IMPACT OF
HPV TESTING ON
CERVICAL CANCER SCREENING

Dorien Rijkaart
Impact of HPV testing on cervical cancer screening. Thesis, VU University Medical Center, Amsterdam, the Netherlands.

The work described in this thesis was performed at the Department of Pathology VU University Medical Center, Amsterdam, the Netherlands.

Financial support for printing of this thesis was kindly provided by Hologic Gen-probe, Sanofi Pasteur MSD, GSK, Greiner Bio-One, Memidis Pharma, Thermo Fisher Scientific, Medical Dynamics, Abbott Diagnostics, Werkgroep cervix uteri and Vrije Universiteit.

ISBN: 978-94-6182-226-0
Layout: ittakesto.nl
Printing: Off Page, Amsterdam

Copyright © 2013 D.C. Rijkaart, Amsterdam, the Netherlands. All rights reserved. No part of this thesis may be reproduced or transmitted in any form or by any means, without the prior written permission of the author.
Impact of HPV testing on cervical cancer screening

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. L.M. Bouter,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de Faculteit der Geneeskunde
op vrijdag 1 maart 2013 om 11.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Dorothea Christine Rijkaart

geboren te Gouda
promotoren: prof.dr. C.J.L.M. Meijer
             prof.dr. P.J.F. Snijders

copromotoren: dr. J. Berkhof
              dr. D.A.M. Heideman
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Introduction and outline of this thesis</td>
<td>7</td>
</tr>
</tbody>
</table>
| 2        | Comparison of HPV and cytology triage algorithms for women with borderline or mild dyskaryosis in population-based screening (VUSA-Screen trial)  
*International Journal of Cancer 2010; 9: 2175-81* | 29   |
| 3        | HPV DNA testing in population-based cervical screening (VUSA-Screen study): results and implications  
*British Journal of Cancer 2012; 5: 975-81* | 41   |
| 4        | Human papillomavirus testing for the detection of high-grade cervical intraepithelial neoplasia and cancer: final results of the POBASCAM randomised controlled trial  
*Lancet Oncology 2012; 1: 78-88* | 57   |
| 5        | Evaluation of 14 triage strategies for HPV DNA positive women in population-based cervical screening  
| 6        | Comparison of Hybrid capture 2 testing at different thresholds with cytology as primary cervical screening test  
*British Journal of Cancer 2010; 7: 939-46* | 95   |
| 7        | General discussion                                                   | 111  |
| 8        | Summary  
Samenvatting  
List of Publications  
Dankwoord  
Curriculum Vitae | 127  
128  
132  
136  
138  
142 |
CHAPTER 1

Introduction and outline of this thesis

1. Cervical cancer
   1.1 Precursor lesions of cervical cancer

2. Human Papillomavirus (HPV)
   2.1 Prevalence of HPV infections
   2.2 The viral life cycle and transforming infections

3. High-risk HPV and cervical carcinogenesis

4. Cervical cancer prevention
   4.1 Primary prevention by vaccination
   4.2 Secondary prevention
      4.2.1 Cytology screening
      4.2.2 Screening in the Netherlands
      4.2.3 Screening in other European countries

5. Improvement of cervical cancer screening
   5.1 HPV detection methods

6. Outline of this thesis
1. Cervical cancer

Cervical cancer is an important public health problem. With 530,000 new cases and 275,000 deaths per year, cervical cancer is the third most common malignancy in women worldwide.\textsuperscript{1,2} Furthermore, a disproportionally high incidence is observed in developing countries, especially in Africa, South Asia, and parts of Latin America, with incidence rates >20 per 100,000 women.\textsuperscript{1} The lowest rates are found in Western Asia, Australia, New Zealand, North America, and Western Europe (Figure 1). In the Netherlands in 2010, cervical cancer was newly diagnosed in 718 women (age-standardised incidence rate of 6.0/100,000) and 205 cervical cancer deaths were observed (age-standardised mortality rate of 1.3/100,000).\textsuperscript{3}

\textbf{Figure 1} World Standard cervical cancer incidence and mortality rates per country (rate per 100,000).\textsuperscript{1}
CHAPTER 1

1.1 Precursor lesions of cervical cancer

According to the classic concept cervical cancer usually arises in the cervical transformation zone. The transformation zone consists of metaplastic squamous epithelium that is located at the site where the squamous epithelial cells of the ectocervix (outer part of the cervix) meet the glandular columnar epithelial cells of the endocervix (inner part of the cervix). The position of the transformation zone is dynamic, shifting outwards during puberty, and, over the following years, gradually shifting towards, and into, the endocervical canal as squamous epithelium replaces the glandular epithelium. However, recent collected data supports the concept that cervical cancer and its high-grade precursor lesion develops from a discrete population of ectoendocervical squamocolumnar junctional cells, rather than cells from the transformation zone. Apparently, these cells are more susceptible to the human papillomavirus (HPV) mediated transformation and therefore prone to cervical cancer development.

Cervical cancer can be classified into different histological subtypes, of which squamous cell carcinoma is the most common one (accounting for about 80% of all cervical cancers). The second most common type is adenocarcinoma, accounting for approximately 15% of cervical cancers. Very rarely, other types, such as neuro-endocrine carcinomas and clear-cell carcinomas, are diagnosed.

Cervical squamous cell carcinomas develop through premalignant precursor lesions called cervical intraepithelial neoplasia (CIN). CIN lesions are classified into three groups: mild (CIN1), moderate (CIN2), or severe (CIN3, including carcinoma in situ) lesions, depending on the extent of replacement of the epithelial lining by atypical cells. In CIN1, which represents productive infections (see 2.2), up to one-third of the cells of the lower epithelial layer is replaced by atypical cells. In CIN3, the most advanced precursor stage representing a transforming HPV infection (see 2.2), from two-thirds up to the whole epithelial layer consists of atypical cells (Figure 2). Moreover, from CIN1 to CIN3, the cells become more atypical. Lesions are graded as carcinoma when atypical cells pass the basal layer (invasion). Whether cervical cancer develops through consecutive CIN1, 2 and 3 is under debate, since CIN2/3 lesions may develop rapidly following a high-risk HPV (hrHPV) infection, leaving only a very limited time frame for a preceding CIN1 lesion. The premalignant lesions can regress, persist, or progress. Lesions graded as CIN1 display a high regression rate, whereas CIN3 has the lowest regression rate. CIN2 lesions, a subset of which represents productive infections, have an estimated risk of cervical cancer of about 40%. CIN3 lesions have the highest risk of about 50% to progress to invasive cervical cancer.

Regression of CIN lesions is always associated with hrHPV clearance.

Together, CIN2 and CIN3 lesions are referred to as high-grade CIN. As mentioned before, the majority of the high-grade lesions would never progress to cancer in absence of treatment. However, at present it is not possible to distinguish morphological non-progressive lesions from progressive lesions. Therefore, women with high-grade lesions (CIN2+) are usually treated, resulting in a certain amount of overtreatment. These women are mainly treated through a loop electrosurgical excision procedure (LEEP). CIN1 lesions are called low-grade CIN. Women with these lesions are not treated but are recalled for a repeat cytology smear.
2. Human papillomavirus (HPV)

Persistent infection of the cervical epithelium with hrHPV is necessary for the development of cervical cancer.\textsuperscript{14-17} hrHPV can be detected in almost all cervical squamous cell carcinomas\textsuperscript{14} and in 94% to 100% of all adenocarcinomas.\textsuperscript{18-20}

Papillomaviruses are small, double-stranded DNA viruses. So far, more than 100 different HPV types have been identified. HPV types that are associated with cervical cancer are classified as ‘high-risk’ (hr) or carcinogenic. Based on epidemiologic criteria at least a dozen high-risk HPV types have been identified (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59).\textsuperscript{16,18,21} In addition, HPV types 26, 53, 66, 68, 73, and 82 are considered probably high-risk.\textsuperscript{22} Low-risk (lr) HPV types such as HPV6 and HPV11 are associated with benign wart-like lesions. The risk for cervical cancer differs between hrHPV types. Women with an HPV16 infection have a significantly greater risk for developing CIN3 or cervical cancer (CIN3+) compared to those infected with other oncogenic types.\textsuperscript{23-25} For example, in the POBASCAM trial, 18-month risk of CIN2+ in hrHPV-positive women was 13% after normal cytology and 34% after borderline or mild cytological abnormalities. If positive for HPV16, CIN2+ risks were 27% and 48%, respectively.\textsuperscript{25} Likewise, HPV18-positive women have an increased risk for CIN3+, although to a lesser extent as HPV16.\textsuperscript{24,25} Longitudinal studies with extensive genotyping have furthermore revealed that HPV31 and 33 conveyed increased risks of CIN2+ or CIN3+.\textsuperscript{24-26} In fact, in a study reported by Naucler et al.\textsuperscript{26} infection with HPV16, 31 and 33 conferred the highest risks of CIN2+ within 4 years of follow-up and were responsible for 33.1%, 18.3% and 7.7% of CIN2+ cases. Given these figures, it is not surprising that HPV16 causes more than half of the cervical cancers worldwide, followed by HPV18 (~16%), and HPV33 (~4%).\textsuperscript{27} In addition, hrHPV infections can also cause cancer in other parts of the anogenital tract, such as anal, vulvar, and penile cancer, as well as in the head and neck, particularly of oral and oropharyngeal origin.\textsuperscript{22,28-31}

2.1 Prevalence of HPV infections

Genital HPV infections are relatively common among sexually active women.\textsuperscript{32,33} It is estimated that about 80% of women are infected with a genital HPV infection during their lifetime.\textsuperscript{33} Since hrHPV is transmitted through sexual contact,\textsuperscript{34} the prevalence of hrHPV infection is highest in young women (ages 20-24) after starting sexual contact.\textsuperscript{32,35,36}

In the Netherlands, the highest prevalence of about 24% is detected in 22-year-old women. The prevalence gradually declines by increasing age to under 3% in women older than 45 years.\textsuperscript{37} Estimates of single-point prevalence of hrHPV infection among women participating in the screening programme in the Netherlands are between 4% and 5%.\textsuperscript{38,39} Risk factors that are associated with acquiring an hrHPV infection are the number of sexual partners, the age at which sexual intercourse was initiated, and smoking.\textsuperscript{17,40,41} In addition, contact with men who have promiscuous sexual behaviour is associated with increased risk for acquiring an hrHPV infection.\textsuperscript{33,34}

Fortunately, most women clear the hrHPV infection within 1-2 years.\textsuperscript{17,42} Persistent infection with hrHPV is a necessary but insufficient cause of cervical cancer.\textsuperscript{14,18} Decreased efficiency of the immune system to clear an hrHPV infection and additional (epi) genetic transforming events are necessary for malignant progression.
2.2 The viral life cycle and transforming infections

The viral life cycle is dependent on the differentiation of infected epithelium, and the virus uses the host replication machinery to generate progeny. The process is as follows: HPV infects the basal cells in the cervical epithelium and replicates in differentiating epithelial cells. Encapsidation takes place in the upper layers of the host epithelium and, finally, viruses are shed when the superficial epithelial cells die. HPV is thought to access the basal cells of squamous epithelium through small tears, known as microtraumas, in the mucosal surface (Figure 2). Besides the viral proteins E1 and E2, which are essential for viral replication, the virus relies entirely on the host cell DNA replication machinery for viral DNA synthesis. Viral E6 and E7 proteins are needed to create conditions that allow viral replication in differentiated, non-dividing epithelial host cells, in which the DNA replication machinery is normally not activated. The location of expression of the latter viral proteins is tightly regulated to the mid-zone of the epithelium. E6 binds to the human tumor-suppressor protein p53 and degrades it; p53 plays an important role in cell cycle control and apoptosis. E7 binds to the retinoblastoma protein (pRb) tumor suppressor protein and thereby disrupts the binding of pRb to E2F, which leads to S-phase entry through released E2F. Thus, E6 and E7 stimulate a condition of DNA synthesis in the infected host cell, thereby supporting viral replication. Many HPV infections are productive infections, in which new viral particles are formed and released. Productive infections of the cervix may give rise to mild to moderate cellular abnormalities, histologically comparable with CIN1/CIN2, but not often to true pre-cancerous stages of cervical cancer.

Under conditions that the viral E6/E7 proteins are improperly expressed in the proliferating basal cells, they may stimulate viral transformation. These so-called transforming infections are associated with histologically CIN2, CIN3 and cervical cancer (CIN2+). The mechanism underlying the deregulation of E6 and E7 expression is not completely understood. A possible explanation is integration of viral DNA in the host cell genome, but methylation of E2 binding sites within the viral long control region (LCR) has also been suggested. Deregulated expression of E6 and E7 in the basal cells will result in chromosomal instability and provides the driving force for further progression towards cancer. Free E2F resulting from interaction of E7 with pRb stimulates uncontrolled cell growth in proliferating cells. With less p53 due to degradation by E6, the cell cannot cope with uncontrolled cell growth, which triggers the development of genetic instability. As a consequence of deregulated E7 expression, the tumour suppressor p16INK4a is up-regulated. P16INK4a is a cyclin dependent kinase inhibitor that normally prevents inactivation of pRb by cyclin D1 and therefore induces cell cycle arrest at G1. However, in the presence of hrHPV E7 protein, upregulated p16INK4a has no effect since pRB is already inactivated by E7. The overexpression of the tumour suppressor p16INK4a throughout the cervical epithelium (i.e. diffuse immunostaining for p16INK4a) can be considered as a marker for lesions that harbour a transforming hrHPV infection.
3. High-risk HPV and cervical carcinogenesis

Cervical cancer develops through the following steps: hrHPV infection, hrHPV persistence, hrHPV transformation and development of precancerous lesions, and finally progression to invasive cervical cancer (Figure 2). Backward steps do also occur, with hrHPV clearance and regression of cervical lesions to normality.

About 80% of all hrHPV infections are cleared and will not result in premalignant lesions. The majority of the remaining 20% will develop into non-progressive low-grade CIN lesions characterized by a productive hrHPV infection. Only a small group of CIN lesions containing a transforming hrHPV infection are at risk of progression to cervical cancer. Next to viral persistence and a molecular switch into a transforming infection accumulation of additive (epi)genetic alterations is necessary for further progression towards invasive cancer.

Figure 2 Schematic representation of cervical cancer development [adapted from 57]. * Activation of oncogenes, loss of tumour suppressor gene function, p16INK4a overexpression, and chromosomal instability.
CHAPTER 1

4. Cervical cancer prevention
Two ways of cervical cancer prevention can be recognised: primary prevention and secondary prevention. Primary prevention is defined as an intervention aimed at taking the risk factor of cervical cancer, i.e. hrHPV infection, away in healthy women. Prophylactic vaccination with HPV16/18 L1 virus-like particles (VLPs), which was introduced in the Netherlands in 2009, is an example of primary prevention. Secondary prevention is an intervention to prevent cervical cancer by treatment of women who have subclinical (non-symptomatic) disease. Cervical cancer screening by detection of abnormal cells in cervical smears (also called a Pap smear) is an example of secondary cancer prevention.

4.1 Primary prevention by vaccination
Two prophylactic vaccines are available, a bivalent vaccine that protects against HPV16 and HPV18 (Cervarix®, GSK) and a quadrivalent vaccine that includes the low-risk types HPV6 and HPV11 as well (Gardasil®, Merck). At present, vaccination does not eliminate the need for cervical cancer screening. Since the HPV vaccine protects against infection of HPV16 and HPV18, which together cause about 70% of cervical cancer cases, screening will still be needed as a supplementary tool for the remaining 30%. Even with cross protection against non-vaccine HPV types, not more than 77% of cervical cancers will be prevented (HPV31 and 45 contribute to 7% of cervical cancer cases). Moreover, by no means will all individuals of the target group come forward for vaccination. In the Netherlands, the HPV vaccine is given to HPV-negative (naïve) 12-year-old girls with an initial catch-up vaccination for 13- to 16-year-old girls, and an average of 56% coverage is observed. As a result, for the majority of women, screening will remain the most important preventative strategy at least for the next 20 years.

4.2 Secondary prevention
Screening is an important cancer prevention tool. It is believed that nearly all cervical cancer-deaths could be prevented if women and their healthcare providers would fully adhere to screening recommendations and follow-up regimens. Success of cervical cancer screening is due to three factors: first, cervical cancer has a long-lasting pre-invasive phase, allowing time for detection. The median time between precursor lesions and cervical cancer is estimated to be more than 10 years. Second, cellular morphological changes on the cervix can be seen in the Pap smear and used for early diagnosis. Third, disease can be simply and effectively treated in the pre-invasive phase.

Tests used in primary cervical screening should fulfill certain requirements. Clinical sensitivity, clinical specificity, positive predictive value (PPV), and negative predictive value (NPV) are statistical measurements to express performance of a test. Clinical sensitivity is the probability that a test correctly classifies people with clinically meaningful disease at a preclinical stage as positive (e.g. the percentage of people with disease who are correctly identified as having the condition). Clinical specificity is the probability that a test classifies people without disease as negative (e.g. the percentage of healthy people who are correctly identified as not having the condition). Predictive
values of test results depend on the prevalence of disease in the population. The positive predictive value (PPV) is the proportion of people with a positive test result who have the disease. The negative predictive value (NPV) is the proportion of people with a negative test result who do not have the disease.

Cervical screening should be performed in organized programmes with quality assurance at all levels and good information should be provided about the benefits and risks. Opportunistic screening activities are usually not acceptable as they may not achieve the potential benefits and may cause unnecessary negative side effects. The benefit of screening on the reduction of cervical cancer cases depends on the clinical sensitivity of the screening test, participation of invited women, and availability of adequate treatment and follow-up algorithms for women with abnormal test results. The test used in screening needs to be robust and highly reliable and should therefore display high intra-laboratory reproducibility in time and inter-laboratory agreement. However, screening also has negative side effects. The negative effects of screening depend on the sensitivity (false negative tests) and specificity of the test (false positive tests, resulting in unnecessary treatment and distress about a positive test) and on the possible side effects of early treatment.

4.2.1 Cytology screening

The development of the Pap smear has resulted in the implementation of cytology-based cervical cancer screening programmes.

For a cytological diagnosis, cells are scraped from the transformation zone of the cervix. These cells are placed on a glass slide, fixed, and coloured. The morphological changes of the cells are graded based on the subjective interpretation of the degree of abnormality. There are several commonly used classifications. In the Netherlands, the CISOE-A coding system is used (in Dutch KOPAC-B). The American Bethesda

<table>
<thead>
<tr>
<th>CISOE-A</th>
<th>S0, O0, E0</th>
<th>S1, O1 E1-2</th>
<th>S1, O1 E1-2</th>
<th>S2-3, O3, E3</th>
<th>S4, O4, E4</th>
<th>S5, O5, E5</th>
<th>S6, O6, E6</th>
<th>S7, E7</th>
<th>S8-9, O7-8, E9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pap</td>
<td>Pap0</td>
<td>Pap1</td>
<td>Pap1</td>
<td>Pap2</td>
<td>Pap3a1</td>
<td>Pap3a2</td>
<td>Pap3b</td>
<td>Pap4</td>
<td>Pap5</td>
</tr>
<tr>
<td>Description</td>
<td>Inadequate</td>
<td>Normal</td>
<td>Borderline</td>
<td>Mild</td>
<td>Moderate</td>
<td>Severe</td>
<td>Carcinoma in Situ</td>
<td>Carcinoma</td>
<td></td>
</tr>
<tr>
<td>Bethesda 2001 for</td>
<td>NILM</td>
<td>Atrophy, NILM</td>
<td>ASC-US/ASC-H</td>
<td>ASC-H/LSIL</td>
<td>HSIL</td>
<td>Dyskaryosis</td>
<td>AGC</td>
<td>AGC favor neoplastic</td>
<td>AIS</td>
</tr>
</tbody>
</table>

Table 1. Cytomorphological classification: the CISOE-A and Pap classification compared to Bethesda 2001 classification.

CISOE-A, C composition, I inflammation, S squamous epithelium, O Other abnormalities and endometrium, and E endo-cervical columnar epithelium; the acronym CISOE-A is KOPAC-B in Dutch. NHIL, negative for intra-epithelial lesions or malignancy; ASC-H, atypical squamous cells cannot exclude HSIL; ASC-US, atypical squamous cells of undetermined significance; AGC, atypical glandular cells; LSIL, low grade squamous intraepithelial lesion; HSIL, high grade squamous intraepithelial lesion; AIS, endocervical adenocarcinoma in situ; SCC, squamous cell carcinoma; AC, adenocarcinoma.
CHAPTER 1

classification is most commonly used in internationally published studies on cervical
cancer screening.72,73 The CISOE-A classification can be converted into the Bethesda
system (Table 1).74,75

Until now, primary cervical cancer screening has been based on cytology. Several
epidemiologic studies have shown that cytological screening has been
successful in reducing the incidence and mortality of cervical cancer.7,76-78 However,
the cytology test has some limitations. The sensitivity of cytology for high-grade
lesions is quite low (50-70%) and the performance is heterogeneous across populations79.
Cervical high-grade lesions are missed by cytology due to sampling and detection
failures.80 To compensate for the low sensitivity of a single cytology test, frequent testing
is necessary. Since the specificity of cytology is about 95%,79,81 a substantial number of
women with minor abnormalities, and who do not harbour any underlying high-grade
disease, have unnecessary follow-up procedures. Moreover, adenocarcinoma and its
precursors are frequently missed by cytology. In addition, cytology is a subjective
test and labour intensive.82

Liquid-based cytology is a technique that has been developed to reduce variation
in quality of cytology. With this technique, the cell sample is collected as in the
conventional cytology test, but cells are immersed in a vial with preservation solution
instead of spread onto a slide. In the laboratory, a concentrated monolayer of cells is
prepared. The advantage of liquid-based cytology is that the slides are easier to
interpret because the process reduces the amount of obscuring material, such as
blood and leucocytes. The number of cytological preparations read by
cytotechnicians per hour can be increased substantially using liquid-based
cytology. Moreover, liquid-based specimens can be used for other ancillary
tests, such as hrHPV tests and tests for other biomarkers. However, in contrast to
what has been claimed in earlier publications, liquid-based cytology is not more
sensitive for detecting high-grade cervical lesions than a conventional smear.83-86
Disadvantages of liquid-based cytology include a lower number of cells and loss
of structural integrity of cell groups, by which cohesion of neoplastic cells disappears.
As a result, high-grade lesions and carcinomas might be missed.

4.2.2 Current screening in the Netherlands
In the Netherlands, cytological screening programmes were introduced in the 1970’s.
In three regions, Nijmegen, Utrecht, and Rotterdam, a pilot-screening programme
was introduced. Nationwide screening was introduced in 1988 for women aged 35-54
with a 3-year interval. In 1996, the cytological screening programme was restructured;
screening ages were extended to women aged 30-60 years, and the screening
interval was widened to 5 years.70 In addition, a cytological smear with cytomorphic signs of inflammation and/or presence of specific microorganisms was no longer
classified as Pap 2 (i.e. borderline dyskaryosis), but classified as within normal
limits.71,87 Moreover, a compact disc with microscopic examples of abnormal cytology
was provided to the all the pathologists. This resulted in a decrease in the number of
equivocal diagnoses from 11.3% to 2.6%.74 Normal smears without endocervical cells
were no longer repeated until a smear with endocervical cells was obtained, as there
was doubt about the importance of these cells in cervical smears. Furthermore, for quality control, the evaluation of the screening programme was improved. In 2006, negative triage with hrHPV testing on repeat smears after a borderline smear was incorporated. Since 2008, both the conventional and the new liquid-based cytology technique have been accepted for the national screening programme.

In the present day, approximately 800,000 women are annually invited for cervical screening in the Netherlands. In 2008, 550,000 women participated in the screening programme (67% of the invited population). About 95% of the participating women had a normal smear. These women were invited for the next screening round after five years. In 1.9% of the women, the smear was inadequate, and these women needed to repeat the smear after six weeks. A smear with borderline or mild dyskaryosis (BMD) was observed in 2.5% of the women. Women with BMD were recalled for repeat cytology after 6 and 18 months and were referred for colposcopy if the repeat test result was positive (BMD or worse). This policy is used because only 5%-15% of women with BMD have or will develop high-grade cervical lesions. Since 2006, an hrHPV test can be added to cytology for the first follow-up smear at 6 months after the baseline BMD smear. This leads to a reduction in the number of repeat smears, as it is considered safe to refer women with normal cytology and a negative hrHPV test back to routine screening. In 0.7% of the women, a smear with moderate dyskaryosis or worse (>BMD) was present. These women were directly referred to the gynaecologist for colposcopy examination.

The detection of cervical lesions classified as CIN1 or worse was 5.3 per 1,000-screened women. Of the women who were directly referred, 60% had CIN3+ and 17% had a CIN2 lesion.

4.2.3. Screening in other European countries
Almost all European countries have cervical cancer screening. However, only seven have a national organized screening programme (Denmark, Finland, Hungary, the Netherlands, Slovenia, Sweden, and the United Kingdom). Although all policies are mainly in line with the European recommendations (screening women every 3-5 years), there is a large variation in screening intensity. The number of smears taken in a lifetime varies between 7-16 per women, with the exception of Germany with a 1-year interval and over 50 smears taken in a lifetime.

The number of opportunistic smears taken in addition to screening varies strongly between countries. In the Netherlands, 77% of women aged 30-64 years had at least one smear in the past 5 years, whereas the response to the screening invitations was 65%. Unlike in the Netherlands, in Finland there are many opportunistic smears taken in addition to screening. Annually, 460,000 smears are made while only 270,000 women per year are eligible for a smear. An explanation for this might be that in the Netherlands the smears taken outside the screening programme are not reimbursed. In addition, in the Netherlands the general practitioner and assistant sample for cytology-screening tests, and they are well informed about the guidelines. Furthermore, many women in Finland have had a smear taken before the first invitation for cervical screening. Opportunistic smears add little to the effectiveness of the regular
programme, and therefore decrease the cost-effectiveness of the screening activities. Also age at which women are screened for the first time varies between countries. In Luxembourg, women are screened starting at the age of fifteen, in Belgium at the age of eighteen and in Germany at the age of twenty. Finland and the Netherlands have the highest age, i.e. thirty years, at which women are screened for the first time. With the upper age limit of 60 years, as also used in Denmark and Sweden.

Cervical cancer incidence rates vary widely across Europe. The highest incidence rates are seen in Eastern European countries. The lowest rates are seen in countries such as Italy, Switzerland, Spain, Finland, and the Netherlands. Despite the presence of a relatively effective screening programme in the Netherlands, cervical cancer continues to be a considerable public health problem. The main reasons for missing cervical cancer cases despite a screening programme are a relatively low adherence to the screening programme (65% per round attendance rate of invited women) and a relatively high number of false-negative cytology tests (low sensitivity). In addition, the follow-up of screen-positive women is not optimal. Therefore, over the last decades, efforts to improve screening have focused on increasing attendance and on development of alternative screening tests that are more sensitive than cytology.

5. Improvement of cervical cancer screening
Several trials have studied the value of hrHPV testing in cervical cancer screening programmes (Table 2). These studies have shown that hrHPV testing has a higher sensitivity for high-grade CIN than cytology testing. However, the hrHPV test has a lower specificity because the hrHPV test detects a substantial number of women with transient hrHPV infections that will not lead to clinically meaningful lesions. This may lead to over-referral for colposcopy and thus over-treatment. As a result, women with a positive hrHPV test result should not be offered colposcopy immediately but should be further stratified by means of triage and repeat testing.

Testing for hrHPV can be used for various purposes: as a primary screening test, as triage for women with minor cytological abnormalities (BMD), and as follow-up test after treatment of pre-invasive lesions.

A seemingly effective method of improving screening coverage is by sending self-sampling devices to women who do not respond to invitation for the screening programme. About 30% of the non-attendees respond by returning their self-sampler. Moreover, the detection of high-grade lesions in the self-sampling group was higher than in the regular screening programme. Studies using interview surveys have shown that women prefer self-collection over physician-collection. Time and place of sampling, privacy, and ease of sampling have been mentioned as advantages of self-sampling.
5.1 HPV detection methods

A variety of hrHPV tests are currently available. HrHPV nucleic acids can be detected by target or signal amplification assays. Target amplification assays mainly comprise polymerase chain reaction (PCR)-based methods and isothermal RNA amplification methods. PCR assays that are used include GP5+/6+, PGMY09/11, SPF10 and the Roche Amplicor HPV test. Examples of signal amplification assays are the Hybrid Capture 2 (HC2) test (Qiagen), Invader technology (Cervista HPV-HR Hologic), and in situ hybridisation (ISH) assays. In HC2, hybridisation of one of the RNA probes to hrHPV DNA is detected by biotin-labelled antibodies that recognize RNA/DNA hybrids following capture in streptavidin-coated microwell plates. The outcome of this test is expressed in relative light units per calibrator cut-off (RLU/CO).

When considering hrHPV tests, it is important to recognize the difference between analytical sensitivity and specificity versus clinical sensitivity and specificity. Analytical sensitivity and specificity refer to the detection of all hrHPV infections, including transient infections and those associated with high-grade lesions. Instead, clinical sensitivity and specificity point to the detection of only those hrHPV infections that are associated with clinically meaningful lesions. Ideally, the hrHPV test used in clinical settings should detect only women at risk for cervical cancer but not those with transient hrHPV infections.

The hrHPV tests GP5+/6+ PCR and HC2 have been used in large randomised controlled trials (Table 2) and have proven to perform well in cervical screening programmes. As such these assays are considered clinically validated. Both assays detect the hrHPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, and, additionally, HPV66 is targeted by GP5+/6+ PCR and detected by HC2 as a result of cross-hybridisation.

