SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES
This thesis attempts to answer a number of research questions. The first question is: Which digital colposcopy techniques are presently available, what is the technical background and how is their clinical efficacy? In chapter 2 we describe various methods of digital colposcopy. These new techniques are all defined by the word ‘digital’ in which ‘digital’ means every method of colposcopy using any form of image enhancement by a computer. To further detail these digital colposcopy methods we discuss them under one of the following headings: digital imaging and telecolposcopy, spectroscopy, computerised colposcopy, optical coherence tomography and confocal microcolposcopy.

Photographing the cervix at colposcopy and digitalising the image was the first step on the path of development of digital colposcopy. Digital image processing techniques permit contrast enhancement of features, such as white epithelium and abnormal vascularisation, and are in this way able to help the colposcopist to identify and to grade a lesion. Digital images of the cervix can also be analysed by a computer for characteristic features and colour patterns, which may enhance the objectivity of the colposcopic examination. This process is known as computerised colposcopy and should be considered as an adjunct to conventional colposcopy, as is digital imaging. For both digital imaging processing and computerised colposcopy techniques, sensitivities and specificities above 90% are reported, but their use in day to day clinical practice is limited.

Optical coherence tomography and confocal microcolposcopy are two techniques that are least commonly used for clinical purposes within the field of gynaecology. Only one or two studies have been conducted for the use of both optical coherence tomography (a non-invasive technique that uses infrared light and provides real-time, in vivo images of the cervix with high resolution) and confocal microcolposcopy (an optical imaging technique that reconstructs 3D images with high contrast, through point illumination and a pinhole conjugate plane in front of a detector) in cervical tissue. Therefore no accurate sensitivity or specificity data can be given.

Among the most promising developments is spectroscopy, allowing a more or less automated analysis and interpretation of the colposcopic image. Spectroscopy is a non-invasive method in which for example light or electric current is used to study the biochemical composition as well as the metabolic and structural features of tissue. Digital colposcopy techniques that use spectroscopy are SpectRx, LUMA™, DySIS™, Trimodal, Truscreen® and impedance colposcopy. Sensitivity of these methods varies between 70% (Truscreen®) and 95% (SpectRx), specificity between 50% (LUMA™) and 83% (SpectRx).

Our second research question was: Are hrHPV-related lesions recognisable by colposcopy? Indeed, in chapter 3 we found that hrHPV positive lesions have other characteristics than hrHPV negative lesions. During colposcopy, the colposcopist evaluates a number of visual features of the cervix and/ or lesion (i.e. mosaic and punctuation patterns, vessels, acetowhitrnening, etc.). Visibility of the transformation
zone, larger lesion size (more than 25% of the visible cervix) and coarse and irregular punctation patterns are the colposcopic features statistically significantly associated with a positive hrHPV status (OR = 2.29 (95%CI 1.41 - 3.73), OR = 1.78 (95%CI 1.08 - 2.94), and OR = 2.37 (95%CI 1.08 - 5.19) respectively).

After correction for histology (a high-grade cervical lesion is more likely to be hrHPV positive than a low-grade lesion), the difference between hrHPV positive and negative lesions for visibility of the transformation zone and lesion size remained statistically significant (OR = 2.44 (95%CI 1.35 - 4.41) and OR = 1.92 (95%CI 1.04 - 3.54) respectively). Thus these criteria, which the colposcopist already uses to form an impression of the cervix and to locate and grade a possible lesion, may help the colposcopist to identify hrHPV positive patients and as such women that are at risk for neoplastic cervical lesions.

The following research question was: Does dynamic spectral imaging colposcopy improve the sensitivity of colposcopic examination? In total 275 women were included in this Dynamic Spectral Imaging (DSI) validation study (chapter 4): 239 women were analysed in the ‘intention to treat’ (ITT) cohort and 183 women in the ‘according to protocol’ (ATP) cohort. In the ATP cohort all cases adhered strictly to the study protocol (proof of principle) and the ATP cohort is a subset of the ITT cohort. In the ATP cohort the sensitivity of DSI colposcopy to identify patients with high-grade (CIN2+) lesions was significantly higher than of conventional colposcopy (79% (95%CI 70 - 88) and 55% (95%CI 44 - 65) respectively (p = 0.0006, asymptotic McNemar test)). When the DSI colour-coded map was combined with conventional colposcopy, the sensitivity increased further to 88% (95%CI 82 - 95). The specificity of DSI colposcopy in the ATP cohort was not significantly different from conventional colposcopy (77% (95%CI 69 - 86) and 85% (95%CI 77 - 92), respectively (p = 0.144, asymptotic McNemar test)).

