General discussion

Development of a patient-friendly, reproducible and accurate approach to identifying women at risk of cervical cancer, and men and women from high-risk populations at risk of anal cancer is the focus of this and other research trying to improve screening and cancer prevention. The aim is that the approach should improve the specificity and sensitivity of existing screening and simplify or circumvent the complex multi-stage triage processes in use in screening in developed countries. It is important that any new test is also cost-effective and easily implementable in current Western health care systems, addressing other current limitations, particularly incomplete coverage. Accurate identification and simplification of the follow-up of screen-positive people with accurate selection for treatment is also an important aim for developing screening in low- and middle-income countries (LMIC). Costs and finding the most optimal screening are subject to various factors and influences and cannot always be determined at an early stage of development. Accurate assessment of test reproducibility and accuracy is the key to initially evaluating new tests.

Optimal risk assessment for an individual and a test has to take into account a thorough understanding of natural history of HPV infection and cancer development, including differences and similarities between the infected sites and relevant cell types, HPV genotypes, subtypes or isolates[1]. Patient related factors such as immune status or demographics need to be considered when searching for a strategy with optimal performance [2]. A full assessment involves the patient, policy makers, clinicians, pathologists, molecular biologists, epidemiologists and other health care providers. This makes it complex to come up with a safe and efficient algorithm for the detection and evaluation for treatment in both cervical and anal cancer prevention [3, 4].

This thesis is focussed on the evaluation of molecular markers that can be used in cervical screening and in anal cancer prevention. One important aspect is the investigation of the performance of molecular markers for use on the primary screening sample in cervical screening. This especially studies those that could be carried out on self-sampled material and provide less ambiguous results than current practice of hrHPV testing with or without limited HPV typing to separate patients in need of direct treatment from patients for whom close follow-up is appropriate and patients with a low cancer risk. Both viral factors such as type and multiple infections, and host factors such as methylation of tumour suppressor genes involved in cancer development were explored in relation to cervical and anal cancer prevention.

The second focus is on the use of cervical and anal biopsy at the stage of colposcopic or anoscopic evaluation of detected lesions. This is a key part of the decision to treat a screen-detected lesion but is based on subjective assessment by a pathologist. More

reproducible tests at this stage that can specifically identify progressive lesions needing surgical treatment are needed to avoid over-treatment. In this general discussion, the findings in both these areas of practice are placed in the light of the wider research progress, and a possible future perspective is outlined.

Biomarkers at the screening sample level

Cervical cancer screening

Very importantly, the change to primary hrHPV screening has made the introduction of self-sampling into the cervical screening programme possible. Self-sampling is more convenient than visiting the doctor's office for a clinician-taken smear and has proven to extend the reach of the screening program by including a proportion of former non-responders [5-9]. Several self-sampling devices have been evaluated and the Evalyn Brush is currently used in the Dutch screening program. This brush-based self-sampling device is inserted in the vagina, collecting cervicovaginal cells which cannot be used for cytological reviewing but are suitable for HPV testing [10]. Urine self-sampling has been found convenient and acceptable by women and is even less invasive [11].

In chapter two, we studied the sensitivity of hrHPV and genotyping in self-collected urine samples in the morning and later on during the day, brush-based self-samples, and clinician-taken smears for the detection of CIN2+ in a cytology-screened colposcopic referral population. We found a high agreement for hrHPV detection and genotyping in paired urine samples, brush-based self-samples and clinician-taken smears. Our study shows that, in a referral population, CIN2+ detection using HPV testing of first-void urine samples shows a sensitivity similar to that of clinician-taken samples or brush-based self-samples. In addition, urine self-sampling was found convenient to use for women.