However, many new hrHPV tests have been developed, and the clinical performance of these tests is mostly unknown. Therefore, standards for hrHPV test performance and characteristics in clinical practice have been formulated. Using these guidelines, three hrHPV DNA tests (cobas® 4800, [Roche Molecular Systems Inc., Alameda, CA, USA], RealTime PCR [Abbott Molecular, Des Plaines, IL, USA] and Papillocheck® [Greiner Bio-One, Frickenhausen, Germany]) have proven to fulfill the sensitivity and specificity criteria for cervical screening purposes and can be considered clinically validated following demonstration of a sufficient reproducibility.
Table 2: Studies with hrHPV testing and cytology in cervical cancer screening

<table>
<thead>
<tr>
<th>Study</th>
<th>Study type</th>
<th>Age</th>
<th>Population</th>
<th>Primary screening test in intervention arm</th>
<th>Primary screening test in control arm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hart study</td>
<td>Multicentre screening study</td>
<td>30-60</td>
<td>10,358 women, United Kingdom</td>
<td>Conventional cytology + HC2</td>
<td>Conventional cytology + HC2</td>
</tr>
<tr>
<td>(Cuzick, 2003)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hart study</td>
<td>RCT</td>
<td>30-60</td>
<td>10,358 women, United Kingdom</td>
<td>Conventional cytology + HC2</td>
<td>Conventional cytology + HC2</td>
</tr>
<tr>
<td>(Cuzick, 2003)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTCC, Phase I</td>
<td>RCT</td>
<td>25-60</td>
<td>45,174 women, Italy</td>
<td>LBC + HC2</td>
<td>Conventional cytology + HC2</td>
</tr>
<tr>
<td>(Ronco 2006, 2010)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTCC, Phase II</td>
<td>RCT</td>
<td>25-60</td>
<td>49,196 women, Italy</td>
<td>HC2</td>
<td>Conventional cytology + HC2</td>
</tr>
<tr>
<td>(Ronco 2008, 2010)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POBASCAM</td>
<td>RCT</td>
<td>29-56</td>
<td>40,105 women, the Netherlands</td>
<td>Conventional cytology + GP5+/6+</td>
<td>Conventional cytology + HC2</td>
</tr>
<tr>
<td>(Bulkmans 2007, Rijkaart 2012)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swedescreen</td>
<td>RCT</td>
<td>32-38</td>
<td>12,527 women, Sweden</td>
<td>Conventional cytology + GP5+/6+</td>
<td>Conventional cytology + HC2</td>
</tr>
<tr>
<td>(Naucler 2007)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCCaST</td>
<td>RCT</td>
<td>30-69</td>
<td>10,154 women, Canada</td>
<td>HC2</td>
<td>Conventional cytology + HC2</td>
</tr>
<tr>
<td>(Mayrand 2007)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARTISTIC</td>
<td>RCT</td>
<td>20-64</td>
<td>24,510 women, United Kingdom</td>
<td>LBC + HC2</td>
<td>LBC</td>
</tr>
<tr>
<td>(Kitchener 2009)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>India screening trial</td>
<td>RCT</td>
<td>30-59</td>
<td>131,746 women, India</td>
<td>HC2 vs. Conventional cytology vs VIA</td>
<td>No screening</td>
</tr>
<tr>
<td>(Sankaranarayanan 2009)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Finnish screening trial</td>
<td>RCT</td>
<td>30-60</td>
<td>38,670 women, Finland</td>
<td>HC2 + Conventional cytology triage</td>
<td>Conventional cytology + HC2</td>
</tr>
<tr>
<td>(Anttila 2011)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VUSA-Screen</td>
<td>Cohort study</td>
<td>30-60</td>
<td>48,088 women, the Netherlands</td>
<td>Conventional cytology + HC2</td>
<td>Conventional cytology</td>
</tr>
<tr>
<td>(Rijkaart 2010)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LBC: liquid-based cytology, RCT: randomised controlled trial, VIA: visual inspection with acetic acid
6. Outline of this thesis

Infection with hrHPV plays a causal role in the development of cervical cancer.\textsuperscript{14,16} This has resulted in the development of hrHPV testing suitable for population-based screening. The goal of this thesis is to examine whether cervical screening can be improved by 1) hrHPV testing in triage of borderline or mild dyskaryotic cytology, and 2) hrHPV testing as a primary screening test. Other goals were to evaluate the potential clinical impact of hrHPV screening compared to cytology. In addition, we studied the optimal follow-up algorithm for hrHPV-positive women.

In most countries that screen for cervical cancer, including the Netherlands, women with borderline or mild dyskaryosis (BMD, similar to Pap2 and Pap3a1, in CISOE-A: S2-4, O3 E3-5) are recalled for repeat testing, and they are only referred for colposcopy if the cytological abnormality persists. This algorithm was developed since only 5\%-15\% of these women develop high-grade lesions (CIN2+). The disadvantage of this repeat testing algorithm is the loss of follow-up and distress for the women because of the uncertain diagnosis. In Chapter 2, we answer the question: is it feasible to use hrHPV triage for women with BMD? The VUSA-Screen study (Vrije Universiteit Medical Center Saltro laboratory population-based cervical screening) was designed to investigate the efficacy of additional testing for hrHPV in the cervical screening programme. In this study, women aged 30-60 years were offered hrHPV testing and cytology (intervention group) or cytology only (control group). In the intervention group, women with BMD and an hrHPV-positive test were immediately referred for colposcopy, whereas hrHPV-negative BMD women were advised to repeat cytology at 6 and 18 months and were referred for colposcopy when the repeat test result was positive. We compared hrHPV and cytology triage algorithms for women with BMD using the end point of histologically confirmed CIN3+, the number of repeat smears, the number of colposcopy referrals, and the medical costs.

Data from randomised controlled trials indicate that hrHPV screening has a higher sensitivity than cytology screening for detecting high-grade cervical lesions.\textsuperscript{79,104-108,130} However, implementation of hrHPV testing as a primary screening instrument in cervical screening is still under debate. In Chapter 3, we present results from the VUSA-Screen study focusing on the following three implementation issues: 1) whether hrHPV testing in primary screening should be offered alone or as an adjunct to cytology, 2) how to manage hrHPV-positive women, and 3) the age of application of hrHPV-based screening.

In Chapter 4, we present the definitive 5-year results of the POBASCAM trial (Population Based Screening Study Amsterdam), a population-based, randomised controlled trial in which women were screened at baseline with either combined hrHPV testing and cytology or conventional cytology alone, from January 1999 to September 2002. At the subsequent screening round after 5 years, all women were screened with both hrHPV testing and cytology. Our main goal was to assess whether hrHPV testing decreases detection of CIN3+, CIN2+, and of cervical cancer in the second screening round. An additional goal was to assess the most appropriate age at which hrHPV testing should start. We also evaluated how detection of high-grade
lesions in two screening rounds was associated with particular HPV genotypes.

Several studies have shown that hrHPV testing has a lower specificity than cytology screening for detecting high-grade cervical lesions.79,104-108 The lower specificity has raised concerns that primary screening with hrHPV leads to increased referral rates for colposcopy and to overtreatment. Therefore, hrHPV-positive women should not be referred for colposcopy immediately. In Chapter 5, we used data from the intervention arm of the VUSA-Screen study to evaluate fourteen triage and repeat testing strategies for hrHPV-positive women. Another potentially cost-saving and simple option is to adapt the threshold for considering an hrHPV test as positive, i.e. increase the relative light unit/cut-off (RLU/CO) threshold of the Hybrid capture 2 (HC2) test. In Chapter 6, we studied the effect of hrHPV testing with higher HC2 thresholds on sensitivity, the colposcopy referral rate, and the false positive rate.

The results of the preceding chapters are discussed in Chapter 7 and summarized in Chapter 8.

References
17. Zielinski GD, Rozendaal L, Vooorhorst FJ, et al. HPV testing can reduce the number of follow-up visits in women treated for cervical intraepithelial neoplasia grade 3. Gynecol Oncol 2003;91:73
Introduction: Impact of HPV DNA testing on cervical screening
CHAPTER 1

Introduction: Impact of HPV DNA testing on cervical screening
CHAPTER 1

89. van Kemenade FJ, Wiersma T, Helmerhorst TJ. [New version of the pathology practice guideline for cervical cytology: sharpened criteria for adequacy; expanded use of new techniques]. Ned Tijdschr Geneeskd 2007;151:1283-86

Introduction: Impact of HPV DNA testing on cervical screening
CHAPTER 1

Introduction: Impact of HPV DNA testing on cervical screening

CHAPTER 1

Introduction: Impact of HPV DNA testing on cervical screening
COMPARISON OF HPV AND CYTOLOGY TRIAGE ALGORITHMS FOR WOMEN WITH BORDERLINE OR MILD DYSKARYOSIS IN POPULATION-BASED SCREENING (VUSA-SCREEN TRIAL)
CHAPTER 2

ABSTRACT

Aim
We studied the effectiveness of high-risk human papillomavirus (hrHPV) triage for immediate colposcopy in women with borderline or mild dyskaryosis (BMD).

Methods
In the Utrecht province of the Netherlands, women aged 30–60 years who participated in the regular cervical screening programme were offered hrHPV testing and cytology (intervention group) or cytology only (control group). In the intervention group (n = 337), women with BMD were immediately referred for colposcopy only if the sample was hrHPV positive. Women with a hrHPV negative test were advised to repeat cytology at 6 and 18 months and were referred for colposcopy if and when the repeat test result was positive (BMD or worse). In the control group (n = 329), referral of women with BMD was delayed until cytology was repeatedly positive at 6 or 18 months.

Results
The CIN3 detection rates were 10.7% (36/337) in the intervention group and 6.4% (21/329) in the control group (p = 0.047). Moreover, hrHPV triaging resulted in shorter time to diagnosis (154 vs. 381 days). Although the number of colposcopy referrals was 51.5% higher in the intervention group than in the control group, the medical costs per detected CIN3 were slightly lower ([euro] 4781 vs. [euro] 6235). If, in addition, hrHPV negative women had been referred back to routine screening at baseline, the CIN3 rate would have been 10.1% (34/337) and colposcopy rate would only have been 30.4% higher than in the control group.

Conclusion
This study shows that hrHPV triaging of women with BMD is at least as effective for detecting CIN3 as repeat cytology, also when hrHPV negative women are referred back to routine screening.
CHAPTER 2

Introduction
In many European population-based screening programmes, women with borderline or mild dyskaryosis (BMD) are recalled for repeat cytology before being referred for colposcopy only if the cytological abnormality persists. This policy is used because only 5–15% of these women have or will develop high-grade cervical lesions.\textsuperscript{1–3} A disadvantage of repeat testing is that women may become lost during follow-up.\textsuperscript{4} Repeat cytology also induces a considerable amount of side effects in terms of psychological distress.\textsuperscript{5,6} Several groups have studied the possible value of hrHPV testing and cytology for the detection of cervical lesions.\textsuperscript{7,8} Most studies show that hrHPV testing is a sensitive instrument to triage women with BMD, but the optimal triaging strategy remains controversial.\textsuperscript{9,10}

In the Netherlands, women with BMD are followed with cytology testing at 6 and 18 months. Since 2006, screening laboratories may choose to include a hrHPV test in the repeat cytology at 6 months.\textsuperscript{11} This leads to a reduction in the number of repeat smears as it is considered safe to refer women with normal cytology and a negative hrHPV test back to the routine screening. A more substantial reduction in the number of repeat smears is expected when women with BMD are tested for hrHPV at baseline. However, population-based prospective evidence, in terms of the end-points CIN3 or cancer (CIN3+), colposcopy referrals and medical costs needed to support implementation of this strategy, is lacking.

To study the feasibility of hrHPV triage at baseline, we compared repeat cytology to direct referral of hrHPV-positive BMD women in a sub-study of the VUSA-Screen study (Vrije Universiteit Medical Centre Saltro laboratory population-based cervical SCREENing study). Outcome measures were CIN3+ and CIN2+ detection rates, number of colposcopy referrals, and medical costs.

Material and Methods
The present cohort study is part of the VUSA-Screen study. VUSA-Screen is an intervention study designed to evaluate the effectiveness of combined cervical cytology screening with hrHPV testing by Hybrid Capture 2 hybridization assay (HC2, Digene Corporation). The VUSA-Screen study has 2 aims. First, we evaluated the effectiveness of hrHPV triage in women with BMD by comparing current screening protocols using conventional cytology with a strategy where women with BMD were advised on the basis of hrHPV test result.

The second aim of the VUSA-Screen study was to evaluate the risk of developing high-grade CIN lesions in cytologically normal women with a hrHPV positive versus a hrHPV negative test result. Therefore, women with normal cytology and a positive hrHPV test were retested for cytology and hrHPV at 12 and 24 months. Women were referred if cytology was abnormal at 12 months and if cytology was abnormal and/or hrHPV positive at 24 months. Each hrHPV positive, cytologically normal woman was matched to 3 randomly chosen hrHPV negative, cytologically normal women of the same age, who were also rescreened at 24 months. In this article, we present the results of the BMD women only.

The study was carried out in the Utrecht province of the Netherlands in the setting of the regular Dutch screening programme. Women aged 30–60 years were advised
to visit their general practitioner (GP) for cytology screening every 5 years by means of a call and recall method. Between October 2003 and August 2005, GPs affiliated to the Saltro laboratory in Utrecht were asked to participate in the VUSA-Screen study. After education of 500 GPs, 254 agreed to participate and recruited women for both cytology and hrHPV testing. Women who agreed to receive cytology and hrHPV testing gave written consent and these women formed the intervention group. This group was compared with women visiting the GP for regular cytological screening who did not participate in the VUSA-Screen study, but were affiliated to the Saltro laboratory (control group). Women in the control group were screened according to the national guidelines\(^\text{12,13}\) and data were analyzed anonymously.

Women were excluded from the analysis if they had a history of cervical intraepithelial neoplasia grade 2 or worse (CIN2+) or abnormal cytology in the preceding 2 years. All cytology and HC2 tests were performed in the Saltro laboratory in Utrecht under the supervision of a pathologist from the department of Pathology of the Vrije Universiteit Medical Center in Amsterdam. All study participants of the intervention group were given written information before screening regarding hrHPV infection and its role in carcinogenesis. A physician staffed telephone help desk was available throughout the study and follow-up period. The Ministry of Public Health obtained approval before the study started (advice nr 2002/02-WBO; ISBN-10: 90-5549-452-6), according to Dutch law. The study was registered in the trial register as NTR215, ISRCTN64621295.

**Figure 1** Flowchart study design. The baseline cytology results of the VUSA-Screen study and study design of women with BMD

Comparison of hrHPV and cytology triage algorithms for women with BMD
CHAPTER 2

The triage design of the study and the baseline characteristics of women with minor cytological abnormalities are presented in Figure 1. Women in the intervention group were triaged according to both cytological testing and hrHPV DNA results. Women with BMD and a positive hrHPV test were directly referred to colposcopy. Women with BMD at baseline and a negative hrHPV test were tested for cytology at 6 and 18 months and referred if cytology was abnormal at one of these occasions. Women in the control group were tested with cytology according to the current guidelines for cervical screening in the Netherlands. Women with BMD were advised to repeat the tests after 6 and 18 months. If one of the repeat tests was abnormal, women were referred to colposcopy.

A scrape was taken using a cytobrush (Rovers, Oss). After preparation of a conventional smear on a glass slide, the brush was placed in a vial containing 1 ml UCM (Universal Collection Medium, Dgene) for hrHPV testing. Cervical cytology results were reported, blinded to the hrHPV DNA testing results, according to the CISOE-A classification, which is routinely used in the Netherlands and can be easily converted into the BSCC classification. Cytological results were grouped as normal, BMD and moderate dyskaryosis or worse (>BMD).

HrHPV testing was performed by the Hybrid Capture 2 (HC2) high-risk HPV DNA test in an automated format on a rapid capture system (RCS) according to the manufacturer’s instructions (Qiagen, Gaithersburg, MD). This test uses a cocktail probe to detect 13 high-risk HPV types: 16, 18, 31,33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. Positive controls containing 1 pg/ml of cloned HPV-16 DNA and negative controls (provided by the manufacturer) were included in each assay. The results of the HC2 assay were expressed as relatively light units per cut-off value (RLU), representing the ratio between the emission from a sample to the average of 3 positive controls. Samples were considered positive if they attained or exceeded threshold of 1.0 RLU/CO (corresponding with 1 pg/ml HPV16 DNA).

Of the women that were referred to a gynaecologist for colposcopy, colposcopy-directed biopsies were taken from suspicious areas of the cervix, according to standard procedures in the Netherlands. Histological examination of biopsies was done at local pathology laboratories and classified as normal, cervical intra-epithelial neoplasia grade 1, 2 or 3 or as invasive cancer, according to international criteria. High-grade lesions were reviewed by 2 independent pathologists. Cytology and histology results of both the intervention and control group were retrieved from the nationwide network and registry of histopathology and cytopathology (PALGA; Bunnik, the Netherlands).

The primary outcome measure of the study was histologically confirmed CIN3+. Secondary outcome measures were histologically confirmed CIN2+, number of colposcopy referrals, and medical costs. The follow-up time was set at 3 years.

Baseline differences between the intervention and control group were examined for age (Mann-Whitney test), the prevalence of BMD in the screened population (Pearson Chisquare test), the percentage of women that attended the previous screening round (Chi-square test), and the socioeconomic status rank score (Mann-Whitney test). A woman was considered as a participant at the previous screening round if she had her last smear within 7 years. The socioeconomic status
score was postcode based and retrieved from the governmental “Sociaal en Cultureel Planbureau” (http://www.scp.nl/onderzoek/statusscores). The outcome measures, number of CIN3+, CIN2+ cases and colposcopy referrals were compared by Pearson’s Chi-square test. Loss to follow-up was defined as no cytological or histological information obtained from women who were eligible for follow-up. The time to reach diagnosis was the time between baseline smear and histological diagnosis.

Women were referred to colposcopy on the basis of the study protocol. A woman could only be counted once for a referral to colposcopy. The colposcopy referral advices given by the gynaecologist after abnormal histology were not taken into account. Some women were referred for colposcopy despite the fact that the advice was repeat testing or return to routine screening programme. These cases were included in the calculation of the total number of colposcopy referrals. All calculations were repeated after omitting the follow-up of hrHPV-negative women in the intervention group. Clopper-Pearson confidence intervals were used for the detection rates, and normal intervals were used for the detection ratios and for the times to referral and diagnosis. The calculations were performed in STATA 10.0. Tests were two-sided and a test was significant if \( p < 0.05 \).

The number of screened women was targeted at 25,000 in both groups. Assuming a BMD prevalence of 2.5%, the sample size is sufficient to achieve 80% power to detect a 5% difference in CIN3+ yield.

The direct medical costs in euros per unit of health care resource utilization are included in the analysis and presented in Table 3. A health care perspective was taken and indirect costs and time and travel costs were not included. All costs were indexed at year 2006. The costs of screening and treatment were published previously and were updated to 2006 using the consumer price index. The utilities for different health states (Table 3) were based on international publications. Following the Dutch guidelines, the discounting rate per year for costs and health effects were set at 4% and 1.5%, respectively.

Results

The patient characteristics of women with BMD are presented in Table 1. The intervention group and control group were comparable on all baseline characteristics.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Intervention group</th>
<th>Control group</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year of intake</td>
<td>2003-2005</td>
<td>2003-2005</td>
<td></td>
</tr>
<tr>
<td>Region</td>
<td>Utrecht</td>
<td>Utrecht</td>
<td></td>
</tr>
<tr>
<td>Median age</td>
<td>40.0</td>
<td>39.0</td>
<td>0.099</td>
</tr>
<tr>
<td>Prevalence of BMD in screening population</td>
<td>1.3%</td>
<td>1.5%</td>
<td>0.095</td>
</tr>
<tr>
<td>Borderline dyskaryosis</td>
<td>73.9%</td>
<td>72.0%</td>
<td>0.591</td>
</tr>
<tr>
<td>Mild dyskaryosis</td>
<td>26.1%</td>
<td>28.0%</td>
<td>0.591</td>
</tr>
<tr>
<td>Attendance at previous screening round(^1)</td>
<td>82.5%</td>
<td>80.9%</td>
<td>0.637</td>
</tr>
<tr>
<td>Median socioeconomic status rank score(^2)</td>
<td>0.43</td>
<td>0.47</td>
<td>0.677</td>
</tr>
</tbody>
</table>

\(^1\) Computed for women who had received an invitation at a previous screening round (i.e. women ≥ 34 years of age). Attendance was defined as having a screening result within the last 7 years. \(^2\) Ranks ranged from 0 to 1.
Table 2 shows the histological results and the total number of gynaecological referrals for all women with BMD. The cumulative 3-year CIN3 detection rate was higher in the intervention group than in the control group (10.7% vs. 6.4%, p = 0.047). No cancers or adenocarcinoma in situ were detected. The CIN2+ rate was not statistically different between both groups (22.3% vs. 18.5%, p = 0.235), whereas the ≤CIN1 rate was higher in the intervention than in the control group (23.4% vs. 12.2%, p < 0.001). Of the 337 women with BMD in the intervention group, 167 (49.6%) were hrHPV positive. Of these, 34 (20.4%, 95%CI 14.5-27.3) had a CIN3 diagnosis. Of 170 hrHPV negative women with BMD, 2 (1.2%, 95%CI 0.1–4.2) had CIN3. Of the 329 women in the control group, 21 were diagnosed with CIN3 (6.4%, 95%CI 4.0–9.6). The colposcopy referral rate was higher in the intervention group than in the control group (57.6% vs. 38.0%, p < 0.001). However, the number of referrals per detected CIN3 was equal in both groups (5.4 vs. 6.0, p = 0.689). There was a marginal difference in the number of referrals per detected CIN2+ (2.6 vs. 2.0, p = 0.074).

Women with hrHPV negative test and women in the control group showed the same cytology follow-up at 6 and 18 months (75.3% vs. 76.9% p = 0.690 and 41.1% vs. 39.4% p = 0.779, respectively). The overall loss to follow-up was not statistically different between both groups (13.5% vs. 13.7%, p = 0.935).

Among women with a colposcopy referral, the mean time to referral was 39 (95%CI 22-55) days in the intervention group and 298 (95%CI 259-336) days in the control group. Among women with a histological diagnosis, the mean time to

---

Table 2. Follow-up results of women with BMD: 3-year cumulative colposcopy referrals and histology

<table>
<thead>
<tr>
<th>Source</th>
<th>Control group n = 329</th>
<th>Intervention group n = 337</th>
<th>Intervention group, hrHPV+ n = 167</th>
<th>Intervention group, hrHPV- n = 170 hr</th>
<th>Intervention vs control group</th>
<th>Intervention (follow-up of hrHPV-negative women omitted) vs control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>No colposcopy referrals</td>
<td>204 (62.0; 56.5-67.2)</td>
<td>143 (42.4; 37.1-47.9)</td>
<td>0 (84.1; 77.7-89.3)</td>
<td>0.68 (0.59-0.80)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colposcopy referrals</td>
<td>125 (38.0; 32.7-43.5)</td>
<td>194 (57.6; 52.1-62.9)</td>
<td>167 (15.9; 10.7-22.3)</td>
<td>1.52 (1.28-1.79)</td>
<td></td>
<td>1.30 (1.10-1.55)</td>
</tr>
<tr>
<td>No biopsy</td>
<td>24 (7.3; 4.7-10.7)</td>
<td>40 (11.9; 8.6-15.8)</td>
<td>33 (19.8; 14.0-26.6)</td>
<td>1.63 (1.00-2.64)</td>
<td></td>
<td>1.34 (0.81-2.22)</td>
</tr>
<tr>
<td>CIN0/1</td>
<td>40 (12.2; 8.8-16.2)</td>
<td>79 (23.4; 19.0-28.3)</td>
<td>64 (38.3; 30.9-46.2)</td>
<td>1.93 (1.36-2.73)</td>
<td></td>
<td>1.56 (1.09-2.25)</td>
</tr>
<tr>
<td>CIN2+</td>
<td>61 (18.5; 14.5-23.2)</td>
<td>75 (22.3; 17.9-27.1)</td>
<td>70 (41.9; 34.3-49.8)</td>
<td>1.20 (0.9-1.62)</td>
<td></td>
<td>1.12 (0.8-1.52)</td>
</tr>
<tr>
<td>CIN2</td>
<td>40 (12.2; 8.8-16.2)</td>
<td>39 (11.6; 8.4-15.5)</td>
<td>36 (21.6; 15.6-28.6)</td>
<td>0.95 (0.63-1.44)</td>
<td></td>
<td>0.88 (0.58-1.34)</td>
</tr>
<tr>
<td>CIN3</td>
<td>21 (6.4; 4.0-9.6)</td>
<td>36 (10.7; 7.6-14.5)</td>
<td>34 (20.4; 14.5-27.3)</td>
<td>1.67 (1.00-2.81)</td>
<td></td>
<td>1.38 (0.90-2.67)</td>
</tr>
</tbody>
</table>
| **hrHPV+= hrHPV positive; hrHPV-= hrHPV negative; CI= confidence interval; ≤CIN1= normal or CIN 1; CIN2+= CIN 2 or worse. Criteria for referral to colposcopy: abnormal cytology or positive hrHPV test. Referrals include colposcopies that were performed despite the fact that the advise was repeat testing or return to routine screening programme.**
diagnosis was 154 days (95% CI 124-184) in the intervention group and 381 days (95% CI 323-440) in the control group.

The medical costs of screening, diagnosis and treatment were €512 per woman in the intervention group and €398 per woman in the control group as shown in Table 3. Per detected CIN3, the medical costs were €4,781 in the intervention group and €6,235 in the control group. To study the impact of negative hrHPV triaging, we repeated the analyses while omitting follow-up after a hrHPV-negative test. In that case, the CIN3 detection ratio was 1.58 (95% CI 0.90–2.67%) compared with the control group and the colposcopy referral ratio was 1.30 (95% CI 1.10–1.55) compared with the control group. The medical costs per woman became €447 and the medical costs per detected CIN3 became €4,429 (Table 3).

Table 3 Medical procedure costs (screening, diagnosis, treatment): unit costs, costs per woman, costs per detected CIN2+ and CIN3

<table>
<thead>
<tr>
<th>Treatment and follow-up of:</th>
<th>Costs per woman with BMD (€)²</th>
<th>Intervention group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>First cytology³</td>
<td>57.0</td>
<td>57.0</td>
<td>57.0</td>
</tr>
<tr>
<td>Repeat cytology(s)⁴</td>
<td>55.0</td>
<td>43.2</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>133.6</td>
<td>102.0</td>
</tr>
<tr>
<td>Colposcopy</td>
<td>158.4</td>
<td>91.2</td>
<td>79.2</td>
</tr>
<tr>
<td>Biopsy</td>
<td>68.1</td>
<td>31.1</td>
<td>27.1</td>
</tr>
</tbody>
</table>

Costs per detected CIN2+ | 2298.3 | 2151.5 | 2146.6
Costs per detected CIN3 | 4781.3 | 4428.8 | 6235.4

¹Unit cost of medical procedures, screening, diagnosis and treatment, based on recent Dutch data. ²The costs of the average number of diagnostic. ³Includes invitation organization, the visit at the GP and the collection of sample material and laboratory costs. ⁴Includes the visit at the GP and the collection of sample material and laboratory costs. ⁵Treatment costs include the charge per type of treatment, preoperative diagnostics and cost of hospital days.

Discussion

HrHPV testing to triage women with borderline or mild dyskaryosis resulted in a much earlier diagnosis and was at least as effective for detection of CIN3 as repeat cytological testing. Although hrHPV triaging led to an increase in the number of colposcopies, this did not lead to an increase in the referral rate per CIN3. The medical costs per woman were similar for hrHPV triaging and repeat cytological testing and the costs per detected CIN3 were even slightly lower for hrHPV triaging.

The high CIN3/CIN2+ detection rate of the hrHPV triaging strategy is in line with a recent meta-analysis,⁷ where it has been shown that hrHPV triaging has a higher sensitivity than repeat cytology (at the ASCUS threshold) for detection of CIN2+, without a marked specificity loss. We, therefore, evaluated the impact of “negative triaging” in which women with BMD and a negative hrHPV test are considered

Comparison of hrHPV and cytology triage algorithms for women with BMD
CHAPTER 2

not to be at increased risk and thus able to return to the regular screening programme. This strategy has been suggested by several authors.\textsuperscript{23–25} Therefore, we recalculated the CIN3 yield after omitting the follow-up of hrHPV-negative women. This yield would still be at least as high as in the repeat cytology arm, indicating that hrHPV testing after BMD has a high-negative predictive value. This ensures that implementation of a protocol in which hrHPV-negative BMD women return to the regular screening programme does not lead to an increase in undetected lesions that are clinically meaningful.

We found higher colposcopy referral rates in the hrHPV triage arm than in the repeat cytology arm (57.6\% vs. 38.0\%). The ≤CIN1 rate was also higher in the hrHPV triage arm (23.4\%) than in the repeat cytology arm (12.2\%). This carries the potential risk of over-treatment in an additional 10\% of cases. Our data did not allow us to analyze whether such was indeed the case. If women had been referred to the regular screening programme after a negative hrHPV test at baseline, the increase in colposcopy rate would have dropped from 51.5\% to 30.4\%. This increase in colposcopy rate, regardless whether it is implemented with or without negative hrHPV triaging, is not expected to lead to capacity problems in a country such as the Netherlands where the annual number of primary smears read as BMD is about 8,700.\textsuperscript{26} On the basis of our study, introducing hrHPV triaging is expected to increase the number of coloscopies by about 1,700 without and 1,000 with a negative hrHPV triage scenario. The latter increase translates into an increase in the annual medical costs of 226,500 euros. In the Netherlands, the BMD prevalence is low and hrHPV positivity in BMD was 50\% in our study. In countries where BMD rates are higher, hrHPV positivity in BMD may be lower in which case hrHPV triaging will be more efficient than in the Netherlands. This argument does not need to hold for a relatively young screening population in which hrHPV positivity and BMD rates are both high. A study by Moss et al.\textsuperscript{10} in which most women were between 20–34 years old, showed higher colposcopy referral rates in the hrHPV triaging arm and lower colposcopy referral rates in the repeat cytology arm than our study.