DSI combined with conventional colposcopy had a specificity of 69% (95%CI 60 - 78).

The data in the ITT cohort, even though not all cases were adhering to protocol criteria (e.g. not all DSI indications for high-grade lesions were sampled or the device was used even though there was a hardware problem) were analysed to approximate the performance of DSI colposcopy under clinical conditions (clinical performance). Again, in the ITT cohort the sensitivity of DSI colposcopy was significantly higher than of conventional colposcopy (65% (95%CI 56 – 74) and 52% (95%CI 42 – 61) respectively (p = 0.039, asymptotic McNemar test)). When the two techniques were combined the sensitivity was 80% (95%CI 72 - 87). The specificity of DSI colposcopy in this cohort was significantly lower than that of conventional colposcopy: 70% (95%CI 62 - 78) versus 82% (95%CI 75 - 88) (p = 0.011, asymptotic McNemar test). The combination of DSI with conventional colposcopy led to a specificity of 63% (95%CI 54 - 71) in the ITT cohort. The lower specificity means that some women are incorrectly identified as having high-grade cervical disease. However, in the clinical setting it is, in contrast to a screening setting, more important not to miss any high-grade cervical disease, which is reflected in a high sensitivity. In conclusion, both in 'according to protocol'
and ‘intention to treat’ analysis DSI colposcopy has a significantly higher sensitivity in detecting high-grade cervical lesions than conventional colposcopy. This signifies that also in suboptimal conditions (i.e. the ITT analysis) the DSI colposcope can be a valuable asset to the colposcopic examination.

The next research questions, resulting from the previous one, were: Is the detection of cervical premalignant lesions dependent of the hrHPV type present and/or lesion size? And is there a difference between conventional colposcopy and dynamic spectral imaging (DSI) colposcopy in detection of these type-specific lesions? HrHPV type 16 positive cervical lesions have been suggested to show more defined abnormalities at colposcopy than lesions positive for other hrHPV types. So, hrHPV type 16 positive lesions should be easier to detect at colposcopy than non type 16 hrHPV positive lesions. For that reason we studied in chapter 5 in a subset of women from the aforementioned DSI validation study the performance of DSI and conventional colposcopy in relation to the agreement between colposcopic impression and final histology. Furthermore, we explored if lesion size influences the colposcopic performance for both DSI and conventional colposcopy.

DSI defined more high-grade cervical lesions among hrHPV type 16 positive women compared to non-type 16 hrHPV positive women. This effect was seen in women with CIN2+ lesions in their final histology (p = 0.009), but also without stratifying for final histology DSI detected more high-grade cervical lesions among hrHPV type 16 positive women compared to non-type 16 hrHPV positive women (p = 0.032). Thus, in hrHPV type 16 positive women the sensitivity of DSI colposcopy for detecting CIN2+ lesions was higher than in non-type 16 hrHPV positive women (97% (95%CI 84 – 100) versus 74% (95%CI 57 – 87)). Contrastingly, this higher sensitivity in hrHPV type 16 positive women was not found for conventional colposcopy.

Moreover, we found that CIN2+ lesions defined as high-grade by DSI were significantly smaller than CIN2+ lesions defined as high-grade by the colposcopist. Since detection of lesions with conventional colposcopy occurs with the naked eye, it is likely that it is easier to miss the smaller lesions. DSI colposcopy, however, detects all high-grade cervical lesions on a per pixel level and is therefore more objective in its analysis of the cervix. This supports the hypothesis that the higher sensitivity of the DSI colposcope results from the detection of smaller high-grade lesions. One could furthermore argue that the even better sensitivity of DSI for hrHPV type 16 positive high-grade lesions is because hrHPV type 16 is a more aggressive hrHPV type causes more often high-grade abnormalities when the lesion is small in size. This is also reflected by the fact that no higher sensitivity was found for conventional colposcopy in hrHPV type 16 positive women. We could not confirm that hrHPV type 16 positive lesions have more distinct features and are therefore more easily recognised.

