

BMJ Open Role of *FAM19A4/miR124-2* methylation analysis in predicting regression or non-regression of CIN2/3 lesions: a protocol of an observational longitudinal cohort study

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ABSTRACT

Introduction The clinical course of high-grade cervical intraepithelial neoplasia (CIN2/3) is characterised by a high spontaneous regression rate. Histological assessment is unable to differentiate between CIN2/3 lesions likely to regress and those likely to persist or progress. Most CIN2/3 lesions are treated by surgical excision, leading to overtreatment of a substantial proportion. In this prospective study, we evaluate the value of DNA methylation of host cell genes, which has shown to be particularly sensitive for the detection of advanced CIN2/3 and cervical cancer, in the prediction of regression or non-regression of CIN2/3 lesions.

Methods and analysis This is a multicentre observational longitudinal study with 24-month follow-up. Women referred for colposcopy with an abnormal cervical scrape, who have been diagnosed with CIN2/3 and a small cervical lesion ($\leq 50\%$ of cervix) will be asked to participate. Participants will be monitored by 6-monthly cytological and colposcopic examination. In case of clinical progression, participants will receive treatment and exit the study protocol. At baseline and during follow-up, self-sampled cervicovaginal brushes and cervical scrapes will be collected for high-risk human papillomavirus (HPV) testing and *FAM19A4/miR124-2* methylation analysis. A colposcopy-directed biopsy will be taken from all participants at the last follow-up visit. The primary study endpoint is regression or non-regression at the end of the study based on the histological diagnosis. Regression is defined as CIN1 or less. Non-regression is defined as CIN2 or worse. The secondary study endpoint is defined as HPV clearance (double-negative HPV test at two consecutive time-points). The association between methylation status and regression probability will be evaluated by means of χ^2 testing.

Ethics and dissemination Ethics approval was obtained in all participating clinics. Results of the main study will be submitted for publication in a peer-reviewed journal.

Trial registration number NTR6069; Pre-results

Strengths and limitations of this study

- Longitudinal data and cervical sample collection from women with untreated CIN2/3 will allow us to study the natural history of these lesions in relation to DNA methylation markers.
- Strict criteria for inclusion and 6-monthly cytological and colposcopic evaluation are applied to minimise the risk of missing carcinomas or progression into cervical cancer.
- Close surveillance allows for monitoring lesion outcome, yet cervical sampling (including cervical biopsies) may influence the clinical course.
- Women are included after diagnosis of CIN2/3 on a cervical biopsy, resulting in the collection of the first study sample after an initial biopsy.
- If *FAM19A4/miR124-2* methylation analysis proves to predict regression or non-regression, overtreatment of CIN2/3 lesions could be prevented by using this test in clinical management decisions.

INTRODUCTION

Current cervical screening programmes serve to detect and treat premalignant lesions (cervical intraepithelial neoplasia, CIN, graded 1–3) to prevent invasive cervical cancer. Clinical management of women with an abnormal screening test is based on the histological diagnosis of a cervical biopsy: CIN1 lesions are managed conservatively, whereas CIN2 lesions or worse are generally treated with surgical excision. However, this diagnostic-treatment trajectory is associated with considerable overtreatment as CIN2/3 lesions have high regression rates. An estimated 44%–50% of CIN2% and 32% of CIN3 regress spontaneously,^{1–3} while ~5% of untreated CIN2 and 12%–31% of untreated CIN3 ultimately progress to cervical cancer.^{1,4}

Because predictive markers for cancer progression of CIN2/3 lesions are lacking, most CIN2/3 lesions are treated similarly by surgical excision, either large loop excision of the transformation zone (LLETZ) or cold knife conisation. While this treatment of CIN2/3 lesions detected through screening programmes led to a dramatic decline in cervical cancer incidence in developed countries, there are several adverse effects. Excisional treatment of cervical lesions is associated with an increased risk of pregnancy-related morbidity due to preterm delivery.^{5–7} As women receiving cervical treatment are often of reproductive age, distinction between CIN2/3 lesions likely to regress and CIN2/3 lesions likely to progress will be of great clinical and social value. Biomarker testing could guide clinical decision-making, treating only those CIN2/3 lesions likely to progress, thus preventing overtreatment.