Our study was a cohort study and not a randomized controlled trial. The study was performed in the setting of a private laboratory, a real life situation, and this made it difficult to organize randomization. Therefore, women may have different baseline characteristics, specifically as both groups were not recruited from the same GPs. To assess the possible effects of baseline differences, we compared the intervention and control group on the available characteristics: age, prevalence of BMD, screening history, and postcode-based socioeconomic status. We did not find any significant differences between the 2 groups, which strengthen the results in our study. In addition, the loss to follow-up was not statistically different between hrHPV negative women in the intervention group and the women in the control group.

The development of screening programme guidelines requires careful consideration of the benefits, burdens, and costs that are associated with the adoption of new technologies. Our study clearly supports the evaluation of new guidelines for management of BMD, as early hrHPV triaging leads to equal or better CIN3 detection and can be implemented against low costs. The use of hrHPV triaging will lead to a faster diagnosis and less distress and is therefore an important step in improving the woman-friendliness of screening.
Acknowledgements

We thank Ms. Angelique Balfoort, Ms. Lianne van de Heuvel, Ms. Annemieke Bijl, Ms. Carien Corstiaenssen, Mr. Ton van Dijk, Ms. Henny Hatzmann, and Mr. Erwin van Houten for performing the hrHPV tests. We thank participating GPs, their assistants and the gynecologists for participating in the study. Qiagen (Digene) is acknowledged for providing collection tubes with UCM and HC2 kits. We thank Ms. Maaike Dijkstra for her help with editing the manuscript.

References

HPV DNA TESTING IN POPULATION-BASED CERVICAL SCREENING (VUSA-SCREEN STUDY): RESULTS AND IMPLICATIONS

Dorien C. Rijkaart
Veerle M.V. Coupe
Rene H.M. Verheijen
Peter J.F. Snijders
Johannes Berkhof
Lawrence Rozendaal
Saskia Bulk
Chris J.L.M. Meijer
Folkert J. van Kemenade
Daniëlle A.M. Heideman
Wim M. Verweij

British Journal of Cancer 2012; 5: 975-81
ABSTRACT

Background
Human papillomavirus (HPV) testing is more sensitive than cytology for detecting high-grade cervical intraepithelial neoplasia (CIN). We evaluated the performance of high-risk HPV (hrHPV) testing in routine screening.

Methods
In all, 25,871 women (29–61) enrolled in our population-based cohort study were offered both cytology and hrHPV testing. High-risk HPV-positive women with normal cytology and an age-matched subcohort of hrHPV-negative women with normal cytology were invited for repeat testing after 1 and/or 2 years and were referred for colposcopy if they presented with abnormal cytology and/or a positive hrHPV test. The hrHPV-positive women with borderline or mild dyskaryosis (BMD) and all women with moderate dyskaryosis or worse (>BMD) were directly referred for colposcopy. Women with BMD and an hrHPV-negative test were advised to repeat cytology at 6 and 18 months and were referred for colposcopy if the repeat cytology test was abnormal. The main outcome measure was CIN grade 3 or worse (CIN3+). Results were adjusted for non-attendance at repeat testing.

Results
The hrHPV-positive women with abnormal cytology had a CIN3+ risk of 42.2% (95% confidence interval (CI): 36.4-48.2), whereas the hrHPV-positive women with normal cytology had a much lower risk of 5.22% (95% CI: 3.72-7.91). In hrHPV-positive women with normal cytology, an additional cytology step after 1 year reduced the CIN3+ risk to only 1.6% (95% CI: 0.6-4.9) if the repeat test was normal. The CIN3+ risk in women with hrHPV-positive normal cytology was higher among women invited for the first time (29–33 years of age) (9.1%; 95% CI: 5.6–14.3) than among older women (3.0%; 95% CI: 1.5–5.5).

Conclusion
Primary hrHPV screening with cytology triage in women aged ≥30 years is an effective way to stratify women on CIN3+ risk and seems a feasible alternative to cytological screening. Repeat cytology after 1 year for hrHPV-positive women with normal cytology is however necessary before returning women to routine screening.
CHAPTER 3

Introduction

Cytological screening has reduced the incidence and mortality of cervical cancer in countries with organised screening programmes. However, cytological screening offers a suboptimal prevention against cervical cancer as cytology has a limited sensitivity for high-grade cervical intraepithelial neoplasia (CIN). Many studies conducted by combined high-risk human papillomavirus (hrHPV) and cytology testing have revealed that testing for hrHPV results in a much higher sensitivity for high-grade CIN and cervical cancer (CIN2+) than cytology.

Although the data collected so far are in favour of implementing hrHPV testing in primary screening there is still debate about the management of hrHPV-positive women and about the screening ages at which hrHPV testing would be most beneficial. In young women, the prevalence of hrHPV is high and as a consequence the management of hrHPV-positive women may be complicated. The higher number of positive primary screening tests in this age group may lead to adverse effects of screening if more unnecessary follow-up tests and colposcopy referrals are made. This is of particular importance for these women of reproductive age, because it has been shown that the rate of serious obstetrical complications, such as preterm deliveries, low birth weight and premature rupture of the membranes, is increased after excisional treatments for precancerous lesions.

Furthermore, young women may have a disproportional high number of regressive CIN2 lesions. Ronco et al have shown that primary hrHPV screening is particularly effective for women 35 years or older, whereas in younger women hrHPV screening would lead to over-diagnosis of regressive CIN2. On the other hand, Bulkmans et al demonstrated that in women between 30 and 60 years the total number of CIN2+ lesions over two screening rounds was equal in both the hrHPV plus cytology arm and the cytology only arm, indicating that there is no CIN2 over-diagnosis in the hrHPV plus cytology arm. Instead, more highgrade lesions were detected earlier in the hrHPV plus cytology arm than in the control arm. This indicates that in this age category hrHPV testing detects non-regressing, clinically relevant CIN2+ lesions earlier than cytology and suggests that primary hrHPV screening in women of ≥30 years is feasible.

To evaluate for the Dutch cervical screening programme the effectiveness of implementing hrHPV testing and to assess future implementation issues, we set up the VUSA-Screen study (Vrije Universiteit Medical Centre-Saltro laboratory population-based cervical screening). The study was carried out within the setting of a routine cervical screening programme. We present the main results of this cohort study in which 3-year follow-up results were related to baseline hrHPV testing and cytology testing to find an optimal primary screening method. Special attention was given to the question whether hrHPV testing should be offered in combination with cytology or as a sole primary screening instrument. In addition, we study how hrHPV-positive women should be managed. Finally, we examine at what age (at 30 years or at older age) it would be most beneficial to start hrHPV testing.
CHAPTER 3

Patients and Methods

Patients and procedures

The VUSA-Screen study is a cohort study within the setting of the Dutch population-based cervical screening programme designed to evaluate the effectiveness of combined cervical cytology screening with hrHPV testing by the HC2 hybridisation assay (Qiagen, Gaithersburg, MD, USA). In the Netherlands, women are invited for cervical cancer screening at 5-year intervals starting in the year in which they reach the age of 30 and with the last invitation in the year in which they turn 60 (age range, 29–61 years). The study was carried out in the province of Utrecht in the Netherlands among women who were invited for the regular cervical screening programme between October 2003 and August 2005. The design of the study, including exclusion criteria, has been described previously. All participants gave written informed consent. The VUSA-Screen study was approved by the Ministry of Public Health (2002/02-WBO; ISBN-10: 90-5549-452-6) and registered in the trial register (NTR215, ISRCTN64621295).

**Figure 1** Flowchart of the screening profiles of women in the VUSA-Screen study. HrHPV = high-risk human papillomavirus; hrHPV+ = positive hrHPV test; hrHPV− = negative hrHPV test; BMD = Borderline or mild dyskaryosis; >BMD = moderate dyskaryosis or worse; CIN = cervical intraepithelial neoplasia (grade 2 or 3); Ca = Cervical carcinoma. *The baseline hrHPV test results of these matched women were blinded.
From all participants, a conventional cytological smear was taken with a cytobrush (Rovers, Oss, the Netherlands). After preparation of the smear on a glass slide, the brush was placed in a vial containing 1 ml UCM (Universal Collection Medium; Qiagen) for hrHPV testing. Cervical cytology results were reported, blinded to the hrHPV testing results, according to the CISOE-A classification, which is routinely used in the Netherlands and can be easily converted into the 2001 Bethesda system. Cytological results were grouped as normal, borderline or mild dyskaryosis (BMD), and moderate dyskaryosis or worse (≥BMD). In the 2001 Bethesda system, BMD corresponds to atypical squamous cells of undetermined significance, atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesions, or low-grade squamous intraepithelial lesions. Moderate dyskaryosis or worse corresponds to high-grade squamous or glandular intraepithelial lesions.

High-risk HPV testing was performed by HC2 high-risk HPV DNA test in an automated format on a rapid capture system according to manufacturer’s instructions (Qiagen). This test uses a cocktail probe to detect 13 high-risk HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. Samples with HC2 outcome of ≥1 RLU/CO were considered as hrHPV positive. HC2-positive samples were tested with GP5+/6+-PCR-EIA, and all specimens tested positive by GP5+/6+-PCR-EIA were typed by reverse line blotting.

Women with BMD or worse were informed about the hrHPV test result. The hrHPV-positive women with BMD and all women with ≥BMD were directly referred for colposcopy (Figure 1). Women with BMD and a negative hrHPV test were offered cytology at 6 and 18 months and referred if cytology was abnormal (threshold BMD) at one of these occasions.

In the women with normal cytology at baseline, a subcohort was selected. In this subcohort, all (n = 1021) hrHPV-positive women as well as a subset of hrHPV-negative, cytologically normal women (n = 3063) were included. To select the hrHPV-negative women, each hrHPV-positive woman was matched to three randomly chosen hrHPV-negative women of the same age. Women were not informed about the hrHPV test result. Women with normal cytology and hrHPV-positive test were offered cytology and a blinded hrHPV test at 12 months and combined hrHPV and cytology testing at 24 months. Women were referred at 12 months if cytology was abnormal and at 24 months if cytology was abnormal and/or the hrHPV test was positive.

Women in the subcohort with normal cytology and hrHPV-negative test were invited for repeat testing with both tests at 24 months. These women were referred at 24 months if cytology was abnormal and/or the hrHPV test was positive. Women with normal cytology who were not included in the subcohort were recalled at the next screening round after 5 years as part of the routine screening programme.

Of the women who were referred to a gynaecologist for colposcopy, colposcopy-directed biopsies were taken from suspicious areas of the cervix, according to standard procedures in the Netherlands. Biopsy results were reported as normal, CIN1, 2, or 3, or as invasive cancer, according to the international criteria. Cytology and histology results were retrieved from the nationwide network and registry of histopathology and cytopathology (PALGA, Bunnik, the Netherlands).
CHAPTER 3

Statistical analysis

The primary outcome measure of the study was histologically confirmed CIN3+, detected cumulatively within 3 years after baseline. A secondary outcome was cumulatively detected CIN2+. In the calculations of the number of CIN3+ and CIN2+ lesions, also cases of cervical adenocarcinoma and cervical adenocarcinoma in situ were included.

Separate CIN3+ and CIN2+ risks were calculated for hrHPV, cytology and age-specific strata. The risks were adjusted for nonattendance at repeat testing. Non-attendance rates at 12 and 24 months may depend on previous screening test results and were read from flow charts (Figure 1).

The sensitivity and specificity of the hrHPV test and cytology were adjusted for non-attendance at repeat testing by writing them as functions of stratum-specific CIN3+ or CIN2+ risks. Ninety-five percent confidence intervals (CIs) for the CIN3+ and CIN2+ risks and for the sensitivities and specificities were obtained from Bayesian posterior distributions. To compute posteriors, Beta (0.5,0.5) priors were imposed on the probabilities of moving from one box to another box in flowchart (Figure 1). The posterior intervals were computed via simulation. The posterior intervals may become narrow when one or more of the point estimates of the probabilities equal 0 (or 1). We accounted for this by imposing a point prior at 0 (or 1) and recomputed the Bayesian posterior interval. The reported CIs are unions of the original and recomputed posterior intervals. This approach has reasonable frequentist properties when estimating a proportion.

Analyses were done with SPSS version 15.0 (LEAD Technologies Inc., Haddonfield, NJ, USA), Excel (Microsoft Corporation, Redmond, WA, USA), and Matlab version 7.9 (The Mathworks Inc., Natick, MA, USA).

Results

Of the 25,871 women recruited for the VUSA-Screen study, 25,658 (99.2%) had adequate baseline cytology and hrHPV HC2 test. The median age of participating women was 44.0 years (range 29–61 years). Among the women with adequate cytology, 97.4% had a normal result, 1.3% had BMD and 0.5% had >BMD. The proportion of women with hrHPV infection(s) was 4.1% in women with normal cytology, 49.6% in women with BMD and 92.0% in women with >BMD. Overall, 5.1% (1,303 out of 25,658) of the women tested hrHPV positive by HC2. In women with BMD and negative hrHPV result, the overall compliance to repeat testing was 86.5%. In the subcohort of women with normal cytology, compliance to repeat testing was similar in the blinded hrHPV-positive and hrHPV-negative group (61.8% and 59.7%, respectively, p = 0.237). For women with normal cytology at baseline with follow-up, the histology follow-up at 24 months showed a higher referral rates after abnormal cytology than after an hrHPV positive, cytologically normal test result (57.0% vs 21.1%, respectively). Among women who attended at repeat testing, the average time to the first follow-up test was 15.0 months with a standard deviation of 4.7 months. The follow-up time ranged from 1.3 to 28.6 months.
We evaluated hrHPV prevalence in seven age groups corresponding to the screening rounds. We found the highest hrHPV prevalence among women between 29 and 33 years of age who were invited for the first time (10.5%; 95% CI: 9.6–11.4%). As the age increased, hrHPV prevalence decreased until age 49 years. The hrHPV prevalence in women aged 59–61 years was 2.0% (95% CI: 1.5–2.8%; Figure 2). Women aged 29–33 years showed a significantly higher hrHPV prevalence (10.5%; 95% CI: 9.6–11.4%) than women aged 34–61 (4.0%; 95% CI: 3.7–4.3%) (p < 0.001). Among women with adequate cytology, 1.8% (95% CI: 1.62.0%) had an abnormal result (≥BMD). The highest proportion of abnormal cytology was found in women aged 29–33 years (i.e., 2.5%; 95% CI: 2.1–3.1%) and the lowest proportion was found in women aged 59–61 years (0.6%; 95% CI: 0.3–1.0%).

The histological follow-up results in relation to baseline cytology and hrHPV test results, stratified by two age groups, are presented in Table 1. Among women with normal cytological results, the proportion of CIN3+ cases was 2.6% (27 out of 1021) if the hrHPV test was positive and 0.07% (2 out of 3063) if the hrHPV test was negative. For women with abnormal cytology, the proportion of CIN3+ cases was 42.2% (119 out of 282) if the hrHPV test was positive and 2.8% (5 out of 180) if the hrHPV test was negative.

Of 1021 women with normal cytology and a positive hrHPV test at baseline, 92 women had abnormal cytology at 12 months follow-up of whom 76 (82.6%) tested hrHPV positive, 6 (6.5%) tested hrHPV negative and 10 (10.9%) women had an...
unknown hrHPV status. Of the women with normal cytology and a positive hrHPV test at baseline, 528 had normal cytology at 12 months follow-up of whom 224 (42.4%) tested hrHPV positive, 219 (41.5%) tested hrHPV negative and 84 (15.9%) had an unknown hrHPV status. The attendance at 24 months was comparable for women with normal cytology and hrHPV-positive test at 12 months (14 women with normal cytology and hrHPV-negative test and 52 women with abnormal cytology and/or hrHPV-positive test) and women with normal cytology and hrHPV-negative test (52 women with normal cytology and hrHPV-negative test and 6 women with abnormal cytology and/or hrHPV-positive test).

The absolute and relative sensitivity and specificity of cytology and hrHPV testing for detection of CIN3+ and CIN2+ are presented in Table 2. The sensitivity of hrHPV testing for CIN3+, adjusted for non-attendance at repeat testing, was 1.42-fold higher than the sensitivity of cytology (91.9% vs 64.6%) at the cost of a lower specificity (95.6% vs 98.7%). The sensitivity of hrHPV testing for CIN2+ was 1.63-fold higher than cytology (82.0% vs 50.5%); however, the specificity was 0.97 fold lower (96.0% vs 98.9%).

The cumulative 3-year CIN3+ and CIN2+ risks, adjusted for non-attendance at repeat testing, are presented in Figure 3. The CIN3+ risk was markedly lower in women negative for hrHPV (0.06%; 95% CI: 0.02–0.46%) than in women with negative cytology (0.26%, 95% CI: 0.20–0.65%). There was only a small, non-significant difference in CIN3+ risks between women with negative results on both tests (0.05%; 95% CI: 0.01–0.42) and women negative for hrHPV only (0.06%; 95% CI: 0.02–0.46). Women with abnormal cytology (≥BMD) result had a CIN3+ risk of 26.2% (95% CI: 22.5–32.2) and those with an hrHPV positive test had a risk of 13.2% (95% CI: 11.4–15.9). The highest CIN3+ risk (i.e., 42.2%; 95% CI: 36.4–48.2) was found in hrHPV-positive women who had abnormal cytology. The HPV16/18 genotyping at baseline showed that hrHPV-positive women for other types than HPV16/18 still had a CIN3+ risk of 6.6% (95% CI: 4.8–9.0). The HPV16+ and/or 18+ women had a CIN3+ risk of 26.1% (95% CI: 21.4–31.4) (Figure 3).

The majority of the hrHPV-positive women had normal cytology and those women still had a CIN3+ risk of 5.22% (95% CI: 3.72–7.91). The HPV16/18 genotyping of hrHPV-positive women at baseline did not result in sufficient low risks for a screenings programme with 5 years interval. Women with hrHPV-positive normal cytology and HPV16 and/or HPV18-positive test had a CIN3+ risk of 13.0% (7.93–23.6), whereas women who tested hrHPV positive for other high-risk types had a much lower risk of 2.44% (95% CI: 1.61–5.25). We also evaluated a baseline triage and follow-up strategy for hrHPV-positive women. Baseline cytology triage followed by repeat cytology testing at 1 year showed that the CIN3+ risk reduced to only 1.6% (95% CI: 0.6–4.9) in women with normal cytology at the repeat test. In women with abnormal cytology at the repeat test, the CIN3+ risk was 25.0% (95% CI: 16.6 – 35.1). This CIN3+ risk is comparable to the CIN3+ risk of hrHPV-positive women with abnormal cytology at baseline.

Analysis using CIN2+ as outcome measure found comparable results, albeit with higher absolute risks. The cumulative 3-year CIN2+ risk was 0.26% (95% CI: 0.14–0.69%) among hrHPV-negative women and 0.68% (95% CI: 0.54–1.13%) among women with negative cytology. The CIN2+ risk was similar for women with negative results on both
CHAPTER 3

Table 1 Three-year cumulative histology outcome by age, baseline cytology and hrHPV results

<table>
<thead>
<tr>
<th>Age</th>
<th>Cytology</th>
<th>hrHPV+</th>
<th>Total</th>
<th>CIN0/1</th>
<th>CIN2</th>
<th>CIN3</th>
<th>AdCa</th>
<th>SCC</th>
<th>CIN3+</th>
<th>CIN2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invited for</td>
<td>Normal in sub-cohort</td>
<td>hrHPV+</td>
<td>387</td>
<td>15</td>
<td>11</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>13</td>
<td>24</td>
</tr>
<tr>
<td>the first time</td>
<td></td>
<td>hrHPV-</td>
<td>1099</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>(29-33 yrs)</td>
<td>Abnormal</td>
<td>hrHPV+</td>
<td>85</td>
<td>19</td>
<td>18</td>
<td>34</td>
<td>0</td>
<td>1</td>
<td>35</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hrHPV-</td>
<td>25</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Others</td>
<td>Normal in sub-cohort</td>
<td>hrHPV+</td>
<td>654</td>
<td>47</td>
<td>19</td>
<td>13</td>
<td>0</td>
<td>1</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td>(34-61 yrs)</td>
<td></td>
<td>hrHPV-</td>
<td>1964</td>
<td>24</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Abnormal</td>
<td>hrHPV+</td>
<td>197</td>
<td>56</td>
<td>33</td>
<td>81</td>
<td>1</td>
<td>2</td>
<td>84</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hrHPV-</td>
<td>155</td>
<td>18</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>7</td>
</tr>
</tbody>
</table>

AdCa = adenocarcinoma; CIN = cervical intraepithelial neoplasia; hrHPV = high-risk human papillomavirus; hrHPV+ = positive hrHPV test; hrHPV- = negative hrHPV test; abnormal cytology = borderline or mild dyskaryosis or worse (≥BMD); normal in subcohort = a cohort of women with normal cytology and hrHPV- was age matched to hrHPV-positive women with normal cytology; SCC = squamous cell carcinoma. Women in the subcohort were invited for combined testing at 24 months and referred if cytology was abnormal and/or the hrHPV test was positive.

Table 2 Absolute and relative sensitivity and specificity of hrHPV testing vs cytology, adjusted for non-attendance at repeat testing

<table>
<thead>
<tr>
<th>Screening test</th>
<th>Endpoint CIN3+ (95% CI)</th>
<th>Endpoint CIN2+ (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hrHPV</td>
<td>91.9% (61.0-96.7)</td>
<td>82.0% (62.9-89.6)</td>
</tr>
<tr>
<td>Cytology</td>
<td>64.6% (43.3-73.1)</td>
<td>50.5% (38.4-58.0)</td>
</tr>
<tr>
<td>Specificity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hrHPV</td>
<td>95.6% (95.3-95.8)</td>
<td>96.0% (95.7-96.3)</td>
</tr>
<tr>
<td>Cytology</td>
<td>98.7% (98.5-98.8)</td>
<td>98.9% (98.7-99.0)</td>
</tr>
<tr>
<td>Relative sensitivity</td>
<td>1.42 (1.19-1.67)</td>
<td>1.63 (1.40-1.89)</td>
</tr>
<tr>
<td>hrHPV vs cytology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative specificity</td>
<td>0.969 (0.966-0.971)</td>
<td>0.971 (0.968-0.974)</td>
</tr>
</tbody>
</table>

hrHPV = high-risk human papillomavirus; CI = confidence interval; CIN = cervical intraepithelial neoplasia (grade 2 or 3 or higher). aCytology positivity was defined as a result of borderline or mild dyskaryosis or worse (≥BMD).

tests (0.24%; 95% CI: 0.12–0.64%) and women negative for hrHPV only (0.26%; 95% CI: 0.14–0.69%). The hrHPV-positive women with normal cytology had a CIN2+ risk of 11.3% (95% CI: 8.90–15.2) and hrHPV-positive women with normal cytology and negative for HPV16/18 genotyping had a CIN2+ risk of 8.01% (95% CI: 5.53–12.6).

When stratifying hrHPV-positive women into two age groups, there was a borderline non-significant difference in CIN3+ risk (adjusted for non-attendance at repeat testing) between women ≥34 years of age and women invited for the first time (aged 29–33 years) (relative risk (RR) 0.78 95% CI: 0.52–1.15). The RR for CIN2+ was 0.87 (95% CI: 0.64–1.18). In hrHPV-positive women with normal cytology, the CIN3+ risk was significantly lower in women aged ≥34 years (4.0; 95% CI: 2.3-6.6) than in women aged 29–33 years (10.9%; 95% CI: 5.9–19.2). The corresponding RR was 3.02 (95% CI: 1.39–7.07). The CIN2+ risk was 10.7 (95% CI: 7.3–15.3) in women with normal cytology and age ≥34 years, 16.6% (95% CI: 10.7–25.3) in women with normal cytology and age 29–33 years, and the corresponding RR was 0.65 (95% CI: 0.37–1.14). When stratifying hrHPV-positive women with abnormal cytology into two age groups, no risk difference between the older and younger age group was observed for CIN3+ and CIN2+ (RR CIN3. 1.03; 95% CI: 0.78–1.42; RR CIN2. 0.95; 95% CI: 0.79–1.18).
Figure 3  Cumulative two-year risk of CIN3+ and CIN2+ stratified by cytology and hrHPV status at baseline, adjusted for non-attendance at repeat testing.  
HrHPV = high-risk human papillomavirus; hrHPV+ = positive hrHPV test; hrHPV- = negative hrHPV test; Abnormal cytology = borderline or mild dyskaryosis or worse (≥BMD); CI = confidence interval; CIN = cervical intraepithelial neoplasia (grade 2 or 3 or higher).
CHAPTER 3

Discussion
Implementation of hrHPV testing as a primary screening instrument in cervical screening is still under debate. The presented study enables us to examine three implementation issues in more detail. First, whether hrHPV testing should be offered in combination with cytology or as a sole primary screening instrument. Second, how hrHPV-positive women should be managed. Third, the screening ages at which hrHPV testing would be most beneficial.

In our study, hrHPV testing has a 27.3% higher sensitivity but a 3.1% lower specificity than cytology for detection of CIN3+. For CIN2+, these figures were 31.5% and 2.9%, respectively. These results are in line with other screening studies \(^6\)\(^7\)\(^19\)\(^24\) which have demonstrated that hrHPV testing is superior to cytology in terms of sensitivity but not in terms of specificity. Women with a negative hrHPV test were found to have a very low risk of an underlying or incipient high-grade CIN lesion and their CIN3+ risk is not markedly lower after ascertainment that cytology is normal. Therefore, from a health-economic perspective, cervical screening with a primary, stand-alone hrHPV test seems preferable. Similar recommendations have been made based on the recent cost-effectiveness studies.\(^25\)

The hrHPV testing in primary screening creates a clinical dilemma for the management of hrHPV-positive women. These women are at mildly but significant risk of CIN3+ (13.2%). However, referring all hrHPV-positive women to colposcopy may result in overdiagnosis and overtreatment.\(^8\) In current cytological screening practice, women with a BMD smear, having a CIN3+ risk of 6.4%,\(^11\) are also not immediately referred for colposcopy but are advised to repeat cytology testing after 6 and 18 months.\(^26\)

In the present study, we have used cytology as a triage tool to identify women at high risk for CIN3+ among the hrHPV-positive women. Women with an hrHPV-positive test and abnormal cytology had a CIN3+ risk of 42.2% and obviously need immediate colposcopy. On the other hand, hrHPV-positive women with normal cytology have a low, but still non-negligible CIN3+ risk of 5.2%. This risk is too high to delay follow-up to the next screening round (in the Netherlands 5 years) but too low to refer them for immediate colposcopy. Therefore, hrHPV-positive women with normal cytology at baseline require further triage testing and/or follow-up. In our study, women were retested after 1 year by means of cytology and after 2 years by both cytology and hrHPV. An analysis of the repeat testing results showed that the decision either referral to colposcopy or return to routine screening can be made after 1 year on the basis of one repeat cytological test. The CIN3+ risk after 1-year normal cytology was only 1.6%. This risk is similar to the CIN3+ risk of women with BMD at baseline and normal cytology at 6 and 18 months follow-up (1.2%), which is presently accepted in the Netherlands. Furthermore, the CIN3+ risk of 1.6% is also below the CIN3+ risk threshold proposed by Castle et al\(^27\) (2%) to justify no further follow-up. The CIN3+ risk after 1-year abnormal cytology was 25% and high enough to warrant referral for colposcopy.

Based on the present data, one may ask what the results are of other triage algorithms for hrHPV-positive women. This post hoc analysis on data of the
present study is beyond the scope of this paper but has been presented in a separate paper.\textsuperscript{28}

We observed in our cohort study that the CIN3+ and CIN2+ detection rate in hrHPV-positive women was similar for women invited for cervical screening for the first time (age 29–33 years) and for older women (≥34 years). The same accounts for hrHPV-positive women with abnormal cytology. However, the CIN3+ detection rate in hrHPV-positive women with normal cytology was significantly higher among younger women (29–33) than among older women (≥34). Ronco et al.\textsuperscript{9} found in the hrHPV arm of women between 25 and 34 years a substantially higher proportion of CIN2 lesions than in women ≥35 years. This coincided with an increase in detection of CIN2+ over two screening rounds compared with women ≥35 years. It was argued that under the CIN2+ lesions detected in women younger than 35 years in the first round, a disproportionate number of regressive CIN2 lesions were present. We argue that such a potential age-related overdiagnosis does not occur in women ≥30 years of age.

First, in the POBASCAM study the CIN2+/CIN3+ baseline detection in women over 30 years of age was higher in the hrHPV testing arm than in the cytology arm but over two screening rounds (interval 5 years), the CIN2+/CIN3+ detection rates were similar in both arms.\textsuperscript{5} These data indicate that the increased detection of CIN2+/3+ lesions in the hrHPV arm at baseline in women over 30 years of age does not lead to overdiagnosis of regressive CIN2+ lesions but that the lesions are merely detected earlier and non-regressive, clinically relevant.\textsuperscript{5}

In addition, in present study the CIN3+ and CIN2+ risk was similar in women invited for the first time (29–33 years) and in women ≥34 years. Moreover, in hrHPV-positive women with normal cytology, the CIN3+ risk was higher in women invited for the first time than in older women. A possible explanation is that hrHPV infections detected in women invited for the first time may have persisted for many years before being identified by screening and therefore more likely to have developed into high-grade lesions. These results are in line with published data from the Guanacaste cohort.\textsuperscript{29} In addition, we recently showed that the detection rate of CIN3+ and CIN2+ did not differ between women aged 29–33 years and women ≥34 years.\textsuperscript{30} Moreover, this study indicates that hrHPV testing in women aged 29–33 years does not result in excessive diagnosis of regressive lesions.

**Limitations and strengths of the study**

A limitation of our study is that women with normal cytology were not informed about the hrHPV status at baseline. This concealment was necessary to maximise attendance at repeat testing among hrHPV-negative women with normal cytology. The repeat testing attendance rate in women with normal cytology was 61.8% in hrHPV-negative and 59.7% in hrHPV-positive women. The attendance at repeat testing has been shown to be particularly poor after a cytologically normal test.\textsuperscript{31} The attendance rate of hrHPV-positive women in the present study might have been higher if women had been informed about their hrHPV test result.

