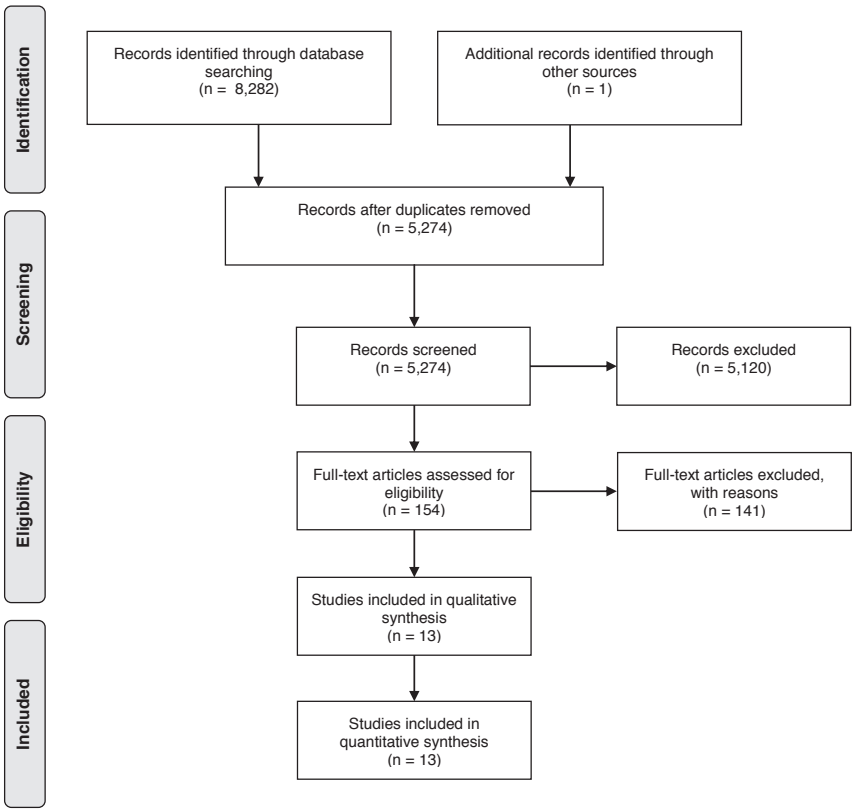

Figure 1. Flowchart of the systematic review process, per Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).

Dynamic spectral imaging is a colposcopy technique that measures the acetowhitening effect of cervical cells to determine the severity of CIN. The characteristics of the different studies on dynamic spectral imaging are summarized in Table 1. All four studies included women from a colposcopy referral population, of which one also included women from a colposcopy follow-up population. Study populations range from 183 to 443 women. Pooling the data from the studies on dynamic spectral imaging results in a sensitivity of 69% (95% CI 48–85) combined with a specificity of 83% (95% CI 76–88) (Figure 3).

Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
DeSantis 2007	135	157	7	273	0.95 (0.92 - 0.99)	0.63 (0.59 - 0.68)
Ferris 2007	18	16	1	76	0.95 (0.74 - 1.00)	0.83 (0.73 - 0.90)
Huh 2004	59	103	5	104	0.92 (0.83 - 0.97)	0.50 (0.43 - 0.57)
Park 2008	15	1	4	7	0.79 (0.54 - 0.94)	0.88 (0.47 - 1.00)
Twiggs 2013	252	767	24	404	0.91 (0.81 - 0.94)	0.35 (0.32 - 0.37)
Werner 2007	19	28	1	54	0.95 (0.75 - 1.00)	0.66 (0.55 - 0.76)
Pooled Total					0.93 (0.89 - 0.95)	0.62 (0.47 - 0.76)

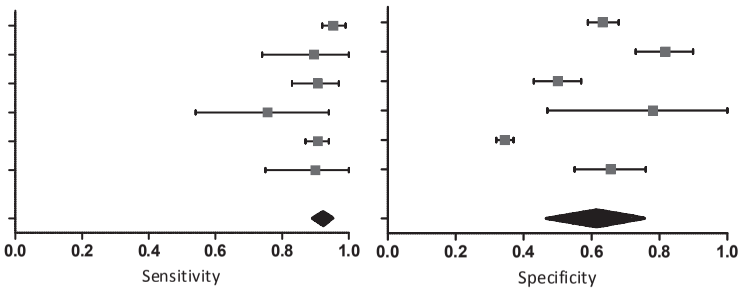

Figure 2. Forest plot of sensitivity and specificity in studies with fluorescence and reflectance spectroscopy.
CI = confidence interval; FN = false negative; FP = false positive; TN = true negative; TP = true positive.

Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Coronado 2015	33	40	8	362	0.80 (0.65 - 0.91)	0.90 (0.87 - 0.93)
Louwers 2011	68	22	18	75	0.79 (0.70 - 0.88)	0.77 (0.69 - 0.86)
Roensbo 2015	22	29	46	142	0.32 (0.72 - 0.45)	0.83 (0.77- 0.88)
Scoutter 2009	57	57	15	179	0.79 (0.68 - 0.88)	0.76 (0.70 - 0.81)
Pooled Total					0.69 (0.48 - 0.85)	0.83 (0.76 - 0.88)

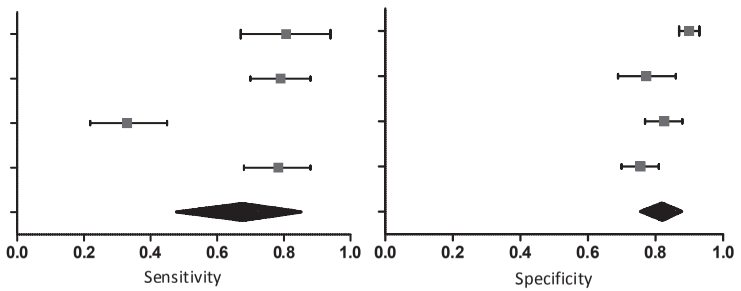

Figure 3. Forest plot of sensitivity and specificity in studies with dynamic spectral imaging.
CI = confidence interval; FN = false negative; FP = false positive; TN = true negative; TP = true positive.

Figure 4. Forest plot of sensitivity and specificity in studies with optical coherence tomography.
CI = confidence interval; FN = false negative; FP = false positive; TN = true negative; TP = true positive.

Table 1. Characteristics of included studies.

Study	Population	Device	No. of women	Sensitivity (95% CI)	Specificity (95% CI)	Colposcopy Sensitivity (95% CI)	Colposcopy Specificity (95% CI)
Fluorescence and Reflectance Spectroscopy							
DeSantis, 2007[31]	Colposcopy population (including follow-up)	LuViva® Advanced Cervical Scan (Guided Therapeutics)	572	95% (92–99)	63% (59–68)		
Ferris, 2001[32]	Colposcopy population (including follow-up)	Multimodal Hyperspectral Imaging System Prototype	111	95% (74–100)	83% (73–90)		
Huh,2004 [33]	Colposcopy population	Preproduction commercial optical system (MediSpectra)	271	92% (83–97)	50% (43–57)		
Park, 2008[34]	Colposcopy population (only HG smear)	Multispectral digital colposcope (Model IDL)	29	79% (54–94)	88% (47–100)		
Twiggs, 2013[35]	Colposcopy population	LuViva® Advanced Cervical Scan (Guided Therapeutics)	1,447	91% (87–94)	35% (32–37)		
Werner, 2007[36]	Colposcopy population	LuViva® Advanced Cervical Scan (Guided Therapeutics)	102	95% (75–100)	66% (55–76)		
Pooled total			**2,530**	**93% (89–95)**	**62% (47–76)**		
Dynamic Spectral Imaging							
Coronado, 2015[24]	Colposcopy population	DySIS (DySIS Medical Ltd)	443	81% (67-94)	90% (87-93)	73% (58-88)	92% (90-95)
Louwers, 2011[25]	Colposcopy population (including follow-up)	DySIS (Forth Photonics)	183	79% (70–88)	77% (69–86)	55% (44-65)	85% (77-92)
Roensbo, 2015[26]	Colposcopy population	DySIS (DySIS Medical Ltd)	239	32% (22–45)	83% (77–88)		
Soutter, 2009[37]	Colposcopy population	Precommercial DySIS model FPC-03 (Forth Photonics)	308	79% (68–88)	76% (70–81)	49% (37-61)	89% (85-93)
Pooled total			**1,173**	**69% (48–85)**	**83% (76–88)**		

Table 1. Characteristics of included studies. (continued)

Study	Population	Device	No. of women	Sensitivity (95% CI)	Specificity (95% CI)	Colposcopy Sensitivity (95% CI)	Colposcopy Specificity (95% CI)
Optical coherence tomography							
Escobar, 2006[38]	Colposcopy population	OCT device (Imalux Corporation)	212	56% (39–72)	59% (52–66)	39% (24-57)	71% (64-77)
Liu, 2010[27]	Colposcopy population	Niris Imaging System (Imalux Corporation)	299	32% (21–46)	93% (89–96)	23% (13-36)	96% (91-93)
Wulan, 2010[39]	Colposcopy population	OCT device (Imalux Corporation)	182	59% (39 –78)	65% (57–73)		
Pooled total			**693**	**48% (32–64)**	**77% (52–91)**		

Table 2. Cost of alternative digital colposcopy compared with conventional colposcopy.[40]

	LuViva (fluorescence and reflectance spectroscopy)	DySIS (dynamic spectral imaging)	Niris (optical coherence tomography)	Conventional colposcopy
Purchase price (£)	11,500	18,000-22,000	Device $49,500 Probe: $2,700	6,000-12,000
Consumables per woman (£)	17.25	3.5	$30.00	2.00
Service and maintenance cost (£)	320 for replacement light source every 2 years	1,600 for maintenance contract after warranty	Not applicable	10% of the list price is typically charged for a service contract
Anticipated life span (y)	5	5	7-10	5
Average time per treatment (min)	1	Less than 15	4	Less than 15

Modified from National Institute for Health and Clinical Excellence. Diagnostics assessment program. Adjunctive colposcopy technologies for examination of the uterine cervix - DySIS, LuViva Advanced Cervical Scan, Niris Imaging System and Zilico APX-100. Final scope. September, 2011. Available at: https://www.nice.org.uk/guidance/DG4/documents/adjunctive-colposcopy-technologies-for-examination-of-the-uterine-cervix-dysis-and-niris-imaging-system-diagnostics-assessment-report2. Retrieved June 7, 2016.

Optical coherence tomography uses near-infrared light to differentiate normal from diseased cells. Table 1 summarizes the characteristics of the different studies with optical coherence tomography, all including only women from a colposcopy referral population. Study populations range from 182 to 299 women. The pooled sensitivity for optical coherence tomography is 48% (95% CI 32–64) with a pooled specificity of 77% (95% CI 52–91) (Figure 4).

DISCUSSION

Fluorescence and reflectance spectroscopy shows the highest pooled sensitivity of the three discussed techniques, which is higher than the sensitivity of conventional colposcopy. However, its specificity is lower than conventional colposcopy. Dynamic spectral imaging shows the highest specificity, similar to conventional colposcopy, with a sensitivity also similar to conventional colposcopy.[2] Because alternative colposcopy techniques are still in development, randomized controlled trials to compare alternative techniques to conventional colposcopy are still lacking, and no recommendation for introduction in clinical practice can be made yet. The choice for a certain digital colposcopy device may depend on the use of the device in clinical setting. When a high sensitivity is desirable to assure as little as possible missed cervical abnormalities,

fluorescence and reflectance spectroscopy could be considered. The combination of two spectrum analyses may be the reason for the high sensitivity of this technique. Fluorescence and reflectance spectroscopy may thus be used as triage method of high-risk human papillomavirus-positive women or women with abnormal cytology, in order to limit colposcopy referrals, resulting in reduced unnecessary colposcopies and costs. The additional value of dynamic spectral imaging is limited given its similar specificity and sensitivity to conventional colposcopy. However, the objective assessment of this digital technique may be an advantage over conventional colposcopy. Additionally, previously performed studies showed that the sensitivity of conventional colposcopy increases by combining it with dynamic spectral imaging.[25] Therefore, a combination of conventional colposcopy and dynamic spectral imaging might be considered but will increase costs. Optical coherence tomography is best used adjunct to colposcopy according to the included studies, because it can give an optical biopsy, but has limited sensitivity and specificity. In countries with a high risk of loss to follow-up, or limited experience in colposcopy, digital colposcopy techniques may be beneficial in screening or triage purposes. An immediate result is shown, which gives the opportunity for treatment in the same visit. All three techniques may be performed by a trained healthcare worker, whereas a colposcopist requires more training to perform colposcopy with the most accurate outcome. Moreover, digital colposcopy techniques provide an objective assessment of cervical epithelia, whereas colposcopy is subjective based on human interpretation.

The main aim of this review was to assess the diagnostic value of alternative digital colposcopy techniques; however, cost estimates are important to give more insight into clinical applicability of the techniques. The price of a conventional colposcopy device varies between £6,000 and £12,000.[40] The price of alternative colposcopy techniques varies widely between $11,500 and $49,500.[40] Table 2 summarizes the costs for alternative digital colposcopy and conventional colposcopy. Of the three techniques, fluorescence and reflectance spectroscopy is the cheapest, but is not cheaper than conventional colposcopy devices. Because it is important to first show additional value of an alternative technique, we did not perform a cost-effectiveness analysis for these devices.

A major strength of this study is that this is a meta-analysis on the diagnostic value of digital colposcopy techniques for detection of cervical neoplasia. We found no other meta-analysis based our systematic search. Other strengths are the systematic approach, number of studies and techniques included, the criteria used for inclusion (i.e. colposcopy population, histology endpoint, cut-off point at CIN2, and a per woman analysis), and the moderate to high quality of the included studies resulting in a low risk of methodological bias.

A limitation is the relatively wide 95% CI for the pooled data on optical coherence tomography, because of limited numbers of included women in this group. This leads to

less reliable results, and makes it difficult to conclude on the diagnostic value and the clinical use of the technique. Additionally, sensitivity of dynamic spectral imaging and specificity of fluorescence and reflectance spectroscopy show a relatively wide 95% CI, combined with small 95% CI's on sensitivity of fluorescence and reflectance spectroscopy and specificity of dynamic spectral imaging. Second, other potential techniques (e.g. Raman spectroscopy, Truscreen, electrical impedance spectroscopy, and confocal microscopy) could not be included in this meta-analysis, because there were not enough or no studies on these techniques to match the inclusion criteria. Third, data on age of the women are not included in the analysis, because this was reported in such a heterogeneous way. Fourth, the study by Park et al. included only women with high-grade cervical results. However, sensitivity analysis excluding this study showed no significant difference in pooled results.

The clinical value of alternative colposcopy techniques varies between techniques, as well as the costs of these alternative devices. Alternative digital colposcopy techniques may result in increased or similar sensitivity and specificity compared with conventional colposcopy and hold aspects attractive for clinical use. However, because these alternative colposcopy techniques are still in development, and randomized controlled trials comparing alternative techniques to conventional colposcopy are still lacking, no recommendation for introduction in clinical practice can be made yet.

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SUPPLEMENTAL MATERIAL

Table S1: Search strategy.

(Cervix Uteri[mesh]) OR (Endocervix[tiab]) OR (Squamocolumnar junction[tiab]) OR (Cervi*[tiab]) OR (CIN[tiab]) OR (Squamous intraepithelial lesion [tiab]) OR (SIL[tiab]) OR (HSIL[tiab]) OR (LSIL[tiab]) NOT Neck AND (Tomography[tiab]) OR (Spectros*[tiab]) OR (Reflectance*[tiab]) OR (Fluorescence*[tiab]) OR (hyperspectr*[tiab]) OR (LuViva[tiab]) OR (Telecolposcopy[tiab]) OR (Multispectr*[tiab]) OR (Dysis[tiab]) OR (Spectra*[tiab]) OR (Optical colpo*[tiab]) OR (Advanced cervical scan[tiab]) OR (Optical ima*[tiab]) OR (Microcolpo*[tiab]) OR (Point probe[tiab]) OR (Confocal endo*[tiab]) OR (Confocal microscopy[tiab]) OR (Computerized colpo*[tiab]) OR (Spectrum analysis [mesh]) OR (Digital[tiab]) OR (Truscreen[tiab]) AND (Sensitivity[tiab]) OR (Specificity[tiab]) OR (Positive predictive value[tiab]) OR (Negative predictive value[tiab]) OR (Receiver operating characteristics [tiab]) OR (Area under the curve[tiab]) OR (Diagnos*[tiab]) OR (Sensitivity and Specificity[mesh]) OR (ROC[tiab]) OR (AUC[tiab]) OR (true positive[tiab]) OR (false positive[tiab])

Terms used for search strategy in MEDLINE, EMBASE, ClinicalTrials.gov and The Cochrane Library from inception to January 11th 2016. Terms with [tiab] reflect free text terms appearing in title or abstract.