Women visiting the outpatient gynaecology clinic often obtain a Pap smear. Given the higher sensitivity of hrHPV-testing for detection of cervical lesions we formulated the following questions: What is the added value of hrHPV-testing to cervical cytology
in a university hospital gynaecology outpatient clinic? And is it possible to define a useful risk profile in relation to the referral reason to identify who needs beforehand testing for cervical premalignant lesions? In chapter 6 we describe our observational cohort study in which all women referred to the gynaecology outpatient clinic undergoing cervical cytology testing were co-tested for hrHPV between January and June 2007. From the Dutch nationwide network and registry of histopathology and cytopathology (PALGA) and the hospital information system all reasons for referral and follow-up data were retrieved 18 and 42 months after the last subject had entered the study. The 21 different referral reasons were categorised in three groups: women with presumed cervix pathology, women with presumed endometrial pathology and women with other referral indications (miscellaneous).

The 18 month risk for CIN2+ or CIN3+ was highest in the group with presumed cervix pathology with adjusted risks of 11.1% and 5.4% respectively. In the group with presumed endometrial pathology the 18 month risk for both CIN2+ and CIN3+ was 3.1%. The women in the miscellaneous group – women without an a priori risk for cervical or endometrial pathology - had CIN2+ and CIN3+ risks of 4.1% and 1.8% respectively. Furthermore, in this diagnostic setting and irrespective of the reason for referral, hrHPV-testing detected more CIN2/3+ lesions than cytology. This is in concordance with other studies. Therefore, for whatever reason cervical testing is performed, co-testing with hrHPV is preferred. The added value of using both hrHPV-testing and cytology lies mainly in detecting lesions in women with normal cytology, who otherwise would not be detected. Furthermore, it seems reasonable not to routinely take smears as part of the intake procedure at a gynaecologic outpatient clinic, except for those women referred for presumed cervical and endometrial pathology.

Although screening with hrHPV as an alternative for cervical cytology has many advantages (i.e. higher sensitivity, the possibility to perform the test in self-sampled material, etc.), sole hrHPV-testing has a high false positive rate. The reason for this is that a transient hrHPV infection that will not cause any premalignant cervical lesions cannot be distinguished from a persistent hrHPV infection that will develop into a premalignant lesion in need of treatment. Therefore, additional triage markers are necessary to identify those women that are at risk, for example cytology combined with hrHPV genotyping, p16/Ki-67 dual-stained cytology or methylation markers. We focussed on methylation markers: single CADM1 and single MAL promoter methylation analysis has been researched, but without satisfactory results for use in a clinical population. This led to the formulation of the last research question: Is it possible to develop a panel of clinically useful methylation markers to identify hrHPV positive women at risk for high-grade cervical lesions and cervical cancer?

In abovementioned study (chapter 7) combined methylation analysis for CADM1 and MAL was tested as a triage marker for hrHPV positive women, instead of using methylation analysis for just CADM1 of just MAL. Two quantitative methylation-specific PCR’s (qMSP) for CADM1 (regions M12 and M18) and two qMSP’s for MAL...
(regions M1 and M2) were applied to 261 cervical tissue specimens, ranging from healthy tissue to cervical carcinoma. Most CIN3+ lesions were detected if combining two qMSPs representing both CADM1 and MAL: up to 99% of the carcinomas were positive. Further analysis of hrHPV positive smears led to the conclusion that a combination of CADM1-M18 and MAL-M1 had the best sensitivity and specificity to detect high-grade cervical lesions and cervical cancer. This panel of methylation markers performed at least as good as cytology in terms of sensitivity and specificity.

The final step in this study was to apply these data to a clinical population. Therefore CADM1-M18 and MAL-M1 methylation analysis was applied to 79 hrHPV positive women visiting an outpatient colposcopy clinic because of abnormal cytology. HrHPV-testing had a sensitivity of 97% and a specificity of 33% for CIN3+ lesions in this population. Adding CADM1-M18 / MAL-M1 methylation analysis increased the specificity to 78% with a sensitivity of 70%. So to conclude, in this study the CADM1-M18 / MAL-M1 methylation panel was at least as good as cytology. Therefore, combination of CADM1 and MAL methylation analysis may be useful as an alternative molecular triage tool for hrHPV positive women. In this way, women can be further stratified into risk profiles for cervical pathology, making the selection for the clinician easier who to refer for colposcopy, although the sensitivity and specificity of this methylation panel should be further improved.