We observed a higher percentage of histology reports after referral on the basis of abnormal cytology than after an hrHPV-positive, cytologically normal test result. This difference may be related to anticipated association between biopsy rate and
colposcopic image of the cervix. If adjusted for, the effect of hrHPV testing on CIN3+ will be somewhat higher than the effect reported in this study. In this regard, several studies have indicated that the effect of hrHPV testing will be higher when a blind biopsy is carried out in women with a normal colposcopic impression.\(^3\)

Another limitation of our study may be the use of a subjective test such as cytology as a triage test for hrHPV-positive women. However, Leinonen et al.\(^23\) reported that the influence of knowing the hrHPV results in reading cytology was small. In this context, it is expected that in the near future molecular biomarkers can be used as objective triage tests of hrHPV-positive women. Suitable candidate novel bio-markers such as HPV mRNA,\(^33\) methylation markers\(^34,35\) or genotyping\(^36\) might further enhance the efficacy of screening with hrHPV DNA.

A strong point of our study is that this study is population based and is integrated in the regular screening programme of eligible women aged 29–61 years. The differences in sensitivity between hrHPV screening and cytological screening are slightly overestimated because in the practice of screening some women will not attend repeat testing after an hrHPV-positive test. Nevertheless, a higher attendance at repeat testing is to be expected once the implication of a positive hrHPV test is well communicated to the women and hrHPV screening becomes routine.\(^37\)

**Conclusions**

Although cytology adds little to the reassurance from a negative hrHPV test against high-grade lesions, it is a very useful risk stratifier in hrHPV-positive women and results in a feasible screening algorithm. We showed that repeat cytology testing after 1 year for hrHPV-positive women with normal cytology at baseline is critical for maximising the benefits of primary hrHPV testing in routine cervical cancer screening.

**Acknowledgements**

The study was supported by the Stichting Researchfonds Pathologie Amsterdam and Saltro Laboratory Utrecht. We thank all the staff of the Saltro Laboratory Utrecht for their expertise, commitment, and invaluable support for the VUSA-Screen study. We thank participating GPs, their assistants, the gynaecologists and the thousands of women who have participated in this study. Qiagen (Digene) is acknowledged for providing collection tubes with UCM and HC2 kits.

**Role of the funding source**

The funding source had no role in study design, data collection, data analysis, data interpretation, writing of the report or in decision to publish the article. The corresponding author had full access to all the data in the study and had final responsibility to submit the manuscript for publication.
CHAPTER 3

References

37. Franco EL. Is the UK ready to embrace HPV testing? Lancet Oncol 2009;10:643-44
HUMAN PAPILLOMAVIRUS TESTING FOR THE DETECTION OF HIGH-GRADE CERVICAL INTRAEPITHELIAL NEOPLASIA AND CANCER: FINAL RESULTS OF THE POBASCAM RANDOMISED CONTROLLED TRIAL
CHAPTER 4

ABSTRACT

Background

Human papillomavirus (HPV) testing is more sensitive for the detection of high-grade cervical lesions than is cytology, but detection of HPV by DNA screening in two screening rounds 5 years apart has not been assessed. The aim of this study was to assess whether HPV DNA testing in the first screen decreases detection of cervical intraepithelial neoplasia (CIN) grade 3 or worse, CIN grade 2 or worse, and cervical cancer in the second screening.

Methods

In this randomised trial, women aged 29–56 years participating in the cervical screening programme in the Netherlands were randomly assigned to receive HPV DNA (GP5+/6+-PCR method) and cytology co-testing or cytology testing alone, from January, 1999, to September, 2002. Randomisation (in a 1:1 ratio) was done with computer-generated random numbers after the cervical specimen had been taken. At the second screening 5 years later, HPV DNA and cytology co-testing was done in both groups; researchers were masked to the patient’s assignment. The primary endpoint was the number of CIN grade 3 or worse detected. Analysis was done by intention to screen. The trial is now finished and is registered, number ISRCTN20781131.

Findings

22 420 women were randomly assigned to the intervention group and 22 518 to the control group; 19 999 in the intervention group and 20 106 in the control group were eligible for analysis at the first screen. At the second screen, 19 579 women in the intervention group and 19 731 in the control group were eligible, of whom 16 750 and 16 743, respectively, attended the second screen. In the second round, CIN grade 3 or worse was less common in the intervention group than in the control group (88 of 19 579 in the intervention group vs 122 of 19 731 in the control group; relative risk 0.73, 95% CI 0.55–0.96; p=0.023). Cervical cancer was also less common in the intervention group than in the control group (four of 19 579 in the intervention group vs 14 of 19 731; 0.29, 0.10–0.87; p=0.031). In the baseline round, detection of CIN grade 3 or worse did not differ significantly between groups (171 of 19 999 vs 150 of 20 106; 1.15, 0.92–1.43; p=0.239) but was significantly more common in women with normal cytology (34 of 19 286 vs 12 of 19 373; 2.85, 1.47–5.49; p=0.001). Furthermore, significantly more cases of CIN grade 2 or worse were detected in the intervention group than in the control group (267 of 19 999 vs 215 of 20 106; 1.25, 1.05–1.50; p=0.015). In the second screen, fewer HPV16-positive CIN grade 3 or worse were detected in the intervention group than in the control group (17 of 9481 vs 35 of 9354; 0.48, 0.27–0.85; p=0.012); detection of non-HPV16-positive CIN grade 3 or worse did not differ between groups (25 of 9481 vs 25 of 9354; 0.99, 0.57–1.72; p=1.00). The cumulative detection of CIN grade 3 or worse and CIN grade 2 or worse did not differ significantly between study arms, neither for the whole study group (CIN grade 3 or worse: 259 of 19 999 vs 272 of 20 106; 0.96, 0.81–1.14, p=0.631; CIN grade 2 or worse:
CHAPTER 4

427 of 19 999 vs 399 of 20 106; 1.08, 0.94–1.24; p=0.292), nor for subgroups of women invited for the first time (CIN grade 3 or worse in women aged 29–33 years: 102 of 3139 vs 105 of 3128; 0.97, 0.74–1.27; CIN grade 2 or worse in women aged 29–33 years: 153 of 3139 vs 151 of 3128; 1.01, 0.81–1.26; CIN grade 3 or worse in women aged 34–56 years: 157 of 16 860 vs 167 of 16 978; 0.95, 0.76–1.18; CIN grade 2 or worse in women aged 34–56 years: 274 of 16 860 vs 248 of 16 978; 1.11, 0.94–1.32).

Interpretation
Implementation of HPV DNA testing in cervical screening leads to earlier detection of clinically relevant CIN grade 2 or worse, which when adequately treated, improves protection against CIN grade 3 or worse and cervical cancer. Early detection of high-grade cervical lesions caused by HPV16 was a major component of this benefit. Our results lend support to the use of HPV DNA testing for all women aged 29 years and older.
CHAPTER 4

Introduction
Infection with high-risk types of human papillomavirus (HPV) has a causal role in the development of cervical cancer.\(^1,2\) This link has stimulated the development of HPV DNA testing, which might be useful in primary cervical screening.\(^3,4\) Furthermore, prophylactic HPV16 and HPV18 vaccines have been developed and introduced in many countries as a primary prevention method.\(^5,6\)

Randomised controlled screening trials of HPV DNA testing \(^7-11\) have shown a decreased detection of high-grade cervical lesions at the second screening round compared with cytology alone. Although the screening protocols, study endpoints, and interval between screening rounds varied in these trials, the consistent results suggest that HPV DNA testing offers better protection against high-grade cervical lesions in second screening rounds than do cytology-based screening methods. Only one study\(^10\) was large enough to also show protection against cervical cancer in the second screening round.

We present the final results of the POpulation-Based SCreening study AMsterdam (POBASCAM) trial, a population-based, randomised controlled trial. Our main goal was to assess whether HPV DNA testing decreases detection of cervical intraepithelial neoplasia (CIN) grade 3 or worse, of CIN grade 2 or worse, and of cervical cancer, in the second screening round. Additionally, to assess the most appropriate age at which HPV DNA testing should start, we analysed women invited for the first time (aged 29–33 years) and older women (34–56 years) separately. We also assessed how detection of high-grade lesions in two screening rounds was associated with particular HPV genotypes.

Methods
Study design and participants
Patients were enrolled between January, 1999, and September, 2002, as part of the nationwide cervical screening programme. In the Netherlands, women are invited for cervical cancer screening at 5 year intervals starting in the year when they reach age 30 years and ending in the year when they reach age 60 years. The design, methods, and baseline results of the trial have been described.\(^7,12\) Detection of neoplasia or cancer in the first 48 months was classed as detected in the first screening round and those detected during 48–108 months were classed as detected in the second screening round. The cutoff after 48 months was used because women in the Netherlands are invited for a new screen in the year they reach 30 years, 35 years, 40 years, and so on. Therefore, the actual screening interval between invited smears is between 4 and 6 years. Women who had a history of CIN grade 2 or worse, had abnormal cytology in the preceding 2 years, or who had had a hysterectomy were excluded. Women aged 57 years or older at baseline were also excluded because they would not routinely receive a smear in the second round. All participants provided written informed consent. The trial was approved by both the Medical Ethics Committee of VU University Medical Centre and the Ministry of Public Health (The Hague, Netherlands).
CHAPTER 4

Randomisation and masking
LR randomly assigned women to the intervention or control groups in a 1:1 ratio with computer-generated random numbers after the cervical specimen had been taken and administrative data entered into the central study database. Neither the molecular technicians nor the cytotechnicians had access to the central study database, and so were unaware of a patient’s assignment. Women in the intervention group were given their HPV DNA and cytology result and managed accordingly. For the control group, the patients and all medical personnel were masked to the HPV DNA test results. At the second screening round, women in both groups were managed according to their newly obtained HPV DNA and cytological test results. In both groups pathologists were aware of the cytology result but not of the HPV result. CIN (CIN grade 1 or worse) biopsies were reviewed by two experienced cervical pathologists (FjvK and LR) who were masked to the HPV and cytology results.

Procedures
Women were screened at baseline with either combined HPV DNA testing and cytology or cytology alone. At the second screening round 5 years later, all women were screened with both HPV DNA testing and cytology so that we could detect high-grade CIN and cancer cases in the control group that we would otherwise have missed with cytology alone.

Conventional cytological smears were taken with a Cervex-Brush (Rovers, Oss, Netherlands) or a cytobrush. The brush was placed in a vial containing 5 mL phosphate-buffered saline for HPV DNA testing. Cytology results were read according to the CI SOE-A classification, which can be roughly converted to the 2001 Bethesda system. Cytological results were grouped as normal, borderline or mild dyskaryosis, or moderate dyskaryosis or worse. In the 2001 Bethesda system, borderline

Figure 1 Management of women in the intervention group at the first screening round. In the second screening round women in both groups were managed in the same way as the intervention group were in the first screening round. BMD=borderline or mild dyskaryosis. HPV=human papillomavirus.
or mild dyskaryosis corresponds to atypical squamous cells of unknown clinical significance, atypical squamous cells and cannot rule out high-grade squamous intraepithelial lesions, low-grade squamous intraepithelial lesions, or atypical glandular cells. Moderate dyskaryosis or worse corresponds to both squamous and glandular high-grade intraepithelial lesions.

HPV DNA testing was done by general primer (GP5+/6+) PCR enzyme immunoassay,\textsuperscript{15} which detects 14 high-risk types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). Positive specimens were typed by reverse line blotting.\textsuperscript{16}

Figure 1 shows how patients were managed, in accordance with Dutch guidelines,\textsuperscript{13,17} and has been described in detail previously.\textsuperscript{7} Of the women who were referred for colposcopy, colposcopy-directed biopsies of suspicious parts of the cervix were taken for histological examination, according to standard procedures in the Netherlands.\textsuperscript{18} Colposcopies were not registered, therefore, data of registered biopsy rates were used to estimate the number of gynaecological tests.

Histology was examined locally and classified as normal, CIN grade 1, 2, 3, or invasive cancer, according to international criteria.\textsuperscript{19,20} Glandular intraepithelial neoplasia grade 1, 2, and 3 were included in their respective CIN categories. In the analyses presented here, the original diagnoses were used. Cytology and histology results were retrieved from the nationwide registry of histopathology and cytopathology results and, when necessary, from individual laboratory records. The reviewed histological results for biopsies of CIN grade 1 or worse are shown in the appendix.

The primary outcome measure was histologically confirmed CIN grade 3 or worse, with a primary endpoint of the number of CIN grade 3 or worse detected. The secondary outcome measures were cervical cancer and CIN grade 2 or worse. Cervical cancer included squamous cell carcinoma, adenocarcinoma, and adenosquamous cell carcinoma; adenocarcinoma in situ were included in the CIN grade 3 group.

Statistical analysis
The main analyses included all randomly assigned women and were done by intention to screen. The overall number of CIN grade 3 or worse identified in each group was calculated for the first screening round, the second screening round, and the two rounds combined. For the second round, we included only those women who did not have CIN grade 2 or worse at baseline and who were eligible for subsequent screening, because those with CIN 2 or worse were managed according to current treatment protocols. When enrolment was complete all women had the opportunity of 108 months of follow-up. Events after 108 months were not included. Because HPV DNA prevalence decreases with age and CIN grade 2 or worse can spontaneously regress in young women, we analysed women invited for the first time (aged 29–33 years) and older women (34–56 years) separately to assess the most appropriate age at which HPV DNA testing should start.

Reason for smear test was not recorded in the nationwide registry of histopathology and cytopathology results. The contribution of opportunistic screening in women with a negative screening test at baseline was estimated by the proportion of women who had an additional smear before receiving a new invitation after 5 years.
Differences in detection between the groups were calculated with Fisher’s exact test. All p values are two-sided. Analyses were done with SPSS version 12.0.

A study size of 44 000 was calculated to be sufficiently large to detect (with 80% power) a significant difference in the number of lesions CIN grade 3 or worse between groups at the second screen after borderline or mild dyskaryosis at baseline. The sample size was also sufficient to show a decrease at the second round of CIN grade 3 or worse lesions in women in the intervention group who had normal cytology at baseline compared with women in the control group who had normal cytology at baseline. For the power calculations, we assumed that 84.5% of the baseline smears were cytologically normal, that 14% of the baseline smears were...
CHAPTER 4

borderline or mild dyskaryosis, and that 4% of the normal and 15% of the borderline or mild dyskaryosis smears were positive for HPV DNA. The prevalences of CIN grade 3 or worse in the second screen were assumed to be 0.4% and 0.9% for women with normal cytology and borderline or mild dyskaryosis, respectively. Furthermore, the relative risk of HPV DNA for CIN grade 3 or worse in the subsequent round was assumed to be 13, which is the lower bound of the 95% CI reported in a previous study, which provided a conservative estimate for the required sample size. The study was not powered to detect a difference in CIN grade 3 or worse at baseline. This trial is registered as an International Standard Randomised Controlled Trial, ISRCTN20781131.

Table 1 Cervical intraepithelial neoplasia and cervical cancers at baseline screen and subsequent screen.

<table>
<thead>
<tr>
<th>Intervention group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline screen</strong></td>
<td></td>
</tr>
<tr>
<td>Inadequate cytology</td>
<td></td>
</tr>
<tr>
<td>HPV DNA positive</td>
<td>2 0 1 0 0 0</td>
</tr>
<tr>
<td>HPV DNA negative</td>
<td>31 1 0 0 0 0</td>
</tr>
<tr>
<td>Normal cytology</td>
<td></td>
</tr>
<tr>
<td>HPV DNA positive</td>
<td>724 48 31 29 2</td>
</tr>
<tr>
<td>HPV DNA negative</td>
<td>18562 218 7 3 0</td>
</tr>
<tr>
<td>Borderline or mild dyskaryosis</td>
<td></td>
</tr>
<tr>
<td>HPV DNA positive</td>
<td>185 29 24 34 3</td>
</tr>
<tr>
<td>HPV DNA negative</td>
<td>330 28 3 3 1</td>
</tr>
<tr>
<td>Moderate dyskaryosis or worse</td>
<td></td>
</tr>
<tr>
<td>HPV DNA positive</td>
<td>146 11 26 87 5</td>
</tr>
<tr>
<td>HPV DNA negative</td>
<td>19 6 4 3 1</td>
</tr>
<tr>
<td>Total</td>
<td>19999 341 96 159 12</td>
</tr>
<tr>
<td><strong>Subsequent round</strong></td>
<td></td>
</tr>
<tr>
<td>Inadequate cytology</td>
<td></td>
</tr>
<tr>
<td>HPV DNA positive</td>
<td>0 0 0 0 0 0</td>
</tr>
<tr>
<td>HPV DNA negative</td>
<td>21 0 0 0 0 0</td>
</tr>
<tr>
<td>No HPV DNA test</td>
<td>21 2 0 0 0 0</td>
</tr>
<tr>
<td>Normal cytology</td>
<td></td>
</tr>
<tr>
<td>HPV DNA positive</td>
<td>284 18 10 9 1</td>
</tr>
<tr>
<td>HPV DNA negative</td>
<td>8941 98 6 1 0</td>
</tr>
<tr>
<td>No HPV DNA test</td>
<td>7025 134 14 1 1</td>
</tr>
<tr>
<td>Borderline or mild dyskaryosis</td>
<td></td>
</tr>
<tr>
<td>HPV DNA positive</td>
<td>67 20 9 12 0</td>
</tr>
<tr>
<td>HPV DNA negative</td>
<td>129 7 1 2 0</td>
</tr>
<tr>
<td>No HPV DNA test</td>
<td>164 23 14 7 0</td>
</tr>
<tr>
<td>Moderate dyskaryosis or worse</td>
<td></td>
</tr>
<tr>
<td>HPV DNA positive</td>
<td>31 3 5 20 0</td>
</tr>
<tr>
<td>HPV DNA negative</td>
<td>8 2 3 1 0</td>
</tr>
<tr>
<td>No HPV DNA test</td>
<td>59 10 10 26 2</td>
</tr>
<tr>
<td>No screening test*</td>
<td>2829 52 0 5 0</td>
</tr>
<tr>
<td>Total</td>
<td>19579 369 72 84 4</td>
</tr>
<tr>
<td><strong>Both rounds</strong></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>19999 710 168 243 16</td>
</tr>
</tbody>
</table>

Cases detected by opportunistic screening are also included. CIN3=cervical intraepithelial neoplasia grade 3. CIN2=cervical intraepithelial neoplasia grade 2. CIN0/1=cervical intraepithelial neoplasia grade 0 or 1.*Women who had no second round smear recorded.
CHAPTER 4

Role of the funding source
The sponsors had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results
Figure 2 shows the trial profile. The median age at recruitment was 40.0 years (IQR 34.0–49.0 years) in both groups. For women without CIN grade 2 or worse, the median time since last cytological results was 5.0 years (4.4–5.5). Compliance to follow-up testing (at least one repeat smear) was higher in the control group (477 of 527 patients [91%]) than in the intervention group (1007 of 1239 patients [81%]). The difference in compliance was related to baseline cytology. In the intervention group, 456 of 515 (89%) women with borderline or mild dyskaryosis at baseline, and 551 of 724 (76%) with normal cytology and a positive high risk HPV test complied with follow-up.

Attendance for the second screen was not significantly different between groups (16 750 of 19 579 [86%] patients in the intervention group vs 16 743 of 19 731 [85%] in the control group). Compliance to follow-up testing did not differ significantly between groups—539 (84%) of 644 patients in the intervention group and 564 (85%) of 667 in the control group complied. Attendance at the second screen of cytologically negative, HPV DNA positive women was much the same in the intervention and control groups (553 of 724 [76%] women vs 588 of 766 [77%] women). 2424 of 18 562 (13%) women who were negative at baseline in the intervention group and 2542 of 19 373 (13%) women who were negative at baseline in the control group had an additional smear before receiving a new invitation after 5 years (opportunistic screening). Table 1 shows the number of cervical lesions by study group, in relation to cytological and HPV DNA results at the first and second screens. Positive HPV DNA (masked in the control group) and abnormal cytology at baseline were much the same for the intervention and control groups.12 The number of CIN grade 0 or 1 detected at the first and second screens was similar in the intervention and control groups. Most CIN grade 0 or 1 were detected after a negative first screen whereas most CIN grade 2 or 3, and cancer cases were detected after a positive first screen.

In the intervention group, 12 cancers (one adenocarcinoma and 11 squamous cell carcinomas) and five adenocarcinomas in situ were identified in the first screen; in the second screen, four cancers (one adenocarcinoma and three squamous cell carcinomas) and one adenocarcinoma in situ were identified. In the control group, six cancers (two adenocarcinomas and four squamous cell carcinomas) and five adenocarcinomas in situ were identified at the first screen; 14 cancers (two adenocarcinoma and 12 squamous cell carcinomas) and four adenocarcinomas in situ were identified in the second screen.

Table 2 shows the number of high-grade cervical lesions in each group at first and second screens. For the first screen, detection of CIN grade 3 or worse did not differ significantly in the intervention group compared with the control group (p=0.239). Additionally, the number of cervical cancer cases did not differ significantly between the intervention and control groups (p=0.166). However, significantly more
CIN grade 2 or worse were detected at baseline in the intervention group than in the control group (p=0.015). In the second screen, fewer CIN grade 3 or worse and cervical cancers were recorded in the intervention group than in the control group (p=0.023 and p=0.031, respectively). However, there was no significant difference in detection of CIN grade 2 or worse in the intervention group compared with the control group in the second screen (p=0.234). When combining both rounds, the detection rates in the intervention and control groups were much the same for CIN grade 2 or worse, CIN grade 3 or worse, and for cervical cancer (table 2). However, over two screening rounds, a third more CIN grade 2 were recorded in the intervention group than in the control group (table 2).

In the first screen, two cancers in the intervention group were missed by HPV DNA testing but had abnormal cytology (in patients aged 32 years with FIGO IB1 and aged 54 years with FIGO IA1). Three cervical cancers were missed by cytology testing (one patient aged 36 years and two patients aged 44 years, all three with FIGO IB1), but tested positive for HPV DNA. Five cervical cancers were diagnosed after borderline or mild dyskaryosis cytology (in patients aged 30 years with FIGO IB1, 32 years with IB1, 35 years with IB1, 40 years FIGO with 1B1, and 50 years with IA1), which could have been missed if patients were not followed up correctly. Only one woman who developed cervical cancer (aged 59 years with FIGO IB1) had a negative HPV DNA test at the second screen. She was also HPV negative in the first screen.

**Table 2 Occurrences of high-grade disease**

<table>
<thead>
<tr>
<th></th>
<th>Intervention group</th>
<th>Control group</th>
<th>Intervention vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n[%; 95% CI])</td>
<td>(n[%; 95% CI])</td>
<td>Risk difference (95% CI)</td>
</tr>
<tr>
<td><strong>Baseline screen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>12 (0.06%; 0.03-0.11)</td>
<td>6 (0.03%; 0.01-0.07)</td>
<td>0.03% [-0.01 to 0.07]</td>
</tr>
<tr>
<td>CIN3 or worse</td>
<td>171 (0.86%; 0.73-1.00)</td>
<td>150 (0.75%; 0.63-0.88)</td>
<td>0.11% [-0.07 to 0.28]</td>
</tr>
<tr>
<td>CIN3</td>
<td>159 (0.80%; 0.68-0.93)</td>
<td>144 (0.72%; 0.61-0.85)</td>
<td>0.08% [-0.09 to 0.25]</td>
</tr>
<tr>
<td>CIN2 or worse</td>
<td>267 (1.34%; 1.18-1.51)</td>
<td>215 (1.07%; 0.93-1.22)</td>
<td>0.27% [0.05 to 0.48]</td>
</tr>
<tr>
<td>CIN2</td>
<td>96 (0.48%; 0.39-0.59)</td>
<td>65 (0.32; 0.25-0.41)</td>
<td>0.16% [0.03 to 0.28]</td>
</tr>
<tr>
<td><strong>Subsequent screen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>4 (0.02%; 0.01-0.06)</td>
<td>14 (0.07%; 0.04-0.12)</td>
<td>-0.05% [-0.09 to -0.01]</td>
</tr>
<tr>
<td>CIN3 or worse</td>
<td>88 (0.45%; 0.36-0.56)</td>
<td>122 (0.62%; 0.52-0.74)</td>
<td>-0.17% [-0.31 to -0.03]</td>
</tr>
<tr>
<td>CIN3</td>
<td>84 (0.43%; 0.34-0.53)</td>
<td>108 (0.55%; 0.45-0.66)</td>
<td>-0.12% [-0.26 to 0.02]</td>
</tr>
<tr>
<td>CIN2 or worse</td>
<td>160 (0.82%; 0.70-0.96)</td>
<td>184 (0.93; 0.81-1.08)</td>
<td>-0.12% [-0.30 to 0.07]</td>
</tr>
<tr>
<td>CIN2</td>
<td>72 (0.37%; 0.29-0.47)</td>
<td>62 (0.31%; 0.24-0.41)</td>
<td>0.05% [-0.06 to 0.17]</td>
</tr>
<tr>
<td><strong>Both screens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>16 (0.08%; 0.05-0.13)</td>
<td>20 (0.10%; 0.06-0.16)</td>
<td>-0.02% [-0.08 to 0.04]</td>
</tr>
<tr>
<td>CIN3 or worse</td>
<td>259 (1.30%; 1.15-1.46)</td>
<td>272 (1.35%; 1.20-1.52)</td>
<td>-0.06% [-0.28 to 0.17]</td>
</tr>
<tr>
<td>CIN3</td>
<td>243 (1.22%; 1.07-1.38)</td>
<td>252 (1.25%; 1.11-1.42)</td>
<td>-0.04% [-0.25 to 0.18]</td>
</tr>
<tr>
<td>CIN2 or worse</td>
<td>427 (2.14%; 1.94-2.35)</td>
<td>399 (1.98%; 1.80-2.19)</td>
<td>0.15% [-0.13 to 0.43]</td>
</tr>
<tr>
<td>CIN2</td>
<td>168 (0.84%; 0.72-0.98)</td>
<td>127 (0.63%; 0.53-0.75)</td>
<td>0.21% [0.04 to 0.38]</td>
</tr>
</tbody>
</table>

*n=19 999 in the intervention group, n=20 106 in the control group. †n=19 579 in the intervention group, n=19 731 in the control group. CIN3=cervical intraepithelial neoplasia grade 3. CIN2=cervical intraepithelial neoplasia grade 2.
For women positive for high-risk HPV, with normal cytology at first screen, the number of high-grade lesions differed between the intervention and control group in both first and second screens (Table 3). These women also have a substantial risk of CIN grade 3 or worse in the second screen in the intervention and control groups. At first screen, CIN grade 3 or worse was not significantly different between the intervention and control groups for women with moderate dyskaryosis or worse (96 of 165 women vs 103 of 179; RR 1.01, 95% CI 0.84–1.21; p=0.9) and for women with borderline or mild dyskaryosis (41 of 515 women vs 35 of 527; 1.20, 0.78–1.85; p=0.5). However, more CIN grade 3 or worse lesions were detected in women with normal cytology in the intervention group than in the control group (34 of 19 286 patients vs 12 of 19 373; 2.85, 1.47–5.49; p=0.001). At the second screen, excluding women who had CIN grade 2 or worse, or who had hysterectomy at baseline, numbers of CIN grade 3 or worse were not significantly different between study groups in women with

<table>
<thead>
<tr>
<th>Table 3 Cervical cancers and high grade intraepithelial neoplasia by test results in the first and second screen rounds.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First screen</strong></td>
</tr>
<tr>
<td><strong>Intervention group</strong></td>
</tr>
<tr>
<td>Inadequate cytology</td>
</tr>
<tr>
<td>HPV positive</td>
</tr>
<tr>
<td>HPV negative</td>
</tr>
<tr>
<td>Normal cytology</td>
</tr>
<tr>
<td>HPV negative</td>
</tr>
<tr>
<td>Borderline or mild dyskaryosis HPV positive</td>
</tr>
<tr>
<td>HPV negative</td>
</tr>
<tr>
<td>Moderate dyskaryosis or worse HPV positive</td>
</tr>
<tr>
<td>HPV negative</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>
| **CIN3**=cervical intraepithelial neoplasia grade 3. **CIN2**=cervical intraepithelial neoplasia grade 2.
baseline moderate dyskaryosis or worse (three of 39 vs six of 48; 0.61, 0.16–2.30; p=0.7) and in women with baseline borderline or mild dyskaryosis (11 of 443 vs 15 of 461; 0.75, 0.35–1.62; p=0.6). However, CIN grade 3 or worse differed between groups in women with normal cytology at the first screen (74 of 19 066 vs 101 of 19 196; 0.74, 0.55–0.99; p=0.049).

The cumulative number of CIN grade 3 or worse lesions detected over two screening rounds was much the same for both study groups in women with moderate dyskaryosis or worse at baseline (99 of 165 vs 109 of 179; 0.99, 0.83–1.17), women with borderline or mild dyskaryosis at baseline (52 of 515 vs 50 of 527; 1.06, 0.74–1.54), and women with normal cytology at baseline (108 of 19 286 vs 113 of 19 373; 0.96, 0.74–1.25). Furthermore, the cumulative number of CIN lesions of grade 3 or worse over two screening rounds was significantly lower for women who tested negative for HPV DNA at baseline in the intervention group than for those who had normal cytology at baseline in the control group (50 of 18 942 vs 113 of 19 373; 0.45, 0.32–0.63).

Figure 3

**Figure 3** Histological results for women aged 29–33 years (A) and 34–56 years (B), by study group and screening round. CIN2=cervical intraepithelial neoplasia grade 2. CIN3=cervical intraepithelial neoplasia grade 3. I=intervention. C=control.
Use of CIN grade 2 or worse as an outcome measure gave much the same result (107 of 18 942 vs 177 of 19 373; 0.62, 0.49–0.79). Use of reviewed histological results did not give different results (appendix).