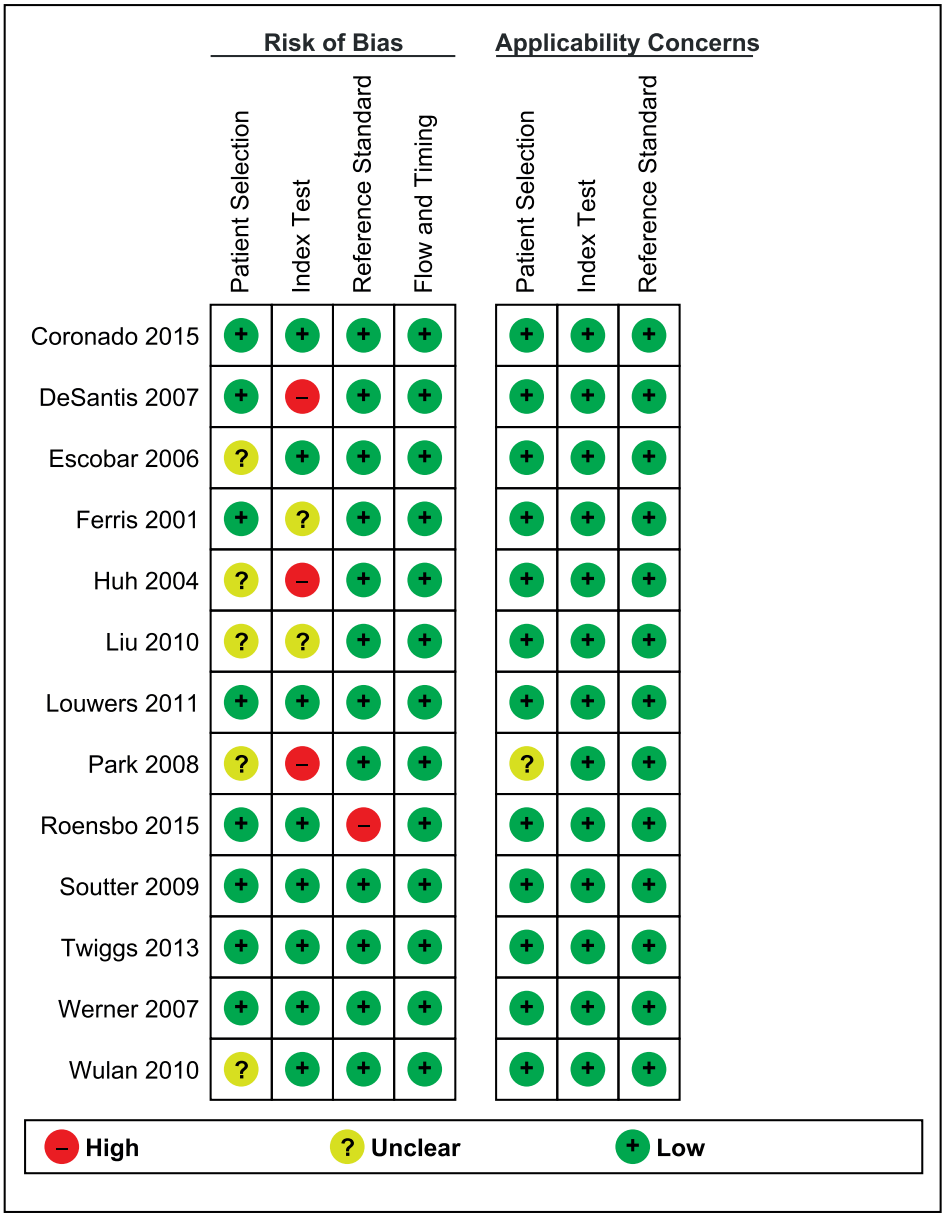

Figure S2. Risk of Bias and Applicability Concerns.

CHAPTER 8

A persistent high-risk HPV infection before the age of 30 is associated with a high risk of HSIL later in life

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Submitted

ABSTRACT

Objective High-risk human papillomavirus (hrHPV) screening is rapidly becoming the cornerstone of cervical cancer prevention. Because of the low specificity of hrHPV screening for young women, the Dutch screening program starts at the age of 30 years. However, young women with cervical cancer are missed with this screening strategy. The objective of this study was to investigate how hrHPV detection before the age of 30, may relate to risk of high-grade cervical lesions after the age of 30.

Methods We retrospectively analyzed follow-up data from a prospective cohort study on HPV prevalence in 2,065 unscreened Dutch women between 18 and 29 years of age. Women performed multiple self-collected cervico-vaginal samples for HPV detection and genotyping from 2007 to 2009. Women 30 years of age before the 1st of June 2015 were invited for the first cervical cancer screening round in the Netherlands, and were included in the present study. Based on the original study, they were categorized as; hrHPV negative, a cleared hrHPV infection, or a persistent hrHPV infection. Anonymized follow-up data for each group was obtained from the Dutch nationwide registry of histopathology and cytopathology (PALGA). Composite outcome measures with cytology and histology were obtained, defined as; normal, low-grade squamous intraepithelial lesion (LSIL) or high-grade squamous intraepithelial lesion (HSIL). The correlation between follow-up results and time of hrHPV clearance, persistence, and genotypes was analyzed.

Results A pathology result was registered for 962 women, of which 841 (87.4%) showed normal cervical smear results, 82 (8.5%) had a LSIL result, and 39 (4.1%) had a HSIL result. The prevalence of HSIL was 19.3% in women who had a persistent HPV infection at a younger age. This is significantly higher ($p<0,001$) compared with the HSIL prevalence of 1.5% in women who were HPV-negative at a younger age, as well as the HSIL prevalence of 3.1% in women who cleared the hrHPV infection in the past.

Conclusion Women who had a persistent hrHPV in their 20s, showed an increased risk of a HSIL lesion in their early 30s. We should therefore consider the advantages of screening for persistent hrHPV infections before the age of 30.

INTRODUCTION

Infections with human papillomavirus (HPV) are common; over 80% of the sexually active women have been genitally infected by one or more HPV types at some point in their life.[1] Most HPV infections are transient and clear spontaneously.[2] However, a persistent infection with a high-risk HPV (hrHPV) is thought to be a prerequisite for the development of cervical precancer and cancer.[3,4] HPV16 and HPV18 are the most carcinogenic hrHPV genotypes and cause 70% of all cervical cancers.[5]

The risk of sexual transmission of HPV generally peaks early in sexual life and declines with higher age.[6] Therefore, younger women test positive for hrHPV more frequently than women over 30 years of age.[7] The majority of infections does not lead to cervical cancer at such a young age. Screening of young women with hrHPV is often discouraged because of the limited specificity of the test with a high risk of unnecessary diagnostics or overtreatment.

From 1996 to 2016 Dutch women were screened in a cytology-based organized 5-yearly cervical cancer screening program, from 30 to 60 years of age. The Dutch National Cancer Registry shows that 7.3% of all cervical cancers are diagnosed in women between 20 and 29 years of age and another 10.1% is diagnosed in women between 30 and 35 years of age. This results in a group of 17.4% of all cervical cancers detected before the age of 35. Starting cytology screening at the age of 30 did not prevent these cases. An hrHPV-based cervical cancer screening strategy with cytology triage for women under 30 could potentially prevent these cancers by detecting hrHPV infections and cervical intraepithelial neoplasia (CIN) before they progress to cancer.[8]

The objective of this study was to investigate how hrHPV detection before the age of 30, may relate to the risk of high-grade cervical lesions after the age of 30 years.

METHODS

In this cohort study, we retrospectively analyzed follow-up data from a large prospective cohort study on HPV prevalence, incidence and clearance in women under the age of 30 which was performed in the Netherlands in 2007.[9,10] In total, 2,065 unscreened women between 18 and 29 years of age were included. Women performed a 3-monthly self-collected cervico-vaginal sample for 12 months. All women received a self-sample kit and questionnaires by mail and performed the cervico-vaginal self-sample in the privacy of their own home. Self-samples were tested for the presence of HPV with full genital HPV genotyping. Polymerase chain reaction (PCR)-based hrHPV testing on self-samples has been shown equally sensitive compared with clinician-based samples.[11] When women were hrHPV-positive after 12 months, another 12 months' follow-up with 6-monthly HPV

testing was offered. If women were hrHPV-positive at the end of 24 months, a clinician-taken smear for cytology testing was advised. Patient characteristics of this group of 2,065 women are previously described.[10,12] The self-samples were tested for the presence of HPV by using the highly sensitive SPF_{10}-DEIA, and genotyping of HPV positive samples was performed with the SPF_{10}-LiPA.[12]

From the total cohort of 2,065 women, 1,333 were over 30 years of age at June 1st 2015 and were included in this study, because at that point they had been invited for the first screening round of the national cervical cancer screening program in the Netherlands. Women who were only tested once in the initial study were excluded because HPV persistence or clearance could not be determined with one single test. Women with two or more hrHPV negative test results, and no hrHPV-positive results were classified as 'hrHPV negative'. Women with one or more hrHPV-positive test result followed by only hrHPV negative test results were classified as 'cleared infection'. Women who still tested hrHPV-positive at the end of the 24-month period were classified as 'persistent infection'. The 'persistent infection' and 'cleared infection' groups were subcategorized according to the presence or absence of HPV16 or HPV18, independent of the presence of the other hrHPV genotypes (i.e., 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68 and 73).

Women were categorized according to their HPV test result. Anonymized 8-year follow-up data for each group were obtained from the Dutch nationwide registry of histopathology and cytopathology (PALGA). Date of birth and first four letters of the surname were used as a personal identifier. For all women, cervical cytology and histology results registered up to June 1st 2015 were collected. Results were anonymized by assigning a random study-number.

If a histology result was available; the most severe histology result was used as outcome measure, otherwise the most severe cytology result was used as outcome measure. The Dutch CISOE-A (composition, inflammation, squamous, other and endometrium, endocervical cyclindrical epithelium, adequacy) classification system was used to report the test results for the cervical smears, which can easily be translated into the Bethesda nomenclature.[13] The first six months of follow-up after the final sample for the initial prospective study were censored from analysis because these were most likely results from the advised clinician-taken sample for cytology and would possibly not have been detected without the study. Women with HSIL or cervical cancer results in these first six months were excluded from follow-up because treatment of these lesions most likely affected the natural follow-up. Furthermore, women for whom no valid cytology or histology result was registered after the first six months, were excluded, as well as women with an uncertain identity. The identity was uncertain when the woman's first name from the study database did not match the first name from the PALGA database, because it is possible that these are two different women with the same surname and date of birth.

Composite outcome measures were obtained, defined as; negative for intraepithelial lesion and malignancy (NILM), including normal histology and normal cytology results; low-grade squamous intraepithelial lesions (LSIL), including CIN1 histology and atypical cells of undetermined significance (ASC-US) and LSIL cytology; and high-grade squamous intraepithelial lesions (HSIL), including histology results of CIN grade 2 or worse and HSIL cytology.

The correlation between composite outcome during follow-up and hrHPV presence, clearance, persistence, and different genotypes was analyzed. Also, the duration of the hrHPV infection until clearance was analyzed in regard to the outcome. Significance was calculated using the Fisher's Exact test, with a $p<0.05$ threshold for significance. All statistical analyses were performed using SPSS version 22.0.

The study was approved by the scientific committee of PALGA. The study was exempt from institutional review board approval because data were gathered retrospectively and analyzed anonymously.

RESULTS

From the cohort of 1,333 women, one or more valid cervical pathology result was obtained from 1,018 women. For 235 women, no valid cervical pathology result was registered, and for 80 women the identity was uncertain. Forty-six women had no registered pathology result after the first six months of follow-up and were excluded. For ten women, a high-grade result was registered in the first six months and these were also censored for further follow-up. This resulted in a group of 962 (72.2%) women >30 years of age for which follow-up data was available (Figure 1). These 962 women were subcategorized in groups according to their previous hrHPV results, as described. Of the total cohort, 591 women (61.4%) were hrHPV negative, 257 (26.7%) showed a cleared hrHPV infection, and 114 (11.9%) had a persistent hrHPV infection (Table 1).

During follow-up, 841 (87.4%) women had a normal cervical smear or normal cervical histology, 82 (8.5%) women had LSIL cytology or histology, and 39 (4.1%) women had HSIL cervical histology or cytology results registered in the PALGA database (Table 2). The prevalence of HSIL in follow-up was 19.3% for women with a 24-month persistent hrHPV infection. This is significantly higher ($p<0.001$) compared with the 1.5%, HSIL prevalence in hrHPV-negative women, as well as the 3.1% HSIL prevalence in women with a cleared hrHPV infection. In HPV16/18 persistent infections the HSIL prevalence was highest with 28.6%. Persistent infections with the other hrHPV types showed a HSIL prevalence of 13.9%, which was not significantly different from the persistent HPV16/18 group ($p=0.84$). Persistent infections also show the highest percentage of LSIL follow-up, although with a lower percentage of LSIL in HPV16/8 infections with 7.1%, compared

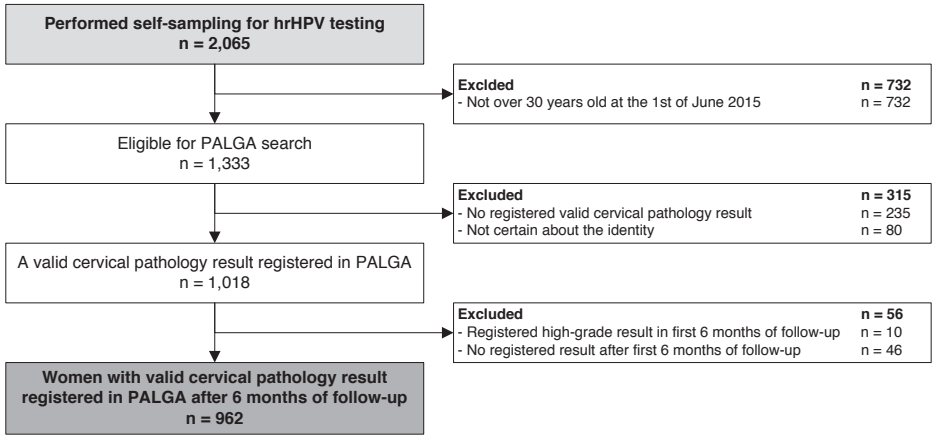

Figure 1. Trial profile.

with an 18.0% incidence of LSIL in persistent other hrHPV infections (Figure 2). Of the hrHPV negative group, one woman was diagnosed with micro invasive cervical cancer. In total, 17 women were diagnosed with CIN grade 3, of which ten in the persistent hrHPV group, and 21 women were diagnosed with CIN grade 2, of which 12 in the persistent hrHPV group (Table 3). As may be expected, hrHPV negative women showed the lowest risk of HSIL (1.5%). The highest risk was estimated for women who still showed a positive hrHPV test after 12 months, with a HSIL prevalence of 19.5% during follow-up. For women who cleared their infection within 12 months, the HSIL risk was significantly lower (p<0,001) with a HSIL prevalence of 3.1%. Differences between HPV16/18 and other hrHPV types were not studied as groups were too small (Table 4).

Table 1. Characteristics of groups of women in regard to groups based on previous hrHPV results.

Groups based on previous hrHPV results	n (%)
hrHPV negative	591 (61.4)
Cleared hrHPV infection	257 (26.7)
Cleared HPV16/18 infection	78 (8.1)
Cleared other hrHPV infection	179 (18.6)
Persistent hrHPV infection	114 (11.9)
Persistent HPV16/18 infection	42 (4.4)
Persistent other hrHPV infection	72 (7.5)
Total	**962 (100)**

hrHPV = high-risk human papillomavirus. Other hrHPV includes HPV types 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68 and 73.

DISCUSSION

This cohort study shows a significant increase in risk of HSIL in the early 30s of women who had a persistent hrHPV in their 20s. Especially women with an hrHPV infection still present after 12 months showed a significantly higher risk of HSIL, compared with women with an hrHPV infection that was cleard within 12 months.