It is still unclear whether a triage test could be carried out on urine. Urine samples work because they collect the shed cells from the cervix, among other cells from the urogenital tract [12]. However, the contribution of cervical cells in relation to that of cells originating from other epithelial sites is not known. In triage tests such as methylation testing, differences in sampled cells might be important. Firstly, because a low proportion of cervical cells might lead to missing cervical lesions. Secondly, because some methylation markers are not tumor specific but are general tumor markers and a positive test might not only detect cervical dysplastic lesions, but also lesions residing in the bladder or elsewhere in the urinary tract [13]. Studies have shown that methylation testing to detect cervical cancer in urine samples is possible when using the right work-up and markers [14, 15]. Whether these markers are also sensitive for the detection of other cancers is not known. In addition to determining the possibilities and limitations of triage testing on urine samples it would also be useful to see if urine samples could

be used for a test-of-cure after LEEP treatment of cervical precancer. Such a test could be self-collected at home after treatment and prevent unnecessary visits to the colposcopy clinic for cervical sampling.

Currently, hrHPV genotyping for HPV16/18 is the most frequently used triage test after cytology-based, hrHPV-based or co-testing-based screening [16-18] and has as a great advantage that it can be performed on both clinician-taken samples and self-collected samples. In the search for an objective and reproducible triage strategy with good clinical performance, we tested various molecular markers on clinician-taken samples and self-samples.

In Chapter 3, the performance of hrHPV-testing and genotyping (GP5+/6+), and methylation testing of human tumor suppressor genes FAM19A4 and/or miR124-2, and different combinations of those, for the detection of CIN3 and cervical carcinoma was compared in women with an ASC-US/LSIL or ASC-H/AGC/HSIL Pap smear result. By combining HPV16/18 genotyping and methylation analysis we found a very sensitive strategy for the detecting of women with HSIL/CIN3+, which offered improved specificity compared to hrHPV alone. The study shows that testing for hrHPV and methylation performed on liquid-based cytology samples in a cytology-screened population could help early and accurate identification of HSIL/CIN3+. It also demonstrated that HSIL/CIN3+ is a very heterogeneous group which consists of both methylation positive and methylation negative lesions.

A methylation negative cytology sample in a woman with HSIL/CIN3+ on biopsy may be found in younger women with a shorter duration of HPV infection or an infection following a different pathway [19, 20]. Also, colposcopy has its limitations with a sensitivity of CIN2+ of 50-70% [21-24] and lesions might be missed, especially when higher up in the cervical canal. hrHPV genotyping or moreover methylation testing of screening sample and histology sample might provide important information about representability of both sampling modalities.

Chapter 4 studied whether genotyping for HPV16/18 as a triage test on hrHPV positive brush-based self-samples can identify the worst underlying lesion. In addition, we studied whether any differences in hypermethylation of FAM19A4/miR124-2 exist between CIN lesions caused by different hrHPV types. We observed that the causative genotype of the worst underlying lesion is detected in almost all self-samples. And we found that there are no significant differences in positivity for these markers between CIN lesions caused by different types of hrHPV [25].

However, this finding does not explain why infections with certain hrHPV genotypes, particularly HPV16 are more prone to develop into cancer. In addition, hypermethylation detects long-lasting hrHPV infections resulting in advanced transforming lesions but far from all of these lesions will actually develop into cancer [26]. The combination of methylation markers FAM19A4/miR124-2 that was used in our studies does not only detect women at risk of progression to cancer, but also detects hypermethylation in women with persisting hrHPV infection and women of older age [27, 28]. Follow-up studies of women with a positive methylation test at baseline are needed to address this important question before methylation testing can be introduced in clinical practice [29].

Anal cancer screening

In anal cancer screening, collecting representative anal cytology sampling and moreover anal self-sampling is challenging because of the anatomy of the anal canal with its folds and circular configuration, because of contamination with faeces. In addition, the prevalence of HPV DNA in the anal canal is high, but a correlation between anal HPV infection and a suspicious anal cytology is infrequently observed [30 31]. Samples are taken with a dacron swab and not with a cervix brush because of the fragility of the anal canal, making it more difficult to collect a sample with sufficient cell density. This limits the tests that can be performed on an anal swab: an anal cytology result of HSIL is specific, but anal cytology lacks sensitivity [32 33]. Immune stainings give the same problem. Low cell count of anal swabs results in low human DNA concentrations, making methylation testing challenging. Special brushes are being developed and possibly better instructions for talking samples and better preservatives could help [34]. In HIV positive MSM, HPV screening in anal samples is not an option because of the high prevalence of HPV in this population [35]. Studies have shown that a genotype specific persistent hrHPV infection could help detect underlying AIN2+ lesions [36 37]. hrHPV detection on anal samples could open the possibility of selective HRA.