373 of 3139 (12%) women aged 29–33 years in the intervention group and 379 of 3128 (12%) women aged 29–33 years in the control group had HPV DNA at baseline (p=0.786). By contrast, HPV at baseline was detected in 684 of 16 860 (4%) women in the intervention group aged 34–56 years and in 739 of 16 978 (4%) of women in the control group aged 34–56 years (p=0.176). 152 of 3139 (5%) women in the intervention group aged 29–33 years at baseline had biopsy samples taken, compared with 129 of 3128 (4%) women in the control group aged 29–33 years. In women aged 34–56 years, baseline screen biopsy samples were taken from 456 of 16 860 (3%) patients in the intervention group and 407 of 16 978 (2%) in the control group. 712 of 3136

Table 4 HPV types at baseline and subsequent screens and number of HPV-positive CIN grade 3 or worse and CIN grade 2 or worse detected.

<table>
<thead>
<tr>
<th>Total number of women</th>
<th>CIN3+</th>
<th>CIN2+</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intervention group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>First screen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV16</td>
<td>333 (32%)</td>
<td>101 (63%)</td>
</tr>
<tr>
<td>Inadequate</td>
<td>1 (0%)</td>
<td>0</td>
</tr>
<tr>
<td>Normal</td>
<td>202 (19%)</td>
<td>23 (14%)</td>
</tr>
<tr>
<td>BMD</td>
<td>55 (5%)</td>
<td>22 (14%)</td>
</tr>
<tr>
<td>&gt;BMD</td>
<td>75 (7%)</td>
<td>56 (35%)</td>
</tr>
<tr>
<td>Other HPV type(s)</td>
<td>724 (68%)</td>
<td>59 (37%)</td>
</tr>
<tr>
<td>Inadequate</td>
<td>1 (0%)</td>
<td>0</td>
</tr>
<tr>
<td>Normal</td>
<td>522 (49%)</td>
<td>8 (5%)</td>
</tr>
<tr>
<td>BMD</td>
<td>130 (12%)</td>
<td>15 (9%)</td>
</tr>
<tr>
<td>&gt;BMD</td>
<td>71 (7%)</td>
<td>36 (23%)</td>
</tr>
<tr>
<td><strong>Second screen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV16</td>
<td>100 (26%)</td>
<td>17 (40%)</td>
</tr>
<tr>
<td>Normal</td>
<td>70 (18%)</td>
<td>6 (14%)</td>
</tr>
<tr>
<td>BMD</td>
<td>19 (5%)</td>
<td>4 (10%)</td>
</tr>
<tr>
<td>&gt;BMD</td>
<td>11 (3%)</td>
<td>7 (17%)</td>
</tr>
<tr>
<td>Other HPV type</td>
<td>282 (74%)</td>
<td>25 (60%)</td>
</tr>
<tr>
<td>Normal</td>
<td>214 (56%)</td>
<td>4 (10%)</td>
</tr>
<tr>
<td>BMD</td>
<td>48 (13%)</td>
<td>8 (19%)</td>
</tr>
<tr>
<td>&gt;BMD</td>
<td>20 (5%)</td>
<td>13 (31%)</td>
</tr>
<tr>
<td><strong>Control group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Second round</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV16</td>
<td>99 (25%)</td>
<td>35 (58%)</td>
</tr>
<tr>
<td>Normal</td>
<td>53 (14%)</td>
<td>7 (12%)</td>
</tr>
<tr>
<td>BMD</td>
<td>24 (6%)</td>
<td>8 (13%)</td>
</tr>
<tr>
<td>&gt;BMD</td>
<td>22 (6%)</td>
<td>20 (33%)</td>
</tr>
<tr>
<td>Other HPV type</td>
<td>293 (75%)</td>
<td>25 (42%)</td>
</tr>
<tr>
<td>Inadequate</td>
<td>2 (1%)</td>
<td>0</td>
</tr>
<tr>
<td>Normal</td>
<td>219 (56%)</td>
<td>7 (12%)</td>
</tr>
<tr>
<td>BMD</td>
<td>53 (14%)</td>
<td>8 (13%)</td>
</tr>
<tr>
<td>&gt;BMD</td>
<td>19 (5%)</td>
<td>10 (17%)</td>
</tr>
</tbody>
</table>

Data are n [%]. CIN3+=cervical intraepithelial neoplasia grade 3 or worse. CIN2+=cervical intraepithelial neoplasia grade 2 or worse. BMD=borderline or mild dyskaryosis.
(23%) women aged 29–33 years in the intervention group had repeat screening test or histology, or both, after the baseline round compared with 554 of 3128 (18%) in the control group. In women aged 34–56 years, follow-up was 3100 of 16 860 (18%) in the intervention group and 2887 of 16 978 (17%) in the control group. If women with a negative first screen were not counted in the numerator, follow-up among women aged 29–33 years was 355 of 3139 (11%) in the intervention group and 172 of 3128 (5%) in the control group, for women aged 34–56 years follow-up was 843 of 16 860 (5%) in the intervention group and 497 of 16 978 (3%) in the control group. For the two screening rounds combined, the two study groups did not differ significantly for CIN grade 3 or worse and CIN grade 2 or worse for both women aged 29–33 and those aged 34–56 (figure 3). The ratio of CIN grade 3 or worse detection between the intervention and control groups was 0.97 (102 of 3139 vs 105 of 3128; 95% CI 0.74–1.27) for women aged 29–33 years and 0.95 (157 of 16 860 vs 167 of 16 978; 0.76–1.18) for women aged 34–56 years. Ratios for CIN grade 2 or worse were 1.01 (153 of 3139 vs 151 of 3128; 0.81–1.26) and 1.11 (274 of 16 860 vs 248 of 16 978; 0.94–1.32) for women aged 29–33 years and 34–56 years, respectively.

Finally, we compared the number of CIN grade 3 or worse and CIN grade 2 or worse at first and second screens for HPV16-positive and HPV16-negative women (table 4). We only included women with a valid HPV DNA test result. Individual HPV types other than HPV16 were not analysed separately because of their low prevalence in cervical lesions. For the first screen in the intervention group 101 (59%) of 171 of the CIN grade 3 worse and 135 (51%) of 267 of the CIN grade 2 or worse lesions were HPV16 positive. In women with normal cytology, 23 (68%) of 34 of CIN lesions of grade 3 or worse were HPV16 positive and in women with abnormal cytology, 78 (57%) of 137 of CIN grade 3 or worse lesions were HPV16 positive. In the second screen, fewer HPV16-positive CIN grade 3 or worse were detected in the intervention group than in the control group (17 of 9481 vs 35 of 9354; RR 0.48, 95% CI 0.27–0.85; p=0.012) whereas detection of non-HPV16-positive CIN grade 3 or worse did not differ (25 of 9481 vs 25 of 9354; 0.99, 0.57–1.72; p=1.00). The proportion of HPV16 in HPV-positive CIN grade 3 or worse and grade 2 or worse lesions was also lower in the intervention group than in the control group but the positivity ratio was not significantly different for either CIN grade 3 or worse (17 of 42 vs 35 of 60, 0.69; 0.45–1.06; p=0.08) or for CIN grade 2 or worse (23 of 66 vs 42 of 92; 0.76, 0.51–1.14; p=0.17).
Panel: Research in context
Systematic review
This trial was designed in 1997, when no data about performance of HPV testing in population-based screening existed. The design was based on several findings. First, HPV prevalence data obtained by general primer PCR in women with normal cervix, cervical intraepithelial neoplasia (CIN) grade 1–3 and cervical cancer led to the hypothesis that HPV testing by general primer PCR could be used for cervical cancer screening. After improvement of our first generation general primer PCR by elongation of the primers and addition of an easy readout, we applied this GP5+/6+ general primer PCR in case-control studies, showing that high-risk HPV was present in nearly all cervical carcinomas. Analysis of normal cervical scrapings of women who later developed cervical cancer showed that the same HPV type as that in the carcinoma could be found in the preceding normal smear. A subsequent prospective study on a gynaecological outpatient population showed that the presence of a persistent HPV infection in women with an abnormal smear was associated with progressive CIN disease. For the design of the POBASCAM trial the Dutch health council, an independent advisory body of the government, required that the study be done in the setting of the nationwide screening programme, which has a screening interval of 5 years, which is why the results are only now available.

Interpretation
The final results accord with those of similar randomised controlled trials that used shorter screening intervals, such as Swedscreen, ARTISTIC, and NTCC, as well as those of our interim analysis, showing that HPV testing significantly reduces detection of CIN grade 3 or worse in the second screening round relative to conventional cytology. Additionally, as was the case in the NTCC trial, the final POBASCAM data also show that HPV screening protects against cervical cancer better than does cytology alone. By contrast with other studies, CIN was diagnosed by pathologists in daily routine practice rather than by a reviewed diagnosis, to study the performance of HPV screening in a routine population-based setting. Review of CIN diagnoses did not affect the results of the study. As such, the study can be classed as an implementation study. Furthermore, the long screening interval as used in POBASCAM allows assessment of whether a cervical lesion is persistent or regressive. Our study also lends support to the notion that HPV screening does not have to be postponed until patients reach age 35 years but can be implemented at age 30 years because the cumulative detection of CIN grade 3 or worse and CIN grade 2 or worse across two screening rounds does not differ between women aged 29–33 years and women 34 years or older.
Discussion

Our results showed that fewer CIN lesions of grade 3 or worse and cervical cancer were detected during second screens in women who were screened for HPV DNA in combination with cytology at first screen than those who had cytology alone. Furthermore, the value of HPV DNA testing is lent support by the finding that women who tested negative for HPV DNA in the first screen had significantly fewer CINs of grade 3 or worse detected over two screening rounds than did women with normal cytology at baseline. These findings accord with previously reported data. However, our study has longer follow-up than other studies did because the screening interval in the Netherlands is longer than that in other countries (5 years vs 3 years). This difference is important because it enables us to assess whether a cervical lesion is persistent or regressive. Our study also suggests that HPV screening does not have to be postponed until age 36 years or older but can be started at age 30 years because the cumulative two screen detection of CIN of grade 3 or worse and grade 2 or worse did not differ between women aged 29–33 years and women older than 33 years. Moreover, we showed that early detection of high-grade cervical lesions caused by HPV16 was a major component of the benefit of adding high-risk HPV testing to cytology.

The interim analysis of 17,155 women from this trial did not show a significant difference between the intervention and control groups in the number of cervical cancers detected at the second screening round. Our final analysis of the whole population was large enough to identify a significant difference in the number of cervical cancers in the intervention group versus the control group at the second screen. Such an effect on cervical cancer in the second screening round because of HPV DNA testing has only been shown in one previous trial. The investigators reported no cervical cancer in the second screen in 47,369 women screened by HPV DNA testing, whereas nine cervical cancers were recorded in 47,001 women in the control group. Reduced mortality from cervical cancer because of primary HPV DNA testing was also shown in a trial in India. 34 deaths from cervical cancer occurred in the HPV DNA testing group (34,126 women) compared with 64 in the control group (31,488 women).

The decreased relative risks of CIN grade 3 or worse and cervical cancer in the second screening round occurred after an increase in relative detection of CIN grade 2 or worse in the baseline screen in the intervention group compared with the control group. By contrast with our interim findings, we failed to show a significant difference in the detection of CIN grade 3 or worse in the first screen in the whole study population. However, our study was powered to detect a significant difference in the number of CIN grade 3 or worse at the second round rather than at the first screen. Also, in some other randomised trials no significant difference in detection of CIN grade 3 or worse at the first screen occurred after HPV DNA testing, despite decreased detection of CIN grade 3 or worse in the second screening round. When we stratified our analysis on the basis of baseline cytology, we did not note a significant difference in the occurrence of CIN grade 3 or worse in women with borderline or mild dyskaryosis or moderate dyskaryosis or worse but report a significant difference...
in the number of CIN grade 3 or worse in women with normal cytology. The latter finding matches our expectations because no additional testing was recommended for women in the control group with baseline normal cytology. The extra CIN grade 3 or worse cases detected by HPV DNA testing compared with cytology are the main benefit of using HPV DNA testing for primary screening. The failure to detect a relative difference in the occurrence of CIN grade 3 or worse could be related to the decision to change the cytollogical classification criteria in 1996, whereas the study was powered by cytolical results collected earlier. Notably, the use of more stringent criteria for borderline or mild dyskaryosis and moderate dyskaryosis or worse resulted in a decrease of borderline or mild dyskaryosis in the Netherlands, from 11% in 1999 to 3% in 2004. This explains the low occurrence of CIN grade 3 or worse in women with borderline or mild dyskaryosis compared with other studies. The cumulative number of women with CIN grade 3 or worse and grade 2 or worse over both screening rounds did not differ substantially between study groups, which lends support to the idea that HPV DNA testing leads to earlier detection of clinically relevant high-grade lesions that, with the exception of a small number of CIN grade 2 lesions, do not regress.

An important issue is the age at which HPV DNA testing should be offered in primary screening. HPV DNA prevalence is age dependent, therefore HPV DNA testing in young women might have a different risk-benefit ratio to HPV DNA testing in older women. Ronco and colleagues reported that HPV DNA testing in women aged 25–34 years could lead to substantial overdiagnosis of regressive CIN grade 2 or worse lesions, particularly when HPV DNA positive women in this age group are directly referred for colposcopy without further triage testing. Our results suggest that with the triage algorithm used in this trial HPV DNA testing in women aged 29–33 years does not result in excessive diagnosis of lesions destined to regress and lends support to the implementation of HPV DNA testing in programmed cervical screening starting at age 30 years.

The protective effect against CIN grade 3 or worse in the second screen in the intervention group was largely attributable to HPV16. HPV16 is the main genotype present in cervical cancer, hence, the early detection of HPV16-associated CIN grade 3 lesions is expected to eventually have an effect on long-term outcomes such as cancer morbidity and mortality. However, our study was not large enough to provide detailed information about the effect of other, non-HPV16 types, in the first and second screening round. This investigation needs a pooled analysis of many screening studies, which is being done (pooling Swedscreen, NTCC, and data from this study). Variation in histological classification of cervical lesions between individual centres might be a limitation of our study. However, we believe that the use of the original diagnosis closely matches treatment guidance and mimics future implementation of HPV DNA testing in population-based screening programmes. Moreover, use of histological classification obtained after review by two experienced cervical pathologists, although leading to more CIN grade 2 or worse and CIN grade 3 or worse diagnoses, did not change our conclusions.
CHAPTER 4

A strength of our study is the large size of the trial and the longitudinal design. We included all cytology and histological follow-up data derived from the nationwide registry within 9 years after the first screen. The trial was done within the regular Dutch nationwide screening programme. Together with the HC2 assay, the GP5+/6+ PCR method is clinically validated and performs much the same as HC2 in sensitivity, specificity, and intra-laboratory and inter-laboratory agreement. Therefore, our results can be considered representative for organised cervical screening.

Our results are from a population-based screening programme in which CIN diagnosis was made in routine pathology laboratories. Therefore, our study can be viewed as an implementation study, representative for organised screening and as such provides the strongest evidence to date in favour of implementation of HPV testing in nationwide cervical screening programmes. On the basis of these data the Health Council of the Netherlands, an independent advisory body of the Dutch Government, has issued advice to the minister of health to convert the current cytology-based cervical screening programme to an HPV testing-based programme starting at 30 years. Additionally, offer of HPV testing using self-collected cervico-vaginal samples to women who do not respond to an invitation for cervical screening results in a response of about 30%, showing that a proportion of women prefer self-sampling to a smear taken by a physician. Studies should investigate the acceptance of this alternative for HPV testing in regular attendees to the screening programme. Ultimately, this option will likely increase attendance to cervical screening programmes.

Acknowledgments
The study was funded by Zorg Onderzoek Nederland (Netherlands Organisation for Health Research and Development). We thank the 242 family doctors and their assistants, the Municipal Health Service Southwest of Amsterdam, Medial, DHV Kennemerland-Haarlemmermeer EO, and the nationwide registry of histopathology and cytopathology. We also thank research staff and technicians of the Unit of Molecular Pathology, VU University Medical Centre, Amsterdam for HPV DNA testing, the cytotechnologists of the Spaarne Ziekenhuis (Hoofddorp), Kennemer Gasthuis (Haarlem), Leiden Cytology and Pathology Laboratory (Leiden), and VU University Medical Centre (Amsterdam) for cytological testing and logistics, and the administrative workers and the information technology team of the Department of Pathology, VU University Medical Centre, Amsterdam, for their supportive work. We also thank the Department of Public Health and Social Medicine of Erasmus University (Rotterdam) for help with the design of the trial, and the gynaecologists in the study region for their contribution.
CHAPTER 4

References
EVALUATION OF 14 TRIAGE STRATEGIES FOR HPV DNA POSITIVE WOMEN IN POPULATION BASED CERVICAL SCREENING

Dorien C. Rijkaart
Veerle M.H. Coupe
Danielle A.M. Heideman
Wim M. Verweij

Johannes Berkhof
Albertus T. Hesselink
Rene H. Verheijen
Peter J.F. Snijders

Folkert J. Van Kemenade
Lawrence Rozendaal
Saskia Bulk
Chris J..L.M. Meijer

International Journal of Cancer 2012; 3: 602-10
CHAPTER 5

ABSTRACT

Introduction
High-risk human papillomavirus (hrHPV) testing has a higher sensitivity but lower specificity than cytology for detection of high-grade intraepithelial neoplasia (CIN). To avoid over-referral to colposcopy and overtreatment, hrHPV-positive women require triage testing and/or follow-up.

Methods
A total of 25,658 women (30–60 years) enrolled in a population-based cohort study had an adequate baseline Pap smear and hrHPV test. The end-point was cumulative two-year risk of CIN grade 3 or worse (CIN3+). In a post-hoc analysis, fourteen triage/follow-up strategies for hrHPV-positive women (n = 1,303) were evaluated for colposcopy referral rate, positive (PPV) and negative predictive value (NPV). Five strategies involved triage testing without a repeat test and nine strategies involved triage testing followed by one repeat testing. The tests were cytology, hrHPV, HPV16/18 genotyping and HPV16/18/31/33/45 genotyping.

Results
Results were adjusted for women in the cohort study who did not attend repeat testing. Of the strategies without repeat testing, combined cytology and HPV16/18/31/33/45 genotyping gave the highest NPV of 98.9% (95%CI 97.6–99.5%). The corresponding colposcopy referral rate was 58.1% (95%CI 55.4–60.8%). Eight of the nine strategies with retesting had an estimated NPV of at least 98%. Of those, cytology triage followed by cytology at 12 months had a markedly lower colposcopy referral rate of 33.4% (95%CI 30.2–36.7%) than the other strategies. The NPV of the latter strategy was 99.3% (95%CI 98.1–99.8%).

Conclusion
Triage hrHPV-positive women with cytology, followed by repeat cytology testing yielded a high NPV and modest colposcopy referral rate and appear to be the most feasible management strategy.
CHAPTER 5

Introduction

Strong evidence is now available that testing for high-risk human papillomavirus (hrHPV) infection is more sensitive than cytology in detecting high-grade cervical intraepithelial neoplasia (CIN). However, hrHPV testing also detects more transient hrHPV infections than cytology, which may lead to over-referral for colposcopy and thus overtreatment. Management of hrHPV-positive women is, therefore, of major concern. In particular, in countries with an efficient cytology-based screening program and a moderate colposcopy referral rate such as the Netherlands and the United Kingdom, the increased burden on healthcare resources upon introduction of a less-specific screening test may be substantial. To control the number of colposcopy referrals, hrHPV-positive women should not be offered colposcopy immediately but should be further stratified by means of triage testing and repeat testing. Several triage suggestions have been made in the literature including cytology, hrHPV geno-typing and hrHPV-type specific persistence analysis and p16 staining, but no uniform algorithm has emerged until now.

To evaluate the effectiveness of hrHPV testing in primary cervical screening, we set up the Vrije Universiteit Medical Centre- Saltro laboratory population-based cervical screening (VUSA-Screen study) within the routine cervical screening program in the Netherlands. Women participating in this cohort received combined hrHPV testing and cytology instead of cytology. We used the data from the cohort study for post-hoc analyses to determine feasible triage and followup testing schemes for hrHPV-positive women. We compared 14 triage/followup testing strategies using cytology, hrHPV testing and/or HPV genotyping. The end points were negative (NPV) and positive predictive values (PPV) for CIN grade 3 or worse (CIN3+) and colposcopy referral rates. The estimates of the end points were adjusted for women in the cohort study who did not attend repeat testing.

Material and Methods

Study design VUSA-screen

VUSA-screen is a population-based cohort study designed to evaluate the effectiveness of combined cervical cytology screening with hrHPV testing by HC2 hybridization assay (Qiagen, Gaithersburg, MD). The study was carried out in the Utrecht province of the Netherlands in the setting of the regular Dutch screening program that invites women aged 30–60 years to be screened every 5 years. The design of the study has been described elsewhere. In brief, women invited for regular cervical screening were recruited between October 2003 and August 2005. Women with a history of cervical intraepithelial neoplasia grade 2 or worse (CIN2+) or abnormal cytology in the preceding 2 years were excluded from analysis. Participating women gave written informed consent. The VUSA-screen study was approved by the Ministry of Public Health (2002/02-WBO; ISBN-10: 90-5549-452-6) and registered in the trial register (NTR215, ISRCTN64621295).

Cervical scrapings were taken by a cytobrush (Rovers, Oss, the Netherlands), and after making a conventional cytological smear, the brush was placed in a container with 1 ml universal collection medium (Qiagen, Gaithersburg) for hrHPV HC2 testing (cocollection for HPV testing and conventional cytology). Cytology
results were reported, blinded to the hrHPV testing results, according to the CIOSE-A classification, which is used in the Dutch screening program and can be easily converted into the 2001 Bethesda system. Cytological results were grouped as normal, borderline or mild dyskaryosis (BMD) and >BMD (moderate dyskaryosis or worse). In the 2001 Bethesda system, BMD corresponds to atypical squamous cells of undetermined significance; atypical squamous cells, cannot rule out high-grade squamous intraepithelial lesions or low-grade squamous intraepithelial lesions and >BMD corresponds to high-grade squamous intraepithelial lesions.

Women with BMD or worse and positive in hrHPV test were directly referred for colposcopy. In women with normal cytology at baseline, a substudy was carried out. In this substudy, all hrHPV-positive women (n = 1,021) were included as well as a subset of n = 3,063 hrHPV-negative, cytologically normal women. Women with normal cytology were not informed about the hrHPV test result. HrHPV-positive women with normal cytology were offered cytology and a blinded hrHPV test at 12 months and combined hrHPV testing and cytology at 24 months. Women were referred at 12 months if cytology was abnormal and at 24 months in case of a positive hrHPV test and/or abnormal cytology (Fig. 1).

Of the women who were referred to a gynecologist for colposcopy, colposcopy-directed biopsies were taken for histological examination of suspicious areas on the cervix, according to standard procedures in the Netherlands. Biopsy results were reported as normal, cervical intraepithelial neoplasia (CIN) grades 1, 2 and 3 or as invasive cancer, according to international criteria. Glandular intraepithelial neoplasia grades 1, 2 and 3 were included in CIN. Cytology and histology results were retrieved from the nationwide network and registry of histopathology and cytopathology (PALGA; Bunnik, the Netherlands). We included all lesions diagnosed after the referral smear and within 3 years after baseline.

hrHPV DNA testing
hrHPV testing was performed by the HC2 hrHPV DNA test (13 hrHPV types) in an automated format on a rapid capture system (RCS; Qiagen, Gaithersburg, MD) as described before. Samples were considered positive if they attained or exceeded threshold of 1.0 RLU/CO (corresponding with 1 pg/mL HPV16 DNA).

HC2 positive samples were tested with GP5+/6+-PCR-EIA, and all specimens tested positive by GP5+/6+-PCR-EIA were typed by reverse line blotting according to established protocols.

Statistical Analysis
The primary endpoint of the study was cumulatively detected CIN grade 3 or worse (CIN3+). A secondary outcome was cumulatively detected CIN2+.

In our post-hoc analysis, 14 triage per followup strategies for hrHPV-positive women with no or a single repeat test were evaluated with respect to NPV, PPV, sensitivity, specificity and the colposcopy referral rate. For the repeat test, the 12-month screening test in the cohort study was used. The 24-month screening test was used if the 12-month test was missing. To gain insight into the impact of replacing cytological screening by hrHPV screening on the colposcopy referral rate in the entire screening
population, we also calculated an overall colposcopy referral rate by multiplying the colposcopy referral rate in hrHPV-positive women by the hrHPV prevalence.

hrHPV-positive women with normal cytology and without a valid repeat combination test result (repeat cytology and hrHPV test result) were considered as lost to followup. We accounted for this loss-to-followup by replacing raw counts by expected counts. The expectations were calculated under the assumption that women with normal cytology at baseline without a valid repeat test result had the same disease risk as women with a valid repeat test result. Furthermore, we classified a woman as having CIN0/1 if she was referred for colposcopy at baseline and had no detected CIN2+ or if she had a valid repeat test result and no detected CIN2+.

![Flowchart of the screening profiles of hrHPV-positive women in the VUSA-Screen study.](image)

**Figure 1** Flowchart of the screening profiles of hrHPV-positive women in the VUSA-Screen study.
The latter implies that women with a double negative test at 24 months, which are returned to routine screening, are classified as CIN0/1.

For each new strategy, we estimated the expected number of colposcopy referrals and the expected number of CIN3+ and CIN2+ detected by the strategy. Furthermore, on the basis of the screening strategy used in the cohort study, we estimated the expected number of CIN3+ and CIN2+ in the whole cohort. We calculated the PPV and NPV for CIN3+ of a new strategy as:

\[
PPV = \frac{\text{Expected number of CIN3+ in women referred for colposcopy}}{\text{Expected number of women referred for colposcopy}}
\]

\[
NPV = 1 - \frac{\text{Expected number of CIN3+ in women not referred for colposcopy}}{\text{Expected number of women not referred for colposcopy}}
\]

The sensitivity and specificity for CIN3+ were calculated as

\[
\text{Sensitivity} = \frac{\text{Expected number of CIN3+ in women referred for colposcopy}}{\text{Expected number of CIN3+ in cohort}}
\]

\[
\text{Specificity} = \frac{\text{Expected number of CIN0=1=2 in women not referred for colposcopy}}{\text{Expected number of CIN0=1=2 in cohort}}
\]

Ninety-five percent confidence intervals were calculated for the outcome measures using the Wilson Score method,\(^{18}\) where the sample size was set equal to the number of cases observed in the cohort study. To evaluate the strategies, we considered a NPV for CIN3+ of at least 98% (corresponding with a 2-year CIN3+ risk of at maximum 2%) to be a minimal requirement.\(^{19}\)

We considered the following five strategies for hrHPV-positive women with triage at baseline but without a repeat test: (i) cytology (threshold BMD), (ii) HPV16/18 genotyping, (iii) HPV16/18/31/33/45 genotyping, (iv) combined cytology and HPV16/18 genotyping and (v) combined cytology and HPV16/18/31/33/45 genotyping (Table 1). We considered nine strategies with baseline triage followed by one round of repeat testing. In the first five of these strategies, baseline triage testing consisted of cytology only: (vi) cytology triage at baseline and cytological testing at 12 months; (vii) cytology triage at baseline and hrHPV testing at 12 months; (viii) cytology triage at baseline and HPV type persistence at 12 months; (ix) cytology triage at baseline and combined cytology and hrHPV testing at 12 months and (x) cytology triage at baseline and combined cytology and HPV16/18 genotyping at 12 months. In the last four strategies, baseline triage testing consisted of cytology combined with hrHPV16/18 genotyping. For the repeat tests, strategies (xi–xiv) were similar to strategies (vi–ix) (Table 2).

Analyses were done with SPSS version 15.0 (LEAD Technologies Inc., Haddonfield, NJ), Excel (Microsoft Corporation, Redmond, WA) and MATLAB version 7.9 (The MathWorks Inc., Natick, MA).
Table 1 Sensitivity, specificity, NPV, PPV and colposcopy referral rate of five baseline triage strategies for hrHPV-positive women, adjusted for non-attendance at repeat testing.

<table>
<thead>
<tr>
<th>Triage strategy</th>
<th>Endpoint CIN3+</th>
<th>hrHPV-positive women</th>
<th>Endpoint CIN2+</th>
<th>Total screening population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
<td>NPV</td>
<td>PPV</td>
</tr>
<tr>
<td>I Cytology</td>
<td>70.6</td>
<td>85.6</td>
<td>95.1</td>
<td>42.2</td>
</tr>
<tr>
<td>(62.7-77.4)</td>
<td>(82.8-88.1)</td>
<td>(93.0-96.7)</td>
<td>(36.6-48.0)</td>
<td>(56.2-68.8)</td>
</tr>
<tr>
<td>II HPV 16/18</td>
<td>65.4</td>
<td>72.5</td>
<td>93.4</td>
<td>26.1</td>
</tr>
<tr>
<td>(57.4-72.7)</td>
<td>(69.0-75.8)</td>
<td>(91.0-95.2)</td>
<td>(21.4-31.4)</td>
<td>(52.1-64.9)</td>
</tr>
<tr>
<td>III HPV</td>
<td>81.2</td>
<td>53.8</td>
<td>95.1</td>
<td>20.7</td>
</tr>
<tr>
<td>(74.1-86.8)</td>
<td>(50.0-57.5)</td>
<td>(92.4-96.8)</td>
<td>(17.2-24.7)</td>
<td>(71.9-82.7)</td>
</tr>
<tr>
<td>IV Cytology &amp;</td>
<td>87.4</td>
<td>63.2</td>
<td>97.1</td>
<td>26.0</td>
</tr>
<tr>
<td>HPV16/18</td>
<td>(81.1-91.9)</td>
<td>(59.5-66.7)</td>
<td>(94.9-98.4)</td>
<td>(22.1-30.4)</td>
</tr>
<tr>
<td>(91.9-98.4)</td>
<td>(44.2-50.6)</td>
<td>(97.6-99.5)</td>
<td>(17.5-24.3)</td>
<td>(91.3-97.2)</td>
</tr>
</tbody>
</table>

HPV = human papillomavirus; CI = confidence interval; CIN = cervical intraepithelial neoplasia (grade 2 and 3 or higher); NPV = negative predictive value; PPV = positive predictive value.
CHAPTER 5

Results
VUSA-screen characteristics
Of the 25,871 women participating in the VUSA-screen study, 25,658 (99.2%) had an adequate baseline Pap smear and hrHPV test. The median age of participating women was 44.0 years (range 29–61 years). Among the women with an adequate Pap smear, 98.2% had normal cytology, 1.3% had BMD and 0.5% had >BMD (Fig. 1). The proportion of women with a positive hrHPV HC2 test was 4.1% among those with normal cytology, 49.6% in those with BMD and 92.0% in women with >BMD. Overall, 5.1% (1,303/25,658) of the women tested hrHPV positive.

