PEER REVIEW HISTORY

BMJ Open publishes all reviews undertaken for accepted manuscripts. Reviewers are asked to complete a checklist review form (**http://bmjopen.bmj.com/site/about/resources/checklist.pdf**) and are provided with free text boxes to elaborate on their assessment. These free text comments are reproduced below.

ARTICLE DETAILS

TITLE (PROVISIONAL)	The 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
AUTHORS	Kremer, Wieke; Berkhof, Johannes; Bleeker, Maaike; Heideman, Daniëlle; van Trommel, Nienke; van Baal, Marchien; Verhoeve, H.R.; Meijer, Chris; Kenter, Gemma

VERSION 1 – REVIEW

REVIEWER	Partha Basu International Agency for Research on Cancer, France
REVIEW RETURNED	23-Feb-2019

GENERAL COMMENTS	The study is addressing an important research question. Well designed with appropriate ethical safeguards. A few comments: 1. It is already known that p16 negative CIN 2 lesions behave more like low grade lesions and have higher regression rate. Information on p16 status at the baseline and exit biopsies will add value 2. The authors have mentioned 'inadequate colposcopy (non-visualisation of TZ)' as an exclusion criteria. As per the IFCPC classification inadequate colposcopy indicates failure of satisfactory visualisation of the TZ due to any reason and the authors are perfectly justified to exclude such cases. The authors have not mentioned if TZ type 3 will be an exclusion criteria or what will be the policy if such a TZ is encountered. In fact, I will be very hesitant to recruit at least a CIN 3 with Type 3 TZ to such follow up study. 3. The younger women (<25 years) have much higher probability of regression. It may be important to stratify the participants by age at least during analysis 4. The authors have not mentioned anything about 'blinding'

REVIEWER	Chi Lam AU YEUNG Instructor The University of Texas MD Anderson Cancer Center USA
REVIEW RETURNED	22-Mar-2019

GENERAL COMMENTS	Is this a planned or ongoing study? The dates of the study is not included in the manuscript. Are 100 women recruited for the study? or will more than 100 women be recruited to start with? Since some of them will be expected to exit the study. This part is not clear in the protocol.

	There is a section for "Strengths and limitations of this study", but limitations of the study was not included/well addressed.

VERSION 1 – AUTHOR RESPONSE

Reviewer 1
The study is addressing an important research question. Well designed with appropriate ethical safeguards. A few comments:
Comment 1:
It is already known that p16 negative CIN 2 lesions behave more like low grade lesions and have higher regression rate. Information on p16 status at the baseline and exit biopsies will add value.

Author response:
It would indeed be very interesting to evaluate p16 immunohistochemical staining in cervical biopsies of study participants at baseline and study exit. In the Netherlands, the use of p16 immunohistochemical staining using the LAST criteria (Darragh, Arch Pathol Lab Med 2012) is current practice and these data are available. Accordingly, p16 negative lesions will not be considered as CIN2, but atypical metaplastic epithelium. An increase in p16 positivity is associated with higher CIN grade and an increase in methylation of host cell genes (Van Zummeren, Modern Pathol 2018).Therefore, we are planning to request all cervical specimens collected at baseline and study exit to perform additional immunohistochemical staining for p16 and other biomarkers to evaluate their potential prognostic value, but since this is not the main study objective and the original histopathological diagnosis will be used to define the primary study endpoint, we did not include this in the study protocol.

Comment 2:
The authors have mentioned 'inadequate colposcopy (non-visualisation of TZ)' as an exclusion criteria. As per the IFCPC classification inadequate colposcopy indicates failure of satisfactory visualisation of the TZ due to any reason and the authors are perfectly justified to exclude such cases. The authors have not mentioned if TZ type 3 will be an exclusion criteria or what will be the policy if such a TZ is encountered. In fact, I will be very hesitant to recruit at least a CIN 3 with Type 3 TZ to such follow up study.
Author response:
If the transformation zone cannot be completely assessed, as is the case in a type 3 transformation zone, these women will not be included. This exclusion criterion has been clarified in the manuscript (page 6, lines 120-122): "…inadequate colposcopy (i.e., transformation zone is not fully visible (type 3 transformation zone according to the International Federation of Cervical Pathology and Colposcopy guidelines (26)))".
Comment 3:
The younger women (<25 years) have much higher probability of regression. It may be important to stratify the participants by age at least during analysis.
Author response:
Age is indeed an important factor that influences the regression probability and will be used to stratify the participants during analysis. However, most women included in the study will be older than 25 years due to the cervical screening programme in The Netherlands. Women are invited for their first screen when they turn 30. Women aged <30 years can be included in the study when they are referred with abnormal cytology, but this group will most likely be smaller.
Comment 4:
The authors have not mentioned anything about 'blinding'.
Author response:

Gynaecologists and study participants have access to cytology and histology results collected during follow-up. These tests are performed during routine diagnostics in the participating clinics. Additional high-risk HPV testing and FAM19A4/miR124-2 methylation analysis is performed blinded to the cytology, HPV and histology results at the main research site (Amsterdam UMC, location VUmc).
The section on Study parameters in the manuscript has been adjusted (page 8, lines 173-174): "…methylation analysis and high-risk HPV testing will be performed blinded to the cytology and histology results from routine clinical diagnostics".

Reviewer 2
Comment 1:
Is this a planned or ongoing study? The dates of the study is not included in the manuscript.
Author response:
The study is ongoing. The starting date of the study has been added to the manuscript (page 6, lines 108-109): "This is an ongoing study multicentre observational longitudinal cohort study with 24 months 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."

Comment 2:
Are 100 women recruited for the study? or will more than 100 women be recruited to start with? Since some of them will be expected to exit the study. This part is not clear in the protocol.
Author response:
In total 100 women will be recruited for the study. We do not expect a high loss to follow-up, as women are actively reminded for their follow-up visits by the research physician. Women who exit the study earlier do to clinical progression will receive excisional treatment and reach the primary study endpoint (non-regression). These women will be included in the analyses. The manuscript has been adjusted to clarify (page 8, line 186): "In total, 100 women will be included in the study…".

Comment 3:
There is a section for "Strengths and limitations of this study", but limitations of the study was not included/well addressed.
Author response:
The maximum bullets allowed for this article type is 5, which allows for only minimal reflection on the strengths and limitations of the study. We have now included the two most important limitations to this study (influence of cervical sampling, i.e. biopsies, on natural history, and the collection of the first study sample after an initial biopsy). We agree that there may be other limitations to this study, but the format of this section does not allow extensive discussion thereof.

VERSION 2 – REVIEW

REVIEWER	Partha Basu International Agency for Research on Cancer, France
REVIEW RETURNED	22-Apr-2019

GENERAL COMMENTS	The authors have reported the protocol of an important study that aims to correlate the methylation analysis outcomes with regression of histopathology proved CIN 2/3 lesions. The protocol has been appropriately designed and described and the ethical

	issues have been adequately addressed. A few suggestions that the authors may consider. 1. The cases with progressive or non-regressing lesions are certainly to be treated by excision. The LEEP/CB histopathology results will be more authenticate final outcome measure. 2. The authors should clearly mention that the non-regressing lesions will be treated at the end of the study 3. Please mention if the women with glandular abnormalities on cytology going to be excluded 4. The histopathology evaluation should be blinded and ideally by an independent panel performed at the conclusion of the study. 5. Please mention the medium that will be used to collect the samples - ? PreservCyt

VERSION 2 – AUTHOR RESPONSE

Response to reviewer's comments

Manuscript ID bmjopen-2019-029017.R1

Reviewer 1

The authors have reported the protocol of an important study that aims to correlate the methylation analysis outcomes with regression of histopathology proved CIN 2/3 lesions. The protocol has been appropriately designed and described and the ethical issues have been adequately addressed. A few suggestions that the authors may consider.

Comment 1:

The cases with progressive or non-regressing lesions are certainly to be treated by excision. The LEEP/CB histopathology results will be more authenticate final outcome measure.

Author response:

We agree with the reviewer that the excision specimens will provide more complete histopathological assessment of the entire lesion and transformation zone. The final outcome measure of this study is regression (in case of CIN1 or less) or non-regression (in case of CIN2 or worse) based on the cervical exit biopsy. If the excision specimen would be used for this final outcome measure instead, this would only result in a different study endpoint, i.e. regression instead of non-regression, in case the histopathological diagnosis of this specimen is CIN1 or less while the cervical biopsy shows a CIN2 or worse. In our opinion, it would not be correct to define such cases as regression, and therefore we decided to use the histopathological diagnosis of the exit biopsy to define the study endpoint.

Comment 2:

The authors should clearly mention that the non-regressing lesions will be treated at the end of the study.

Author response:

Women with non-regressing lesions at the end of the study will indeed receive treatment according to regular care. This is stated in the manuscript on page 7, lines 147-149: "All participants with a CIN2 or worse at this last study visit will receive treatment according to regular care."

Comment 3:

Please mention if the women with glandular abnormalities on cytology going to be excluded.

Author response:

Women with glandular lesions on cytology may be included in the study. Women with adenocarcinoma in situ (AIS) on a cervical biopsy will not be included in the study and study participants who are diagnosed with AIS on histology during the study will receive appropriate treatment and exit the study protocol. This information has now been added to the study protocol (page 6, lines 119-120; page 7, lines 154-155).

Comment 4:

The histopathology evaluation should be blinded and ideally by an independent panel performed at the conclusion of the study.

Author response:

Histopathological evaluation of all cervical tissue samples collected during the study will be performed by local pathologists, blinded of the methylation results, and within routine diagnostics. These diagnoses will be used for the primary study endpoints.

We have clarified the blinding of the histopathological assessment in the manuscript (page 8, line 164-165).

Comment 5:

Please mention the medium that will be used to collect the samples - ? PreservCyt

Author response:

Cervical scrapes and self-collected cervicovaginal cells collected during the study will be collected in ThinPrep PreservCyt® Solution (Hologic Inc., Marlborough, Massachusetts, USA). We have now added this information to the manuscript (page 8, lines 172-174).