Table 2. Histology and cytology follow-up results in regard to groups based on previous hrHPV results.

Groups based on previous hrHPV results	Normal follow-up n (%)	LSIL follow-up n (%)	HSIL follow-up n (%)	Total n
hrHPV negative	537 (90.9)	45 (7.6)	9 (1.5)	591
Cleared hrHPV infection	228 (88.7)	21 (8.2)	8 (3.1)	257
Cleared HPV16/18 infection	73 (93.6)	4 (5.1)	1 (1.3)	78
Cleared other hrHPV infection	155 (86.6)	17 (9.5)	7 (3.9)	179
Persistent hrHPV infection	76 (66.7)	16 (14.0)	22 (19.3)	114
Persistent HPV16/18 infection	27 (64.3)	3 (7.1)	12 (28.6)	42
Persistent other hrHPV infection	49 (68.1)	13 (18.0)	10 (13.9)	72
Total	**841 (87.4)**	**82 (8.5)**	**39 (4.1)**	**962 (100)**

Note that numbers and percentages of the subgroups in cleared and persistent infections are added up in the total group of cleared and persistent infections, and therefore do not add up to the total columns. hrHPV = high-risk human papillomavirus; HSIL = high-grade squamous intraepithelial lesion; LSIL = low-grade squamous intraepithelial lesion. Other hrHPV includes HPV types 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68 and 73.

Table 3 HSIL endpoints in regard to groups based on previous hrHPV results.

Groups based on previous HPV results	CIN2	CIN3	Invasive carcinoma	Total n (%)
hrHPV negative	5	3	1	9 (23.1)
Cleared hrHPV infection	4	4	0	8 (20.5)
Cleared HPV16/18 infection	0	1	0	1 (2.6)
Cleared other hrHPV infection	4	3	0	7 (17.9)
Persistent hrHPV infection	12	10	0	22 (56.4)
Persistent HPV16/18 infection	6	6	0	12 (30.8)
Persistent other hrHPV infection	6	4	0	10 (25.6)
Total	**21 (53.8)**	**17 (43.6)**	**1 (2.6)**	**39 (100)**

Note that numbers and percentages of the subgroups in cleared and persistent infections are added up in the total group of cleared and persistent infections, and therefore do not add up to the total columns. hrHPV = high-risk human papillomavirus; HSIL = high-grade squamous intraepithelial lesion; LSIL = low-grade squamous intraepithelial lesion. Other hrHPV includes HPV types 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68 and 73.

In line with our results, previous studies have shown that women with a persistent hrHPV infection have a significantly higher risk of developing high-grade CIN lesions

compared with those who cleared their infection.[14,15] Data from this study adds the increased future HSIL risk for these young women. HPV prevalence and persistence have especially been shown to be high among sexually active young women; with a HPV prevalence up to 54%, of which 34% was a persistent infection.[16] It is however known that these infections only rarely cause cervical cancer at such young age,[8] and that overtreatment especially at young age is undesirable because of the risk of cervical insufficiency in future pregnancies.[17] HrHPV-based screening between the age of 20 and 30 years is therefore debatable and not performed in all countries. In the Netherlands screening starts at the age of 30 in order to prevent overtreatment of young women.

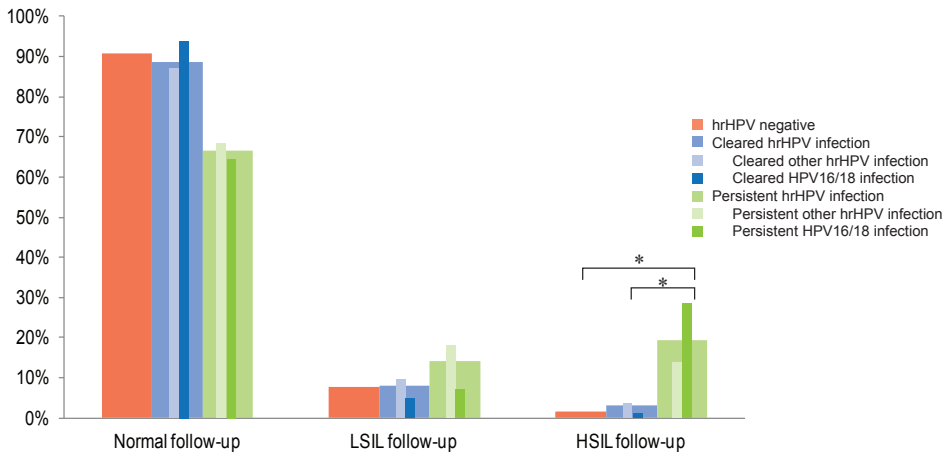

Figure 2. Histology and cytology follow-up results in regard to groups based on previous hrHPV results. hrHPV = high-risk human papillomavirus; HSIL = high-grade squamous intraepithelial lesion; LSIL = low-grade squamous intraepithelial lesion. Differences marked with an * are statistically significant with a p value <0.05.

Table 4. Follow-up results in regard to frequency of subsequent hrHPV detection.

	Normal (%)	LSIL (%)	HSIL (%)	Total (%)
hrHPV negative	537 (90.9)	45 (7.6)	9 (1.5)	591 (100)
1-4x hrHPV-positive (0-9 months)*	230 (89.1)	20 (7.8)	8 (3.1)	258 (100)
5-7x hrHPV-positive (12-24 months)**	74 (65.5)	17 (15.0)	22 (19.5)	113 (100)
Total	**841**	**82**	**39**	**962**

*1-4 hrHPV infections represent infections that cleared within 3-12 months. **5-7 hrHPV infections represent infections that did not clear within 12-24 months. hrHPV = high-risk human papillomavirus; HSIL = high-grade squamous intraepithelial lesion; LSIL = low-grade squamous intraepithelial lesion.

Various countries differ in the organization of their cervical cancer screening program; in terms of type of screening test, invitation methodology, population-based screening or opportunistic screening, target population, screening intervals, but also in age of

starting cervical cancer screening. In the Netherlands and Finland screening is offered by the government from the age of 30 years with a 5-yearly schedule. Belgium, France, Australia, the UK and Italy start earlier and invite women every 3-5 years starting at their 25th birthday. Sweden starts at the age of 23, and Germany, Canada, and the USA start even earlier at 20 or 21 years old and use a 1-5 yearly schedule. Some countries use an hrHPV-based screening program, others use Pap cytology as primary screening method, and others combine them and use co-testing. However, the cervical cancer incidence in these countries is not inevitably linked to the start of screening or screening interval.[18] Germany offers the most screening rounds, but the cervical cancer incidence is lowest in Finland where women are screened 5-yearly from the age of 30.[17] Participation to the screening program is known to be important in lowering cervical cancer incidence, and young age is a risk factor for non-participation.[18] Participation to screening programs may be improved by offering self-sampling for hrHPV testing,[19] which has been shown to be equally sensitive to clinician-taken samples for hrHPV testing and may be an attractive option for screening young women.[20]

In the Netherlands in 2015, the 17.4% of all cervical cancers diagnosed between 20 and 35 years. The majority of these cancers may potentially be avoided if a persistent infection in a girls' 20s was detected and present CIN lesions were treated in time. It could for example be considered to start hrHPV-based screening for all women at the age of 20 or 25. Data from this study show that the risk of a HSIL lesion is only 3.1% in women with an hrHPV infection which is cleared within 12 months, and no carcinomas were found in this group. The risk of HSIL is much higher with 19.5% when women still test hrHPV positive 12 months after their first positive hrHPV test. Performing cytology triage after one single hrHPV-positive test may therefore result in referral of too many young women for colposcopic examination with possibly unnecessary diagnostics and unnecessary treatment. It may be valuable to individualize screening for young women. Young women with one hrHPV-positive result can then be monitored with a watchful waiting policy to see whether the infection will clear within 12 months. When these infections do not clear within the 12 months, cytology triage should be performed to detect the presence of CIN lesions in need of treatment, to prevent development of cervical cancer before the age of 30 years.

On the contrary to the increased risk of HSIL lesions in young women with a persistent hrHPV infection, this study shows that women who are hrHPV negative in their early adulthood have a low risk (1.5%) of developing a HSIL lesions in the next eight years of follow-up. Women who are hrHPV negative before the age of 30, might even benefit from longer screening intervals. However, further studies should be performed to see if extending screening intervals would be safe. Also, the discovery of one micro-invasive squamous cell carcinoma in the hrHPV negative group of this study might contradict this suggestion. Because all follow-up data was anonymized we could not identify how

many hrHPV negative tests preceded this carcinoma. These were at least two negative tests because all women with two or more hrHPV negative test results and no hrHPV-positive results were scored in this group. Also, we could not identify when these tests were hrHPV negative, or if the carcinoma was hrHPV-positive or negative. As follow-up in this study was eight years, this carcinoma may also have developed quickly.

From the total group of 1,333 eligible women, at least 1,018 (76.4%) women had a cervical smear taken. This percentage is high compared with cervical cancer screening participation in young women in the Netherlands which ranges between 50-60% in women from 30 to 35 years of age.[21] The knowledge on hrHPV obtained by participating in the study or knowledge of their hrHPV status may have affected women in their choice of having a cervical smear taken in the first cervical cancer screening round.

Another potential source of bias in this study is the possibility that an infection classified as persistent is not truly a persistent infection, but could also be a re-infection with hrHPV. Persistence in this study was purely based on the presence of hrHPV. A re-infection however might still indicate increased susceptibility for hrHPV and additional cytology triage might be needed. Also, ASC-US cervical smears were categorized as LSIL in this study. In fact these two results are not directly comparable, which could also have caused potential bias with increased numbers of LSIL in different groups. This was however done in all groups, so potential bias would be present in all groups equally. Furthermore, the first six months of follow-up after the final sample for the initial prospective study were censored from analysis, and 10 women with HSIL or cervical cancer results in these first six months were excluded because treatment of these lesions most likely affected the natural follow-up. Censoring of these first six months and exclusion of 10 women with HSIL or cervical cancer might have caused bias which could cause an underestimation of our results.

From 2009 on, prophylactic hrHPV vaccination with the bivalent vaccine is offered to girls in the Netherlands, with a coverage-rate of 61% in 2016.[22] These vaccinated women will first enter the organized screening program at the age of 30, which will be the case in 2023. Screening strategies for women under the age of 30 will therefore still be beneficial as not all girls are vaccinated, and it is unknown to which extent vaccinated girls will be protected for cervical cancer caused by hrHPV types other than HPV16 and HPV18. These differences in risk-estimates for an hrHPV infection with HPV16 or HPV18 might be a reason for individualizing screening in the future. Earlier research has shown that young women between 21 and 30 years of age in the United States who had not initiated HPV vaccination were also less likely to have a cervical smear taken, compared with women who initiated vaccination.[23] The option of hrHPV self-sampling in the privacy of their own homes might persuade these young women to indeed participate in cervical cancer screening.[19]

Multiple studies have shown the value of HPV16/18 genotyping in triage of hrHPV-positive women.[24,25] This study also shows the highest risk of HSIL in women with a

persistent HPV16/18 infection, which is twice as high compared with the HSIL risk for women with a persistent infection with one of the other hrHPV types. This confirms that HPV16/18 genotyping may indeed be useful in individualized screening, triage, and follow-up strategies of hrHPV-positive women. However, the numbers in this study are too small to draw specific conclusions in this group of young women.

In conclusion, this study shows that women who had a persistent hrHPV infection in their 20s, showed an increased risk of a HSIL lesion in their early 30s. We should therefore consider the advantages of screening for persistent hrHPV infections before the age of 30.

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CHAPTER 9

Long-lasting increased risk of human papillomavirus-related carcinomas and premalignancies after cervical intraepithelial neoplasia grade 3: a population-based cohort study

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ABSTRACT

Objective The aim of this study was to determine the risk of human papillomavirus (HPV)-related carcinomas and premalignancies in women diagnosed with cervical intraepithelial neoplasia grade 3 (CIN3). Knowledge on this risk is important to preventing development and progression of other HPV-related premalignancies and carcinomas, by considering HPV vaccination and/or by paying increased attention to other HPV-related carcinomas and premalignancies when CIN3 is identified.

Methods Women diagnosed with a CIN3 between 1990 and 2010 were identified from the Dutch nationwide registry of histopathology and cytopathology (PALGA) and matched with a control group of women without CIN3. Subsequently, all cases of high-risk (hr)HPV-associated high-grade lesions and carcinomas in the anogenital region and oropharynx between 1990 and 2015 were extracted. Incidence rate ratios (IRR) were estimated for carcinomas and premalignancies of the vulva, vagina, anus and oropharynx.

Results A total of 178,036 women were identified: 89,018 with a previous diagnosis of CIN3 and 89,018 matched control subjects without a history of CIN3. Women with a history of CIN3 showed increased risk of HPV-related carcinomas and premalignancies with IRRs of; 3.85 (95% CI 2.32-6.37) for anal cancer, 6.68 (95% CI 3.64-12.25) for anal intraepithelial neoplasia grade 3 (AIN3), 4.97 (95% CI 3.26-7.57) for vulvar cancer, 13.66 (95% CI 9.69-19.25) for vulvar intraepithelial neoplasia grade 3 (VIN3), 86.08 (95% CI 11.98-618.08) for vaginal cancer, 25.65 (95% CI 10.50-62.69) for vaginal intraepithelial neoplasia grade 3 (VAIN3), and 5.51 (95% CI 1.22-24.84) for oropharyngeal cancer. This risk remained significantly increased, even after long-term follow-up of up to 20 years.

Conclusion This population-based study shows a long-lasting increased risk for HPV-related carcinomas and premalignancies of the anogenital and oropharyngeal region after a CIN3 diagnosis. Studies that investigate methods to prevent this increased risk in this group of patients, such as intensified screening or vaccination, are warranted.

INTRODUCTION

Infections with a high-risk human papillomavirus (hrHPV) are estimated to be the cause of 5.2% of all cancers worldwide.[1] HrHPV infections in women cause cervical cancer and cervical intraepithelial neoplasia (CIN), as well as carcinomas and premalignancies of the oropharynx, and anogenital region including the vulva, vagina, and anus.

The prevalence of hrHPV in cervical cancer is close to 100%. In vaginal cancers, the prevalence of hrHPV is 60 to 70%, and in vulvar cancer it ranges from 20 to 50%. In anal cancer, an hrHPV infection is found in 88 to 95% of all cases, and 15 to 65% of the oropharyngeal cancers are related to the HPV.[2,3]

It has been shown that women with a diagnosis of CIN grade 3 (CIN3) are at increased risk of carcinomas of the anogenital and head and neck region, probably as the result of an infection with an hrHPV.[4–8] However, none of those studies included the risk for high-grade premalignancies after a diagnosis of CIN3.[4,5,8–10] Knowledge of this risk is important to preventing the development and progression of other HPV-related premalignancies and carcinomas, by considering prophylactic HPV vaccination and/or by paying increased attention to other HPV-related carcinomas and premalignancies when CIN3 is identified. Primary prevention of HPV-related carcinomas and premalignancies by prophylactic vaccination is now only available for young women. Older and unvaccinated women are therefore still at risk for HPV-related disease in the next decades. Treating premalignancies in a cohort may prevent malignancies from developing, and the risk for HPV-related carcinomas and premalignancies combined represents the possible preventive effect of HPV vaccination in women with a CIN3. The aim of this study was therefore to determine the risk of HPV-related carcinomas and premalignancies in women with a previous histologic diagnosis of CIN3.

METHODS

Women with a diagnosis of CIN3 between January 1, 1990 and December 31, 2010, were identified from the Dutch nationwide registry of histopathology and cytopathology (PALGA). Date of birth and first four letters of the surname were used as a personal identifier. Women registered in the database with a benign dermal nevus, but who were never diagnosed with CIN3 or cervical carcinoma before or after the diagnosis of nevus, were selected from the database as a control group. Registration of a pathology result in the database was needed to obtain a control group from the database; a registered benign dermal nevus was chosen because it is not related to HPV and can be diagnosed in all women. Women with both a CIN3 and a benign nevus diagnosis were included in the CIN3 group. Control subjects were assigned a random number and sorted.

Subsequently, from the total group of 547,924 matched women with a nevus, random frequency matching was done by age and year of detection (both within a range of five years), and population density areas; smaller cities with <100,000 inhabitants were considered low-density areas, and those with >100,000 inhabitants higher-density cities. For all identified women, histology results for carcinomas and high-grade premalignancies of the vulva, vagina, perineum, anus, and oropharynx between January 1, 1990 and March 1, 2015, were retrieved, resulting in 4 to 25 years of follow-up. In addition, patient age and date of diagnosis were retrieved.