**GENERAL DISCUSSION**

**Aspects of colposcopy**

This thesis describes different ways to improve detection of premalignant cervical lesions. As has become clear, conventional screening with cervical cytology and subsequent colposcopy if the test result is abnormal is a suboptimal way of detecting premalignant cervical lesions. Due to the low to average sensitivity of cytology and conventional colposcopy of approximately 55 – 65%, high-grade premalignant cervical lesions are missed.(1-5) On the other hand, due to over- or underestimation of the severity of the lesion at colposcopy, ‘see and treat’ procedures might be carried out either unnecessarily or unwantingly. For these reasons, histology remains the gold standard. But the accuracy of the gold standard itself is hindered by the variability in histological diagnosis among pathologists and the obvious sampling errors through the inaccuracy of colposcopic tissue sampling.(6-8)

Treatment of CIN lesions, mainly loop electrosurgical excision procedure (LEEP) is not without (obstetric) complications. A study by Armarnik et al. found substantial higher risk of preterm delivery under 34 weeks of gestation. Others had the same findings and some also found an association between a LEEP procedure and preterm, premature rupture of membranes (PPROM).(9-13) Other known complications of a LEEP procedure are bleeding, infection, cervical stenosis (that could lead to fertility problems) and even bowel injury is described.(14-16)
On the other hand, missing or not treating a high-grade cervical lesion with the potency to become cervical cancer is an adverse outcome as well. Therefore in clinical practice we tend to treat premalignant lesions before they have the chance to become malignant and accept the possible complications of the LEEP procedure. This is especially true when the lesion originates from the columnar epithelium of the cervix (e.g. adenocarcinoma in situ). Of all cervical carcinomas, approximately 80% is of squamous origin, but 10-20% is an adenosquamous- or adenocarcinoma. Although the incidence of squamous cell carcinomas has drastically decreased with the implementation of population based cytology screening programs, the frequency of adenocarcinomas has remained the same or has even increased in developed countries. (17-19) It is hypothesised that adenocarcinomas are more difficult to detect by cytology and/or colposcopy because they can be located out of reach and sight in the cervical canal. Furthermore, there is no well-defined precursor lesion. This worrying increase in incidence asks for better, i.e. more sensitive and more discriminative detection methods for cervical lesions in general and specifically for lesions of adeno(squamous) origin.

Digital colposcopy as an adjunct to and perhaps as a replacement for traditional colposcopy seems very promising. Although some studies report (very) high sensitivities and specificities, until now most digital colposcopy techniques have not been used on a large scale.(20-25) Factors that might play a role are the relative high purchase and maintenance costs (especially for low resource countries) and lack of knowledge of the possibilities that digital colposcopy might provide. Furthermore, of the various techniques for digital colposcopy described only a few have extensively been tested and validated in well-defined, sufficiently large patient populations (chapter 2). Some studies seem very promising, but after only one or perhaps a few publications, the publications end. This may be because preliminary results may not have been validated in follow-up studies or simply that funds may be lacking for further research.

The digital colposcopy techniques that use spectroscopy (including fluorescence, trimodal, contact probe and impedance spectroscopy) are the ones that are best researched.(26-28) Spectroscopy is a non-invasive method in which for example light or electric current is used to study the biochemical composition as well as the metabolic and structural features of tissue. The Dynamic Spectral Imaging (DSI) colposcope (DySIS, DySISmedical, Livingston, UK) is such a colposcope. Conventional colposcopy, by which a colposcopist examines the cervix after the application of acetic acid and identifies the sites most likely to harbour premalignant abnormalities, has only a moderate sensitivity and specificity.(1-5) Digital colposcopy, like DSI, is able to increase the sensitivity of the colposcopic examination and Soutter et al. (25) were the first to publish their validation analysis of this device in an outpatient population.

In this study, DSI and conventional colposcopy was carried out simultaneously: one colposcopist performed DSI colposcopy in the examination room, while another colposcopist sat in another room and evaluated the digital images the DSI colposcope made of the cervix, without the spectroscopic analysis. A sensitivity of 79% (95%CI
68 – 88) for DSI colposcopy was found and a specificity of 76% (95%CI 70 – 81). In comparison, in this study conventional colposcopy had a sensitivity of 49% (95%CI 37 – 61) and a specificity of 89% (95%CI 85 – 93).(25) Despite these good results, of the 447 women included in the test set of this study, 139 women (31.1%) had to be excluded, mainly because no biopsies were taken or the view of the cervix was unsatisfactory, compromising the use of this DSI colposcope in clinical practice.