To accurately predict the individual cancer risk, adjunct methods are needed. Although several potential prognostic factors, such as human papillomavirus (HPV) type 16 positivity,^{2,8,9} high HPV viral load^{10,11} and overexpression of cell cycle regulatory proteins p16^{INK4A12–18} and Ki-67,¹⁹ have been evaluated, none of these have proven their true clinical prognostic value in CIN2/3 lesions.²⁰ DNA methylation analysis of host cell genes has emerged as a promising biomarker that can distinguish between advanced transforming CIN2/3, with a high short-term risk of cervical cancer, and productive or early transforming CIN2/3 lesions, with a low short-term risk of cervical cancer.^{21–23} Among other genes, hypermethylation of host cell genes *FAM19A4* and *miR124-2* has been studied extensively. An increase in methylation levels of these genes is not only related to the degree but also to the duration of CIN2/3 disease, and levels are exceptionally high in cervical samples of women with cervical cancer.^{22,24} Additionally, a negative *FAM19A4/miR124-2* methylation test provides a low long-term cancer risk among HPV-positive women.²⁵ This suggests that the *FAM19A4/miR124-2* methylation test is particularly sensitive for CIN2/3 lesions with an increased short-term risk of progression.

In this multicentre observational longitudinal cohort study, we will clinically validate whether *FAM19A4/miR124-2* methylation analysis can distinguish CIN2/3 lesions likely to persist and progress from those likely to regress, thus determining the need of immediate treatment versus active surveillance. This could prevent overtreatment and the associated cervical morbidity, which is especially relevant for women of childbearing age.

METHODS AND ANALYSIS

The aim of this study is to clinically validate whether hypermethylation of host cell genes can predict regression or non-regression of CIN2/3, and, consequently, allows distinction between advanced transforming CIN2/3, in need of treatment, and productive or early

transforming CIN2/3, for which an active surveillance approach is acceptable.

This is an ongoing multicentre observational longitudinal cohort study with 24-month follow-up. Study inclusion started in May 2017 and takes place in three participating clinics in The Netherlands: OLVG (Amsterdam), Flevoziekenhuis (Almere) and Bergman Clinics (Amstelveen). HPV testing and methylation analysis takes place at the Department of Pathology of Amsterdam UMC, Vrije Universiteit Amsterdam.

Inclusion and exclusion criteria

Women referred to the participating clinics for colposcopy because of an abnormal cervical scrape, who have been diagnosed with a CIN2 or CIN3 on a cervical punch biopsy and who have a small cervical lesion (covering $\leq 50\%$ of the visible cervix), will be asked to participate in the study. A total of 100 women will be included in this group. In order to be eligible for inclusion, women must meet all of the following criteria: non-pregnant and aged 18–55 years. Women who meet one or more of the following criteria will be excluded from study participation: cervical adenocarcinoma in situ (AIS) on histology, history of cervical pathology (ie, CIN1 or worse) in the preceding 2 years, inadequate colposcopy (ie, transformation zone is not fully visible (type 3 transformation zone according to International Federation of Cervical Pathology and Colposcopy guidelines²⁶)), prenatal diethylstilboestrol exposure, concomitant cancer or insufficient Dutch or English language skills.