The 1,021 hrHPV-positive women with normal baseline cytology received a recommendation to return at 12 and 24 months for a smear and a hrHPV test. A total of 535 of these women had at least one round of followup with a hrHPV test and a smear for evaluation of cytology. Among women who attended at repeat testing, the average time to the first followup test was 15.0 months with a standard deviation of 4.7 months. The followup time ranged from 1.3 to 28.6 months.

Evaluation of triage strategies for hrHPV-positive women
Detailed results of the five strategies without a repeat test are presented in Table 1. The highest NPV of 98.9% (95%CI: 97.6–99.5%) was obtained with the strategy of combined cytology and HPV16/18/31/33/45 genotyping. None of other four strategies met the NPV threshold for CIN3+ of 98% and, therefore, these strategies were not acceptable. However, the strategy with combined cytology and HPV16/18/31/33/45 genotyping resulted in a high colposcopy referral rate of 58.1% (95%CI: 55.4–60.8%) among hrHPV-positive women. This would translate into a referral rate of 2.95% (95%CI: 2.75–3.17%) in the total screening population, which is almost threefold higher than obtained with baseline cytology triage only (colposcopy referral rate of 1.09%; 95%CI: 0.96–1.22%).

Table 2 shows detailed results of the nine strategies with baseline triage testing followed by one round of repeat testing. Eight strategies had a NPV for CIN3+ above the threshold of 98%. The PPVs of these strategies were acceptable, ranging from 17.7 to 37.5%. Strategies with hrHPV testing at followup showed high colposcopy referral rates (ranging from 65.7 to 73.0%).

The lowest colposcopy referral rate in hrHPV-positive women was obtained with cytology triage at baseline followed by repeat cytology testing at 12 months (33.4%). In the total screening population, this would result in a colposcopy referral rate of 1.70% (95%CI: 1.54–1.85%), a 1.55-fold increase compared with baseline cytology triage only.

The NPV and PPV of the 14 strategies are graphically displayed in Figure 2. Nine strategies had an estimated NPV greater than 98%, eight strategies with and one without repeat testing. Strategies with hrHPV testing in followup (strategies vii, ix, xii and xiv) showed not only the highest NPV but also the lowest PPV. The strategy with cytology triage followed by cytology testing showed both a high PPV and high NPV (Fig. 2, upper right corner).
**Table 2** Sensitivity, specificity, NPV, PPV and colposcopy referral rate of nine triage strategies for hrHPV-positive women based on baseline and one round of repeat testing, adjusted for non-attendance at repeat testing.

<table>
<thead>
<tr>
<th>Baseline</th>
<th>Repeat test at 12months</th>
<th>hrHPV-positive women</th>
<th>Endpoint CIN3+</th>
<th>Endpoint CIN2+</th>
<th>Repeat tests, Colpo referral rate</th>
<th>Total screening population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sensitivity, % (95% CI)</td>
<td>Specificity, % (95% CI)</td>
<td>NPV, % (95% CI)</td>
<td>PPV, % (95% CI)</td>
<td>Sensitivity, % (95% CI)</td>
</tr>
<tr>
<td>vi</td>
<td>Cytology</td>
<td>96.6 (92.3-98.6)</td>
<td>76.0 (72.7-79.1)</td>
<td>99.3 (98.1-99.8)</td>
<td>37.5 (32.4-42.6)</td>
<td>90.9 (86.3-94.0)</td>
</tr>
<tr>
<td>vii</td>
<td>hrHPV (HC2)</td>
<td>98.9 (95.5-99.7)</td>
<td>39.2 (35.6-42.9)</td>
<td>99.6 (97.6-99.9)</td>
<td>19.5 (16.5-22.9)</td>
<td>98.6 (96.0-99.5)</td>
</tr>
<tr>
<td>viii</td>
<td>HPV type persistence</td>
<td>89.8 (83.8-93.8)</td>
<td>58.0 (54.3-61.7)</td>
<td>97.5 (95.2-98.7)</td>
<td>24.1 (20.5-28.2)</td>
<td>88.0 (83.1-91.7)</td>
</tr>
<tr>
<td>ix</td>
<td>Cytology</td>
<td>100 (94.7-100)</td>
<td>38.4 (34.8-42.1)</td>
<td>100 (98.3-100)</td>
<td>19.4 (16.4-22.8)</td>
<td>100 (98.3-100)</td>
</tr>
<tr>
<td>x</td>
<td>Cytology</td>
<td>97.7 (93.8-99.2)</td>
<td>62.4 (58.7-66.0)</td>
<td>99.5 (98.1-99.9)</td>
<td>27.9 (23.9-32.2)</td>
<td>94.4 (90.5-96.7)</td>
</tr>
<tr>
<td>xi</td>
<td>Cytology</td>
<td>98.9 (95.5-99.7)</td>
<td>57.4 (53.6-61.1)</td>
<td>99.7 (98.4-99.9)</td>
<td>25.6 (21.9-29.7)</td>
<td>94.3 (90.5-96.7)</td>
</tr>
<tr>
<td>xii</td>
<td>hrHPV (HC2)</td>
<td>100 (97.4-100)</td>
<td>31.5 (28.1-35.1)</td>
<td>100 (98.0-100)</td>
<td>17.8 (15.0-21.0)</td>
<td>100 (98.3-100)</td>
</tr>
<tr>
<td>xiii</td>
<td>HPV type persistence</td>
<td>94.3 (89.2-97.0)</td>
<td>47.9 (44.2-51.7)</td>
<td>98.3 (96.0-99.3)</td>
<td>21.2 (17.9-24.8)</td>
<td>92.2 (87.9-95.0)</td>
</tr>
<tr>
<td>xiv</td>
<td>Cytology</td>
<td>100 (97.4-100)</td>
<td>31.0 (27.6-34.6)</td>
<td>100 (97.9-100)</td>
<td>17.7 (14.9-20.8)</td>
<td>100 (98.3-100)</td>
</tr>
</tbody>
</table>

HrHPV = high-risk human papillomavirus; CI = confidence interval; CIN = cervical intraepithelial neoplasia (grade 2 or 3 or higher); Gray: the strategies which met the criteria for NPV at a CIN3+ threshold of 98%.
**Figure 2** NPV and PPV for CIN3+ of the 14 triage/follow-up strategies for hrHPV-positive women, adjusted for non-attendance at repeat testing. PPV = positive predictive value; NPV = negative predictive value; o: triage strategy without a repeat test; n: triage strategy followed by one round of repeat testing; Bars represent 95% confidence intervals; cyto = strategy (i); HPV16/18 = strategy (ii); HPV16/18/31/33/45 = strategy (iii); cyto&HPV16/18 = strategy (iv); cyto&HPV16/18/31/33/45 = strategy (v); cyto + cyto = strategy (vi); cyto + hrHPV = strategy (vii); cyto + HPV type pers = strategy (viii); cyto + cyto&hrHPV = strategy (ix); cyto + cyto&HPV16/18 = strategy (x); cyto&hrHPV16/18 + cyto = strategy (xi); cyto&hrHPV16/18 + hrHPV = strategy (xii); cyto&hrHPV16/18 + HPV type pers = strategy (xiii); cyto&hrHPV16/18 + cyto&hrHPV = strategy (xiv).

**Discussion**

Implementation of hrHPV testing as a primary screening test in cervical screening may soon be reality. However, at the moment there is still debate about how to manage hrHPV test-positive women, because hrHPV testing is less specific than cytology. Referring all hrHPV-positive women to colposcopy will result in over-diagnosis, over-treatment and high costs. Therefore, triage and/or follow-up strategies for hrHPV-positive women are needed. The VUSA-Screen cohort study enabled us to examine this issue in more detail.

Five triage strategies without repeat test were investigated, with cytology, hrHPV, HPV16/18 genotyping and HPV16/18/31/33/45 genotyping as triage tests. A triage strategy was considered feasible if the NPV was equal to or exceeded a predefined threshold of 98%. Of the five triage strategies, one strategy (cytology combined with HPV16/18/31/33/45) met the NPV criterion of 98%. This strategy would be attractive for implementation, because no follow-up is needed. However, a
major disadvantage is the high overall colposcopy referral rate in the total population (2.95%). In addition to the increase in the costs that would result from this strategy, it also poses a large burden on the colposcopy capacity of gynecologists. By adding one repeat test at 12 months, it is possible to reduce this referral rate markedly. The most attractive strategy was cytology triage at baseline followed by repeat cytology testing at 12 months, with an overall colposcopy referral rate in the total population of 1.70 and a NPV of 99.3 (95%CI: 98.1–99.8%).

Loss-to-followup may be a problem when implementing a screening strategy with a repeat test. In this study, the loss-to-followup of women with normal cytology at baseline was about 40%. Other studies have also shown that attendance at repeat testing is poor, particularly after a cytologically normal test. Therefore, adequate communication is necessary to improve attendance at repeat testing. In the POBASCAM trial in the Amsterdam area in the Netherlands, follow-up at repeat testing was 77%. Depending on the magnitude of envisaged loss to follow-up, a direct triage strategy without a repeat visit could be an alternative option if the almost threefold increase in colposcopy rate compared with baseline cytology only triage testing could be accommodated. Such an increase in colposcopy rate is expected to lead to capacity problems in the Netherlands where the annual number of colposcopy referrals is low due to a well-functioning cytological screening program (about 12,000 per year). Therefore, for the Netherlands, it is preferable to implement the strategy with cytology triage at baseline and a repeat cytology test at 12 months. This can be implemented against much less extra coloscopies. Moreover, this increase in colposcopy rate is accompanied by an almost similar increase in the number of detected CIN3+. Furthermore, after a few rounds of hrHPV screening a decline in colposcopy referrals is expected because the extra detection of high-grade lesions (with subsequent treatment) will lead to a reduced rate of abnormalities in subsequent screening rounds.

A point of consideration in our current study is the low followup at repeat testing. HrHPV-positive women with normal cytology and a representative age-matched subcohort of hrHPV-negative women with normal cytology were invited for repeat testing after 1 and/or 2 years and were referred for colposcopy if they presented with abnormal cytology and/or positive hrHPV test. In our study, women with normal cytology were not informed about the hrHPV status at baseline. The concealment was necessary to maximize attendance at repeat testing among hrHPV-negative women with normal cytology. It is likely that the attendance rate in our study would have been higher if women had been informed about their hrHPV test result. In addition to the women, cytotechnicians were not informed about the hrHPV test result. In a single hrHPV testing strategy with cytology triage for hrHPV-positive women, the hrHPV test result may be known to the cytotechnician. Awareness of the negative or positive hrHPV test result may affect the criteria for defining abnormalities because the interpretation of cytology is subjective. In a recent Finnish randomized trial, cytotechnicians in the hrHPV screening arm were informed about the hrHPV screening test result. However, the hrHPV test information in that study only had a small effect on cytology assessment, and, therefore, on the CIN3+ detection rate and the number of coloscopies.
Furthermore, implementation of HPV vaccination may have an effect on the cost effectiveness of screening programs. In particular, it is expected that vaccination reduces the PPV of cytological screening since fewer women will develop high-grade cervical lesions. In particular, the reading performance for cytological slides is likely to deteriorate when the occurrence of an abnormal smear becomes less common. However, HPV vaccination will have a stronger effect on PPV of a primary cytological screening test than on the PPV of triage or repeat cytology in hrHPV-positive women. The reason is that the prevalence of hrHPV will also decrease after vaccination, and this partly compensates the decrease in PPV if cytological evaluation is limited to hrHPV-positive women. Therefore, we think that the proposed strategy with cytological triage at baseline and repeat cytology at 1 year will also be an interesting triage strategy for vaccinated women.

A strong point of our study is the longitudinal design and the older age range of study participants (30–60 year), which is the age for which hrHPV testing is most widely advocated. In our study, we took into account all the cytology and histological follow-up data derived from the national wide network of registry within 3-years after baseline. The study was population-based and part of a routine organised screening activity in a low-risk population, indicating that the results could be implemented in routine practice.

Other studies have suggested to follow-up hrHPV-positive women with normal cytology by one repeat hrHPV test or by a combined cytology and hrHPV test. Our study indicates that implementation of these strategies lead to a substantial increase in colposcopy referral rate, eventually referring two third of all hrHPV-positive women. However, it should be kept in mind that in the Netherlands, the cytological screening program is very efficient, which is demonstrated by a cytology abnormality rate of 1.8% (Fig. 1) with a similarly low colposcopy referral rate. However, in countries with already higher cytological abnormality, and related colposcopy rates, such triage strategies would, depending on the screening interval, have a much lower impact on the current number of colposcopies in those countries.

Finally, in addition to CIN3+ risk considerations, implementation of a triage and follow-up strategy for hrHPV-positive women asks for acceptability by physicians and women. To prevent anxiety among women who are directed back to routine screening, it is essential that the last screening test is negative. A repeat visit for cytology only at 1 year, meets this requirement, but, e.g., implementation of HPV16/18 genotyping after 1 year seems less straightforward as women who will be hrHPV-positive but HPV16/18 negative at a repeat visit, may feel uncomfortable about their mixed test result even if their CIN3+ risk is low enough to return to the next screening round. This may be alleviated with a triage test that directly detects HPV16/18 at baseline, rather than using a consensus test first. Finally, the logistics of a triage and follow-up strategy in national programme should preferably be simple.

In conclusion, our post-hoc analysis of triage strategies for hrHPV-positive women, based on the VUSA-screen cohort strongly points to the use of cytological testing both at baseline and at 12 months in countries with low rates of cytological abnormalities and long screening intervals (5 years). This is a feasible triage strategy because it has a high NPV for CIN3+, modest colposcopy referral rate and is easy to communicate to physicians and women. However, in countries with higher rates of
cytological abnormalities, shorter screening intervals and higher colposcopy rates, other triage strategies involving for instance combined cytology and HPV16/18/31/33/45 genotyping at baseline without repeat testing might be considered for implementation.

Acknowledgements
The authors thank all the staff of the Saltro Laboratory Utrecht for their expertise, commitment and invaluable support for the VUSA-Screen study. The authors thank participating GPs, their assistants, the gynaecologists and the thousands of women who have participated in this study. Qiangen (Digene) is acknowledged for providing collection tubes with UCM and HC2 kits.

References


27. Cox T, Cuzick J. HPV DNA testing in cervical cancer screening: from evidence to policies. Gynecol Oncol 2006; 103: 8–11.


COMPARISON OF HYBRID CAPTURE 2 TESTING AT DIFFERENT THRESHOLDS WITH CYTOLOGY AS PRIMARY CERVICAL SCREENING TEST

Dorien C. Rijkaart  ■  Veerle M.H. Coupe  ■  Folkert J. van Kemenade
Danielle A.M. Heideman  ■  Albertus T. Hesselink  ■  Wim M. Verweij
Lawrence Rozendaal  ■  Rene H. Verheijen  ■  Peter J.F. Snijders
Johannes Berkhof  ■  Chris J.L.M. Meijer
CHAPTER 6

ABSTRACT

Background
We evaluated the performance of primary high-risk human papillomavirus (hrHPV) testing by hybrid capture 2 (HC2) with different thresholds for positivity, in comparison with conventional cytology.

Methods
We used data of 25,871 women (aged 30–60 years) from the intervention group of the VUSA-Screen study (VU University Medical Center and Saltro laboratory population-based cervical screening study), who were screened by cytology and hrHPV. Primary outcome measure was the number of cervical intraepithelial neoplasia grade 3 or higher (CIN3+), detected within 3 years. We compared baseline cytology testing with three possible hrHPV screening strategies at different relative light unit/cutoff (RLU/CO) thresholds.

Results
Compared with baseline cytology testing, hrHPV DNA testing as a sole primary screening instrument did not yield a superior sensitivity, as well as lower colposcopy referral rate and lower false positivity rate at any RLU/CO threshold. The hrHPV screening at 1 RLU/CO threshold with cytology triage at baseline and at 12 months revealed the highest sensitivity for CIN3+ (relative sensitivity of 1.32), although still displaying a lower colposcopy referral rate than cytology testing (relative colposcopy rate of 0.94). Higher thresholds (>1RLU/CO) yielded lower colposcopy rates, but resulted in substantial loss in sensitivity.

Conclusion
The hrHPV testing at the commonly used threshold of 1 RLU/CO with cytology triage at baseline and at 12 months showed a much higher sensitivity with a lower colposcopy referral rate compared with cytology testing.
CHAPTER 6

Introduction

New promising methods of cervical cancer prevention have been introduced since the recognition that infection with high-risk human papillomavirus (hrHPV) is the necessary cause of cervical cancer. The recently introduced prophylactic HPV vaccine may have a major impact on preventing this global disease. The prophylactic vaccines have shown to be highly effective in preventing premalignant lesions. However, it is generally agreed upon that cervical cancer screening will need to continue even for vaccinated women.

Although cytological screening has reduced the incidence and mortality of cervical cancer, it has a limited sensitivity. The much more sensitive hrHPV test has been suggested as an alternative primary screening instrument, given that a clinically validated hrHPV assay is used. At present, the FDA approved hybrid capture 2 (HC2) assay is most commonly used. However, hrHPV testing using such a test has also shown a 4–6% lower specificity than conventional cytology, because many detected infections are transient and regress without developing high-grade lesions.

In population-based screening, specificity is of utmost importance, as it basically determines the costs of the programme and the amount of unwanted adverse effects (anxiety, repetitive and confirmatory tests, as well as unnecessary colposcopy referrals and treatments) in the generally healthy population. Cytological triage of hrHPV DNA-positive women has been found to improve the specificity of the screening test. Another, easier and potentially cost saving option is to adapt the threshold, that is, increase the relative light unit/cutoff (RLU/CO) threshold, of the HC2 test. This would particularly be useful if it obviates the need for repeat testing. Several studies have examined baseline hrHPV testing strategies with triage of hrHPV-positive women at higher thresholds than the one conventionally used (i.e., RLU/CO of ≥1). This did not result in a strategy that ensured higher sensitivity as well as higher specificity in terms of lower colposcopy referral rates compared with cytological screening.

The aim of this study was to investigate the effect of hrHPV HC2 testing with higher thresholds on the sensitivity and specificity in terms of colposcopy referral rate and false positivity rate, considering a number of different hrHPV screening strategies. We searched for strategies that improved the specificity of hrHPV screening by increasing the RLU/CO threshold, while maintaining a higher sensitivity than baseline cytological testing. We used data from the intervention group of the VUSA-Screen study (VU University Medical Center and Saltro laboratory population-based cervical screening study), a study performed within the routine cervical programme of the Netherlands. Women participating in this cohort received combined hrHPV testing and cytology. The primary end points were cervical intraepithelial neoplasia grade 3 or higher (CIN3+), detected within 3 years.
Materials and methods

Study design VUSA-screen

The VUSA-Screen is a population-based study designed to evaluate the effectiveness of combined cervical cytology screening with hrHPV testing by HC2 hybridisation assay (Qiagen, Gaithersburg, MD, USA). The study was carried out in the Utrecht province of the Netherlands in the setting of the regular screening programme that invites women, aged between 30 and 60 years of age, to be screened every 5 years. The design of the study has also been described elsewhere. Between October 2003 and August 2005, women invited for the regular cervical screening programme were asked to participate in the VUSA-Screen study. Women were excluded from the analysis if they had a history of cervical intraepithelial neoplasia grade 2 or higher (CIN2+) or abnormal cytology in the preceding 2 years. Women who agreed to receive cytology and hrHPV testing gave written informed consent.

Conventional cytological smears were taken with a cytobrush (Rovers, Oss, the Netherlands). After preparation of a conventional smear on a glass slide, the brush was placed in a vial containing 1ml UCM (Universal Collection Medium, Digine Corp., Gaithersburg, MD, USA) for hrHPV testing. Cervical cytology results were reported, blinded to the hrHPV testing results, according to the CISOE-A classification, which is routinely used in the Netherlands and can be converted into the 2001 Bethesda system. Cytological results were grouped as normal, BMD (borderline or mild dyskaryosis) and >BMD (moderate dyskaryosis or worse). In the 2001 Bethesda system, BMD corresponds to atypical squamous cells of undetermined significance; atypical squamous cells cannot rule out high-grade squamous intraepithelial lesions; or low-grade squamous intraepithelial lesions and >BMD corresponds to high-grade squamous intraepithelial lesions.

Women with BMD or worse were informed about the hrHPV test result. The hrHPV-positive women with BMD and all women with >BMD were directly referred for colposcopy (Figure 1). Women with BMD at baseline and a negative hrHPV test were offered cytology testing at 6 and 18 months and referred if cytology was abnormal (threshold BMD) at one of these occasions.

In the women with normal cytology at baseline, a sub-study was carried out. In this sub-study, all (n = 1021) hrHPV-positive women as well as a subset of hrHPV-negative cytologically normal women (n = 3063) were included. To select the hrHPV-negative women, each hrHPV-positive woman was matched to three randomly chosen hrHPV-negative women of the same age. Women with normal cytology were not informed about the hrHPV test result. The hrHPV-positive women with normal cytology were offered cytology and a blinded hrHPV test at 12 months, and combined hrHPV testing and cytology at 24 months. A woman was referred at 12 months if cytology was abnormal and at 24 months if the hrHPV test was positive and/or cytology was abnormal. The hrHPV-negative, cytologically normal women in the sub-study were invited for combined testing at 24 months, and referred if cytology was abnormal and/or the hrHPV test was positive. If a woman with normal cytology and a negative hrHPV test was not invited for repeat testing after 24 months, cytological and/or histological follow-up results was not included.
The VUSA-Screen study was approved by the Ministry of Public Health (2002/02-WBO; ISBN-10: 90-5549-452-6) and registered in the trial register (NTR215, ISRCTN64621295).

**Colposcopy**

Of the women who were referred to a gynaecologist for colposcopy, colposcopy-directed biopsies were taken from suspicious areas of the cervix, according to standard procedures in the Netherlands.\(^28\) Biopsy results were reported as normal, cervical intraepithelial neoplasia grade 1, 2, 3, or as invasive cancer, according to the international criteria.\(^29,30\) Cytology and histology results were retrieved from the nationwide network and registry of histopathology and cytopathology (PALGA: Bunnik, the Netherlands).

**hrHPV testing**

The hrHPV testing was performed by the HC2 high-risk HPV DNA test in an automated format on a rapid capture system according to the manufacturer’s instructions (Qiagen). This test uses a cocktail probe to detect 13 high-risk HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. Positive controls containing 1pgml-1 of cloned HPV-16 DNA and negative controls (provided by the manufacturer) were included in each assay (Qiagen). The results of the HC2 assay were expressed as RLU/CO ratio, representing the ratio between the emission from a sample to the average emission of three positive controls. Initially, the threshold of 1 RLU/CO, as proposed by the manufacturer, was used to classify a specimen as positive or negative.

---

**Figure 1** Flowchart of the study design. BMD, borderline or mild dyskaryosis; colpo, colposcopy; cyto, cytology; hrHPV, high-risk human papillomavirus; m, months.
CHAPTER 6

Statistical analysis

The primary outcome measure of the study was histologically confirmed CIN3+, detected cumulatively within 3 years after baseline. A secondary outcome was cumulatively detected CIN2+. In the calculations of the number of CIN3+ and CIN2+ lesions, cases of cervical adenocarcinoma and cervical adenocarcinoma in situ were also included.

The absolute specificity of hrHPV testing with RLU/CO thresholds between 1 and 100 and the absolute specificity of cytology were computed as follows. Specificities were adjusted for nonattendance at repeat testing by applying Bayes’s rule, which means that the specificities were computed from the positive and negative predictive values for CIN3+ (and CIN2+), as well as the baseline prevalences of HC2 and cytology test outcome strata. For this purpose, the baseline test outcomes were grouped into seven strata: (1) >BMD and hrHPV+, (2) >BDM and hrHPV-, (3) BMD and hrHPV+, (4) BMD and hrHPV-, (5) normal and hrHPV+, (6) normal and hrHPV-, and age ≤35 years, and (7) normal and hrHPV-, and age >35 years. We defined two separate age strata for hrHPV-negative women with normal cytology at baseline because hrHPV-negative normal women were age-matched to hrHPV-positive normal women, and women ≤35 years were therefore overrepresented in follow-up. The positive and negative predictive values were computed only on the basis of women with at least one repeat test. For hrHPV-positive, cytologically normal women, the 12-month screening tests were used as repeat tests. The 24-month screening tests were used if the 12-month tests were missing. For BMD hrHPV-negative women, the 6-month results were used as repeat test results and the 18-month results were used if the 6-month results were missing. The specificities presented were therefore adjusted for women without repeat testing, but were not adjusted for differences in intensity of follow-up testing among women with at least one repeat test.

Furthermore, we compared baseline cytology (threshold BMD) with three possible hrHPV screening strategies at RLU/CO thresholds between 1 and 100. The

<table>
<thead>
<tr>
<th>HC2 cutoff</th>
<th>Test positive</th>
<th>Test negative</th>
<th>Histology</th>
<th>End point CIN3+</th>
<th>End point CIN2+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (% of total n=25 658)</td>
<td></td>
<td></td>
<td>Detected</td>
<td>Missed</td>
</tr>
<tr>
<td>Baseline cytology (threshold BMD)</td>
<td>462 (1.80)</td>
<td>1021</td>
<td></td>
<td>124</td>
<td>27</td>
</tr>
<tr>
<td>1</td>
<td>1303 (5.08)</td>
<td>180</td>
<td></td>
<td>146</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>1147 (4.47)</td>
<td>336</td>
<td></td>
<td>144</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>1006 (3.92)</td>
<td>477</td>
<td></td>
<td>141</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>954 (3.72)</td>
<td>529</td>
<td></td>
<td>137</td>
<td>14</td>
</tr>
<tr>
<td>10</td>
<td>877 (3.42)</td>
<td>606</td>
<td></td>
<td>131</td>
<td>20</td>
</tr>
<tr>
<td>30</td>
<td>654 (2.55)</td>
<td>829</td>
<td></td>
<td>118</td>
<td>33</td>
</tr>
<tr>
<td>50</td>
<td>572 (2.23)</td>
<td>911</td>
<td></td>
<td>104</td>
<td>47</td>
</tr>
<tr>
<td>100</td>
<td>433 (1.69)</td>
<td>1050</td>
<td></td>
<td>89</td>
<td>62</td>
</tr>
</tbody>
</table>

BMD=borderline or mild dyskaryosis; CIN=cervical intraepithelial neoplasia (grade 2 or 3 or higher); HC2=hybrid capture 2.
following hrHPV screening strategies were used: (1) baseline hrHPV testing only; (2) cytology triage of hrHPV-positive women at baseline and one repeat cytological test for cytologically normal women; and (3) cytology triage of hrHPV-positive women at baseline and one repeat combined cytology and hrHPV HC2 test (with RLU/CO values as used at baseline) for cytologically normal women. For each comparison, we computed the relative sensitivity for CIN3+ (and CIN2+), relative false positivity rate and relative colposcopy referral rate. Analogous to the calculation of the specificity, the relative rates were calculated by combining positive and negative predictive values (here for CIN3+, CIN2+ and colposcopy referral) and baseline test outcomes. Because double-negative women cancel out when calculating relative rates, we only needed to define five baseline strata: (1) >BMD and hrHPV+, (2) >BDM and hrHPV-, (3) BMD and hrHPV+, (4) BMD and hrHPV- and (5) normal and hrHPV+.

The 95% confidence intervals (CIs) were calculated for absolute specificity using the Wilson Score method (Brown et al, 2001), in which the sample size was set equal to the number of cases observed in the cohort study.

Analyses were done with SPSS version 15.0 (LEAD Technologies Inc, Haddonfield, NJ, USA), and Excel (Microsoft Corporation, Redmond, WA, USA).

Results

Study subjects

Of the 25,871 women from the intervention group of VUSA-Screen study, 25,658 (99.2%) had an adequate baseline Pap smear. Among women with adequate Pap smears, 25,196 had normal cytology of whom 1,021 (4.1%) tested hrHPV-positive, 337 women had a BMD result of whom 167 (49.6%) tested hrHPV-positive and 125 women had a >BMD result of whom 115 (92.0%) tested hrHPV-positive. The median age of participating women was 44.0 years (range, 29–61 years). The hrHPV results of positive RLU/CO (i.e., RLU/CO ≥1) showed a mean of 224.5 (range, 1.0–2,565.7).

The number of test positives and negatives, CIN3+ and CIN2+ detected, stratified for cytology and HC2 thresholds are presented in Table 1.

### Table 2

Comparison of specificity between baseline hrHPV test with different RLU/CO thresholds and baseline cytology testing for CIN3+ and CIN2+, adjusted for non-attendance at repeat testing.