In our large, prospective, multicentre, comparative study we used a newer type of the DSI colposcope. Although we aimed for less exclusions, we discovered that clinical practice can be unruly. This is reflected in the large ‘intention to treat’ (ITT) cohort compared to the ‘according to protocol’ (ATP) cohort. In the ATP cohort (a subset of the ITT cohort) all cases adhered strictly to the study protocol (proof of principle). In the ITT cohort not all cases adhered to protocol criteria (e.g. not all DSI indications for high-grade lesions were sampled or the device was used even though there was a hardware problem) and these data were analysed to approximate the performance of DSI colposcopy under clinical conditions (clinical performance). In total, 239 women were analysed in the ITT cohort, and 183 in the ATP cohort. This means that in one out of every four women the study protocol was not strictly adhered to.

Although the largest difference in sensitivity between DSI and conventional colposcopy was seen in the ATP cohort (79% versus 55%, p=0.0006), also in the ITT cohort a significant difference was observed (65% versus 52%, p=0.039). However, application of DSI colposcopy was most effective when combined with a trained colposcopist, attaining a sensitivity of up to 88% in the ATP cohort and 80% in the ITT cohort. An explanation for the higher sensitivity of the DSI colposcopy is that DSI probably detects relatively smaller lesions.

Both in the Soutter-trial(25) and in our DSI validation trial (chapter 4) DSI colposcopy was compared to videocolposcopy. Conventional colposcopy with a binocular and videocolposcopy are two different techniques. The latter requires the colposcopist to base a clinical decision on a flat screen television image which will be in part dependent on the technology used. Conventional colposcopy with a binocular colposcope allows direct visualisation of the cervix and the response of the tissue to acetic acid. In our study two of the three participating colposcopy clinics were already using videocolscopes in their day-to-day clinical practice prior to the start of the study. In addition, a training phase had taken place to familiarise the colposcopist with the digital images made by the DSI colposcope. And, although limited, there is some data suggesting that videocolposcopy and conventional colposcopy have similar sensitivity and specificity.(29;30)

As we know from the Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesions Triage (ALTS) Study conventional colposcopy detects approximately two thirds of all CIN3+ lesions.(31) The sensitivity of the colposcopy does not vary significantly with the training level of the colposcopist. However, in abovementioned ALTS trial the sensitivity is significantly greater when the
colposcopist takes two or more biopsies instead of one (p < 0.01). Nurse practitioners were most likely to take more than one biopsy, gynaecologic oncologists were less likely. So, although the sensitivity of the procedure does not differ significantly by type of medical training, it is greater when two or more biopsies are taken. These findings were confirmed by Bekkers et al. who also observed no difference in overall colposcopic performance between experienced and relatively inexperienced colposcopists, provided the colposcopist sampled one or more biopsies. These data show that conventional colposcopy is not an adequate tool to estimate the severity of the lesion and support the need for more advanced and objective colposcopy techniques, such as DSI colposcopy, independent of experience or visual skill in order to improve the evaluation of a cervical lesion.

As a side phenomenon we found that in the ATP cohort of the DSI validation trial only one of the 18 women (5.6%) whose high-grade lesion was missed by DSI was hrHPV type 16 positive. In contrast, 15 out of 39 women (38.5%) of whom the high-grade lesion was missed by conventional colposcopy were hrHPV type 16 positive. This suggests a trend that DSI colposcopy is better able than conventional colposcopy to detect hrHPV type 16 positive lesions, which tend to be more aggressive. To further evaluate this finding, we studied a subset of women included in the DSI validation trial. The subset consisted of women from the ATP cohort of whom a hrHPV test result was available. The objective of the study was to estimate the agreement between colposcopic impression and final histology for hrHPV type 16 positive and non type 16 hrHPV positive women.