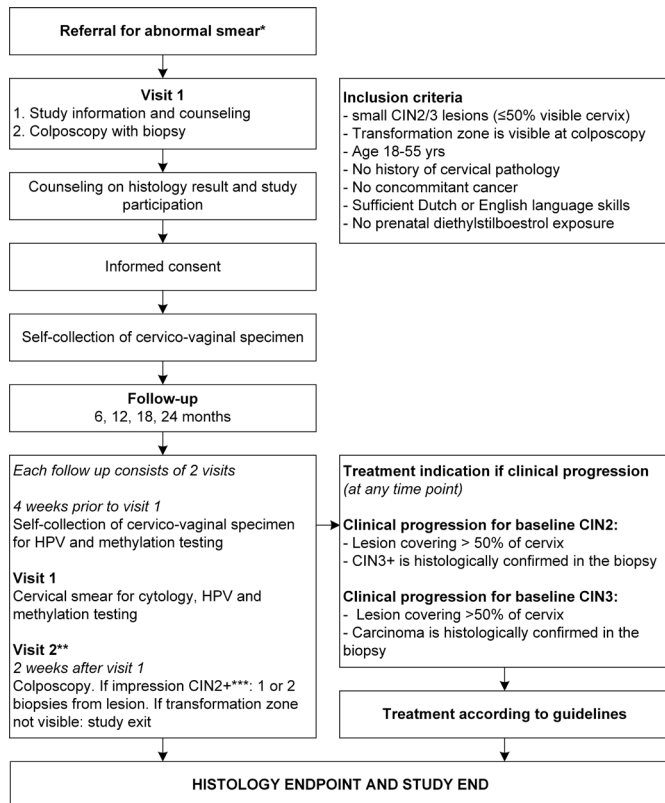
Informed consent procedure

Women will be informed about the study during their first colposcopy visit to the clinic. During this visit, routine colposcopic examination is performed, the size of the cervical lesion is assessed and a diagnostic cervical punch biopsy for histopathology is taken. Approximately 2 weeks after this initial visit, women will be informed about their histology result of the cervical punch biopsy by their gynaecologist. Women who meet the inclusion criteria and who are willing to participate will be asked to give oral and written informed consent.

Study procedures

Study participants will receive an Evalyn brush (Rovers Medical Devices B.V., Oss, The Netherlands) directly after inclusion for the self-collection of a cervicovaginal sample which will be used for baseline high-risk HPV testing and methylation analysis. Clinical information regarding medical history, cytological diagnosis, colposcopic impression and a digital colposcopy photo of the lesion will be retrieved through the participating clinics. Participants will be monitored by an intense follow-up schedule with 6-monthly visits to the colposcopy clinic for 2 years. The study flowchart (figure 1) shows all study procedures schematically.

Follow-up will take place at 6, 12, 18 and 24 months after the first visit for colposcopy. Each follow-up consists



* Referral smear will be requested for baseline methylation analysis

** At second (T=12) and third (T=18) follow-up: colposcopy may be omitted if cytology is normal

*** At the last follow-up visit (T=24) 2 biopsies are taken irrespective of colposcopic impression

Figure 1 Flowchart of study procedures.

of two visits. At the first visit of each follow-up, a cervical scrape will be taken by a specialised nurse or gynaecologist for routine cytological evaluation according to CISOE-A classification by a local pathologist.^{27 28} A few days prior to this visit, participants are requested to use the Evalyn brush for the self-collection of a cervicovaginal sample. The second visit of each follow-up consists of colposcopic examination by an experienced gynaecologist, who will annotate the colposcopic impression, record an image of the cervix and indicate the location of the biopsy (if applicable). Cervical biopsies will be taken according to the colposcopic impression of the gynaecologist. At the last follow-up visit, two exit biopsies are taken from a lesion, or at random if there is no visible lesion. All participants with a CIN2 or worse at this last study visit will receive treatment according to regular care.

If routine cytological evaluation of the cervical scrape shows no abnormalities at 12-month or 18-month follow-up, colposcopic examination may be omitted. This can be decided by the gynaecologist together with the study participant.

Treatment indication

If at any time during follow-up the transformation zone is not completely visible or AIS is found on cervical histology, participants will be excluded from the study protocol and treated. Furthermore, a participant will exit the study and receive treatment if the lesion shows clinical progression.

For women with a CIN2 lesion at baseline, progression is defined as: (1) increase in colposcopic volume of the lesion (covering $> 50\%$ of visible cervix) at follow-up or (2) follow-up histology of a cervical biopsy showing CIN3 or carcinoma. For women with a CIN3 lesion at baseline, progression is defined as: (1) increase in colposcopic volume of the lesion (covering $> 50\%$ of visible cervix) at follow-up or (2) follow-up histology of a cervical biopsy showing carcinoma.

Study endpoints

The primary study endpoint is regression or non-regression at the end of the study based on histology of the cervical exit biopsy. All cervical biopsies will be examined by local pathologists, who are blinded to the methylation results, and classified as no dysplasia, CIN1, CIN2, CIN3 or cervical carcinoma according to international standards.²⁹ Regression is defined as a \leq CIN1 diagnosis in the exit biopsy. Non-regression is defined as a CIN2+ diagnosis in the exit biopsy.

It has been shown that HPV-clearance precedes regression of cervical lesions by an average of 3 months.²⁶ Therefore, the secondary study endpoint is defined as HPV clearance (double-negative HPV test at two consecutive time points).