We identified all HPV-related carcinomas and premalignancies diagnosed after the CIN3 or benign dermal nevus. Only results with a clear diagnosis of malignancy were classified as cancer; when invasiveness was uncertain in a high-grade lesion, the result was classified as high-grade premalignancy. Moderate (CIN2) and low-grade (CIN1) premalignancies were not included in the high-grade group. Women were censored after diagnosis of an HPV-related carcinoma of any site because treatment might influence the subsequent risk of other carcinomas or premalignancies. Women with an HPV-related carcinoma or premalignancy before the CIN3 or benign dermal nevus were excluded from analysis, as well as results indicating recurrence or metastases of disease. Differentiated vulvar intraepithelial neoplasia (VIN) and vulvar cancer after a previous diagnosis of lichen sclerosis, clear cell carcinomas, serous adenocarcinoma, lymphomas, melanomas, and basal cell carcinomas were also excluded because these are not likely to be related to HPV.[11,12]

We calculated person-years at risk and the number of observed carcinomas and premalignancies in each group, taking into account censoring as described in the previous paragraph. Using Poisson regression with an offset on the basis of the person-years, incidence rates (IR) per 100.000 person-years were estimated for both cases and their matched controls. Incidence rate ratios (IRR), comparing women with a CIN3 diagnosis with the matched control group, were computed, first by analyzing the whole follow-up period and secondly by excluding the first year of follow-up, to account for possibly prevalent HPV-related carcinomas and premalignancies in the cohort. HPV-related carcinomas and premalignancies were analyzed separately and combined into clusters of any HPV-related carcinoma and/or any HPV-related premalignancy. The cluster of any HPV-related carcinoma includes anal cancer, vulvar cancer, vaginal cancer, and oropharyngeal cancer. The cluster of any HPV-related premalignancy includes anal intraepithelial neoplasia grade 3 (AIN3), VIN grade 3, and vaginal intraepithelial neoplasia grade 3 (VAIN3). The cluster of any malignancy or premalignancy includes anal cancer, AIN3, vulvar cancer, VIN3, vaginal cancer, VAIN3, and oropharyngeal cancer. In all analyses, person-years were only considered before the first diagnosed carcinoma or premalignancy.

Age-adjusted IRRs for 5-year follow-up intervals were estimated for up to 25 years of follow-up for each carcinoma and premalignancy separately and for the clustered carcinomas and premalignancies, by means of a Poisson model with an interaction term for the group with a history of CIN3 and follow-up period and a continuous variable for age. In addition, IRRs were estimated for women ≤29 years, 30-49 years, 50-69 years, and ≥70 years for the clustered carcinomas and premalignancies. Kaplan-Meier curves were used to visualize the risk of HPV-related carcinomas and premalignancies over time.

All statistical analyses were performed with SAS version 9.2. Kaplan-Meier curves were obtained with SPSS version 22.0.0.1.

The study was approved by the scientific committee of PALGA. The study was exempt from institutional review board approval because data were gathered retrospectively and analyzed anonymously.

Table 1. Characteristics of study population at inclusion.*

	Number of patients	Percentage	Person-years of follow-up	
			CIN3 history (%)	No CIN3 history (%)
Age cohort				
<20 years	143	0.2%	1,894	1,905
20-24 years	2,160	2.4%	32,625	32,722
25-29 years	10,523	11.8%	161,919	161,814
30-34 years	25,426	28.6%	350,931	351,628
35-39 years	20,828	23.4%	307,335	306,170
40-44 years	13,390	15.0%	185,361	186,021
45-49 years	7,749	8.7%	104,492	105,295
50-54 years	3,896	4.4%	54,638	54,461
55-59 years	2,156	2.4%	28,199	28,272
60-64 years	1,257	1.4%	16,642	16,703
65-69 years	505	0.6%	7,602	7,683
70-74 years	418	0.5%	5,540	5,570
75-79 years	303	0.3%	3,113	3,220
≥80 years	264	0.3%	1,511	1,535
Total	89,018	100%	1,261,804	1,262,998
Calendar period				
1990-1994	20,354	22.9%	458,469	457,284
1995-1999	20,785	23.3%	364,119	365,151
2000-2004	19,063	21.4%	239,718	239,056
2005-2010	28,816	32.4%	199,498	201,507
Total	89,018	100%	1,261,804	1,262,998

*The number of patients and percentages of age distribution and calendar period of inclusion are similar in both groups because of 5-year matching. CIN3 = cervical intraepithelial neoplasia grade 3.

Table 2. Observed numbers of carcinomas and premalignancies, person-years, estimated incidence rates per 100,000 person-years (IR) and incidence rate ratios (IRR) of carcinomas and premalignancies of the anus, vulva vagina and oropharynx of women with a history of CIN3, compared with women without a history of CIN3.

	All periods				Excluding first year after inclusion			
	ON	Person-years	IR (95% CI)	IRR (95% CI)	ON	Person-years	IR (95% CI)	IRR (95% CI)
Anal cancer								
CIN3 history	73	1,261,804	5.79 (4.60-7.28)	3.85 (2.32-6.37)	69	1,172,923	5.88 (4.65-7.45)	4.32 (2.51-7.44)
No CIN3 history	19	1,262,998	1.50 (0.96-2.36)		16	1,174,066	1.36 (0.84-2.22)	
AIN3								
CIN3 history	80	1,261,219	6.34 (5.10-7.90)	6.68 (3.64-12.25)	73	1,172,344	6.23 (4.95-7.83)	7.31 (3.78-14.16)
No CIN3 history	12	1,262,912	0.95 (0.54-1.67)		10	1,173,982	0.85 (0.46-1.58)	
Vulvar cancer								
CIN3 history	129	1,265,176	10.20 (8.58-12.12)	4.97 (3.26-7.57)	114	1,173,004	9.72 (8.09-11.68)	4.56 (2.96-7.04)
No CIN3 history	26	1,266,520	2.06 (1.40-3.02)		25	1,174,066	2.13 (1.44-3.15)	
VIN3								
CIN3 history	476	1,257,676	37.85 (34.60-41.41)	13.66 (9.69-19.25)	395	1,168,874	33.79 (30.62-37.30)	13.22 (9.12-19.17)
No CIN3 history	35	1,262,759	2.77 (1.99-3.86)		30	1,173,832	2.56 (1.79-3.66)	
Vaginal cancer								
CIN3 history	86	1,261,804	6.82 (5.52-8.42)	86.08 (11.99-618.08)	63	1,172,923	5.37 (4.20-6.88)	63.06 (8.75-454.66)
No CIN3 history	1	1,262,998	0.08 (0.01-0.56)		1	1,174,066	0.09 (0.01-0.61)	
VAIN3								
CIN3 history	128	1,260,387	10.16 (8.54-12.08)	25.65 (10.50-62.69)	76	1,171,559	6.49 (5.18-8.12)	15.23 (6.16-37.65)
No CIN3 history	5	1,262,982	0.40 (0.17-0.95)		5	1,174,051	0.43 (0.18-1.02)	
Oropharyngeal cancer								
CIN3 history	11	1,261,804	0.87 (0.48-1.57)	5.51 (1.22-24.84)	11	1,172,923	0.94 (0.52-1.70)	11.01 (1.42-85.28)
No CIN3 history	2	1,262,998	0.16 (0.04-0.63)		1	1,174,066	0.09 (0.01-0.61)	

Table 2. Observed numbers of carcinomas and premalignancies, person-years, estimated incidence rates per 100,000 person-years (IR) and incidence rate ratios (IRR) of carcinomas and premalignancies of the anus, vulva vagina and oropharynx of women with a history of CIN3, compared with women without a history of CIN3. (continued)

	All periods				Excluding first year after inclusion			
	ON	Person-years	IR (95% CI)	IRR (95% CI)	ON	Person-years	IR (95% CI)	IRR (95% CI)
Any HPV-related carcinoma*								
CIN3 history	299	1,265,176	23.63 (21.10-26.47)	6.24 (4.60-8.46)	257	1,173,004	21.91 (19.39-24.76)	5.98 (4.33-8.26)
No CIN3 history	48	1,266,520	3.79 (2.86-5.03)		43	1,174,066	3.66 (2.72-4.94)	
Any HPV-related premalignancy**								
CIN3 history	634	1,259,306	50.35 (46.58-54.42)	12.75 (9.56-17.00)	501	1,167,269	42.92 (39.32-46.85)	11.72 (8.58-16.00)
No CIN3 history	50	1,266,192	3.95 (2.99-5.21)		43	1,173,745	3.66 (2.72-4.94)	
Any HPV-related carcinoma or premalignancy†								
CIN3 history	847	1,259,306	67.26 (62.88-71.95)	9.68 (7.77-12.05)	683	1,167,269	58.51 (54.29-63.07)	8.81 (6.97-11.13)
No CIN3 history	88	1,266,192	6.95 (5.64-8.57)		78	1,173,745	6.65 (5.32-8.30)	

* any HPV-related carcinoma includes anal cancer, vulvar cancer, vaginal cancer and oropharyngeal cancer. ** any HPV-related premalignancy includes AIN3, VIN3 and VAIN3. After the first premalignancy women were censored. † any HPV-related carcinoma or premalignancy includes anal cancer, AIN3, vulvar cancer, VIN3, vaginal cancer, VAIN3 and oropharyngeal cancer. After the first carcinoma or premalignancy women were censored. AIN3 = anal intraepithelial neoplasia grade 3; CI = confidence interval; CIN3 = cervical intraepithelial neoplasia grade 3; IR = incidence rate; IRR = incidence rate ratio; ON = observed number; VAIN3 = vaginal intraepithelial neoplasia grade 3; VIN3 = vulvar intraepithelial neoplasia grade 3.

RESULTS

We identified 178,036 women, of whom 89,018 had a histologic diagnosis of CIN3, between 1990 and 2010, and an equal number of women in the matched control group of women without a history of CIN3. The median age at diagnosis was 35 years in women with a previous diagnosis of CIN3, and 36 years in the control group. Characteristics of the study population at inclusion are listed in Table 1. During a median follow-up of 14 years in both groups (range, 4 to 25 years), 1,261,804 person-years were accrued in the group with a previous diagnosis of CIN3. After censoring, a total of 299 HPV-related carcinomas and 634 HPV-related premalignancies were found in the group with a previous diagnosis of CIN3. In the control group, 1,262,998 person-years were accrued, with 48 HPV-related carcinomas and 50 HPV-related premalignancies (Table 2).

The risk of developing HPV–related carcinomas and premalignancies of the anus, vulva, vagina, and oropharynx is strongly associated with a previous diagnosis of CIN3, with a significant increase in the group with a history of CIN3 (Figure 1). The IRRs for anal cancer and AIN3, comparing women with a previous diagnosis of CIN3 with those without a previous diagnosis of CIN3 were 3.85 (95% CI 2.32-6.37) and 6.68 (95% CI 3.64-12.25), respectively. For vulvar cancer and VIN3, the IRRs were 4.97 (95% CI 3.26-7.57) and 13.66 (95% CI 9.69-19.25), respectively. Vaginal cancer and VAIN3 showed the highest IRRs with 86.08 (95% CI 11.99-618.08) for vaginal cancer, and 25.65 (95% CI 10.50-62.69) for VAIN3, as the result of low IRs in the control group. Oropharyngeal cancer showed an IRR of 5.51 (95% CI 1.22-24.84). The IRR for any HPV-related carcinoma was 6.24 (95% CI 4.60-8.46), for any HPV-related premalignancy the IRR was 12.75 (95% CI 9.56-17.00), and for any HPV-related carcinoma or premalignancy the IRR was 9.68 (95% CI 7.77-12.05). Excluding the first year after inclusion resulted in IRR estimates that were not notably different (Table 2).

The age-adjusted IRR stratified by time interval since first diagnosis showed a short-term and long-lasting increased risk of developing HPV-related carcinomas and premalignancies in women with a previous diagnosis of CIN3, when compared with the control group of women without a CIN3 diagnosis. Even up to 20 years after the CIN3 diagnosis, this risk remained increased (Table 3). The increased cumulative incidence over time is clearly visualized in the Kaplan-Meier curves (Figure 2). For the cluster of any HPV-related carcinoma or premalignancy, the IRR decreased over time; however, an increased risk was still visible after long-term follow-up (Figure 3).

IRR stratified by attained age showed that the risk of carcinoma or premalignancies for women with a hitstory of CIN3 might be dependent on age. The IRR of any HPV-related premalignancy and of any carcinoma or premalignancy was highest in women ≤29 years of age, both with an IRR > 30. The IRR for women with any HPV-related carcinoma was not available. In the group of women between 30 and 49 years of age, the risk was lower

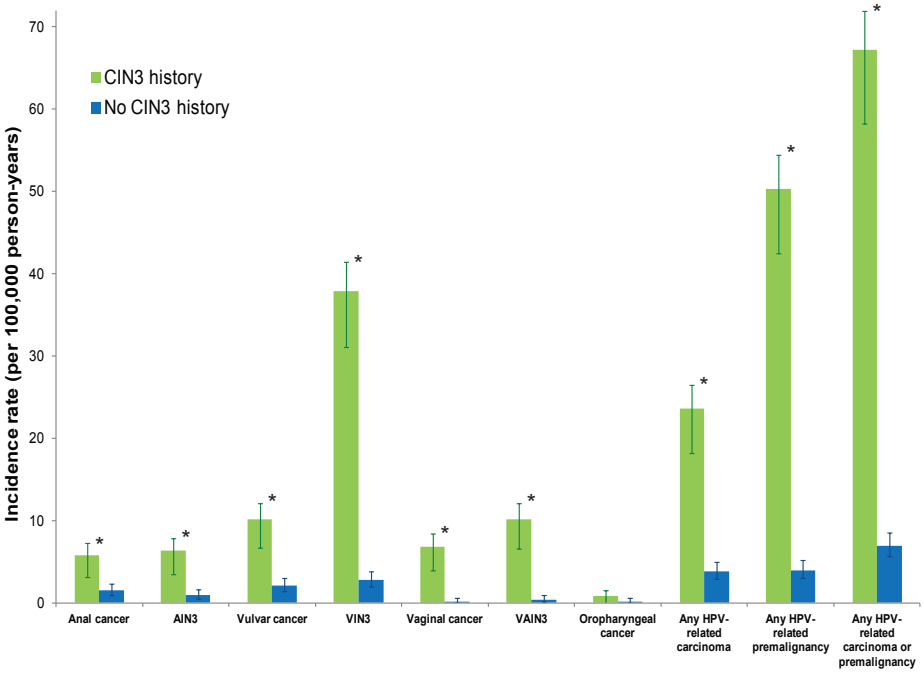

Figure 1. Estimated Incidences rate of HPV-related carcinomas and premalignancies, comparing women with and without a history of CIN3.
The first year after diagnosis of CIN3 or benign dermal nevus was included in the incidence rate visualized in this figure. Error bars indicate 95% confidence intervals of estimated incidence rates. * indicates a significant difference in incidence rates of the group with a history of CIN3 compared with the group without a history of CIN3. Data are in Table 2. AIN3 = anal intraepithelial neoplasia grade 3; CIN3 = cervical intraepithelial neoplasia grade 3; VAIN3 = vaginal intraepithelial neoplasia grade 3; VIN3 = vulvar intraepithelial neoplasia grade 3.

compared with women ≤29 years of age; however, the risk was higher compared with older age groups, with IRRs of 8.09 (95% CI 4.81-13.58) for any HPV-related carcinoma, 16.68 (95% CI 11.14-24.96) for any HPV-related premalignancy, and 13.62 (95% CI 9.76-19.01) for any HPV-related carcinoma or premalignancy (Supplemental material Table S1).