<table>
<thead>
<tr>
<th>Test (Threshold BMD)</th>
<th>End point CIN3+</th>
<th>End point CIN2+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specificity (%)</td>
<td>95% CI</td>
</tr>
<tr>
<td>Baseline cytology</td>
<td>98.7</td>
<td>98.5–98.8</td>
</tr>
<tr>
<td>Baseline HC2 positivity threshold, RLU/CO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>95.5</td>
<td>95.3–95.8</td>
</tr>
<tr>
<td>2</td>
<td>96.2</td>
<td>95.9–96.4</td>
</tr>
<tr>
<td>5</td>
<td>96.7</td>
<td>96.5–96.9</td>
</tr>
<tr>
<td>7</td>
<td>96.9</td>
<td>96.7–97.1</td>
</tr>
<tr>
<td>10</td>
<td>97.2</td>
<td>96.9–97.4</td>
</tr>
<tr>
<td>30</td>
<td>98.0</td>
<td>97.8–98.1</td>
</tr>
<tr>
<td>50</td>
<td>98.2</td>
<td>98.0–98.4</td>
</tr>
<tr>
<td>100</td>
<td>98.7</td>
<td>98.5–98.8</td>
</tr>
</tbody>
</table>

BMD=borderline or mild dyskaryosis; CI=confidence interval; CIN=cervical intraepithelial neoplasia (grade 2 or 3 or higher); HC2=hybrid capture 2; hrHPV=high-risk human papillomavirus; RLU/CO=relative light unit/cutoff.
Table 2 presents the specificity for detected CIN3+ and CIN2+ lesions for baseline cytology testing (threshold BMD) and for a strategy of primary HC2 testing without follow-up testing at different RLU/CO thresholds. Compared with baseline cytology testing, hrHPV testing at the standard threshold of 1 RLU/CO had a lower specificity for CIN3+ (95.5 vs 98.7%) and CIN2+ (95.9 vs 98.9%). The specificity for CIN3+ and CIN2+ increased with increasing RLU/CO thresholds. Only at a RLU/CO threshold of 100, hrHPV testing reached the same specificity for CIN2+ (i.e., 98.9%) and CIN3+ (i.e., 98.7%) as cytology.

The relative colposcopy referral rate, relative sensitivity and relative false positivity rate of primary HC2 testing at different RLU/CO thresholds vs baseline cytology testing are presented in Table 3. Compared with cytology, hrHPV testing at a threshold of 1 RLU/CO would result in a 2.8-fold higher number of colposcopy referral rates. With increasing HC2 threshold, the relative colposcopy referral rates decreased, resulting in a relative rate of 1.9 at 10 RLU/CO and 0.94 at 100 RLU/CO.

Table 3 Relative colposcopy referral rates, relative sensitivity, relative false positivity rate of HC2 RLU/CO thresholds at baseline alone vs baseline cytology testing, adjusted for non-attendance at repeat testing.

<table>
<thead>
<tr>
<th>Test</th>
<th>Relative colposcopy referral rate</th>
<th>End point CIN3+</th>
<th>End point CIN2+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative referral rate</td>
<td>Relative</td>
<td>Relative false</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sensitivity</td>
<td>positivity rate</td>
</tr>
<tr>
<td>Baseline cytology (threshold BMD)</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Baseline HC2 positivity threshold, RLU/CO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.82</td>
<td>1.36</td>
<td>3.35</td>
</tr>
<tr>
<td>2</td>
<td>2.48</td>
<td>1.34</td>
<td>2.89</td>
</tr>
<tr>
<td>5</td>
<td>2.18</td>
<td>1.31</td>
<td>2.49</td>
</tr>
<tr>
<td>7</td>
<td>2.06</td>
<td>1.28</td>
<td>2.34</td>
</tr>
<tr>
<td>10</td>
<td>1.90</td>
<td>1.22</td>
<td>2.14</td>
</tr>
<tr>
<td>30</td>
<td>1.42</td>
<td>1.08</td>
<td>1.53</td>
</tr>
<tr>
<td>50</td>
<td>1.24</td>
<td>0.95</td>
<td>1.34</td>
</tr>
<tr>
<td>100</td>
<td>0.94</td>
<td>0.80</td>
<td>0.99</td>
</tr>
</tbody>
</table>

BMD=borderline or mild dyskaryosis; CIN=cervical intraepithelial neoplasia (grade 2 or 3 or higher); HC2=hybrid capture 2; RLU/CO=relative light unit/cutoff.

At the standard test positivity threshold for HC2 (i.e., 1 RLU/CO), the relative sensitivity of hrHPV was superior to that of cytology, both for CIN3+ (relative sensitivity of 1.36) and CIN2+ relative sensitivity of 1.50. With increasing HC2 threshold values, the relative sensitivity for CIN3+ decreased, resulting in a relative sensitivity of 1.22 at 10 RLU/CO and 0.80 at 100 RLU/CO. Results were comparable using CIN2+ as outcome measure.

There was no HC2 threshold that resulted in an improved false positivity rate and concomitant colposcopy referral rate, without compromising its sensitivity. In fact, the HC2 threshold (i.e., 100 RLU/CO) at which a lower colposcopy referral rate was reached compared with cytology, also revealed lower sensitivities for CIN3+ and CIN2+ (Table 3). As no strategy of sole hrHPV testing at baseline improved on baseline cytology testing, some form of triage or follow-up testing is required.
Table 4 shows the impact of raising the HC2 threshold in the context of two triage and follow-up strategies for HC2-positive women compared with baseline cytology testing. For the strategy with cytology triage at baseline and at 12 months, HC2 screening at RLU/CO thresholds between 1 and 30 resulted in higher sensitivities for both CIN2+ and CIN3+, compared with baseline cytology testing. This strategy showed lower colposcopy referral rates and false positivity rates at all analysed RLU/CO thresholds (1–100).

For the strategy with cytology triage at baseline and combined cytology and HC2 testing (with the same threshold as used at baseline) at 12 months, only at threshold 30, a lower false positivity and colposcopy rate in combination with higher sensitivities for CIN3+ and CIN2+ than baseline cytology testing was found. However, at RLU/CO 30, the gain in sensitivity compared with cytology was only marginal.

The relative sensitivity vs relative false positivity rate of the three investigated screening strategies, compared with baseline cytology testing, is graphically shown in Figure 2. Baseline cytology testing is presented in the origin (relative sensitivity = 1, relative specificity = 1). Quadrant II represents combinations of sensitivity and false positivity rates that are superior to baseline cytology testing. The strategy with base-

<table>
<thead>
<tr>
<th>Test</th>
<th>Relative colposcopy referral rate</th>
<th>Relative sensitivity</th>
<th>Relative false positivity rate</th>
<th>Relative sensitivity</th>
<th>Relative false positivity rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline cytology</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Baseline HC2 positivity threshold, triage test RLU/CO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Cytology</td>
<td>Cytology</td>
<td>0.94</td>
<td>1.32</td>
<td>0.80</td>
</tr>
<tr>
<td>2</td>
<td>Cytology</td>
<td>Cytology</td>
<td>0.89</td>
<td>1.29</td>
<td>0.74</td>
</tr>
<tr>
<td>5</td>
<td>Cytology</td>
<td>Cytology</td>
<td>0.84</td>
<td>1.25</td>
<td>0.69</td>
</tr>
<tr>
<td>7</td>
<td>Cytology</td>
<td>Cytology</td>
<td>0.82</td>
<td>1.22</td>
<td>0.68</td>
</tr>
<tr>
<td>10</td>
<td>Cytology</td>
<td>Cytology</td>
<td>0.79</td>
<td>1.17</td>
<td>0.65</td>
</tr>
<tr>
<td>30</td>
<td>Cytology</td>
<td>Cytology</td>
<td>0.65</td>
<td>1.02</td>
<td>0.52</td>
</tr>
<tr>
<td>50</td>
<td>Cytology</td>
<td>Cytology</td>
<td>0.57</td>
<td>0.90</td>
<td>0.45</td>
</tr>
<tr>
<td>100</td>
<td>Cytology</td>
<td>Cytology</td>
<td>0.46</td>
<td>0.75</td>
<td>0.36</td>
</tr>
</tbody>
</table>

BMD=borderline or mild dyskaryosis; CIN=cervical intraepithelial neoplasia (grade 2 or 3 or higher); HC2=hybrid capture 2; RLU/CO=relative light unit/cutoff. aHC2 RLU/CO threshold as at baseline.
line HC2 testing alone was inferior to baseline cytology testing for all RLU/CO thresholds. The strategy with cytology triage of HC2-positive women and cytology testing at 12 months showed the best combination of relative sensitivity and false positivity rates for RLU/CO between 1 and 30. The RLU/CO data points for this strategy form a steep curve. This indicates that at increasing RLU/CO thresholds, the reduction in false positivity rate in this strategy is relatively small, whereas the decrease in sensitivity is substantial. Thus, low RLU/CO values are required to maintain high sensitivity.

**Figure 2** Relative sensitivity vs relative false positivity rate for three strategies for hybrid capture 2 (HC2)-positive women at different relative light unit/cutoff (RLU/CO) thresholds compared with baseline cytology (cyto) testing, for detection of cervical intraepithelial neoplasia grade 3 or higher (CIN3+). Relative sensitivity for detection of CIN3+ is plotted on the y-axis, against the relative false positivity rate on the x-axis. The used HC2 RLU/CO thresholds are indicated at the respective positions above each plot. I: quadrant with relative sensitivity and relative false positivity rate greater than cytology; II: panel with relative sensitivity greater than and relative false positivity rate lower than cytology; III: panel with relative sensitivity lower than and relative false positivity rate lower than cytology; IV: panel with relative sensitivity and relative false positivity rate lower than cytology. BMD = borderline or mild dyskaryosis.
Discussion
In this study, we evaluated three possible cervical screening strategies that are based on hrHPV testing with different HC2 thresholds and compared them with baseline cytology testing (threshold BMD). We aimed to improve the specificity of hrHPV screening by increasing the RLU/CO threshold, while maintaining a higher sensitivity than baseline cytological testing. The results are based on data from the VUSA-Screen study, a population-based cohort study carried out in the Utrecht province of the Netherlands. We found that compared with baseline cytology testing, there was no HC2 RLU/CO threshold for which a screening strategy of hrHPV testing as a sole primary screening instrument resulted in both superior sensitivity as well as similar (or lower) colposcopy rate and equal (or higher) specificity. As baseline hrHPV testing cannot improve baseline cytology testing, even at increased RLU/CO thresholds, we conclude that some form of triage and follow-up is required in hrHPV screening.

Given that follow-up testing is required in hrHPV-positive women, we searched for a strategy that did not increase colposcopy referral rate compared with cytological testing. A screening strategy that was clearly superior to baseline cytological testing was primary hrHPV screening, with RLU/CO thresholds between 1 and 30 and cytology triage at baseline and repeated cytology testing at 12 months. This strategy was not only more sensitive than baseline cytology testing but also resulted in lower false positivity rates and in fewer colposcopy referrals. Using HC2 RLU/CO thresholds between 1 and 5 results in higher sensitivity (relative sensitivity between 1.32 and 1.25, respectively) and a reduced colposcopy referral rate (between 6 and 16%, respectively) compared with baseline cytology testing. The current threshold of 1 RLU/CO makes optimal use of the superior sensitivity of the hrHPV test for CIN3+/CIN2+, without actually increasing the colposcopy referral rate compared with baseline cytology. The colposcopy referral rate of this strategy is therefore substantially lower than a screening scenario with baseline hrHPV testing only. In addition, a screening strategy with a high sensitivity may allow for extension of the screening interval, which in turn reduces colposcopy referral rates.34

An important issue in the debate about implementation of hrHPV testing has been the increased adverse effects in terms of unnecessary referrals for colposcopy among women with a positive hrHPV test. The issue of overdiagnosis and overtreatment is of particular importance for women of reproductive age, because it has been shown that the rate of serious obstetrical complications, such as preterm deliveries, low birth weight and premature rupture of the membranes, is increased after excisional treatments for precancerous lesions.35 Therefore, there is a need to identify strategies that minimise the need for colposcopy referrals with hrHPV testing, while maintaining its advantage in terms of sensitivity. A number of studies have evaluated the optimisation of cervical screening by studying the different hrHPV HC2 cutoff levels for test positivity.36;20;22;24;25;37-39 Kotaniemi-Talonen et al 24 concluded that when used as a sole screening test, the hrHPV test cutoff level can be increased to 10 RLU/CO. The specificity of hrHPV screening, however, remained lower than that with conventional cytology testing even at the threshold of 10 RLU/CO. This is in line with our findings for the strategy of baseline HC2 testing only. It should be noted that the hrHPV
CHAPTER 6

test may compare to be more favourable with cytology in other countries. In the Netherlands and Finland, the specificity of cytology is quite high. This is also the case for other European screening programmes, but worldwide the specificity of cytology is highly variable. Ronco et al. proposed only a slight increase of the threshold up to 2.00 RLU/CO when HC2 is used for population-based screening. The same threshold has been proposed by Sargent et al. A minimal increase in threshold reflects a preference for a sensitive screening strategy. We found that, in an hrHPV DNA screening strategy with cytology triage and cytology testing at one follow-up visit, an increased sensitivity as well as decreased colposcopy referral rate is possible with RLU/CO thresholds up to 30. Given our observation that hrHPV testing cannot be used as a sole screening instrument and that triage and repeated testing is necessary anyhow, we also prefer a low threshold of 1 to maintain the highest sensitivity.

There are some limitations in our study. In this study, women received cytology and hrHPV testing, and based on both results, women were referred for colposcopy. Therefore, we were not able to compare different RLU/CO thresholds outcomes with current cytology screening programme but only with baseline cytology testing. Furthermore, we adjusted for non-attendance at repeat testing, but the results were not adjusted for differences in intensity of follow-up testing. In addition, actual colposcopies were not reported. Another limitation of our study may be the use of a subjective test, such as cytology, as a triage test for hrHPV-positive women. Awareness of the negative or positive hrHPV test result may affect the criteria for defining cellular abnormalities. However, in this study, the cytotechnicians were not informed about the hrHPV test result. Nevertheless, even in case cytotechnicians were aware of the hrHPV test results, as in a Finnish trial, the hrHPV test information only had a small effect on cytology assessment, and therefore on the CIN3+ detection rate and the number of colposcopies. In this context, it may be expected that in the near future molecular biomarkers may be used as objective triage tests of hrHPV-positive women. Suitable candidate novel biomarkers such as HPV mRNA, p16 ink4a, methylation markers, or genotyping might further enhance the efficacy of screening with hrHPV DNA. Presently, we are investigating the possible value of such alternative triage tests in hrHPV-positive women and preliminary results show that better results can be obtained than with cytology.

Our finding that hrHPV testing alone at the predefined assay threshold of 1 RLU/CO had a somewhat lower specificity than cytology for CIN2+ and CIN3+ is consistent with results from other randomised and nonrandomised studies using HC2 testing, or another clinically validated hrHPV test. However, compared with these studies, our observed specificity of the hrHPV test was relatively high (i.e., 95.9% (95% CI 95.7–96.2) for CIN2+ and 95.5% (95% CI 95.3–95.8) for CIN3+). At least in part, this difference in specificity estimates may be explained by differences in the design of hrHPV screening studies and study populations. Our study included hrHPV testing combined with cytology. In addition, it was conducted within the setting of an organised cervical screening programme with high invitational coverage and low incidence of cervical cancer.
A strong point of this study is the longitudinal design and the older age range of study participants (30–60 years), which is the age for which hrHPV testing is most widely advocated. The study was population-based and part of a routine organised screening activity in a low-risk population, indicating that the results could be implemented in routine practice.

To conclude, no RLU/CO threshold was found for which HC2 testing at baseline resulted in a similar or lower colposcopy referral rate than baseline cytology, while maintaining a higher sensitivity.

Superior combinations of sensitivity and colposcopy rate are possible for HC2 testing with cytology triage at baseline and repeated cytology testing after 1 year. As increasing the RLU/CO threshold only marginally decreases colposcopy referral rate while substantially reducing sensitivity, we suggest maintaining the currently used RLU/CO threshold of 1. This results in more than 30% higher sensitivities for CIN3+ than cytology testing at a 20% lower false positivity rate and 6% lower colposcopy referral rate.

References

33. Pepe MS, Alonzo TA. Comparing disease screening tests when true disease status is ascertained only for screen positives. Biostatistics 2001;2:249-60
CHAPTER 7

General Discussion

The role of hrHPV testing in cervical cancer screening

In many developed countries, cytology-based cervical cancer screening programmes have decreased the incidence and mortality of cervical cancer. However, in recent years, this decreasing effect on the incidence of cervical cancer has levelled off. This can be explained by the varying screening uptake among women, a relatively high number of false-negative cytology tests (lack of sensitivity) and suboptimal follow-up of screen-positive women. In addition, although the incidence of squamous cell carcinoma has decreased, the incidence of cervical adenocarcinoma does not display a decreasing trend. These findings confirm that adenocarcinoma and its precursor lesions are difficult to detect by cervical cytology. In addition, the incidence of adenocarcinoma seems to be increasing in young women.

Since hrHPV testing has a higher sensitivity and offers better protection against high-grade cervical lesions and cervical cancer than cytology, implementation of hrHPV testing as primary cervical cancer screening test is presently considered in many countries.

Here, we will discuss whether cervical screening can be improved by 1. hrHPV testing to triage women with borderline or mild dyskaryosis and 2. implementing hrHPV testing as a primary screening test. Furthermore, we addressed the following questions: at what age should hrHPV testing be started? What is the appropriate triage algorithm for hrHPV-positive women to minimize the loss of specificity with hrHPV screening compared to cytology screening? In addition, we will discuss how cervical screening can be improved by hrHPV testing on self-collected cervicovaginal specimens. Finally, effects of HPV vaccination on screening and future aspects of hrHPV-based screening are discussed.

HrHPV testing to triage women with borderline or mild dyskaryosis

Currently, cervical cancer screening programmes are based on cytology. About 2.5% of the women participating in the Dutch population-base screening programme have a borderline or mild dyskaryosis (BMD) test result. Of these women, only 10%-20% harbor CIN2+ lesions. These women are therefore not directly referred for colposcopy but are advised to repeat cytology at 6 and 18 months and are only referred for colposcopy if any of the repeat cytology tests is abnormal (threshold ≥ BMD). The majority of referred women, however, will have meaningless lesions that will regress spontaneously. To reduce the number of referrals and colposcopies, the New Dutch guidelines allowed laboratories to choose to include hrHPV testing at the repeat visit at 6 months (Figure 1). This guideline change was a first step in the transition towards hrHPV testing. The complexity of this follow-up testing strategy is considerable since women with a repeat smear of BMD and who test positive for hrHPV are referred to a gynecologist, while those with a negative hrHPV test still get a repeat smear at 18 months after the first test (Figure 1). Moreover, only women with a normal repeat smear and negative hrHPV test are referred back to the screening programme, whereas their hrHPV-positive counterparts are offered repeat cytology at 18 months. Women with a repeat smear of >BMD are, independent of the hrHPV test result, directly referred to the gynecologist.
We studied whether a less complex referral strategy is possible where women with BMD are triaged at baseline with hrHPV testing (Chapter 2). In this algorithm women with BMD and a positive hrHPV test are directly referred for colposcopy while the women with BMD and a negative hrHPV test are advised to repeat cytology at 6 and 18 months.

Our study showed that hrHPV triaging of women with BMD resulted in a high CIN3 and CIN2+ detection rate (10.7% and 22.3%, respectively). This finding is consistent with meta-analyses that have shown that hrHPV triaging has a higher sensitivity than repeat cytology for detection of CIN2+. Furthermore, in our study women with BMD and a negative hrHPV test had a low three-year risk to develop high-grade lesions (CIN3 of 1.2% and CIN2+ of 2.9%) and therefore could be directly referred to the routine screening. The next regular screening round (interval 5 year) could serve as a safety net, at least for women aged ≤ 55 years who will still be invited for screening.
Disadvantage of triage by hrHPV testing at baseline is that approximately 20% of the women will have cleared their hrHPV infection in the first 6 months of follow-up. As a consequence, hrHPV triaging at baseline might result in 51.5% higher colposcopy referral rates than repeat cytology (colposcopy referral rates of 57.6% versus 38.0%, respectively). However, our study showed that hrHPV triaging did not lead to an increase in the referral rate per detected CIN3. The medical costs per detected CIN3 were even slightly lower for hrHPV triaging than for repeat cytology testing.

Advantages of baseline triaging instead of repeat cytology are ease of implementation, low loss of follow-up, fast diagnosis and low distress for participating women.

Recommendations

- HrHPV triaging of women with BMD has shown to be at least as effective for detection of CIN3 as repeat cytology. Women with BMD should be directly triaged by hrHPV testing instead of by repeat cytology testing to achieve a fast diagnosis and therefore reduce unnecessary distress for women.
- Women with BMD and hrHPV-positive test should be referred for colposcopy, since these women have a high risk to develop CIN3 and CIN2+ (20.4% and 41.9%, respectively).
- Women with BMD and hrHPV-negative test result have an acceptable low 3 year CIN3+ risk of 1.3% and CIN2+ risk of 2.9% and therefore could be referred to routine screening.

Primary hrHPV based screening

We evaluated hrHPV testing in two prospective population-based screening studies: the POBASCAM trial that followed 40,105 women aged 29-56 for two screening rounds (9 year interval) and the VUSA-screen study that followed 48,088 women aged 29-61 for 3 years.

Both studies showed that hrHPV testing has a higher cross-sectional sensitivity, but lower specificity than cytology for detecting CIN2+ (Chapter 3 and 4). These results are in line with other screening studies. These trials showed that the cross-sectional sensitivity of hrHPV testing for CIN2+ was 49% and for CIN3+ was 30% higher than that of cytology, while the specificity of hrHPV testing was 2.5-4% lower.

The POBASCAM trial furthermore showed that hrHPV testing in combination with cytology resulted in higher detection of CIN2+ lesions compared to cytology testing alone at the first round. This resulted in an improved protection against CIN3+ at the second screening round compared with cytology alone (relative risk 0.73, 95% CI 0.55-0.96) (Chapter 4). These findings are in agreement with other randomised controlled trials, and our interim analysis. Additionally, as was the case in the NTCC trial, our study showed that hrHPV testing provides better protection against cervical cancer in the second screening round than cervical cytology (relative risk 0.29, 95% CI 0.10-0.87). Collectively, all trials showed that hrHPV testing significantly reduces detection of CIN3+ lesions in the second screening round relative to cytology.
These results suggest that an extension of the screening interval may be considered when hrHPV testing is implemented.\textsuperscript{22,26} This is also consistent with the results of the VUSA-Screen study and other studies,\textsuperscript{28-31} showing a very low detection of CIN3+ and CIN2+ lesions after a negative hrHPV test (Chapter 3).\textsuperscript{17} A model study based on data from the POBASCAM study\textsuperscript{32} indicate that an extension of screening interval to 7.5 years is possible without increasing the cancer risk as presently observed with 5-yearly cytology.

Questions have risen whether the increased sensitivity of hrHPV testing results in over-diagnosis of lesions that otherwise would have regressed spontaneously. The long screening interval used in the POBASCAM trial (5 years) may provide some information on whether a cervical lesion is persistent or regressive. The POBASCAM trial showed that over-diagnosis in the women studied (30-60 year) does not seem a clinically relevant problem since the cumulative number of women with CIN3+ over both screening rounds did not differ between intervention (hrHPV & cytology) and the control (cytology only) group (259 of 19,999 vs 272 of 20,106; 0.96, 95%CI 0.81-1.14). This result supports the idea that hrHPV testing leads to earlier detection of clinically relevant lesions.

Another important question is whether hrHPV testing should be offered in combination with cytology or as a single, primary screening instrument. Because hrHPV testing has a very high sensitivity, others and we showed that combined testing with cytology was not better than hrHPV screening alone in detecting CIN3+ and CIN2+ lesions (pooled detection ratios of 1.04 (95%CI 0.92-1.17 for CIN3+ and 1.06 (95%CI 0.97-1.16 for CIN2+, respectively).\textsuperscript{16,17,23,27} For this reason, cervical screening with a primary stand-alone hrHPV test seems preferable. This recommendation is confirmed by cost-effectiveness studies.\textsuperscript{32-34}

About 20% of all cervical cancers are adenocarcinomas.\textsuperscript{35} A problem of the present cytology-based programmes is that in contrast to squamous cell carcinomas the incidence of adenocarcinomas is not decreasing.\textsuperscript{36-38} Reasons for this finding are 1) the poor cytological definition of the precursor lesions of adenocarcinomas except for adenocarcinoma in situ resulting in poor recognition by the cyto-pathologists and 2) the localization of these lesions higher in the endocervical canal. Exfoliation of representative abnormal cells is therefore limited and abnormal cylinder cells are more difficult to detect by the cytopathologist. Studies demonstrated that hrHPV testing has a higher sensitivity for precursor lesions of adenocarcinoma of the cervix than cytology testing.\textsuperscript{39,40} The NTCC trial\textsuperscript{27} reported a higher occurrence of adenocarcinomas after cytology screening compared to hrHPV screening, confirming the notion that cytology is particularly less effective in preventing adenocarcinomas. However, in the POBASCAM trial no significant differences in detection of adenocarcinomas between intervention (hrHPV & cytology) and control arm (cytology) was detected, probably because the number of cervical adenocarcinomas in this study was small. Therefore, the results from prospective trials need to be pooled to determine whether hrHPV testing prevents adenocarcinomas better than cytology. Presently, these pooled trial data are analysed in the PreHdict consortium (www.ecca.info/campaigns/prehdict.html).
CHAPTER 7

Another issue is how much each of the HPV genotypes contributes to the detection of CIN3+ and CIN2+. The POBASCAM study showed that hrHPV testing mainly resulted in early detection of HPV16 associated CIN2+ lesions in the first screening round followed by a reduction of HPV16 CIN3+ lesions in the second round. This finding is in agreement with the finding that HPV16 is the most common genotype in cervical cancer. The contribution of other non-HPV16 genotypes could not be assessed because of the limited numbers of women in the study.

Recommendations

- HrHPV testing in cervical screening provides better protection against CIN3+ and cervical cancer in the second screening round than cytology by earlier detection of clinically relevant CIN2+ lesions in the first round.
- HrHPV testing alone should become the primary screening method.
- Women with a negative hrHPV test have an extremely low risk to develop high-grade cervical lesions. Longer screening intervals (up to 7.5 years) may therefore be used.

Age to begin screening

An important issue is the age at which hrHPV testing should be offered in primary screening. Women under the age of 30 years have a high prevalence of hrHPV infections and the majority of these infections are generally cleared in a relatively short time. Screening young women under 30 years by hrHPV testing results in detection of mostly transient hrHPV infections. As women become older, the prevalence of hrHPV decreases and stabilizes at the age of 35 years. Older women are more likely to have a persistent infection and they are therefore more likely to benefit from intervention. In addition, incidence and mortality rates for cervical cancer among women younger than 30 are low. In the Netherlands, 4.5% of all carcinomas are found under the age of 30 years. The benefit of screening all women between 25-30 years does not outweigh the negative screening effects (high referral rates for colposcopy, which in turn might result in unnecessary treatment and the risk of preterm births and substantial anxiety). Therefore, it has been decided in the Netherlands that hrHPV testing should not be used to screen women younger than 30 years.

Ronco and colleagues reported that hrHPV testing in women aged 25-34 years could lead to substantial over-diagnosis of regressive CIN2+ lesions. As a result, they suggested that hrHPV screening should not start in women younger than 35 years. In the POBASCAM trial, our separate analyses of women attending screening for the first time (age 29-33 years) and older women (age 34-56 years) revealed that the CIN3+ detection rates over two rounds were similar for the intervention and control group within both age subgroups. Also for CIN2+, the detection rates were similar for the intervention and control group (see Figure 3, Chapter 4). These data therefore indicate that hrHPV testing in women aged 29-33 does not result in excessive diagnosis of cervical lesions and argues for implementation of hrHPV testing in programmed cervical screening at a starting age of 30 years.
CHAPTER 7

In addition, we observed in the VUSA-Screen study that the CIN3+ and CIN2+ detection rate in hrHPV-positive women was similar for women invited for cervical screening for the first time (age 29–33 years) and for older women (>34 years). Thus also the data from the VUSA-Screen study support the notion that screening should start at the age of 30.

Recommendations
• Screening by primary hrHPV testing should start at the age of 30 years.

Management of hrHPV-positive women
An adverse effect of using an hrHPV test for cervical screening is that transient infections are detected. In population-based screening, specificity of the screening test is of utmost importance, as it basically determines the costs of the programme and the occurrence of adverse effects (repeat screening test(s), colposcopy referrals, treatment of regressive lesions, anxiety) in the generally healthy population. At first, it is therefore important to use a clinically validated hrHPV test in primary cervical screening. Secondly, management of hrHPV-positive women deserves attention.

In the Netherlands, about 5% of the women between 30 and 60 years of age are hrHPV-positive, and approximately 13% of them have an underlying CIN3+ and about 22% a CIN2+ lesion. Therefore, not all hrHPV-positive women should be directly referred because this would result in a substantial increase in colposcopies and may result in overtreatment. The latter is particularly problematic because unnecessary excisional treatment of cervical lesions may result in preterm delivery in subsequent pregnancies.

An option to improve the specificity of an hrHPV-based screening algorithm is to narrow the definition for a positive hrHPV screening test, for example by increasing the threshold of the hybrid capture 2 HPV DNA test (> 1 RLU). Kotaniemi-Talonen et al. and Ronco et al. concluded that the cutoff can be increased to, respectively 10 RLU/CO and 2 RLU/CO. However, in our VUSA-Screen study (Chapter 6), we found that there was no HC2 threshold for which single hrHPV screening resulted in both superior sensitivity and specificity compared to cytology screening. Therefore, we conclude that changing the threshold of the HC2 test is not sufficient and that some form of triage and/or follow-up testing is required for hrHPV-positive women.

Several triage suggestions for hrHPV-positive women have been made in the literature. Because cytology has a relatively high specificity, it seems suitable to triage hrHPV-positive women. Also genotyping for HPV16 and HPV18 has been considered as triage option as those types are associated with strongly increased CIN2+ risk.

We compared 14 triage/follow-up testing strategies using cytology, hrHPV testing and/or HPV genotyping (Chapter 5). A triage strategy was considered feasible if the 2-year CIN3+ risk was equal to or lower than 2% (corresponding with a NPV of at least 98%), which is presently considered acceptable according to the Dutch screening guidelines. The most attractive strategy was cytology testing at baseline and at 12 months because it has a low 2-year CIN3+ risk (below 1%), a high PPV of 37.5% and
results in only a modest colposcopy referral rate in the total population (1.70%, 95% CI 1.54-1.85). In addition, this strategy is easy to communicate to physicians and women. Another attractive but more costly strategy is combined cytology and HPV16/18 genotyping at baseline followed by repeat cytology at 12 months. This strategy has a low CIN3+ risk (0.3%) and a lower PPV of 25.6% but results in a relatively high colposcopy referral rate in the total population (2.53%, 95CI 2.34-2.73). Both strategies entail short-term recalls of hrHPV-positive (for the second strategy hrHPV-positive but HPV16/18 negative), cytology negative women and demand a high level of compliance to follow-up. One baseline strategy without repeat testing, i.e. combined cytology with HPV16/18/31/33/45 genotyping may be considered as an alternative because it resulted in a CIN3+ risk of test negative women of <1% and does not depend on follow-up compliance. However, such a strategy has a low PPV of 20.7% and will lead to a high overall colposcopy referral rate in the total population (2.95% (95%CI: 2.75-3.17).