Biomarkers at the histology sample level

There are a lot of improvements that could be made regarding identification of men and women with a true high-grade lesion that might progress to cancer. Firstly, we need better and more objective and reproducible markers to grade lesions, so that we can better compare lesions and results between centres, studies and countries. These markers should provide us with more information about the natural history. Current nomenclature groups lesion types that are molecularly significantly different under the same diagnosis [38, 39] . We need improved differentiation of lesions to discriminate those that require treatment from those that can be followed-up.

The immunohistochemical markers that were studied in this thesis, p16 and HPV E4, could provide part of the solution. Besides the fact that these immunohistochemical markers are easier to interpret, are more objective and more reproducible [40], they also tell us something about the current status of the lesion.

In Chapter 5, we used p16, HPV E4 and Ki-67 to describe different immunohistochemical staining patterns in anal biopsies. The patterns that we found suggest that division of lesions into LSIL and HSIL is simplistic and that by combining p16 and Ki-67 with E4 productive AIN lesions can be distinguished from advanced transforming AIN. Ki-67 helps separate LSIL from normal tissue, while the combination of HPV E4 and p16 helped identify productive infections, which are E4 positive, and advanced transforming infections which are diffusely p16 positive and E4 negative.

In AIN in HIV+ MSM, both lrHPV and hrHPV can be found in LSIL and HSIL.

In Chapter 6, we used the combination of HPV E4 and p16 immunohistochemistry to improve definition of lr- and hr-HPV associated AIN in HIV+ MSM. Our study showed that combined p16/E4 staining identifies both productive and non-productive LSIL associated with lrHPV and HSIL associated with hrHPV, proving detailed information about AIN which is not provided by H/E staining alone. E4 positivity in the worst lesion on biopsy identifies a productive infection, while absence of E4 in a diffusely p16 positive HSIL uncovers a possibly advanced transforming infection which might be methylation positive.

The exact position of concurrent use of both immunohistochemical markers in routine pathology practice is not clear yet, p16/E4 dual staining and better define progression risk of the different biomarker expression patterns, performing this double stain on all ≥CIN1/AIN1 would be highly recommendable. Before we will be able to do this, more research on the p16/E4 staining patterns in relation to other markers such as other immunohistochemical markers (SCJ markers), methylation markers (both human tumour suppressor gene methylation and viral methylation markers) and markers that can tell us more about immune response and cell origin (HPV E6/E7 serology, messenger RNA) should be done.

The use of additional immunohistochemical (IHC) markers such as Ki-67 have shown to not significantly improve histological diagnosis of HSIL or improve reproducibility of a HSIL diagnosis when interpreted together with H/E and p16 [41]. When interpreting all together, p16 staining seems to be leading in detecting HSIL and Ki-67 staining patterns are more difficult to interpret [42]. However, when H/E morphology is no longer considered

and p16 and Ki-67 are used to provide an objective immunoscore, van Zummeren et al found that CIN3+ lesions can be detected in a highly sensitive and specific manner when a maximum score for both markers is set as the cut-off, and that this immunoscore improves agreement between pathologists [43]. Thus, this immunoscore was not developed to provide additional information on biology of the lesion or progression risk, but to improve agreement between pathologists. An objective and reproducible gold standard diagnosis is important for epidemiologic research studying risk factors for progression risk, and when introduced in routine pathology could reduce overtreatment.