Jeronimo et al. were the first to suggest that hrHPV type 16 causes more clear visual abnormalities than other hrHPV types, regardless of the final histological diagnosis. Therefore, lesions caused by this hrHPV type may be more easily and accurately recognised by conventional colposcopy. These findings were more or less confirmed by another study derived from the ALTS trial by Safaeian et al. They concluded that a far greater proportion of hrHPV type 16 related CIN3+ lesions were diagnosed at the enrolment visit compared to non-type 16 hrHPV associated CIN3+ lesions, indicating a more definite lesion at colposcopy that is easier recognisable. Drawbacks of both abovementioned studies are that only women with borderline or mildly dyskaryotic smears have been included. Furthermore, biopsy taking was not compulsory at all colposcopy visits in the ALTS study protocol; therefore CIN3+ lesions could have been missed, especially when the lesion is less distinct, as seems to be the case in non-type 16 hrHPV positive lesions.

As DSI colposcopy does not rely on subjective visual interpretation, we determined whether hrHPV16 related lesions are more clearly delineated by digital colposcopy than by conventional colposcopy. We also studied if lesion size was related to hrHPV type 16 infection. Little data is available about the relation between lesion size and hrHPV status. One of the difficulties is to accurately and reproducibly measure the size of the lesion on the sphere shaped cervix. We tried to overcome this by counting the
number of pixels in the colour-coded map covering a cervical lesion, indicated by the DSI colposcope.

We found that with DSI colposcopy among hrHPV type 16 positive women, more defined lesions are detected, the classification of high-grade cervical lesions is better and agreement with final histology is stronger than among non-16 hrHPV positive women. This difference in detection rate between hrHPV type 16 and other hrHPV types could not be found for conventional colposcopy. No significant differences in sensitivity for CIN2+ were observed between hrHPV type 16 positive and non type 16 hrHPV positive women. Another finding was that CIN2+ lesions defined as high-grade by DSI were significantly smaller than CIN2+ lesions defined as high-grade by the colposcopist.

We hypothesised that the more efficient detection of small hrHPV16 positive premalignant cervical lesions by DSI could be because of a more defined acetowhitening effect, since the intensity of the acetowhitening effect over time is what the DSI colposcope measures. Another possible explanation for the better sensitivity of DSI colposcopy among hrHPV type 16 positive lesions is that hrHPV type 16 causes smaller high-grade cervical lesions than other oncogenic HPV types. Identification of lesions at conventional colposcopy occurs with the naked eye, so it is likely that smaller lesions are more easily overlooked. DSI colposcopy on the other hand, detects cervical lesions on a per pixel level on the cervix and is therefore more objective in its analysis of the cervix. The fact that DSI defined more smaller CIN2+ lesions as high-grade than conventional colposcopy supports the hypothesis that the higher sensitivity of the DSI colposcope results from the detection of smaller high-grade lesions. This suggests that the higher sensitivity of DSI for hrHPV type 16 positive high-grade lesions is because these lesions are more often smaller in size. This is also reflected in the fact that no higher sensitivity was found for conventional colposcopy in hrHPV type 16 positive women. We therefore could not confirm the data from Jeronimo et al.(32) that hrHPV type 16 positive lesions have more distinct features and are therefore more easily recognised.

**Triaging hrHPV positive women**

Multiple studies have focussed on the use of hrHPV-testing in screening settings. A meta-analysis and overviews of several studies showed that hrHPV-testing has a substantial higher sensitivity for high-grade cervical disease (approximately 95%) than cytology (maximum 65%), while the specificity only slightly decreases.(34) Based on these data, the Dutch Health council has recently written a report in which it is advised to change screening in the national population-based screening program from cervical cytology to hrHPV-testing.(35)

If, however, hrHPV-testing will be implemented, triage testing will be needed to distinguish between hrHPV positive women who need further testing (i.e. colposcopy) and those who should undergo close surveillance. Otherwise too many women with
transient hrHPV infections will be referred for colposcopy, leading to higher health care costs and over-treatment. Such a triage test could be reflex (i.e. a follow-up test is automatically initiated when certain results are observed in the baseline specimen) cytology, reflex hrHPV genotyping, reflex cytology combined with hrHPV genotyping or a reflex test combined with a 6 or 12 month follow-up test. An evaluation of 14 different strategies revealed that triaging hrHPV positive women with cytology, followed by repeat cytology testing resulted in a high negative predictive value and an acceptable colposcopy referral rate. Therefore, this strategy appears to be the most feasible management strategy for screening programs. However, it remains unclear what the most effective strategy is for hrHPV positive women in a clinical setting (e.g. a gynaecology outpatient clinic) and whether hrHPV-testing should be implemented in a clinical setting in the first place.