Study parameters

All self-sampled cervicovaginal cells and all cervical scrapes (liquid-based cytology) collected during the study will be stored in ThinPrep PreservCyt Solution (Hologic, Marlborough, Massachusetts, USA). Methylation analysis and high-risk HPV testing will be performed on these samples blinded to the cytology and histology results from routine clinical diagnostics. DNA will be isolated from these samples using the Microlab STAR robotic system (Hamilton, Reno, Nevada, USA) according to the manufacturer's protocol. HPV DNA detection will be performed using the clinically validated HPV-risk assay (Self-screen B.V., Amsterdam, The Netherlands), a multiplex real-time PCR-based assay that targets the E7 region of 15 (probable) high-risk HPV types (ie, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66, HPV67 and HPV68),^{30 31} and enables partial genotyping for HPV16 and HPV18.^{30 31} For methylation analysis, cervical DNA will be subjected to bisulphite treatment using the EZ DNA Methylation kit (Zymo Research, Irvine, California, USA), and a commercially available, CE-labelled, multiplex quantitative methylation-specific PCR kit (QIAure Methylation Test, QIAGEN, Hilden, Germany) will be used to measure the methylation status of host cell genes *FAM19A4* and *miR124-2*.²¹

Sample size calculation

In total, 100 women will be included in the study yielding a width of the 95% CI $< 20\%$ when assuming a regression probability of 30%. If we assume that the regression probability is 15% for methylation-positive, and 45% for

methylation-negative women, and assume that 50% of the women are methylation positive, then a sample size of 100 provides a power of 87% to detect a significant difference in regression probability (significance level 0.05, two-sided).

Statistical analysis

The regression probabilities will be estimated by a binomial proportion. CIs will be constructed by Wilson's score method. The association between methylation status and regression probability will be evaluated by means of χ^2 testing. The results will be adjusted for presence of HPV16 and HPV18.

Monitoring

The study will be monitored for quality and regulatory compliance by Amsterdam UMC. The frequency depends on inclusion rates, questions and pending queries from earlier audits and will be once or twice a year.

Patient and public involvement

No patient advisors were involved in the development and design or conduct of this study. Study results will be disseminated to the study participants via an information letter.

ETHICS AND DISSEMINATION

Ethics approval was obtained in The Netherlands at the Medical Ethics Committee VU University Medical Center (Amsterdam, The Netherlands, 2016/471). Additional approval was obtained from the participating clinics. The trial is registered with The Netherlands National Trial Registry.

Dissemination to the medical and scientific community will be achieved through publication in peer-reviewed scientific journals and presentation at international scientific conferences.

On completion of the trial and after publication of the primary manuscript, data requests can be submitted to the researchers at Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands.

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Contributors WWK, JB, MCGB, DAMH, NEvT, CJLMM and GGK were involved in conception and study design. WWK, CJLMM and GGK were involved in drafting of the article. JB, MCGB, DAMH, NEvT, MWB and HRV were involved in critical review of the article for important intellectual content. All authors critically reviewed the manuscript and approved the final version. JB provided statistical expertise. WWK, MWB and HRV will be involved in data acquisition. Collection, management, analysis and interpretation of data, writing of the report, and the decision to submit

the report for publication is the responsibility of GGK, the principal investigator of the study.

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Competing interests DAMH and CJLMM are minority shareholders of Self-screen B.V., a spin-off company of VU University Medical Center; Self-screen B.V. holds patents related to the work (ie, high-risk HPV test and methylation markers for cervical screening); DAMH has been on the speakers bureau of Qiagen and serves occasionally on the scientific advisory boards of Pfizer and Bristol-Myers Squibb; JB received consultancy fees from Roche, GlaxoSmithKline and Merck, and received travel support from DDL. All fees were collected by his employer; CJLMM has received speakers fee from GSK, Qiagen, and SPMSD/Merck, and served occasionally on the scientific advisory board (expert meeting) of GSK, Qiagen, and SPMSD/Merck; CJLMM has a very small number of shares of Qiagen and holds minority stock in Self-screen B.V.; CJLMM is part-time director of Self-screen B.V. since September 2017.

Patient consent for publication Not required.

Provenance and peer review Not commissioned; externally peer reviewed.

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