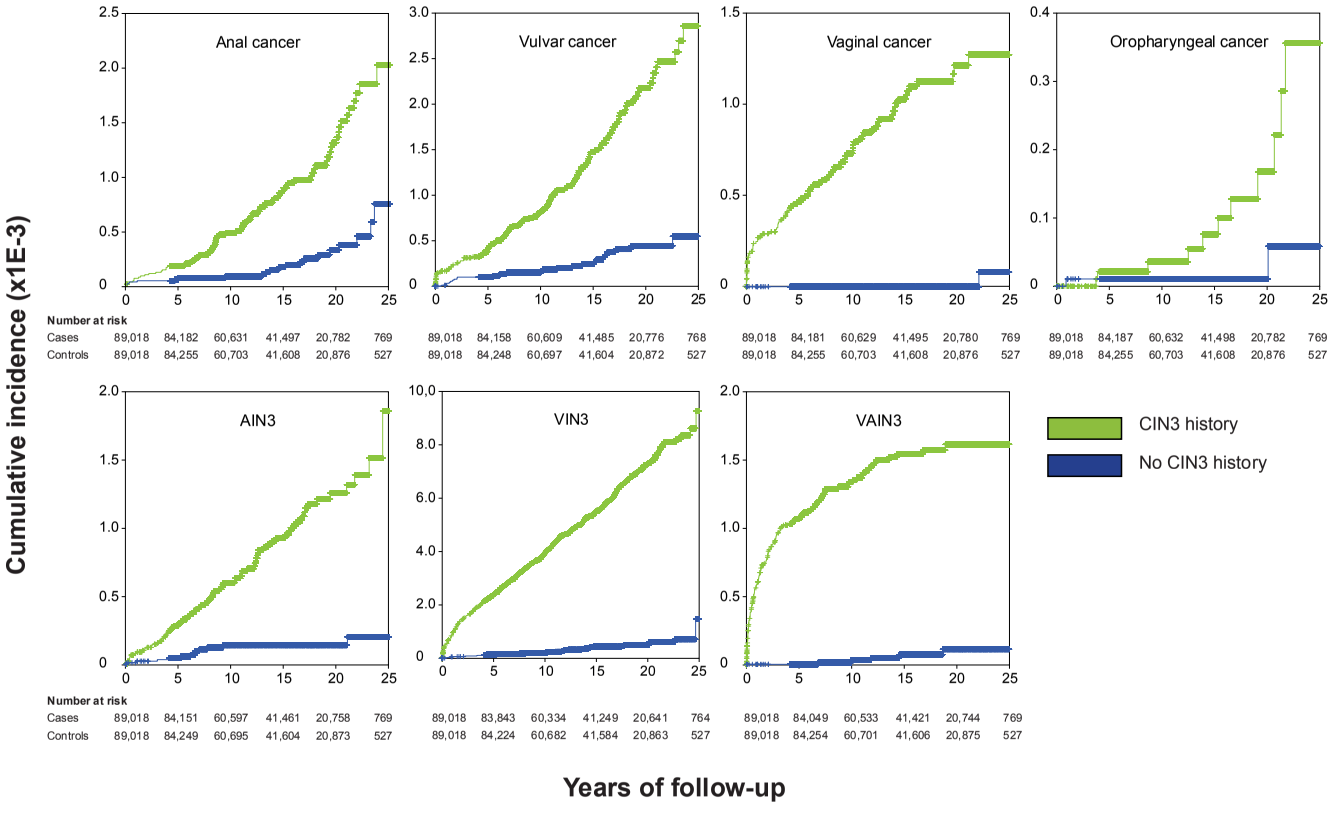

Figure 2. Kaplan-Meier curves estimating the cumulative incidence of HPV-related carcinomas and premalignancies after diagnosis of CIN3 or benign dermal nevus, comparing women with a previous diagnosis of CIN3 with women without CIN3.

Note that the y-axis scale is not the same in all graphs. AIN3 = anal intraepithelial neoplasia grade 3; CIN3 = cervical intraepithelial neoplasia grade 3; VAIN3 = vaginal intraepithelial neoplasia grade 3; VIN3 = vulvar intraepithelial neoplasia grade 3.

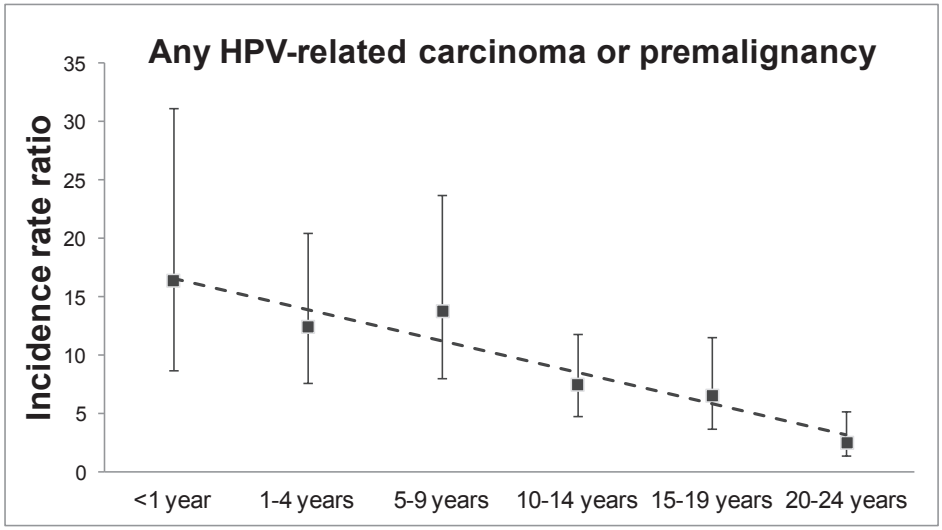

Figure 3. Incidence rate ratio of any HPV-related carcinoma or premalignancy comparing women with a previous diagnosis of CIN3 with women without CIN3, versus time.
Any HPV-related carcinoma or premalignancy includes anal cancer, anal intraepithelial neoplasia grade 3, vulvar cancer, vulvar intraepithelial neoplasia grade 3, vaginal cancer, vaginal intraepithelial neoplasia grade 3, and oropharyngeal cancer. Squares represent the incidence rate ratio at certain follow-up points. Error bars indicate 95% confidence intervals of estimated incidence rates. The dotted line is based on simple linear regression for visualization purposes. Data are in Table 3.

DISCUSSION

This population-based cohort study, which included 178,036 women, showed an increased risk of premalignancies as well as carcinomas of anus, vulva, vagina, and oropharynx in women with a previous diagnosis of CIN3 compared with women without a previous diagnosis of CIN3. The increased risk was still present up to 20 years after the CIN3 diagnosis.

Our results are consistent with previously published studies on HPV-related carcinomas in women with a CIN diagnosis, with risks ratios ranging from 2.2 to 4.4 for vulvar cancer, 6.7 to 18.5 for vaginal cancer, 1.8 to 5.9 for anal cancer, and 1.2 for oral/pharyngeal tumors.[4,5,7–9] No studies have analyzed the risk of HPV-related high-grade premalignancies. In general, high-grade premalignancies are treated before cancer can occur. Our data on IRs of high-grade premalignancies showed high IRRs, indicating that in the group of women with CIN3, carcinomas may have been prevented by early detection and treatment of high-grade premalignancies. Our data overall show higher IRRs compared with published studies. This might be caused by differences in our study design compared with published studies. Some studies used expected IRs on the basis of national averaged IRs, which may also include carcinomas in women with a CIN3 in

Table 3. Observed numbers of carcinomas and premalignancies, person-years of follow-up, and estimated age-adjusted incidence rate ratios (IRR) of carcinomas and premalignancies of anus, vulva vagina and oropharynx of women with a history of CIN3, compared with women without a history of CIN3, stratified by time since first diagnosis of CIN3 or benign dermal nevus.

	<1 year		1-4 years		5-9 years	
	Person-years (ON)	IRR (95% CI)	Person-years (ON)	IRR (95% CI)	Person-years (ON)	IRR (95% CI)
Anal cancer						
CIN3 history	88,938 (4)	1.33	353,476 (10)	5.01	357,688 (21)	21.14
No CIN3 history	99,988 (3)	(0.30-5.96)	353,797 (2)	(1.10-22.85)	359,894 (1)	(2.84-157.12)
AIN3						
CIN3 history	88,932 (7)	3.50	353,413 (19)	9.52	357,546 (22)	3.17
No CIN3 history	88,986 (2)	(0.73-16.86)	353,787 (2)	(2.22-40.86)	359,863 (7)	(1.35-7.41)
Vulvar cancer						
CIN3 history	88,940 (15)	14.94	353,488 (19)	2.37	357,698 (32)	8.04
No CIN3 history	88,988 (1)	(1.98-112.98)	353,797 (8)	(1.04-5.41)	359,894 (4)	(2.84-22.74)
VIN3						
CIN3 history	88,859 (81)	16.22	352,773 (128)	16.05	356,618 (112)	28.26
No CIN3 history	88,983 (5)	(6.58-40.03)	353,765 (8)	(7.86-32.78)	359,832 (4)	(10.42-76.61)
Vaginal cancer						
CIN3 history	88,938 (23)	NA	353,476 (20)	NA	357,688 (20)	NA
No CIN3 history	88,988 (0)		353,797 (0)		359,894 (0)	
VAIN3						
CIN3 history	88,885 (52)	NA	353,103 (43)	NA	357,268 (20)	20.31
No CIN3 history	88,988 (0)		353,797 (0)		359,891 (1)	(2.72-151.51)
Oropharyngeal cancer						
CIN3 history	88,938 (0)	NA	353,476 (2)	NA	357,688 (1)	NA
No CIN3 history	88,988 (1)		353,797 (0)		359,894 (0)	
Any HPV-related carcinoma*						
CIN3 history	88,940 (42)	8.38	353,488 (51)	5.08	357,698 (74)	14.87
No CIN3 history	88,988 (5)	(3.32-21.19)	353,797 (10)	(2.58-10.01)	359,894 (5)	(6.01-36.77)
Any HPV-related premalignancy**						
CIN3 history	88,805 (133)	19.05	352,388 (175)	17.60	356,146 (140)	12.89
No CIN3 history	88,981 (7)	(8.91-40.73)	353,755 (10)	(9.30-33.28)	359,801 (11)	(6.98-23.81)
Any HPV-related carcinoma or premalignancy†						
CIN3 history	88,805 (164)	16.44	352,388 (211)	12.49	356,146 (191)	13.83
No CIN3 history	88,981 (10)	(8.68-31.13)	353,755 (17)	(7.62-20.47)	359,801 (14)	(8.04-23.79)

* any HPV-related carcinoma includes anal cancer, vulvar cancer, vaginal cancer and oropharyngeal cancer. ** any HPV-related premalignancy includes AIN3, VIN3 and VAIN3. After the first premalignancy women were censored. † any HPV-related carcinoma or premalignancy includes anal cancer, AIN3, vulvar cancer, VIN3, vaginal cancer, VAIN3 and oropharyngeal cancer. After the first carcinoma or premalignancy women were censored. AIN3 = anal intraepithelial neoplasia grade 3;

10-14 years		15-19 years		20-24 years	
Person-years (ON)	IRR (95% CI)	Person-years (ON)	IRR (95% CI)	Person-years (ON)	IRR (95% CI)
253,774 (20)	5.00	153,874 (11)	2.21	54,053 (7)	1.69
253,496 (4)	(1.71-14.62)	154,776 (5)	(0.77-6.37)	52,048 (4)	(0.49-5.76)
253,615 (17)	NA	153,721 (11)	NA	53,992 (4)	3.86
253,471 (0)		154,766 (0)		52,041 (1)	(0.43-34.53)
253,794 (33)	6.54	153,904 (22)	3.09	54,063 (8)	7.56
253,496 (5)	(2.55-16.76)	154,776 (7)	(1.32-7.24)	52,048 (1)	(0.95-60.47)
252,701 (82)	6.85	153,001 (55)	27.81	53,725 (18)	4.36
253,432 (12)	(3.74-12.56)	154,718 (2)	(6.78-114.02)	52,029 (4)	(1.48-12.88)
253,774 (16)	NA	153,874 (6)	NA	54,053 (1)	0.98
253,496 (0)		154,776 (0)		52,048 (1)	(0.06-15.69)
253,460 (11)	3.70	153,672 (2)	2.04	53,999 (0)	NA
253,488 (3)	(1.03-13.25)	154,771 (1)	(0.19-22.47)	52,047 (0)	
253,774 (2)	NA	153,874 (3)	NA	54,053 (3)	2.89
253,496 (0)		154,776 (0)		52,048 (1)	(0.30-27.76)
253,794 (71)	7.81	153,904 (42)	3.43	54,063 (19)	2.56
253,496 (9)	(3.90-15.62)	154,776 (12)	(1.80-6.51)	52,048 (7)	(1.07-6.08)
252,308 (102)	7.34	152,722 (63)	21.31	53,648 (21)	4.08
253,407 (14)	(4.20-12.83)	154,705 (3)	(6.69-67.85)	52,022 (5)	(1.54-10.82)
252,308 (157)	7.54	152,722 (91)	6.60	53,648 (33)	2.57
253,407 (21)	(4.78-11.88)	154,705 (14)	(3.76-11.59)	52,022 (12)	(1.38-5.18)

CI = confidence interval; CIN3 = cervical intraepithelial neoplasia grade 3; IRR = incidence rate ratio; NA = not available; ON = observed number; VAIN3 = vaginal intraepithelial neoplasia grade 3; VIN3 = vulvar intraepithelial neoplasia grade 3.

the control group, whereas others used relative risks. Furthermore, the studies by Kalliala et al.[4] and Gaudet et al.[8] included women with CIN1 and/or CIN2, of which CIN1 is known to show a lower prevalence of hrHPV, compared with CIN2-3.[13]

Our results showed the highest IRRs for vaginal cancer and VAIN3 and the lowest IRR for anal cancer, which was still 3.85 (95% CI of 2.32-6.37). Vaginal abnormalities are in proximity to cervical abnormalities; this makes a vaginal co-infection, with development of a vaginal carcinoma or premalignancy, more likely in women with a CIN3. This risk is much lower in women with no history of CIN3, which was shown by a low IR in the control group, resulting in a high IRR in vaginal cancer and VAIN after a CIN3. Also, in vulvar carcinomas and premalignancies, a loco-regional effect of HPV may explain an increased incidence of these lesions in women diagnosed with CIN3. Women diagnosed with a CIN3 are commonly followed up with cervical smears or colposcopic examination. This may also result in increased detection of premalignancies of the vagina and vulva, with a higher IR in women with a CIN3 compared with women without a CIN3; this could result in an overestimation of IRR, especially in these two premalignancies. Also, not all anal, vulvar, vaginal, and oropharyngeal carcinomas and premalignancies are related to HPV; therefore, not all malignancies and premalignancies detected during follow-up in this study are related to HPV. We attempted to exclude carcinomas and premalignancies that were definitely not related to HPV. However, in the majority of cases, HPV tests were not performed on the histology samples; thus, some carcinomas and premalignancies not caused by HPV still would have been included. However, these carcinomas and premalignancies have been included in both the CIN3 group and in the control group of women without CIN3, with an expected similar number of cases; therefore, in estimated IRRs, these numbers cancel each other out. The overall increased IRR might also be explained by a woman's inability or limited ability to clear an hrHPV infection. A limited ability or inability to clear hrHPV infections did result in a persistent hrHPV infection with development of a CIN3 lesion, but could have also resulted in other HPV-related carcinomas and premalignancies. The long-lasting increased risk might also be explained by increased susceptibility to new hrHPV infections and recrudescence of disease because of an inability to clear the infection. It is known that smoking and immunodeficiency increases the risk of persistent hrHPV infections with an increased risk of (pre)malignant development.[14-16] Data on smoking or immune status were not registered in the PALGA database, and could therefore not be included in our analysis. Previously published results, however, show no increased risk explained by smoking.[9]

When looking at IRR over time, almost all IRRs showed a steady decline of increased risk during the long-term follow-up, with the lowest increased risk after 20 to 24 years. The Kaplan-Meier figures of all HPV-related carcinomas and premalignancies also confirmed this long-term increased risk. The increased risk of the cluster of any HPV-related carcinoma or premalignancy over time showed a decrease over time with an IRR of 16.44

(95% CI 8.68-31.13) in the first year, and an IRR of 2.57 (95% CI 1.38-5.18) after 20 years. This indicates that although the risk of HPV-related carcinomas and premalignancies decreased over time, it was still significantly increased up to 20 years after the CIN3 diagnosis. A previous study by Gaudet et al.[8] also showed dropping incidences of malignancies in time, but to the contrary, Kalliala et al.[4] showed an increased standardized IR for malignancies over time after CIN3 treatment. For clusters of all carcinomas and/or all premalignancies, we also observed decreasing IRRs with advancing age. This data should be interpreted with some caution because the effect of a low incidence of malignancies in younger age groups might also be a confounding factor.

