Cervical cancer prevention based on biomarkers

van Zummeren, M.

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SUMMARY

The global burden of cervical cancer is an important public health problem. Cervical cancer is the fourth most common cancer worldwide, with relatively high incidence and mortality rates in low- and middle-income countries (LMIC). This difference between countries is caused by the lack of effective cervical screening strategies and poor access to health care in low-resource settings. In high-income countries, the introduction of cervical screening by cytology in the 1960s has led to a drastic reduction in the incidence of and mortality from cervical cancer. With the recognition that high risk types of the Human Papillomavirus (HPV) are the causative agent of cervical cancer, the following levels in the prevention of cervical cancer can be identified: 1. primary prevention: prophylactic vaccination with HPV virus like particles (VLPs) of two, four or 9 high-risk HPV types to prevent development of cervical intraepithelial neoplasia (CIN) and cancer in healthy individuals; 2. secondary prevention: HPV-based or cytology-based nation-wide cervical screening for the detection of asymptomatic CIN lesions followed by ablative therapy; and 3. tertiary prevention: post-treatment surveillance for high-grade CIN lesions or cancer by cytology or combined cytology and HPV testing.

The prevention of cervical cancer by cytology screening is based on the detection and subsequent treatment of precancerous CIN lesions. The reproducibility of the classification of the different grades of CIN lesions (CIN 1 to 3) is only moderate and pathologists are unable to predict whether a lesion will regress or progress. Consequently, clinical management of CIN lesions varies, especially of CIN2, and physicians tend to overtreat to be sure that no cancer could develop. The use of biomarkers involved in cervical carcinogenesis may result in a more accurate screening for and grading of CIN lesions. The work presented in this thesis describes the possible impact of such biomarkers on the detection, classification, and management of cervical disease, and provides a basis for more accurate, reliable and standardised cervical cancer prevention.

Chapter 1 provides a general introduction on cervical cancer, HPV, primary, secondary, and tertiary cancer prevention, and an overview of potential molecular biomarkers in cervical cancer prevention.

PART 1 SCREENING BY PRIMARY HPV WITH MOLECULAR TRIAGE TESTING

An important improvement of cervical screening has been the introduction of HPV testing as primary screening tool. Compared to cytology, primary HPV testing leads to earlier detection of cervical neoplasia grade 3 or worse (CIN3+), and provides a better protection against cervical cancer. Therefore, in the Netherlands primary cytology-based screening has been replaced by

HPV-based screening in 2017. In the new Dutch screening programme, women are invited to participate at five-year screening intervals between the age of 30 to 60 years. Intervals have been extended up to 10 years for HPV screen negative women aged 40 years and older, thereby skipping screening rounds at 45 and 55 years. However, the specificity of primary HPV testing is 3-5% lower compared to cytology, because most detected HPV infections are transient. To identify those HPV positive women with clinically relevant disease (cervical cancer or high-grade CIN), a second, so-called triage test is necessary. Cytology, with repeat cytology 6 months after baseline, is the triage strategy of choice in the Dutch programme. In **Part 1** of this thesis, we focus on the longitudinal safety of primary HPV-based screening, and recently developed innovative molecular triage strategies.

In **Chapter 2**, we describe the longitudinal 14 year follow-up data of the large population-based POBASCAM trial, which compares screening by HPV testing and cytology with cytology-based screening. The data support recent policy decisions made in the Netherlands. Cumulative incidences of cervical cancer (0.09%) and CIN3+ (0.56%) among HPV screen negative women after three screening rounds, were comparable to those incidences among cytology screen negative women after two screening rounds (0.09% and 0.69% respectively). The very low long term risk for cancer and CIN3+ found among HPV screen negative women supports the extension of the screening interval for these women up to 10 years. However, for HPV positive women with a negative triage test, i.e. baseline and repeat cytology, HPV16/18 genotyping or a combination of these two, the CIN3+ risks were too high to justify an extension of the screening interval over five years. Due to the low number of cancers found in the POBASCAM trial, the cervical cancer risks in women aged >40 years compared to younger women were inconclusive. Therefore, the risk for interval cancers with extension of the screening interval should be closely monitored.

Analysis of the hypermethylation status of promoter regions of host-cell genes involved in cervical carcinogenesis can be used as molecular biomarker-based triage tool in cervical scrapes of HPV positive women. A hypermethylation positive status identifies CIN lesions that have a high short-term progression risk for cervical cancer. **Chapter 3** evaluates the performance of a multiplex assay to assess hypermethylation status of *CADM1*, *MAL*, and *miR124-2* genes (PreCursor-M®, Self-screen, The Netherlands) in HPV positive cervical scrapes. Hypermethylation levels and the number of hypermethylated genes were found increased in proportion to the severity of the underlying cervical lesion. Furthermore, in a large series of cervical scrapes from women with cervical cancer (n = 79), hypermethylation levels were extremely high and the assay tested positive in all scrapes. Interestingly, a substantial subset of scrapes from women with endometrial cancer also tested positive (n = 16 / 21, 76%).