In Chapter 7**,** the relationships between the immunohistochemical expression patterns of markers p16 and HPV E4 in biopsies and methylation markers FAM19A4/miR124-2 in cervical smears of women with different grades of CIN and negative controls associated with hrHPV infection was studied. The inverse relation that we demonstrated between HPV E4 expression on biopsy and methylation marker positivity on both biopsy and cervical swab is important, but implications for carcinogenesis and clinical management are not clear yet. HSIL lesions that are both E4 and methylation positive are not well understood. New hypotheses suggest that productive lesions arise from other cell types than advanced transforming infections, and that lesion development is possibly not a continuum but represents a difference in origin with different progression risk. The use of IHC markers to identify lesions arising from HPV infections of the squamocolumnar junction (SCJ), with a higher chance of progression to cancer, has been studied by Herfs et al. And although other groups have not found associations as strong, the idea that different cell origins produce lesions with different progression risks is carried widely across the scientific population [19]. Doorbar et al have used immunofluorescence to show differences between lesions located at the ectocervix and lesions called atypical metaplasia, arising from the reserve cells in the squamocolumnar junction [44]. In these studies, proliferation marker MCM and p16 and E4 showed different biomarker expression patterns between the different lesion types using immunofluorescence. Such a study in respect to immunohistochemical marker scoring of p16 and E4 has not yet been performed but might help explain the significance of the different biomarker expression patterns found in this thesis. For example, HSIL with p16 up to 1/3 and no E4 expression in the upper cell layers could represent a regressing ectocervical lesion and its recognition could lead to an important reduction in overtreatment.

In addition, we need to better understand what the molecular differences mean for cancer risk. As long as all women with CIN2+ are treated, removing the entire transformation zone, it is very hard to discover markers of progression or regression. This however is extremely important, not only for marker discovery but also for the validation of existing

and future markers. The first studies of methylation markers in women undergoing active surveillance for CIN2/3 are now being conducted [45, 46].

AIN treatment can only be done through local ablation and AIN therefore has a high recurrence rate after treatment [47]. The efficacy of treatment has therefore not very well been defined and so AIN natural history studies not treating anal HSIL do exist, with two large trials currently ongoing (SPANC and ANCHOR) [48, 49]. Results from those studies might also teach us about both AIN and CIN since there are various great similarities between AIN and CIN, but they are not the same and the patient populations are not the same. Although the same types of epithelium cover both the anal canal and the cervix, they are different sites with different microbiomes [50, 51]. Another difference, which possibly has to do with the fact that a lot of the AIN patients in studies are HIV+ MSM, is that lrHPV is often found in AIN lesions [52-54]. Most lrHPV infections will cause condylomas and flat LSIL, but some of these condylomas also harbour a HSIL area with hrHPV, or even cause a HSIL lesion on their own [55]. Therefore, genotyping might not be sufficient as a stand-alone test for risk assessment, but it is a very helpful tool in getting better understanding of natural history and in diagnostics can also help us find the worst lesion.

Use of biomarkers at the patient level

With all new developments in both primary and secondary cervical cancer prevention, cervical cancer screening guidelines are in constant evolution. So far, extended screening intervals for HPV-negative women above the age of 40 years have been the first step towards more personalized screening. Findings from this thesis regarding sampling modality, triage strategy and risk assessment based on molecular profiling might contribute to a future model in which individual patient preferences and patient risks are taken into account. Such a model should also take into account differences between populations.

Firstly, there is a difference between responders and non-responders, and moreover people who are screened and people who are not. People who do not regularly attend screening have a higher risk of developing cancer [56]. Immune status, as demonstrated by differences between patient with and without HIV, also alters the risk of developing cervical or anal cancer [57, 58]. A combination of the two factors mentioned above is most likely the main but not sole reason for differences in cervical cancer between the developed and developing world. Factors such as ethnicity, smoking status, age of sexual debut, number of lifetime sexual partners and contraceptive of choice also influence the risk of cancer [59-63]. In addition, because of the different cancer risks of different hrHPV genotypes and because of the altered risk of progression to cancer in case of genotype specific persisting hrHPV infection, genotyping could become increasingly important in

population based and personalized screening. Every HPV genotype has its own risk of developing into a cancer, with >70% of all cervical cancers caused by HPV16, and most likely there are subtypes and isolates that are more cancerous than others. Mirabello et al found that the more the HPV E7 region of an HPV16 is conserved, so the less SNPs it has, the more likely it is to cause a cancer [1]. In addition, most women will have the same HPV16 isolate on multiple sites of their genital tract (cervical, vulvar, anal). Over time, women might have the same isolate present as a persisting infection or gain a new HPV16 infection with a different isolate.