Ideally, we want to prevent the development of HPV-related carcinomas or high-grade premalignancies, or at least discover premalignancies before they progress to cancer. Higher alertness for HPV-related lesions in women with CIN3 might therefore be advisable. During colposcopy, it is relatively easy to look for vaginal carcinoma and premalignancies. Current guidelines advise to also examine the vaginal walls during colposcopic examination, and when an abnormal smear is not explained by cervical abnormalities, a vaginoscopy can be considered. When VAIN is diagnosed, it can be treated with surgical excision, laser evaporation or imiquimod; however, recurrent disease is a known problem.[17] Also, screening for vulvar and anal carcinoma and premalignancies might be easily combined with cervical diagnostics. Visual inspection will be easy to implement, but presently data are lacking that screening of these areas results in a lower cancer incidence. Indeed, there are no current screening programs for VIN, AIN, or oropharyngeal lesions, but if premalignancies are encountered, they are treated.[18,19]

Because an infection with hrHPV is causal to the development of other HPV-related carcinomas and high-grade premalignancies, prophylactic HPV vaccination may have a preventive effect on development of these lesions. However, the role of prophylactic vaccination in adult women is debated. Previous vaccination trials have shown that noninfected adults could potentially benefit from HPV vaccination.[20,21] Garland et al.[22] showed that women undergoing surgical therapy for cervical lesions after vaccination with the bivalent vaccine may benefit from vaccination, with a reduced risk of developing subsequent ≥CIN2 positivity. Studies in women with a current infection with hrHPV showed varying results. Two recent studies showed a reduced incidence of subsequent HPV-related disease in women vaccinated with the quadrivalent HPV vaccine after surgical treatment for CIN3 when compared with surgically treated women who did not receive the HPV vaccination.[23,24] On the contrary, a recent study by Hildesheim et al.[25] showed no evidence for a vaccine effect on detectable HPV infections in hrHPV-positive women and women undergoing treatment for cervica premalignancy. Also, the small potential harms of vaccination should be taken into account, alongside the fact that HPV infections leading to premalignancies or malignancies may have been acquired before the HPV vaccination. It is therefore not fully clarified how clinically and

cost-effective prophylactic hrHPV vaccination would be in women treated for CIN3. Randomized controlled trials and cost-effectiveness studies might answer these questions. Our study, however, showed that short- and long-term risks of HPV-related abnormalities are strongly increased in women diagnosed with CIN3. Patients should be informed on an individual basis about this increased risk. The Dutch guideline presently advises to consider hrHPV vaccination in women diagnosed with CIN3, until more conclusive evidence on vaccine effect after treatment for CIN3 is available.[17]

The introduction of prophylactic HPV vaccination is expected to reduce the incidence of HPV-related cancers; postvaccination surveillance studies have supported this by showing a significant reduction of 68% in type 16 and 18 infections in countries with female vaccination coverage of ≥50%.[26-29] However, monitoring remains essential to identify possible waning of efficacy, or replacement of HPV-type. Because vaccination programs for young women only started in 2007, it will take many more years before the effects of HPV vaccination will become visible in the total adult female population.

In conclusion, this population-based data set shows further evidence of the increased risk of HPV-related carcinomas and premalignancies of the anogenital and oropharyngeal regions after a diagnosis of CIN3, and gives a clear view of women at risk for HPV related disease. Studies that investigate methods to prevent this increased risk in this group of patients, such as intensified screening or vaccination, are warranted.

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SUPPLEMENTAL MATERIAL

Table S1. Observed numbers of carcinomas and premalignancies, person-years of follow-up and estimated incidence rate ratios (IRR) of carcinomas and premalignancies of anus, vulva vagina and oropharynx for women with a history of CIN3, compared with women without a history of CIN3, stratified by attained age.

	≤29 years		30-49 years		50-69 years		≥70 years	
	Person-years (ON)	IRR (95% CI)	Person-years (ON)	IRR (95% CI)	Person-years (ON)	IRR (95% CI)	Person-years (ON)	IRR (95% CI)
Any HPV-related carcinoma*								
CIN3 history	34,739 (4)	NA	836,486 (133)	8.09	355,154 (123)	4.75	38,796 (39)	8.03
No CIN3 history	42,885 (0)		813,687 (16)	(4.81-13.58)	370,017 (27)	(3.13-7.20)	39,931 (5)	(3.16-20.37)
Any HPV-related premalignancy**								
CIN3 history	34,616 (29)	35.93	833,203 (427)	16.68	353,158 (150)	7.48	38,329 (28)	9.72
No CIN3 history	42,884 (1)	(4.89-263.72)	813,557 (25)	(11.14-24.96)	369,847 (21)	(4.74-11.81)	39,905 (3)	(2.95-31.96)
Any HPV-related carcinoma or premalignancy†								
CIN3 history	34,616 (32)	39.64	833,203 (516)	13.62	353,158 (245)	5.70	38,329 (54)	11.24
No CIN3 history	42,884 (1)	(5.42-290.09)	813,557 (37)	(9.76-19.01)	369,847 (45)	(4.15-7.84)	39,905 (5)	(4.50-28.11)

* any HPV-related malignancy includes anal cancer, vulvar cancer, vaginal cancer and oropharyngeal cancer. ** any HPV-related premalignancy includes AIN3, VIN3 and VAIN3. After the first premalignancy women were censored. † Any HPV-related malignancy or premalignancy includes anal cancer, AIN3, vulvar cancer, VIN3, vulvar cancer, VAIN3 and oropharyngeal cancer. After the first malignancy or premalignancy women were censored. AIN3 = anal intraepithelial neoplasia grade 3; CI = confidence interval; CIN3 = cervical intraepithelial neoplasia grade 3; IRR = incidence rate ratio; ON = observed number; VAIN3 = vaginal intraepithelial neoplasia grade 3; VIN3 = vulvar intraepithelial neoplasia grade 3.

CHAPTER 10

General discussion and future perspective

GENERAL DISCUSSION

Multiple aspects of improving cervical cancer prevention have been explored in this thesis, providing a multidimensional approach towards improving cervical cancer prevention programs. The Netherlands is among the first countries that recently implemented hrHPV-based population screening, and it can be expected that hrHPV-based screening will rapidly become the cornerstone of cervical cancer screening in many countries. With this major shift in cervical cancer screening, we should look closely at the steps following a high-risk human papillomavirus (hrHPV) positive test result. Improvement of these steps may eventually lead to a more accurate, women-friendly, and personalized cervical cancer prevention program. In this general discussion, conclusions from the previously described studies are put into perspective from different point-of-views, and clinical implications are explored alongside a future prospect on cervical cancer prevention.

AS I AM A WOMAN

As a woman, most importantly I do not want to get cervical cancer. Unfortunately, at this point we cannot totally prevent women worldwide from getting cervical cancer. Therefore, an optimized cervical cancer prevention program should be obtained with current knowledge, to make sure cervical cancer incidences and mortality will be lowered as much as possible. For me as a woman, this would be a cervical cancer screening program in which a negative screening and triage test is maximally conclusive in having no risk of cervical cancer, at least until the next screening round. On the other hand, I also do not want a high chance of an unnecessary referral advice, which could result in unwanted anxiety or overtreatment. I definitely would not want to get cervical cancer at a young age. If screening of women below the age of 30 would be needed to prevent getting cancer at a young age, I might consider participating in such a screening program, even at the cost of unnecessary diagnostics. Future research should be focused on optimizing cervical cancer prevention programs. As hrHPV-based cervical cancer screening can be performed on self-sampled specimens, it may be expected that self-sampling will play an increasing role in hrHPV-based cervical cancer screening programs.[1] Cervicovaginal self-sampling has shown to be well tolerated by women and it increases participation of non-responders to a screening program, however, this is only shown in a study setting.[2,3] I would want to have the opportunity of using a cervicovaginal self-sampling device as a safe cervical screening method.

If a colposcopic evaluation is required, I would want to be treated based on my specific situation, thus on an individualized basis. I expect to be informed about all possible

options and would want to have a say in the choice of additional diagnostics, treatment, or a watchful waiting policy, based on a personalized evidence based risk assessment.

If my risk on hrHPV related disease is increased, I expect to be informed of this specific risk and would want to be informed about any possible preventive measurements for developing a carcinoma. As described in this thesis, some women show an increased risk for hrHPV-related disease and may therefore require different screening or treatment. First, unvaccinated women with a persistent hrHPV infection at young age do show a significantly increased risk of developing high-grade cervical intraepithelial neoplasia (CIN) lesions during the first years of follow-up, compared with hrHPV negative women and women who cleared their hrHPV infection. This might indicate a possible increased susceptibility for an hrHPV infection, due to a shortcoming immune system, so new infections or recrudescence after viral latency are more common. Secondly, women diagnosed with a high-grade CIN lesion also show an increased risk for other HPV induced carcinomas of the head-and-neck and anogenital region. This risk has been confirmed with Dutch population-based data showing an increased risk even after 20 years of follow-up. Additionally, a strongly increased long-term risk for hrHPV-related premalignancies is shown. Knowledge on this risk on premalignancies is important to prevent development of hrHPV-related premalignancies and prevent progression of hrHPV-related premalignancies into carcinomas, by considering for example prophylactic HPV vaccination after treatment for high-grade CIN lesions. For now, only young women are being vaccinated, and older women still remain at risk for hrHPV-related premalignancies and carcinomas. The role of prophylactic vaccination in adult women is however debated. Some studies have shown a reduced incidence of subsequent HPV-related disease in women receiving prophylactic hrHPV vaccination after treatment for CIN3; however, this has not been confirmed by all studies.[4-6] As a woman, I would want to be informed about evidence on prophylactic HPV vaccination and increased attention on other HPV-related lesions, and discuss the about pros and cons for these two preventive strategies with my physician. I would strongly consider these options when my personal risk of HPV-related lesions would be increased.

IF I WERE A GYNECOLOGIST

As a gynecologist, I want to inform my patients on the risks of hrHPV infections and the chances of clearance and developing CIN lesions or other hrHPV-related disease, as well as offer them the best treatment options. I also need to be up-to-date on the pros and cons of hrHPV self-sampling offered in this new cervical cancer screening program in the Netherlands, as I expect questions from my patients on this subject. For now, self-sampling will only be available for women in the cervical cancer screening program.

I think self-sampling should also become available for women in a follow-up setting in whom taking a cervical smear for cytology is difficult or not possible because of anxiety or other reasons.

With the limited specificity of the hrHPV test, and Pap cytology triage in case of an hrHPV-positive result, referral rates for colposcopic examination will rise. Especially in the first few years with an estimated increase in colposcopy referrals of 131%, with the highest expected increase in low-grade CIN lesions.[7] I think it is therefore essential to improve colposcopic examination as this is the next step after a positive triage result.[8] Individualized treatment should be applied in the colposcopy clinic; first by considering a watchful waiting policy to prevent overtreatment in hrHPV-positive women with low-grade cytology. Especially in young and fertile women I would advise to be cautious with excisional treatment; and second I would propose to consider see-and-treat management in women with a high-grade cervical smear result and high-grade colposcopic impression as this thesis has shown this is safe and acceptable in this group.

As a gynecologist, I would expect to see progress in the development of digital colposcopy techniques and would like to stay up-to-date on this subject during meetings or conferences on colposcopy, to see if these techniques might be further improved to be valuable for my colposcopy clinic. A major advantage of these techniques is the objective assessment of the cervix. However, presently their added value is too low for introduction within the colposcopy program, because some techniques show limited sensitivity and others show low specificity. The sensitivity and specificity of these strategies have to be high in order to be useful as triage method or in follow-up diagnostics.

Furthermore, as a gynecologist my knowledge on alternatives to surgical treatment in case of a CIN lesion should be up-to-date. New emerging therapies like Imiquimod treatment, or therapeutic vaccination are being investigated. Imiquimod is a Toll-like 7 receptor agonist, that works as a topical immune response modulator, effecting upregulation of interferon and activation of dendritic cells. Imiquimod application has significantly increased histological regression of CIN lesions and clearance of HPV.[9] Also, early studies on therapeutic vaccination for treating CIN lesions show promising results. Additionally, some studies have shown some effectiveness of prophylactic vaccines in preventing CIN recurrence. These two vaccine strategies should be further explored to see if prophylactic vaccines have the ability to prevent new CIN lesions from developing, and if therapeutic vaccines have enough potential for the treatment of cervical lesions.[5,10]

IF I WERE A POLICY MAKER

I think the new hrHPV-based cervical cancer screening program contributes to better healthcare. This new program is expected to be 13-15% more effective in preventing

cervical cancer and cervical cancer deaths. The hrHPV-based program is more cost-effective with a decrease of 35% for screening costs, and a 20% decrease in costs combining diagnostics and treatment. In quality-adjusted life years (QALY) the new program shows a good cost-effectiveness of €3,497.- per QALY (39% less), and it is therefore expected to improve cervical cancer screening effectiveness and coverage combined with lowered costs compared with the Pap cytology-based screening program.[7] I strongly believe women should be in control of their own welfare and think the offering of self-sampling is a major contribution to the program. As a policy maker, I would focus on closely monitoring this new cervical screening program, to see how these adjustments eventually affect participation, and detection of cervical cancer and high-grade CIN lesions. This information is valuable for further improvement of our own program as well as for other countries considering implementing hrHPV-based cervical cancer screening and include hrHPV self-sampling in their screening program.

I would work on creating funds to stimulate collaboration between policy makers, researchers and doctors to improve an aligned screening and treatment strategy, resulting in a more women-friendly cervical cancer prevention program. As I expect self-sampling to become an important screening tool, I would promote collaboration of policy makers and researchers on improving triage directly on self-samples to determine the risk for hrHPV-positive women and to lower unnecessary colposcopies. Also by informing general practitioners and gynecologists on the content of the new screening program and the increased colposcopy referral rates, medicalization and unnecessary treatment should be prevented.

On the longer term, I would investigate adjustments to the screening program that should be made once vaccinated girls will enter the program. In the Netherlands, girls are vaccinated since 2009 with the bivalent prophylactic HPV vaccine, which protects against HPV16 and HPV18. Vaccination coverage in the Netherlands has climbed from 52% in 2009 to almost 61% in 2016, and the first vaccinated women will enter the organized screening program at the age of 30, which will be the case in 2023. This group of young women is vaccinated with the bivalent vaccine and it is yet unknown to which extent these women will be protected for cervical cancer caused by types other than HPV16 and HPV18 by cross protection. Also, the extent of type-replacement is still not fully known. Screening will therefore also remain necessary for these vaccinated women, alongside screening for unvaccinated women. The future screening population will therefore consist of women with different risk profiles on hrHPV infections and development of HPV-related lesions. It will then be important to work towards individualized screening based on risk prediction by combining vaccination status, previous hrHPV results and other factors as smoking or immune status of each woman, and base an individualized screening program on this knowledge. This will increase individualized patient-centered care as women with a low risk might need less or no screening, compared with women

with a higher risk. When the vaccination program will possibly switch to another vaccine with broader HPV coverage, screening programs should also be adjusted accordingly, or might eventually even become unnecessary for these women.

IF I WERE A POST-DOCTORAL RESEARCHER

If I were a post-doctoral researcher one of my major research topics would be the discovery and use of new molecular markers for the triage of hrHPV-positive women. In the coming cervical cancer screening program, Pap cytology will be used as triage method for hrHPV-positive women in the Netherlands. This technique has a limited sensitivity, excludes the use of self-sampled material, and requires 6-month follow-up of hrHPV-positive women with normal cytology to limit the risk of a missed lesion.[11] Studies have shown that triage could be improved on short notice by combining Pap cytology with HPV16/18 genotyping, or replacing it with p16/Ki-67 dual staining, as shown in this thesis.[12,13] This improves the specificity of Pap cytology triage of hrHPV-positive women, and thus limits unnecessary colposcopy referral rates. These strategies are however still morphology-dependent. Molecular biomarkers on the other hand are objective, highly reproducible, do not need high cellularity and some have already been proven to be applicable on self-samples.[12,14-19] Methylation levels are known to increase with a longer duration of a HPV infection, and with the severity of cervical lesions. Unfortunately, at this point in time, none of the studied triage strategies can differentiate all women with a high risk from women with a low risk for high-grade CIN. Molecular triage of hrHPV-positive women might also differentiate productive from transforming infections and some studies have already shown to be able to predict development of high-grade lesions.[20] Thus, a molecular triage test could predict which hrHPV infections have caused or will eventually cause a high-grade CIN or cervical cancer. In this way, unnecessary treatment during colposcopic examination and continuous follow-up could possibly be avoided. If I were a post-doctoral researcher, I would work on the discovery of new molecular markers to further personalize screening. The ultimate goal for me would be a technique that gives a highly-individualized result with predictive potential, and a screening or triage technique based on integration of HPV may show these abilities.

One of the major other but more basic research areas as a post-doctoral researcher would therefore be to study the integration of HPV into the human genome. There are three main research questions in this aspect. The first question to answer is; why does integration take place? Although many women acquire an hrHPV infection at a certain point in their life, in only a minority the HPV DNA integrates into the human host. Integration is considered as a first step in the oncogenic pathway leading to cervical cancer. It is not exactly known why HPV integrates into the human DNA in some infections,

while it does not in others. A second and third research question in line with the former are; when does integration take place, and how does integration take place? It is well-known that the majority, if not all, cervical carcinomas contain integrated hrHPV DNA, the exact time of integration is unknown. Integration takes place more-or-less randomly in the human host DNA but has always a relation with a disruption of the viral E2 gene. Is there a structural DNA component or sequence involved as a hot-spot for integration? What specifically differentiates hrHPV types from low-risk HPV types in this context? This knowledge could possibly be used to prevent HPV from integrating, or to better detect integrated HPV which can be used to develop a more sensitive and more specific screening or triage method based on molecular characteristics.

FUTURE PERSPECTIVE

Cervical cancer prevention programs are on the verge of major adjustments after the introduction of primary hrHPV-based cervical cancer screening. Alternative opportunities for triage of hrHPV-positive women are widely available and current research increases knowledge on these different techniques. The increased numbers of referrals for colposcopic examination, with many women with low-grade cytology with a relatively low risk of high-grade CIN lesions, will require adjustments from gynecologists to their current colposcopy clinic, to be able to give the best care available for the future. Vaccinated girls will enter the screening program and extending knowledge on increased risk of HPV-related disease in certain groups will eventually lead to individualized screening based on personalized risk estimates, combined with individualized treatment, to finally lead to a more accurate, women-friendly, and personalized cervical cancer prevention program.

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CHAPTER 11

Summary & Samenvatting

SUMMARY

Studies on multiple dimensions of improving cervical cancer prevention are described in this thesis. Research focusses on three important aspects of cervical cancer prevention, and may lead to an improved more women-friendly cervical cancer prevention program. **Chapter 1** introduces the background and different aspects of cervical cancer prevention, followed by research aims and the outline of this thesis.

Three chapters in **part I** of this thesis describe studies on triage of high-risk human papillomavirus (hrHPV) positive women. HrHPV testing is expected to replace Pap cytology as primary screening method for cervical cancer screening in an increasing number of countries. The high sensitivity, combined with a low specificity makes triage of hrHPV-positive women necessary to limit unnecessary referrals for colposcopic examination. In **chapter 2** we present an overview of the state-of-the-art on triaging hrHPV-positive women. This chapter differentiates between morphological biomarkers, molecular biomarkers and combined triage strategies. We analyze worldwide literature on clinical value of a large variety of biomarkers, and compare available triage strategies to a yet non-existing ideal strategy. This ideal strategy consists of biomarkers that can be applied on the primary screening sample, and which would result in a highly sensitive and specific test that differentiates women with cervical cancer or high-grade cervical intraepithelial neoplasia (CIN) lesions from women with a low risk for these lesions who can safely return to the next screening round. This overview of worldwide literature shows that experience with morphology-based Pap cytology makes this a valuable triage method. However, molecular biomarkers show more promising results in recent research. These markers are more objective, are highly reproducible, and can be combined to improve qualities of different single biomarkers. It is expected that cervical cancer screening will transform to a full molecular screening program in the future.

Chapter 3 describes a study on the clinical value of HPV16/18 genotyping combined with Pap cytology as triage method for women with hrHPV-positive self-samples. Physician-taken samples from 520 women who tested hrHPV-positive on a self-sample were evaluated for Pap cytology with previous knowledge of the hrHPV-positive status, and HPV16/18 genotyping with the Cobas 4800 test (Roche). Eighteen baseline triage strategies were evaluated for sensitivity, specificity, predictive values and referral rates. Three strategies combining HPV16/18 genotyping and Pap cytology were considered improved, in regard to the comparator strategy of Pap cytology at an atypical cells of undetermined significance (ASC-US) threshold which yielded a sensitivity of 93.5% (95% CI 87.7-97.1) and specificity of 58.5% (95% CI 53.7-63.2). These three strategies are; the presence of HPV16 and/or a Pap cytology result of low-grade squamous intraepithelial lesion or worse (≥LSIL), the strategy with presence of HPV16 and/or HPV18, and/or a Pap cytology result of ≥HSIL, and the strategy with presence of HPV16 and/or a Pap cytology

result of high-grade squamous intraepithelial lesion or worse (≥HSIL). This final strategy yielded the highest specificity of 74.9% (95% CI 70.5-78.9) with a sensitivity of 94.4% (95% CI 89.0-97.7) similar to the comparator strategy, and a decrease in referral rate from 52.2% to 39.5%. These results indicate that in case of prior knowledge of hrHPV presence, triage by Pap cytology may be improved by adjusting its threshold, and combining it with HPV16/18 genotyping.

In **chapter 4** we evaluated the clinical utility of p16/Ki-67 dual staining for the identification of CIN in hrHPV-positive women from a non-responder screening cohort. P16/Ki-67 dual staining, Pap cytology and HPV16/18 genotyping were performed on physician-taken liquid-based samples from 495 women who tested hrHPV-positive on self-sampled material. Different triage strategies involving p16/Ki-67 dual staining were evaluated for sensitivity, specificity, and predictive value for ≥CIN2 and ≥CIN3, and compared with Pap cytology with an ASC-US threshold. Centrally revised histology or an adjusted endpoint with combined hrHPV negative and cytology negative follow-up at six months was used as gold standard. Triage of hrHPV-positive samples with Pap cytology with an ASC-US threshold showed a sensitivity of 93% (95% CI 85-98) with a specificity of 49% (95% CI 41-56) for ≥CIN3. Three triage strategies with p16/Ki-67 showed a significantly increased specificity with similar sensitivity for ≥CIN3: P16/Ki-67 triage of all hrHPV-positive samples had a specificity of 61% (95% CI 54-69). Applying p16/Ki-67 triage to only hrHPV-positive women with low-grade Pap cytology showed a specificity of 64% (95% CI 56-71). For hrHPV-positive women with low-grade and normal Pap cytology, triage with p16/Ki-67 showed a specificity of 58% (95% CI 50-65). Differences in sensitivity and specificity are limited between the three selected strategies. Because the quality of Pap cytology worldwide varies this study concludes that 16/Ki-67 triage of all hrHPV-positive samples would be the most reliable strategy in triage of hrHPV-positive women with an increased specificity and similar sensitivity compared with Pap cytology triage.

Part II of this thesis contains three studies on improving colposcopic examination following a positive screening and/or triage result. First, **chapter 5** describes a systematic review of literature and a meta-analysis of current data on see-and-treat management of CIN. Three major literature databases were searched for studies on see-and-treat management in women with a reported cervical smear result, colposcopic impression and final histology result, to determine overtreatment rates in see-and-treat management of women referred for colposcopic examination because of suspected CIN. Thirteen studies, with a total of 4,611 women were included. By an inverse variance method, incidences were pooled and a random-effects model was used to account for heterogeneity between studies. The overall overtreatment rate in women with a low-grade smear and low-grade colposcopic impression was highest with 72.9% (95% CI 68.1-77.7). In case of a high-grade cervical smear an a high-grade colposcopic impression the overtreatment

rate was lowest with 11.6% (95% CI 7.8-15.3). This was at least comparable with the overtreatment rate of a two-step procedure which varied between 11-35% in literature, therefore justifying see-and-treat management in this group of women. In case of a discrepancy between the cervical smear an colposcopic evaluation, age, wish to have children in the future, and risk of loss-to-follow-up should be taken into account when considering see-and-treat management.

The second chapter in this part is **chapter 6**. This chapter focusses on an alternative colposcopy technique, multimodal hyperspectroscopy (MHS), that combines fluorescence and reflectance spectroscopy. The objective of this prospective single-center cohort study was to evaluate the clinical value of MHS for detecting high-grade cervical intraepithelial neoplasia in a colposcopy referral population and colposcopy follow-up population, to assess whether MHS could be safely used to improve care for women at risk for high-grade CIN. A total of 125 women from a colposcopy referral population and colposcopy follow-up population were evaluated with MHS and tested for the presence of hrHPV with HPV16/18 genotyping. Spectroscopic measurements of the cervix were taken and compared with an endpoint based on histology, hrHPV and cytology. Evaluable data for analysis were collected for 102 of the subjects. Sensitivity, specificity and predictive values were calculated for MHS and colposcopic impression based on conventional colposcopic examination. MHS yielded a sensitivity of 93.6% (95% CI 78.6-99.2), with a corresponding specificity of 42.3% (95% CI 30.6-54.6) in the group with a composite endpoint. No adverse effects occurred and patient acceptability was high. With these results, we can conclude that MHS is a digital colposcopy technique that offers an easy, rapid, well tolerated point-of-care assessment with a high sensitivity for the presence of high-grade cervical intraepithelial lesions, however, with a low specificity, resulting in limited clinical value.

Thirdly, **chapter 7** presents the results of a systematic review and meta-analysis on alternative colposcopy techniques. This study was performed to assess diagnostic value of alternative (digital) colposcopy techniques for detection of CIN lesions in a colposcopy population. Four major data sources were searched for studies with the following inclusion criteria: a digital colposcopy technique was used in a colposcopy population, a histologic outcome was reported differentiating between mild dysplasia or less, and moderate dysplasia or worse, the entire cervix was scanned at once or a per-woman analysis was performed, no other topical application than acetic acid and Lugol's solution was used, and, at least three eligible studies had to be available within a single technique. Thirteen studies met the inclusion criteria of which six on fluorescence and reflectance spectroscopy including 2,530 women, four on dynamic spectral imaging including 1,173 women, and three studies on optical coherence tomography including 693 women. Fluorescence and reflectance spectroscopy showed the highest pooled sensitivity of 93% (95% CI 89-95) with a specificity of 62% (95% CI 47-76). Dynamic spectral

imaging showed the highest pooled specificity of 83% (95% CI 76-88), with a sensitivity of 69% (95% CI 48-85). Previously published conventional colposcopy results showed a sensitivity of 61% (95% CI 58-63) and specificity of 85% (83-86%), indicating that alternative (digital) colposcopy techniques may result in similar or increased sensitivity or specificity. The choice for a certain digital colposcopy device may therefore depend on the use of the device in a clinical setting. However, because alternative colposcopy techniques are still in development, and randomized controlled trials to compare alternative techniques with conventional colposcopy are still lacking, no recommendations for introduction in clinical practice can be made yet.

Two studies in **part III** of this thesis focus on the future risks of high-risk HPV infections.

The aim of the study in **chapter 8** was to investigate future risks of high-grade CIN lesions and cervical cancer in young women who tested hrHPV-positive before the age of 30. We retrospectively investigated how hrHPV detection before the age of 30, might be associated with the risk of high-grade CIN lesions after the age of 30. Follow-up data from a cohort study on HPV prevalence in 2,065 unscreened Dutch women between 18 and 29 years of age was obtained. Women were asked to perform 3-monthly self-samples for hrHPV testing. Women were categorized as; either hrHPV negative, a cleared hrHPV infection, or a persistent hrHPV infection, and anonymized follow-up data for each group was obtained from the Dutch nationwide registry of histopathology and cytopathology (PALGA). A pathology result was registered for 962 women. The prevalence of HSIL was 19.3% in women who had a persistent HPV infection at a younger age. This was significantly higher ($p<0,001$) compared with the HSIL prevalence of 1.5% in women who were HPV-negative at a younger age, as well as the HSIL prevalence of 3.1% in women who cleared the hrHPV infection in the past. With this retrospective study can be concluded that women who had a persistent hrHPV in their 20s, showed an increased risk of a HSIL lesion in their early 30s. The advantages of screening for persistent hrHPV infections before the age of 30 should be considered to lower cervical cancer incidence in young women.

Chapter 9 takes future risks of hrHPV infections one step further, and focusses on determining the risk of HPV-related carcinomas and premalignancies in women who are already diagnosed with high-grade CIN lesions. Knowledge on this risk is important to consider HPV vaccination and/or intensified screening for other HPV-related carcinomas and premalignancies when CIN3 is identified. Women diagnosed with a CIN3 between 1990 and 2010 were identified from the Dutch nationwide registry of histopathology and cytopathology (PALGA) and matched with a control group without CIN3. Subsequently, all hrHPV associated high-grade lesions and carcinomas of the anogenital region and oropharynx between 1990 and 2015 were extracted. Incidence rate ratios (IRR) were estimated for carcinomas and premalignancies of the vulva, vagina, anus and oropharynx. This resulted in a total group of 178,036; 89,018 with a previous diagnosis

of CIN3, and 89,018 matched controls without a history of CIN3. Women with a history of CIN3 showed increased risk of all HPV-related carcinomas and premalignancies with IRRs ranging from 3.85 for anal cancer, to 86.08 for vaginal cancer. The risk of hrHPV-related lesions remained significantly increased, even after long-term follow-up, up to 20 years. It can therefore be concluded that women with a high-grade CIN diagnosis show a long-lasting increased risk on HPV-related carcinomas and premalignancies of the anogenital and oropharyngeal region. Studies investigating successful methods to prevent this increased risk in this group of patients, like intensified screening or vaccination, are warranted.

Finally, the content of this thesis is discussed in broader sense in **chapter 10**. The main focus of this chapter is on different point of views on multiple aspects of cervical cancer prevention. Furthermore, future perspectives of cervical cancer prevention are discussed.

SAMENVATTING

Dit proefschrift beschrijft studies naar verschillende mogelijkheden voor het verbeteren van preventie van cervixcarcinoom. De focus ligt op drie aspecten van de preventie van cervixcarcinoom die kunnen zorgen voor een verbeterd, meer vrouwvriendelijk preventieprogramma. In **Hoofdstuk 1** worden verschillende aspecten van preventie van cervixcarcinoom beschreven, tezamen met onderzoeksdoelen en de hoofdlijnen van dit proefschrift.

Deel I van dit proefschrift bestaat uit drie hoofdstukken over triage van hoog-risico humaan papillomavirus (hrHPV) positieve vrouwen. In een groeiend aantal landen zal screening middels Pap cytologie vervangen worden door primaire screening met een hrHPV test. Omdat de hoge sensitiviteit van hrHPV screening gecombineerd is met een lagere specificiteit, is triage van hrHPV positieve vrouwen noodzakelijk om onnodige verwijzingen voor colposcopie te voorkomen. **Hoofdstuk 2** toont een overzicht van wereldwijde literatuur over triage van hrHPV positieve vrouwen, waarbij wordt gedifferentieerd tussen morfologische biomarkers, moleculaire biomarkers en gecombineerde triage strategieën. De klinische waarde van verschillende biomarkers wordt beschreven en vergeleken met een nog niet bestaande ideale triage strategie. Deze ideale strategie zou bestaan uit een biomarker die op het primaire screening materiaal met een hoge sensitiviteit en specificiteit differentieert tussen cervixcarcinoom of hooggradige cervicale intra-epitheliale neoplasieën (CIN) en vrouwen met een laag risico op deze afwijkingen die geen verder onderzoek behoeven en veilig terug kunnen keren naar het bevolkingsonderzoek. Dit hoofdstuk laat zien dat Pap cytologie, ten gevolge van de uitgebreide ervaring met deze techniek, een waardevolle triage methode is. Moleculaire biomarkers tonen echter veelbelovende resultaten in recent onderzoek. Deze markers zijn objectief, reproduceerbaar en kunnen gecombineerd worden waarbij de goede eigenschappen van verschillende biomarkers gecombineerd zorgen voor een verbeterde triage methode. De verwachting is dat screening op cervixcarcinoom in de toekomst zal veranderen in een volledig moleculair screening programma.

In **hoofdstuk 3** beschrijven we een studie naar de klinische waarde van triage van hrHPV positieve vrouwen met HPV16/18 genotypering en Pap cytologie. Van 520 vrouwen die hrHPV positief getest waren op een zelftest, werden uitstrijkjes afgenomen door huisartsen. Deze uitstrijkjes werden beoordeeld op Pap cytologie met kennis van hrHPV positieve status en HPV16/18 genotypering met de Cobas 4800 test (Roche). De sensitiviteit, specificiteit, voorspellende waarden en het aantal verwijzingen werd bekeken voor 18 triage strategieën. In vergelijking met de standaard triage methode met alleen Pap cytologie met een afkapwaarde van atypische cellen van onzekere significantie (ASC-US), toonden drie van de 18 strategieën betere resultaten in triage. De standaard strategie toonde een sensitiviteit van 93.5% (95% CI 87.7-97.1), specificiteit

van 58.5% (95% CI 53.7-63.2) en percentage verwijzingen van 52.2%. De strategie met HPV16 en/of Pap cytologie van minimaal laaggradige squameuze intra-epitheliale neoplasie (≥LSIL), de strategie met HPV16 en/of Pap cytologie met minimaal hooggradige squameuze intra-epitheliale neoplasie (≥HSIL) en de strategie met HPV16 en/of HPV18 en/of Pap cytologie ≥HSIL toonden een vergelijkbare sensitiviteit met een verhoogde specificiteit en een verlaagd aantal verwijzingen. De laatstgenoemde strategie toonde een sensitiviteit van 94.4% (95% CI 89.0-97.7) met de hoogste specificiteit van 74.9% (95% CI 70.5-78.9) en een verlaagd percentage verwijzingen van 39.5%. Deze resultaten indiceren dat bij bekende hrHPV positieve samples, triage middels alleen Pap cytologie verbeterd zou kunnen worden door de afkapwaarde van cytologie aan te passen en te combineren met HPV16/18 genotypering.

De klinische bruikbaarheid van p16/Ki-67 dubbelkleuring voor identificatie van CIN in hrHPV positieve vrouwen van een non-responder screening cohort wordt beschreven in **hoofdstuk 4**. Van 495 vrouwen die hrHPV positief getest waren op een zelftest, werden uitstrijkjes afgenomen door huisartsen. Deze uitstrijkjes werden getest middels p16/Ki-67 dubbelkleuring, Pap cytologie en HPV16/18 genotypering. De sensitiviteit, specificiteit en voorspellende waarden voor ≥CIN2 en ≥CIN3 werden bepaald voor verschillende gecombineerde triage strategieën en vergeleken met de standaard triage van Pap cytologie met een afkapwaarde van ASC-US. De gouden standaard was centraal gereviseerde histologie of een aangepast eindpunt waarbij het uitstrijkje dat na zes maanden genomen werd hrHPV negatief was met een normale Pap cytologie. Triage van hrHPV positieve vrouwen op de standaard methode middels cytologie met een afkapwaarde van ASC-US toonde een sensitiviteit van 93% (95% CI 85-98), met een specificiteit van 49% (95% CI 41-56) voor ≥CIN3. Drie triage strategieën met p16/Ki-67 tonen een verhoogde specificiteit met vergelijkbare sensitiviteit voor ≥CIN3: p16/ki-67 triage van alle hrHPV positieve samples toont een specificiteit van 61% (95% CI 54-69), p16/Ki-67 triage van alleen hrHPV positieve vrouwen met laaggradige Pap cytologie toont een specificiteit van 64% (95% CI 56-71) en p16/Ki-67 triage van normale en laaggradige cytologie toont een specificiteit van 58% (95% CI 50-65). Omdat de verschillen in sensitiviteit en specificiteit erg klein zijn tussen de drie groepen en de kwaliteit van Pap cytologie wereldwijd erg wisselend is, zou de meest betrouwbare strategie zijn om p16/Ki-67 triage toe te passen op alle hrHPV positieve vrouwen.

Deel II van dit proefschrift bestaat uit een drietal studies over verbeteren van colposcopisch onderzoek na een positieve screening en/of triage test. **Hoofdstuk 5** beschrijft een systematisch review en meta-analyse over see-and-treat behandeling van CIN-laesies. Het doel van deze studie was om te bepalen wat het risico is op overbehandeling bij de see-and-treat benadering. We doorzochten drie grote literatuur databases naar studies over see-and-treat management bij vrouwen met een geregistreerde Pap cytologie uitslag, colposcopische impressie en het uiteindelijke histologie resultaat. Dit resulteerde

in 13 studies met in totaal 4,611 vrouwen. Middels omgekeerde variantie werden er gepoolde incidentie waarden bepaald en met een random-effect model werd er rekening gehouden voor heterogeniteit tussen de studies. Het risico op overbehandeling is het groots bij een laaggradig Pap cytologie resultaat en een laaggradige colposcopische impressie met 72.9% (95% CI 68.1-77.7). Bij een hooggradig Pap cytologie resultaat en een hooggradige colposcopische impressie is het risico op overbehandeling het laagst met 11.6% (95% CI 7.8-15.3). Dit risico is vergelijkbaar met het risico op overbehandeling (11-35%) bij een aanpak waarbij eerst een biopt wordt afgenomen om de diagnose te bevestigen. Dit maakt dat see-and-treat management veilig toegepast kan worden in een groep vrouwen met een hooggradig Pap cytologie resultaat en een hooggradig colposcopisch beeld. In het geval van een discrepantie tussen de Pap cytologie en het colposcopisch beeld zouden leeftijd, kinderwens en risico van uitval bij follow-up meegenomen moeten worden in besluit wel of geen see-and-treat toe te passen.

Hoofdstuk 6 legt de focus op multimodale hyperspectroscopie (MHS), een digitale colposcopie techniek die fluorescentie- en reflectie spectroscopie combineert en in plaats van conventionele colposcopie gebruikt zou kunnen worden. Het doel van de prospectieve cohortstudie beschreven in dit hoofdstuk, was om de klinisch waarde te bepalen van MHS in het opsporen van CIN-laesies in een colposcopie populatie bestaande uit nieuw verwezen patiënten en follow-up patiënten, om zo te bepalen of MHS veilig gebruikt kan worden om zorg voor vrouwen at risk voor hooggradige CIN-laesies te verbeteren. Spectroscopische metingen middels MHS en hrHPV test met HPV16/18 genotypering werden uitgevoerd bij 125 vrouwen van een colposcopie populatie. Deze resultaten werden vergeleken met een eindpunt gebaseerd op histologie, hrHPV en cytologie. Voor 102 vrouwen waarvan complete data beschikbaar was werd de sensitiviteit, specificiteit en voorspellende waarden berekend voor MHS en voor de colposcopische impressie bij conventionele colposcopie. MHS toonde een sensitiviteit van 93.6% (95% CI 78.6-99.2), met een specificiteit van 42.3% (95% CI 30.6-54.6) in de groep met een samengesteld eindpunt. De acceptatie van de techniek door patiënten was hoog en er was geen sprake van bijwerkingen. Concluderend kan gesteld worden dat MHS een digitale colposcopie techniek is die een gemakkelijke, snelle en goed getolereerde beoordeling geeft met een hoge sensitiviteit voor de aanwezigheid van hooggradige CIN-laesies, echter met een lage specificiteit, waardoor de klinische waarde beperkt is.

Hoofdstuk 7 gaat een stapje verder op het gebied van digitale colposcopie technieken. In dit hoofdstuk wordt een systematisch review met een meta-analyse gepresenteerd over verschillende alternatieve (digitale) colposcopie technieken, die gebruikt zouden kunnen worden voor detectie van CIN-laesies in een colposcopie populatie. Middels vier grote databases werden studies geïncludeerd waarbij: een digitale colposcopie techniek gebruikt was in een colposcopie populatie, een histologische uitkomst beschreven stond die differentieerde tussen ≤CIN1 en ≥CIN2, de volledige cervix gescand

werd, of een analyse per vrouw was gedaan, behoudens Lugol en azijnzuur geen middel gebruikt was om de cervix aan te kleuren en minimaal drie studies van één specifieke techniek beschikbaar waren. Dertien studies voldeden aan de inclusiecriteria waarbij zes studies naar fluorescentie- en reflectie spectroscopie met in totaal 2,530 vrouwen, vier studies naar dynamische spectrale beeldvorming met totaal 1,173 vrouwen en drie studies naar optische coherentietomografie met totaal 693 vrouwen. Fluorescentie- en reflectie spectroscopie toonde de hoogste gepoolde sensitiviteit met 93% (95% CI 89-95) met een specificiteit van 62% (95% CI 47-76). Dynamische spectrale beeldvorming toonde de hoogste specificiteit van 83% (95% CI 76-88), met een sensitiviteit van 69% (95% CI 48-85). Eerder gepubliceerde data toonde een sensitiviteit van 61% (95% CI 58-63) en specificiteit van 85% (83-86%) voor conventionele colposcopie. Toepassing van alternatieve digitale colposcopie technieken zou dus kunnen leiden tot vergelijkbare of verhoogde sensitiviteit en specificiteit. De keuze voor een bepaalde techniek zou afhangen van de klinische setting. Er kan echter nog geen aanbeveling voor klinisch gebruik gedaan worden, omdat de technieken nog in ontwikkeling zijn en gerandomiseerde studies die alternatieve colposcopie technieken vergelijken met conventionele technieken ontbreken.

In **deel III** van dit proefschrift wordt met twee studies de focus gelegd op de toekomstige risico's van HPV-infecties.

Het doel van de studie beschreven in **hoofdstuk 8** was om de risico's te onderzoeken op het ontwikkelen van hooggradige CIN-laesies en cervixcarcinoom bij jonge vrouwen die hrHPV positief zijn voor hun 30^{e} levensjaar. Hiervoor wordt retrospectief gekeken hoe hrHPV detectie voor het 30^{e} levensjaar geassocieerd zou kunnen zijn met risico op hooggradige CIN-laesies na het 30^{e} levensjaar. Om dit te onderzoeken werd follow-up data verzameld van een cohortstudie naar HPV-prevalentie in 2,065 niet eerder gescreende Nederlandse vrouwen tussen 18-29 jaar, waarbij vrouwen 3-maandelijks een zelftest voor hrHPV test afnamen. Vrouwen werden ingedeeld in drie groepen: hrHPV negatief, een geklaarde hrHPV infectie, of een persisterende hrHPV infectie. Uit de landelijke Nederlandse pathologie registratie (PALGA) werd geanonimiseerde follow-up data verkregen van 962 vrouwen. In vrouwen met een persisterende infectie op jonge leeftijd was de HSIL-prevalentie 19.3%. Dit was significant hoger ($p<0,001$) dan de HSIL-prevalentie in hrHPV negatieve vrouwen (1.5%) en vrouwen die hun hrHPV infectie geklaard hadden (3.1%). Hiermee kan geconcludeerd worden dat vrouwen met een persisterende infectie voor het 30^{e} levensjaar een verhoogd risico laten zien op een HSIL-laesie na het 30^{e} levensjaar. De voordelen van screening op persisterende hrHPV infecties voor het 30^{e} levensjaar zouden overwogen moeten worden om de incidentie van cervixcarcinoom bij jonge vrouwen te verlagen.

In **hoofdstuk 9** worden de toekomstige risico's van hrHPV infecties bij vrouwen met een CIN3 laesie bestudeerd, waarbij specifiek gekeken wordt naar het risico op andere

hrHPV gerelateerde ernstige premaligniteiten en maligniteiten. Kennis hierover is belangrijk om HPV-vaccinatie of intensievere screening op andere hrHPV gerelateerde maligniteiten en premaligniteiten te overwegen bij vrouwen met een CIN3. Vrouwen met een CIN3 tussen 190 en 2010 werden geïdentificeerd uit de landelijke Nederlandse pathologie registratie (PALGA) en gematcht met een controlegroep zonder CIN3. Alle hrHPV geassocieerde maligniteiten en hooggradige premaligniteiten van anogenitale regio en orofarynx na de inclusiediagnose tussen 1990 en 2015 werden geïdentificeerd en incidentie rate ratio's (IRR) werden berekend. Dit resulteerde in een groep van 178,036 vrouwen waarvan 89,018 met een CIN3, en 89,018 gematchte controles zonder CIN3. Vrouwen met een CIN3 toonden een verhoogd risico op hrHPV-gerelateerde maligniteiten en ernstige premaligniteiten met een IRR van 3.85 voor anuscarcinoom tot 86.08 voor vaginacarcinoom. Het risico op hrHPV gerelateerde afwijkingen was significant verhoogd, zelfs tot 20 jaar na de CIN3 diagnose. Geconcludeerd kan worden dat vrouwen met een CIN3 een langdurig verhoogd risico laten zien op HPV-geassocieerde maligniteiten en ernstige premaligniteiten van de anogenitale regio en orofarynx. Er is aanvullend onderzoek nodig naar methoden als intensieve screening of vaccinatie, die dit verhoogd risico eventueel zouden kunnen voorkomen.

In **hoofdstuk 10** van dit proefschrift worden bovenstaande onderwerpen in een breder perspectief geplaatst. De focus van dit hoofdstuk ligt op verschillende standpunten ten aanzien van de aspecten van preventie van cervixcarcinoom besproken in dit proefschrift. Ook wordt in dit hoofdstuk het toekomstperspectief voor screening op cervixcarcinoom verder uitgelicht.

APPENDIX

Curriculum Vitae
Bibliography
Abbreviations
Dankwoord

CURRICULUM VITAE

Renée Ebisch werd geboren op 09 april 1988 in Venlo als oudste van drie en groeide op in het Limburgse Panningen. In 2006 behaalde zij haar VWO diploma aan het Bouwens van der Boijecollege.

Nadat ze uitgeloot was voor de studie Geneeskunde, begon ze aan de studie Biologie aan de Radboud universiteit te Nijmegen. Na het afronden van haar Bachelor Medische Biologie is ingestroomd in de studie Geneeskunde aan dezelfde universiteit. Ze deed een keuzecoschap Gynaecologie in het Caribische Grenada en sloot haar opleiding af met een keuze-coschap SEH in het VieCuri in Venlo en een senior-coschap Gynaecologie in het Radboud universitair medisch centrum te Nijmegen. Tijdens haar studie werkte ze mee aan een onderzoek op het gebied van zelftesten binnen screening op baarmoederhalskanker. In april 2014 nam zij haar artsenbul in ontvangst.

Vanaf april 2014 werkte zij onder begeleiding van promotor prof. dr. Leon Massuger en co-promotoren, dr. Ruud Bekkers, dr. Willem Melchers en dr. Bert Siebers als onderzoeker aan onderzoek op het gebied van screening op baarmoederhalskanker, hetgeen resulteerde in dit proefschrift. Vanaf 1 januari 2017 is Renée gestart als ANIOS Gynaecologie in het Catharina Ziekenhuis te Eindhoven.

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ABBREVIATIONS

AGC	atypical glandular cells
AIN	anal intraepithelial neoplasia
ASCCP	American society for colposcopy and cervical pathology
ASC-H	atypical squamous cells, cannot exclude HSIL
ASC-US	atypical squamous cells of undetermined significance
CDK	cyclin-dependent kinase
CI	confidence interval
CIN	cervical intraepithelial neoplasia
CISOE-A	composition, inflammation, squamous, other and endometrium, endocervical cyclindrical epithelium, adequacy
COM	comparator
EFC	European federation for colposcopy and pathology of the lower genital tract
ELISA	enzyme-linked Immuno sorbent assay
FN	false negative
FP	false positive
HC2	hybrid capture 2
HPV	human papillomavirus
hrHPV	high-risk human papillomavirus
HSIL	high-grade squamous intraepithelial lesion
hTERC	human telomerase RNA gene
IR	incidence rate
IRR	incidence rate ratio
LBC	liquid-based cytology
LCR	long control region
LEEP	loop electrical excision procedure
LLETZ	large loop excision of the transformation zone
LSIL	low-grade squamous intraepithelial lesion
MCM2	minichromosome maintenance protein 2
MHS	multimodal hyperspecroscopy
miRNA	micro RNA
MOOSE	meta-analysis of observational studies in epidemiology
mRNA	messenger RNA
NA	not available
NHSCSP	national health services cervical screening programme
NILM	negative for intraepithelial lesion or malignancy
NPV	negative predictive value

NRND	number of referrals needed to diagnose
p16	$p16^{INK4A}$
Pap	papanicolaou
PPV	positive predictive value
PRISMA	preferred reporting items for systematic reviews and meta-analyses
PROHTECT	protection by offering HPV testing on self-sampled cervicovaginal specimens trial
Rb	retinoblastoma
REF	referral rate
RR	risk ratio
siRNA	small interfering RNA
TN	true negative
TOP2A	topoisomerase II-a
TP	true positive
VAIN	vaginal intraepithelial neoplasia
VIN	vulvar intraepithelial neoplasia

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