It should be kept in mind that in the Netherlands, cytology has a high quality. The cytology test shows a relatively high sensitivity and specificity and the cytological abnormality rates are low. As a result, the most attractive strategy to triage hrHPV-positive women is cytology testing at baseline and at 12 months. However, in countries where the quality of cytology is worse, cytology and HPV16 and HPV18 genotyping might be an alternative triage tool.

Loss to follow-up is a major risk factor of a screening strategy with a repeat test. Several studies have shown that attendance at repeat testing may be poor, particularly after a cytologically normal test result. Appropriate communication strategies are therefore necessary to establish high attendance at repeat testing. In addition, the logistics of a triage and follow-up strategy should preferably be simple.

Although cytology is an appropriate triage tool for hrHPV-positive women, triage can be improved since the test is subjective and has a rather low reproducibility. Several new molecular biomarkers identified by molecular carcinogenesis research are developed to use as triage test. One of them is p16/ki67 dual-stain cytology. This test may be used as surrogate marker of cell cycle deregulation mediated by transforming hrHPV infections. Promising results have been shown and the test (CINTEC®) seems to perform better than cytology. However, application of the CINTEC® test still requires cyto-pathological experience and prospective studies are needed to validate the published data. Another marker may be the HPV-Proofer E6/E7 mRNA test (Norchip®), detecting an active infection with cell- transforming potential for five hrHPV types. Indeed a positive E6/E7 mRNA test is associated with CIN3+/CIN2+ but the risk of women with a negative E6/E7 mRNA test is too high to dismiss these women from further follow-up. In addition, promoter methylation analyses of tumor suppressor genes have been proposed as triage tool for hrHPV-positive women. CADM1 and MAL methylation markers showed to be promising candidates but prospective studies are needed. These studies are presently ongoing. These molecular biomarkers may pave the way to a complete molecular-based cervical screening program, potentially also suitable to medium/low resource countries.
Recommendations

• HrHPV-positive women need to be triaged to prevent over-diagnosis, overtreatment and high costs.
• In countries with a high quality of cytology, cytology testing at baseline followed by repeat cytology testing at 12 months is an attractive triage tool.
• In countries where the quality of cytology is less good, triage by combined cytology and HPV16/18 genotyping at baseline followed by repeat cytology at 12 months might be considered for implementation.

Improve screening participation by hrHPV testing on self-collected material

In the Netherlands about 35% of the invited women do not respond to a screening invitation. More than half of the cervical cancer cases detected in the Netherlands did not have a previous cervical smear. Therefore, attempts should be made to increase the number of women participating in the programme. Offering women a user-friendly self-sampling device for collecting cervico-vaginal material for hrHPV testing has shown to increase participation rate.

Self-sampling devices enable women to take their own (cervico-)vaginal sample at a suitable time and place. About 30% of the non-participating women responded actively by returning a self-sampling device, which is considerably higher than the rates of 10-17% obtained when women were sent one or two reminders for regular screening. Furthermore, the yield of high-grade lesions was higher in self-sampling responders (non-responders to an invitation of the regular screening program who positively reacted to offering hrHPV testing on self-collected material) compared to screening participants. The hrHPV test performed comparable in terms of sensitivity for CIN2+ on self-sample material and on smears taken by the general practitioner.

In addition, self-sampling may improve participation in countries with cultural and religious programme barriers by increasing acceptance and access to cervical cancer screening. Furthermore, self-sampling can improve screening in countries with lack of medical staff and screening facilities. Indeed, self-sampling has shown to facilitate access to cervical screening in developing regions and may well lead to increased screening participation.

However, cytology cannot be performed on self-collected material, most likely due to the fact that self-sampled material mostly contain admixed vaginal cells and relatively few intact cervical cells (so called cervical indicator cells) indicative for a high-grade precursor lesion of the cervix. For triage testing the women who test hrHPV-positive on self-collected (cervico-)vaginal material have therefore to be referred to the general practitioner for a cervical cytology smear. This extra visit to the physician results in discomfort for the women and loss to follow-up , and is unfeasible in under-resourced settings lacking medical services. Application of triage testing directly on the self-sampled specimens by a non-morphological biomarker test would...
be an ideal alternative to select hrHPV-positive women in need of colposcopy. Studies nowadays evaluate new molecular triage markers that are directly applicable on self-sampling tests, such as methylation markers. In this way a complete molecular objective non-morphological cervical screening can be offered. This will make cervical screening also available to medium and low resource countries.

Recommendations
• Offering self-sampling for hrHPV testing is a promising, effective alternative to sampling by clinicians and improves screening compliance rates.

HPV vaccination
The HPV vaccination programme started in the Netherlands in 2009 for girls of 12 years old with a catch-up for 13-16-year old girls. The prophylactic HPV vaccines have shown to be highly efficacious to prevent high-grade cervical lesions at a population level. It is assumed that HPV16/18 VLP L1 vaccines will prevent about 70%-80% of the squamous cell carcinomas and 90% of the adenocarcinomas. Since the HPV vaccines only have a prophylactic effect when women are hrHPV-negative, they are given to young, hrHPV naïve girls. However, HPV vaccination coverage for the first dose in the Dutch population targeted by the catch-up campaign was about 55%, lower than the aimed 70%. There was a lower uptake among women in conservative religious and ethnic communities and among women with lower socioeconomic status. Therefore, better communication strategies are needed to increase the vaccination attendance rate.

It is, however, of note that the potential benefit of HPV vaccination might be larger than the coverage would indicate because of indirect (herd immunity) protection to non-vaccinated women.

Vaccination does not eliminate the need for cervical cancer screening. The vaccinated women remain at risk of developing cervical cancer associated with non-HPV16/18 genotypes. Even if hrHPV cross-protection is taken into account, about 20% of the cervical carcinomas will not be prevented by vaccination.

HPV vaccination is expected to have an impact on the effectiveness of cytology and hrHPV screening. Vaccination probably decreases hrHPV positivity, cytological abnormalities and reduces the number of high-grade cervical lesions. In countries with effective screening programmes and high coverage rates (i.e., Denmark, Finland, Iceland, the Netherlands, and the UK), the impact of vaccination on the reduction of cervical cancer cases is expected to be relatively small. The impact of vaccination would be further attenuated if vaccination leads to a false sense of security resulting in lower adherence to the screening programme. Therefore, it is very important to educate and motivate women to attend cervical screening programme, even if they are vaccinated. The effect of vaccination is expected to be higher on the incidence of adenocarcinomas because HPV16 and 18 are associated with more than 90% of the adenocarcinomas. HPV vaccination may negatively influence current cytology
based screening since the decrease of cytological abnormalities and high-grade lesions may deteriorate the quality of smear reading.\textsuperscript{94} This is an additional reason to implement hrHPV testing as primary screening test since this test is objective. Cytology could be used as triage test for hrHPV-positive women, because in this group the occurrence of abnormal smear is increased.

**Recommendations**

- Besides HPV vaccination screening for cervical abnormalities will remain important to prevent cervical cancer because 1. the current vaccines protect against about 70%-80% of the cervical cancer cases and 2. the participation rate of vaccination will never reach 100% leaving a considerable number of women at risk of getting infected by hrHPV.

**Future perspectives**

We showed that hrHPV testing is superior to cytology as a primary screening test in cervical cancer screening programmes for women 30 years and older. Additionally, we presented feasible triage algorithms for hrHPV-positive women. Taken together, time has come for implementing hrHPV testing in nationwide cervical screening programmes.

Offering self-sampling to women who do not attend regular screening resulted in a response rate of about 30\%.\textsuperscript{67-68,70,71} Moreover, the number of CIN3+ and CIN2+ lesions detected by hrHPV testing in non-responders was significantly higher than in women responding to the regular screening programme. These results support implementation of self-sampling in the cervical screening programme, at least for non-attendees.\textsuperscript{68,70}

In the future, self-sampling may be offered as primary screening test to all women eligible for cervical screening. Ideally, women would be given the choice for either self-sampling or physician-collection. These options need to be further analysed in implementation studies followed by cost-effectiveness analyses.

Topics of further research include ways to improve triage testing of hrHPV-positive women. Although cytology has shown to be an effective triage method in an hrHPV screening setting, this approach is labor intensive, subjective and unfeasible in under-resourced settings lacking medical services. Objective and reproducible biomarkers may improve triage of hrHPV-positive women. Studies have shown that methylation markers (methylation of the promoter regions of tumor suppressor genes involved in cervical carcinogenesis) are promising triage tools to distinguish women with clinically meaningful cervical disease amongst those who are hrHPV-positive without an underlying cervical high-grade lesion. Further research about the potential of different biomarkers in triaging hrHPV-positive women is needed.

Appropriate communication and education of both women and physicians about hrHPV and cervical cancer is very important to increase vaccination coverage and to motivate women to participate in the screening programme. In this way cervical cancer morbidity and mortality can be reduced.
References


44. van der Aa MA, de Kok IM, Siesling S, et al. Does lowering the screening age for cervical cancer in the Netherlands make sense? Int J Cancer 2008;123:1403-06
64. Berkhof J, Bultman NW, Bleeker MCG, et al. HPV type-specific 18-month risk of high-grade cervical intraepithelial neoplasia in women with a normal or borderline/mildly dyskaryotic smear. Cancer Epidemiol Biomarkers Prev 2006;15:1268-73

General discussion
86. Villa LL. Prophylactic HPV vaccines: reducing the burden of HPV-related diseases. Vaccine 2006;24 Suppl 1:S23-S28
94. Franco EL, Ferenczy A. Cervical cancer screening following the implementation of prophylactic human papillomavirus vaccination. Future Oncol 2007;3:319-27
CHAPTER 8

Summary

Worldwide, cervical cancer is the third most common female malignancy with the highest incident rates reported in low resource countries. In countries with cervical cancer screening, the incidence and mortality rates of cervical cancer have decreased. Cytology-based screening enables cancer prevention by early detection of pre-malignant lesions, which can be treated effectively.

The recognition that a persistent infection with hrHPV is the necessary cause of cervical cancer has resulted in the development of new methods of cervical cancer prevention. These comprise secondary prevention by hrHPV testing for early detection of cervical cancer and primary prevention through HPV vaccination. This thesis presents recent work exploring the possibilities of hrHPV testing in triage of women with borderline or mildly dysplastic smears (BMD, Pap2/3a1) and the potential clinical impact of using the hrHPV test in primary screening.

Chapter 1 provided a general introduction of cervical cancer epidemiology, human papillomavirus infections, cervical carcinogenesis and cervical cancer prevention.

In Chapter 2, we studied whether it is feasible to use the hrHPV test as baseline triage test for women with BMD. In many European countries, including the Netherlands, women with BMD in screening are recalled for repeat testing and they are only referred for colposcopy if the cytological abnormality persists. Because the majority (>80%) of these women do not have a high-grade lesion, a significant burden is imposed on women and the health care system. Therefore, we compared repeat cytology testing at 6 and 18 months to direct referral of women with BMD and a positive hrHPV test. In this study, almost all CIN3 and CIN2+ lesions were found in hrHPV-positive women, whereas women with a negative hrHPV test had an acceptably low CIN3 and CIN2+ risk. Compared to repeat cytology testing, baseline triaging by hrHPV testing can be implemented against low costs, and leads to a faster diagnosis and less distress for women. We therefore support the strategy of referring hrHPV-positive women with BMD immediately for colposcopy and to refer those who are hrHPV-negative to routine screening.

In Chapter 3, we presented the main results of the population based VUSA-Screen study (VU University Medical Center SAltro laboratory population-based cervical Screening) in which 3-year follow-up results were related to baseline hrHPV testing and cytology testing to find an optimal primary screening method. In line with other studies, this study demonstrated that hrHPV testing is superior to cytology in terms of sensitivity but not in terms of specificity. Women with a double (cytology and hrHPV) negative test did not have a markedly lower CIN3+ and CIN2+ risk than women with a single negative hrHPV test. Therefore, from a health-economic perspective, cervical screening with a primary, stand-alone hrHPV test seems preferable. HrHPV-positive women have a non-negligible risk for CIN3+ (13.2%) and CIN2+ (21.9%). To prevent unnecessary colposcopy referrals, hrHPV-positive women should not be offered
colposcopy immediately but should be further stratified by means of triage/repeat testing. Since cytology in the Netherlands has a high specificity for threshold CIN3+, we used cytology for this purpose. Women with an hrHPV-positive test and abnormal cytology had a high CIN3+ (42.2%) and CIN2+ (60.3%) risk and need immediate colposcopy. HrHPV-positive women with normal cytology have a low, but still non-negligible CIN3+ (5.2%) and CIN2+ (11.3%) risk. We showed that repeat cytology testing after 1 year for hrHPV-positive women with normal cytology at baseline is however necessary before returning women to routine screening. In present study the CIN3+ and CIN2+ risk was similar in women invited for the first time (29-33 years) and in women ≥34 years. Moreover, the CIN3+ risk in women with hrHPV-positive normal cytology was higher among women invited for the first time (29-33 years) than among older women.

In Chapter 4, we presented the final data from the POBASCAM (POpulation BAsed SCreening study AMsterdam) trial. In this randomised trial, women were randomly assigned to receive hrHPV and cytology co-testing (intervention group) or cytology testing alone (control group). At the second round 5 years later, hrHPV and cytology co-testing was done in both groups. At baseline, hrHPV testing detected more clinically relevant, CIN2+ lesions compared with screening using cytology only. This improved detection of cervical lesions at baseline led to reduced detection of CIN3+ and cervical cancers in the second round. The higher protection of CIN3+ lesions in the second round by hrHPV testing appeared to be largely attributable to HPV16. HrHPV testing in women aged 29-33 did not result in excessive detection of regressive cervical lesions. Collectively, these results support hrHPV testing in cervical screening starting at age 30.

In Chapter 5, we showed the results of triage strategies for hrHPV-positive women. HrHPV testing as a primary screening test requires efficient management of hrHPV-positive women. Most hrHPV infections will clear spontaneously and only a minority of hrHPV-positive women will have or develop clinically meaningful lesions. Thus, an effective triage strategy that determines which hrHPV-positive women should be referred for colposcopy is crucial to prevent unnecessary colposcopies and treatment. Our analysis of triage strategies for hrHPV-positive women based on the data from the VUSA-Screen study points to use cytological testing at baseline, followed by repeat cytology testing at 12 months. This is a feasible triage strategy because hrHPV-positive women with 2 times negative cytology have an acceptably low CIN3+ and CIN2+ risk (0.7% and 2.9%, respectively), and it is accompanied with a modest colposcopy referral rate (33.4%). Moreover, this strategy is easy to communicate to participating women and physicians.

In Chapter 6 we studied the effect of increasing the threshold level of hrHPV testing by HC2 on the sensitivity and specificity for CIN3+ and on the colposcopy referral rate. We found that increasing the HC2 threshold could result in similar or lower colposcopy referral rates than cytology screening but only at the cost of a lower sensitivity.
However, superior performance in terms of both sensitivity and colposcopy rate was possible if HC2 testing, at the standard threshold, was combined with cytology triage at baseline and repeat cytology testing after 1 year as earlier defined in Chapter 5.

Finally, in Chapter 7 we provided a general discussion of the results presented in this thesis, and discusses possible future developments, prospects and clinical consequences of hrHPV testing. We conclude that hrHPV testing is superior to cytology as a primary screening test in cervical cancer screening programmes for women 30 years and older. Additionally, we presented feasible triage algorithms for hrHPV-positive women. In the future, new objective biomarkers may improve triage of hrHPV-positive women.
Baarmoederhalskanker is na borst- en darmkanker de meest voorkomende vorm van kanker onder vrouwen wereldwijd, met de hoogste incidentie in ontwikkelingslanden. In landen met een bevolkingsonderzoek voor het opsporen van baarmoederhalskanker zijn de incidentie- en sterftecijfers van deze vorm van kanker afgenomen. Het huidige screeningsprogramma maakt gebruik van cytologisch onderzoek van de baarmoederhals met als doel om de voorstadia van baarmoederhalskanker op te sporen en tijdig te behandelen.

De ontdekking dat een infectie met het Humaan Papillomavirus (HPV) een voorwaarde is voor het ontstaan van baarmoederhalskanker heeft geleid tot de ontwikkeling van nieuwe methoden voor de preventie van baarmoederhalskanker. Deze omvatten zowel nieuwe methoden van vroege opsporing van baarmoederhalskanker als van primaire preventie via vaccinatie tegen HPV. In dit proefschrift wordt recent werk gepresenteerd waarin de mogelijkheden en effecten worden onderzocht van het gebruik van HPV testen bij primaire screening en bij triage van licht afwijkende uitstrijkjes (BMD, Pap2/3a1).

Hoofdstuk 1 bevat een algemene introductie over baarmoederhalskanker, HPV infecties, de ontstaanswijze van baarmoederhalskanker en de verschillende methoden die gebruikt kunnen worden om baarmoederhalskanker te voorkomen.

In hoofdstuk 2 hebben we onderzocht of het gebruikelijke follow-up advies voor vrouwen met een BMD uitstrijk kan worden vervangen door een risicoselectie te maken op basis van de hoog risico (hr)HPV test. In veel Europese landen, waaronder Nederland, worden vrouwen met een BMD uitstrijk in het screeningsprogramma opgeroepen voor herhaal uitstrijkjes. Alleen vrouwen met persistent afwijkende uitstrijkjes worden verwezen naar de gynaecoloog voor colposcopie. Omdat de meerderheid (>80%) van deze vrouwen geen hoogwaardige laesie (CIN2+) heeft is dit een behoorlijke belasting voor de vrouwen en voor de gezondheidszorg. Daarom hebben we het standaard follow-up advies voor BMD uitstrijkjes (herhaal uitstrijk na 6 en 18 maanden) zoals toegepast in een controle groep vergeleken met baseline hrHPV triage in een interventie groep. In de interventie groep werden vrouwen met een BMD uitstrijk en hrHPV-positieve test direct doorgestuurd voor colposcopie en vrouwen met een hrHPV-negatieve test kregen het standaard follow-up advies. In dit onderzoek werden bijna alle CIN3 en CIN2+ laesies gevonden in hrHPV-positieve vrouwen, terwijl vrouwen met een negatieve hrHPV-test een laag CIN3 en CIN2+ risico hadden. Vergeleken met herhaal uitstrijkjes kan baseline triage met hrHPV tests ingevoerd worden tegen lage kosten en resulteert het in een snellere diagnose en minder belasting voor de betreffende vrouwen. Daarom is het testen van vrouwen met een BMD uitstrijk op de aanwezigheid van hrHPV aan te bevelen. Wij adviseren vrouwen met een BMD en hrHPV-positieve test direct door te sturen voor colposcopie en vrouwen met een hrHPV-negatieve test terug te sturen naar het bevolkingsonderzoek.
In hoofdstuk 3 hebben we de belangrijkste resultaten van de VUSA-Screen studie (VU University Medical Center SAltro laboratory population-based cervical Screening) gepresenteerd. De drie-jaar follow-up resultaten werden gekoppeld aan de baseline hrHPV en cytologie uitslag voor het vinden van een optimale primaire screeningsmethode. Overeenkomstig met andere studies, toonde deze studie aan dat de hrHPV test een hogere sensitiviteit heeft dan cytologie maar een lagere specificiteit. Omdat het risico na alleen een negatieve hrHPV test nagenoeg gelijk was aan het risico na een gecombineerd (hrHPV en cytologie) negatief test resultaat, heeft testen met uitsluitend hrHPV de voorkeur. Vrouwen met een positieve hrHPV test hebben een verhoogd risico op CIN3+ (13.2%) en CIN2+ (21.9%). Om onnodige colposcopie doorverwijzingen te beperken moeten niet alle hrHPV-positieve vrouwen direct doorgestuurd worden maar moet er een risico stratificatie plaats vinden. Aangezien cytologie in Nederland een hoge specificiteit voor CIN3+ heeft is het een goed hulpmiddel voor risico stratificatie van hrHPV-positieve vrouwen. De vrouwen met hrHPV-positieve test en afwijken- de cytologie dienen verwezen te worden voor colposcopie vanwege het sterk verhoogde risico op CIN3+ en CIN2+. Vrouwen met hrHPV-positieve test en normale cytologie hebben een weerspiegeling van het foute gebruiken van cytologie in Nederland een risico vergelijkbaar voor vrouwen die voor het eerst uitgenodigd werden voor het bevolkingsonderzoek (29-33 jaar) en voor oudere vrouwen (≥34 jaar). Het CIN3+ risico was zelfs hoger voor vrouwen met hrHPV-positieve test en normale cytologie die voor het eerst uitgenodigd werden (29-33 jaar) dan voor oudere vrouwen (≥34 jaar).

De vijf-jaars follow-up resultaten over twee screeningsronden van de totale POBASCAM trial (POpulation BAsed SCreening study AMsterdam) zijn in hoofdstuk 4 gepresenteerd. In deze gerandomiseerde trial kregen vrouwen ofwel een combinatietest van hrHPV en cytologie (interventie groep), ofwel alleen de cytologische test (controle groep). In de eerste screeningsronde detecteerde de groep met de hrHPV test meer klinisch relevante CIN2+ laesies in vergelijking met screening met behulp van cytologie alleen. In de tweede ronde vijf jaar later werden in beide groepen een combinatie van hrHPV en cytologie uitgevoerd. De verbeterde detectie van CIN2+ laesies door hrHPV testen in de eerste ronde resulteerde in een verminderde detectie van CIN3+ en baarmoederhalskanker in de tweede ronde. Dit klinisch voordeel is grotendeels te danken aan een vroege detectie van hooggradige cervicale laesies die worden veroorzaakt door HPV16. Het screenen van vrouwen die voor het eerst worden uitgenodigd voor bevolkingsonderzoek (29-33 jaar) moet behoud van de hrHPV test resulteerde niet in detectie van regressieve cervicale laesies. Deze veronderstelling is gebaseerd op de observatie dat over twee screeningsronden de CIN3+ en CIN2+ detectie niet verschilde tussen vrouwen 29-33 jaar en vrouwen 34 jaar en ouder. Kortom, deze resultaten ondersteunen de implementatie van de
hrHPV test in het reguliere bevolkingsonderzoek voor vrouwen vanaf de leeftijd van 30 jaar.

Primair screenen met hrHPV in het bevolkingsonderzoek vereist een efficiënte follow-up van hrHPV-positieve vrouwen. Omdat de meeste hrHPV infecties spontaan verdwijnen en slechts een minderheid van de hrHPV-positieve vrouwen een klinisch relevante afwijking ontwikkelen. Een effectieve triage strategie die bepaalt welke hrHPV-positieve vrouwen moeten worden doorverwezen is van cruciaal belang om onnodige colposcopie onderzoek en behandeling te voorkomen. In hoofdstuk 5 analyseren we triage strategieën voor hrHPV-positieve vrouwen op basis van de gegevens van de VUSA-Screen studie. Uit deze studie komt de volgende triage strategie naar voren: cytologische onderzoek bij aanvang gevolgd door herhaalde cytologie na 12 maanden. Dit is een haalbare triage strategie omdat hrHPV-positieve vrouwen met 2 maal een negatieve cytologie test een aanvaardbaar laag CIN3+ en CIN2+ risico hebben en het resulteert in een beperkte hoeveelheid colposcopie doorverwijzingen. Bovendien is deze strategie eenvoudig uit te leggen aan de deelnemende vrouwen en artsen.

In hoofdstuk 6 evalueerden we het effect van primair hrHPV screening gebruik makend van HC2 met verschillende afkapwaarden voor een positieve test in vergelijking met cytologie. We onderzochten het effect op sensitiviteit en specificiteit voor CIN3+ en op het aantal colposcopie doorverwijzingen. Het verhogen van de HC2 afkapwaarde resulteerde in een vergelijkbare of lager aantal colposcopie doorverwijzingen dan cytologie maar dit ging ten kost van de sensitiviteit. Superieure prestaties in termen van zowel de sensitiviteit als het aantal colposcopie doorverwijzingen werden verkregen met de HC2 toegepast met de standaard afkapwaarde gecombineerd met cytologie triage op baseline en herhaalde cytologie testen na 1 jaar zoals eerder omschreven in hoofdstuk 5.

Tenslotte beschrijft hoofdstuk 7 een algemene discussie van de resultaten van dit proefschrift, en bespreekt mogelijke toekomstige ontwikkelingen, vooruitzichten en klinische gevolgen van hrHPV testen. Dit proefschrift toont nader bewijs dat het testen op hrHPV superieur is aan cytologie als primaire screeningstest naar baarmoederhalskanker voor vrouwen 30 jaar en ouder. Daarnaast hebben we haalbare triage strategieën gepresenteerd voor hrHPV-positieve vrouwen. In de toekomst kan de triage van hrHPV-positieve vrouwen mogelijk verbeterd worden door nieuwe objectieve biomarkers.
List of publications


Dankwoord

Nu is het moment aangebroken om terug te kijken op mijn promotietijd, ongelofelijk hoeveel er in zo’n periode is gebeurd. Met veel plezier kijk ik erop terug, ik heb veel geleerd en veel verschillende mensen ontmoet. Graag wil ik iedereen bedanken die op welke manier dan ook een bijdrage aan dit proefschrift heeft geleverd.

Allereerst wil ik alle vrouwen bedanken die hebben deelgenomen aan de studies. Tevens wil ik alle huisartsen, gynaecologen, pathologen en analisten bedanken voor al het werk dat zij voor de studies hebben verricht.


De leden van de promotiecommissie prof.dr. Fleuren, prof.dr. Kenter, prof.dr. Massuger en prof.dr. Verheijen wil ik bedanken voor het beoordelen van het manuscript en het plaatsnemen in de commissie. I would like to thank prof.dr. Bosch and dr. Franceschi for their willingness to critically read my thesis and for attending the ceremony.

Dankwoord

Veerle Coupe, bedankt voor de prettige samenwerking. Je was er altijd voor data-analyses en hulp bij het schrijven, maar ook voor een goed gesprek onder het genot van een kop koffie. Onze eerste samenwerking (RNA stuk) is eindelijk gepubliceerd, dit gaan we vieren!

Al hoorde ik er niet helemaal bij, toch wil ik de leden van de HumaVac bedanken voor de leerzame en gezellige maandagmiddag besprekingen.


Alle analisten van SALTRO wil ik bedanken, vooral Angélique en Lainne. Beste Wim Verweij bedankt voor de samenwerking en het mogelijk maken van de VUSA-Screen studie.

De PA-uitjes, AIO retraites, weekenden en borrels waren een leuke afwisseling. Alle (oud-) AIO's, Saskia's, Renee, Floor, Marisca, Rieneke, Tineke, Jelle, Wouter, Annemieke, Jessica en Linda en vele anderen, bedankt voor de gezellige tijd.

Het Leidse borrelclubje, Hedy, Saskia Wilting, Rinus, Saskia Cillissen, Cindy en Jessica wil ik bedanken voor de gezellige treinreizen en borrels. Ik hoop dat er nog veel feestjes volgen.

Collega arts-onderzoekers van de HPV-groep, Maaike, Murat, Jacqueline, Marielle, Afra, Jacolien, Romy, Viola, Margot en Roosmarijn, bedankt jullie enthousiasme, congresbezoeken, feestjes en lunches. Murat bedankt voor de kopjes koffie in de koffiebar. Je stond altijd klaar om me te helpen bij het omzetten van bestanden, ook ‘s avonds als we samen een artikel gingen submitten. Voor diegene die nog niet zijn gepromoveerd; ‘Zet hem op! Voor je het weet ben je met de lay-out van je boekje bezig.’

Prof.dr. Maarten Frens en dr. Jos van der Geest, mijn Master of Neuroscience begeleiders, jullie positieve houding, enthousiasme voor onderzoek en jullie vertrouwen in mij hebben ervoor gezorgd dat ik ben gestart met promotie-onderzoek.

Gynaecologen, collega A(N)IOS en alle andere medewerkers van de afdeling verloskunde en gynaecologie van het Kennemer Gasthuis en het VUmc wil ik bedanken voor het warme welkom, de goede sfeer en leerzame periode.


Lieve vrienden en vriendinnen, veel dank voor al jullie interesse, motiverende gesprekken en de regelmatige afleiding in de vorm van borrels, etentjes en weekendjes weg. Lieve Tekla, bedankt voor al je lay-out werk en je onuitputtelijke geduld.

Lieve familie en schoonfamilie bedankt voor jullie betrokkenheid de afgelopen jaren. Arnold, Jaco en Ellen, Eduard en Saskia, Lenneke, mijn broers en schoonzussen, dank voor de belangstelling en steuntje in de rug.

Mijn lieve ouders, dank voor jullie onvoorwaardelijke steun en liefde. Jullie hebben mij altijd het vertrouwen en vrijheid gegeven om mijn eigen keuzes te maken. Inmiddels begrijp en waardeer ik ook de uitspraak van mijn vader; ‘Het maakt niet uit wat je bereikt in het leven, wij zijn trots op je.’

Lieve Stefan en Sofie het leven is mooi met jullie.

In 1999 werd zij ingeloot voor de studie geneeskunde en studeerde zij de opeenvolgende jaren aan de Erasmus Universiteit te Rotterdam. Tijdens haar studie geneeskunde behaalde zij een Master of Neuroscience, eveneens aan de Erasmus Universiteit. In het kader van deze Master opleiding heeft ze onderzoek verricht naar oculomotor reflexen onder begeleiding van prof.dr. MA Frens in het Erasmus MC.


Dorien is getrouwd met Stefan Pool en woont in Leiden. Zij hebben sinds september 2011 samen een dochter Sofie.