Another important question that needs to be addressed is: who to screen? For cervical cancer, this is mostly clear. In the majority of screening countries, women are screened starting at the age of 30. Younger women more often have HPV infections but are less likely to have CIN lesions that need treatment [64]. In Chapter 3, we have shown that there is a difference in performance of methylation markers FAM19A4/miR124-2 between women under and over the age of 30, which can partly be explained by the fact that hypermethylation is a result of persistent HPV infection and, in addition, the process increases with age [65-67]. However, there is a group of women that develops cervical cancer at a young age (<25) and carcinogenesis in this group might follow a different pathway than in older women and developing more quickly, requiring other tumour markers for risk assessment [68, 69].

For anal cancer, it is unclear how to screen, and moreover who and when to screen. Targeted screening of high-risk populations such as HIV+ MSM is done increasingly, however, anal cancer is more frequently found in women and therefore identification and screening recommendations of women at risk could result in better disease control [30, 70-72]. Feasibility of expansion of targeted screening, or even incorporation into the cervical cancer screening programme, will largely depend on costs, invasiveness and performance of the screening test, which is yet to be selected. [32]

Impact of HPV vaccination on cervical screening

In the Western world, prophylactic vaccination will change cervical cancer screening when the first vaccinated cohorts reach the screening age. Women who have been vaccinated will have a lower cancer risk, allowing for longer screening intervals and less lifetime screens [73]. Until full herd-immunization has been reached, personalized risk assessment and screening algorithms, possibly including other clinical risk factors such as smoking status and number of sexual partners, might therefore apply and registration of vaccination status is important. To eradicate HPV related cancers, including penile, vulvar, vaginal and oropharyngeal cancer, boys also need to be vaccinated in order to reach a high coverage level with herd-immunity for both sexes [74]. In several countries such

as Australia and Austria, gender neutral vaccination has already been implemented. In addition, studies have shown that targeted vaccination of high-risk populations such as MSM is achievable and cost-effective and could substantially reduce the burden of HPV-related diseases among men [75, 76]. Seroconversion and protection against HPV-related disease after prophylactic vaccination has been studied in girls and boys and have shown comparable results [77]. Studies comparing heterosexual men and MSM under the age of 26 showed that immunogenicity is lower among MSM [78, 79]. Research on efficacy and safety of HPV vaccination of men, both heterosexual and MSM, over 26 years of age is ongoing.

Improving colposcopy

Colposcopy and high resolution anoscopy both rely on the subjective identification of visual features of dysplasia by a clinician, resulting in a sensitivity of 50-70% for HSIL detection [22-24, 80-82]. Alternative or supportive tools for colposcopy, such as fluorescence and reflectance spectroscopy, might result in increased sensitivity and specificity, but have not been introduced in standard clinical practice [83]. Digital colposcopy has given us the opportunity to build a digital database of colposcopic images. In combination with histological data [84], this database could be used to develop artificial intelligent algorithms that can identify at risk lesions. Techniques are still under development and have to be evaluated in randomized controlled trials comparing them with conventional colposcopy in order to determine their clinical value.

The future of methylation markers

Many tumor suppressor gene-based methylation markers have been discovered. So far, over 100 human genes have been proposed as markers of cervical precancer and cancer but none of the found markers can be used as a sole marker to detect CIN3+ lesions: a combination of at least 2 markers always has to be made to reach acceptable sensitivity and specificity [85]. Discovery of an accurate and acceptable endpoint for malignant transformation could help to identify new methylation markers. With the help of next generation sequencing, relevant methylated regions between controls and cancers, but also between progressing and regressing precursor lesions can be identified. Most desirably, a method with high coverage that only requires low DNA input would be used [86]. This way, markers that are specific for one histological cancer type or a general tumour marker for anogenital cancer could be identified, allowing anogenital cancer risk assessment through the testing of a single (self)sample of epithelial cells or a blood sample (liquid biopsy).

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