We evaluated the use of hrHPV-testing in a university hospital gynaecologic outpatient clinic. Addition of hrHPV-testing to cytology, results in the detection of more CIN2+ and CIN3+ lesions. Although this finding might have been expected, this study was the first to evaluate the use of hrHPV-testing versus cervical cytology in a well-sized gynaecology outpatient setting. While these results are promising, the extra costs of implementation of cytology/hrHPV co-testing are a concern, especially in light of the relative small number of additionally detected CIN2+ cases.

Another interesting finding in this study was that the majority of smears taken at the gynaecology outpatient clinic were taken without a clear indication for cervical testing. We demonstrated that the CIN3+ risk for women without a clear indication for cervical testing was less than 2%. Therefore, testing should be limited to patients in follow-up or examination for CIN or patients suspected of endometrial pathology. Other patients, such as women with benign conditions and fertility problems should not be tested as a standard procedure at intake. Even the necessity for cervical testing in young women with for example postcoital bleeding can be questioned. The risk of cervical cancer for a woman aged 20 – 24 years with postcoital bleeding is approximately 1 in 44,000. Of course, an individual assessment should always be made, e.g. women with persistent recurrent blood loss or women above the age of 30 who never attended the screening program should also be offered a smear test.

Nonetheless, primarily hrHPV-testing or co-testing for hrHPV and cytology at intake in those women visiting the outpatient clinic with an a priori high risk for cervical disease has as effect that at risk women amongst those with normal cytology will be identified. Close surveillance, such as repeat cervical testing in 12 months, is needed for the hrHPV positive women with normal cytology and both physicians and women should be made more aware of their increased risk of premalignant cervical lesions. Another possibility for hrHPV positive, cytology negative women could be triaging them by p16/Ki-67 dual-stained cytology. P16ink4a is a cell cycle regulatory protein that induces cell cycle arrest under normal conditions. Ki-67 is a proliferation marker and expression of such a marker in combination with p16ink4a expression in the
same cervical epithelial cell may be used as a surrogate marker of cell cycle deregulation mediated by transforming hrHPV infections. Sensitivity of p16/Ki-67 dual-stained testing for CIN2+ lesions was 91.9% and 96.4% for CIN3+ lesions. Specificity was 82.1% and 76.9% for CIN2+ and CIN3+ lesions, respectively. Therefore, p16/Ki-67 testing holds a promise for triaging hrHPV positive, cytology negative women. However, since a clinical population is different from the population in screening settings, the results of analyses of triaging methods are not necessarily translatable to the clinical population of our abovementioned study and therefore further research in a clinical setting is necessary.

Another possible marker set to triage hrHPV positive women is methylation analysis for CADM1 and MAL genes. We found that a two-marker panel consisting of CADM1-M18 and MAL-M1 was most discriminative in smears. Therefore, this marker panel was applied in a colposcopy clinic population where it achieved a sensitivity of 70% and a specificity of 78% for CIN3+ lesions. However, extrapolating data from cervical tissue to cervical scrapings, as we did in this study, can be challenging and the sensitivity of the marker panel was not as high as we had expected, although it performed as good as cytology.

As has become clear from this and other studies, methylation data obtained from tissue samples cannot be directly extrapolated to cervical scrapings. A likely explanation for this phenomenon is that there are differences in cell type composition, resulting in different levels of background methylation. Cervical tissue samples often contain substantial amounts of non-epithelial (stromal) cells, while cervical scrapings mainly contain superficial epithelial cells. However, this difference can be tackled by adjusting the methylation target site and/or the assay threshold, as has been found in our study. Another issue is that we were hindered by our sampling method, where a cervical scraping was taken directly prior to colposcopy. This might have led to more cautious brushing of the cervix in order to prevent bleeding and so this might have hampered the collection of abnormal cells in the sample. Possibly this has resulted in suboptimal cytology and suboptimal methylation analysis. When we corrected for this sampling bias, the specificity of the marker panel increased significantly. So, combined methylation analysis for CADM1 and MAL may be a promising, objective triage tool for hrHPV-positive women, providing that an adequate sample is taken and the correct threshold is used.

**FUTURE PERSPECTIVES**

**Methods to improve detection of cervical disease**

In summary, the system of cervical cytology and/or hrHPV-testing and subsequent colposcopy with or without biopsies is cumbersome. Thus, there is an obvious need for additional markers that can be used to identify women at risk who will need treatment. Ideally, there should be an algorithm that will allow performing a ‘see and
treat’ procedure without, or minimising, the risk of over- or undertreatment. However, such an algorithm is not very likely in the near future.