PART 2 SCREENING BASED ON BIOMARKERS IN LOW- AND MIDDLE INCOME COUNTRIES

Implementation of cervical screening is particularly challenging in LMIC. Here, morbidity rates are high because of the lack of population-based screening, combined with a high prevalence of human immunodeficiency virus (HIV). HIV infection is an important risk factor for HPV persistence and the development cervical (pre)cancer. In Part 2 of this thesis, we assessed the performance of biomarker-based screening tools in women living with HIV (WLHIV).

Chapter 4 presents the clinical performance of primary HPV testing combined with reflex hypermethylation analysis of *CADM1*, *MAL*, and *miR124-2* genes, applied on cervical scrapes of WLHIV from a South African study cohort. HPV testing alone showed a CIN3+ sensitivity of 83.6%, at a specificity of 67.7%. When HPV testing was combined with reflex hypermethylation analysis, CIN3+ sensitivity was 73.8%, at a specificity of 81.5%. Sole hypermethylation analysis showed a good sensitivity of 85.2%, however at a significantly lower specificity of 49.6%. All strategies showed a 100% cancer detection rate (n = 44). The low specificity of primary hypermethylation analysis might be explained by the relatively high baseline methylation levels in cervical scrapes of HIV seropositive women without cervical disease compared to HIV seronegative women. Therefore, primary hypermethylation analysis needs further optimization before implementation would be feasible in populations with a high HIV prevalence.

In **Chapter 5**, we were able to develop of a methylation assay with positivity thresholds fine-tuned for WLHIV. Hypermethylation analysis of the recently discovered methylation markers *ASCL1* or *LHX8* on cervical scrape material of WLHIV, showed an acceptable CIN3+ sensitivity (72.1% and 73.8% respectively), at a good specificity of 75%. Again all scrapes from women with cancer tested positive. These findings warrant further development of such a hypermethylation assays into an affordable and rapid point-of-care test, that could be implemented in an effective screen-and-treat strategy. The detection of all cancers and specifically advanced CIN lesions is relevant for low-resource settings where women are at high risk for cervical (pre)cancer and have only one or two life-time screening opportunities.

PART 3 IMPROVING THE CLASSIFICATION OF CIN LESIONS BY THE USE OF BIOMARKERS

Cervical screening aims for the detection of cervical lesions in a pre-cancerous stage, followed by treatment. Accurate and reproducible grading of these lesions is essential. In Part 3 of this thesis, we analysed the additive value of biomarkers involved in cervical carcinogenesis in the substantiation and standardisation of the diagnosis of CIN. Chapter 6 compares the accuracy and reproducibility of current CIN grading, to that of an immunoscore grading system, which is based solely on the expression of immunohistochemical (IHC) biomarkers Ki-67 and $p16^{ink4a}$.The accuracy and reproducibility was higher for immunoscoring compared to current CIN grading, in particular for CIN3 (treatment) and CIN1 (no treatment). Hereby, the heterogeneous group of CIN2 could be largely split up into CIN3 and CIN1, potentially preventing under- and over-treatment. In clinical practice, the use of the more accurate Ki-67 and $p16^{ink4a}$ immunoscore system in CIN grading could assist the clinician in decision making on treatment.

Chapter 7 describes the expression of HPV-E4 protein, a biomarker indicative for the onset of a productive HPV infection, and the analysis of hypermethylation status of *CADM1*, *MAL*, and *miR124-2* genes, a biomarker indicative for a transforming HPV infection, within lesions characterised by both classical CIN- and immunoscore grading. Extensive E4 expression was found most frequently in low-grade CIN and decreased with increasing CIN grade, to absence in carcinomas. Hypermethylation status increased with increasing CIN grade, up to a 100% positivity rate in all carcinomas. Lesions with extensive E4 expression were associated with a very low hypermethylation status. The expression profiles of both E4 and hypermethylation illustrate the gradual transition of early productive CIN, with a low short-term progression risk for cervical cancer, towards advanced transforming CIN, with a high short–term progression risk for cervical cancer, and might further substantiate the characterisation of CIN.

PART 4 POST-TREATMENT SURVEILLANCE

Women treated for high-grade CIN, are at increased risk for recurrent disease. Therefore strict follow-up is necessary. In Part 4 of this thesis, we evaluate the value of biomarkers in post-treatment surveillance.

Chapter 8 describes a multicentre prospective clinical cohort study, wherein women were followed-up up to one year after treatment. Post-treatment disease was split up in persistent (i.e. incompletely treated) and incident (early onset) CIN lesions. In the follow-up, the *CADM1*/*MAL*-hypermethylation assay was able to identify the majority of persistent recurrent CIN3 (rCIN3) and rCIN2. Furthermore, all incident rCIN tested hypermethylation negative, whereas all post-treatment

detected carcinomas tested hypermethylation positive. Also, a positive hypermethylation status at baseline and follow up, was associated with a significantly increased risk for rCIN2/3+, compared to a hypermethylation negative status. Post-treatment monitoring for rCIN2/3 can therefore be further improved by analysis of the hypermethylation status of *CADM1*/*MAL*.

Finally **Chapter 9** provides a general discussion of the results presented in the different parts in this thesis in the context of the available literature, and also points out future perspectives and possible clinical implications of our research.