The first step would be to implement hrHPV-testing in the population based screening program, just as the Dutch National Health Council has recently advised. (35) HrHPV positive women need further triage testing to distinguish those who are at risk of premalignant cervical disease from women with just transient hrHPV infections. At this moment reflex cytology and repeat cytology after 12 months seem most feasible. But if molecular triage tests, like methylation analysis, are improved, these tests might replace cytology. Furthermore, these tests might also be applicable to clinical settings. Other tests of interest are hrHPV genotyping, hrHPV viral load, hrHPV mRNA, p16/Ki-67 dual stained testing, Telomerase RNA Component (TERC) gain and other proliferation/ cell cycle markers.(42) Where sole hrHPV-testing and/or cervical cytology does not differentiate between incident infection, persistent infection or histological abnormalities, this differentiation might be obtained by application of such tests. When also colposcopy itself is improved, for example with digital colposcopy techniques like dynamic spectral imaging, fewer women with cervical lesions will be missed. This proposed strategy is outlined in Figure 1.

Prophylactic hrHPV vaccination and self-sampling
In the Netherlands the prophylactic hrHPV vaccine was implemented in the national vaccination program in 2009 for girls of 12 years of age. Therefore, it is to be expected that the burden of cervical cancer and its precursor lesions will lessen over time.(43) As a result, the number of colposcopies will drop and it will become more difficult for physicians to obtain enough experience for a reliable colposcopic exam, underlining the need for better selection of women at risk and better colposcopy techniques.

Another point of interest is the use of self-sampled cervico-vaginal material. Self-sampling seems to be as sensitive for the detection of hrHPV as a physician-obtained sample. (44-46) So, cervico-vaginal self-sampling appears to be a reliable and feasible method to detect hrHPV infection of the cervix. Moreover, even hrHPV detection in urine samples might be feasible.(47) However, most studies have been performed among non-attendees of cervical cancer screening programs.(45;48-52) In these studies, screening with hrHPV in self-sampled material is mostly compared to regular pap smear. The results from these studies are very promising. Sending a self-sampling device to women who do not attend the screening program is an effective method to increase coverage of the screening program. Response rates up to 39% have been reported.(49;50;52) Furthermore, the number of high-grade cervical lesions found in this group supports the implementation of self-sampling within the screening program. (49;50;52)

Moreover, feasibility studies have been performed to study the analysis of additional markers in self-sampled material.(51;53) Although the numbers are small, methylation analysis in cervico-vaginal self-sampling material seems to be feasible and its diagnostic
Figure 1: Proposed strategy for the detection of premalignant cervical lesions.
* Preferably in self-sampled material
performance appears to be at least comparable to the detection of cervical neoplasia by cervical cytology and hrHPV. (53) Also, the use of viral load measurement in self-sampled material has been studied, which also seems promising for the detection of premalignant cervical lesions. (51)

A systematic review on women’s acceptance, attitudes, preferences and willingness towards hrHPV vaginal self-sampling has been published in 2010. (54) Twenty studies were included and 8 of them found more women (varying from 63% to 94%) preferred self-sampling to clinician-collected sampling. Furthermore, women were positive towards the idea of self-sampling instead of a clinician-collected sample as part of future screening programs. The drawback of self-sampling was that some women were uncertain whether they had performed the self-sampling correctly. (54)

So, if women prefer self-sampling over a clinician-collected sample, and if the results are similar in self-sampled material as in a clinician-collected sample, self-sampling might replace physician-sampling in the future. Furthermore, if hrHPV-testing in self-sampled material is combined with additional molecular markers for cervical disease, the sensitivity can be increased, but of course this needs to be the topic of further research.

CONCLUSION

We anticipate a future where the colposcopist is assisted by various digital imaging enhancement techniques and where computer software will be able to detect premalignant cervical lesions that are difficult to identify by the naked eye alone. Probably the advances in digital colposcopy will occur in unison with high-technology biomarkers for cervical pathology. In such a future, women with cervical lesions are identified by a panel of markers, preferably in self-sampled material, with such a high sensitivity and specificity that the risk of over treatment is minimised, but that also, with the help of high-quality digital colposcopy, no women with cervical disease are missed.

REFERENCES


SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES