Author’s response to reviews

Title: Cervical microbiota in women with cervical intra-epithelial neoplasia, prior to and after local excisional treatment, a Norwegian cohort study.

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Author’s response to reviews:

Dear Prof. Echo L. Warner,

Thank you for considering our paper "Cervical microbiota in women with cervical intra-epithelial neoplasia, prior to and after local excisional treatment" (BMWH-D-18-00449) for publication in BMC Women's Health.

We wish to thank Yi-Chang Su and James Malone-Lee for their valuable comments on our manuscript.

We have modified the manuscript and incorporated the suggested improvements. We believe that the paper has become more focused, better structured and easier to read thanks to the reviewers suggestions. Please, see the changes highlighted in the enclosed manuscript. A detailed response to all comments follows below.
Editorial comments and our response:

Reviewer 1, Yi-Chang Su:

Major Essential Revisions:

This prospective observational study aimed to compare the cervical microbiota in women with CIN before and after LEEP and to assess whether cone depth and/or HPV persistence affects bacteria composition post-treatment. This research topic is interesting, and Zhang et al. had reported the same topic with the opposite result this March. (Zhang H, Lu J, Lu Y, Cai Q, Liu H, Xu C. Cervical microbiome is altered in cervical intraepithelial neoplasia after loop electrosurgical excision procedure in china. Sci Rep. 2018 Mar 21;8(1):4923. doi: 10.1038/s41598-018-23389-0) Though the sample size of this study is larger than Zhang's, there are several obvious flaws in the study design, which makes the results not solid and convincing. There are also some inadequate assumptions and inferences in the manuscript. The authors are suggested to revise and add more details about the participants, and analyze the effects of the related factors that may influence the cervical microbiota.

Thank you for directing our attention to the article by Zhang H et al., which was not published when this study was performed. We do not find that Zhang et al report opposite results compared to our study, - both studies found a less diverse microbiota after LEEP.

We have added a paragraph comparing our results to the results of the Zhang study in the discussion section, page 15 line 7-14.

1) Since the cervical microbiota is the most important variables observed in this study, and the microbiota variety is closed related to the menopausal status, use of hormone replacement therapy, the time of menstruation, menstrual hygiene practices, use of vaginal douching, sexual behaviors, use of hormonal or barrier contraceptive products, numbers of sexual partners, history of recent sexual activity and antibiotics/probiotics (oral or topical) for other diseases; the authors should record and analyze the effects of these related factors of each participants instead stating these may impact the results as the study limitations.

Thank you for pointing out this important issue. We have included further data on the study population´s characteristics and conducted additional analysis as far as possible.

Menopausal status: Unfortunately, we do not have data on menopausal status. However, we have performed a subanalysis for women under 46 years (assumed premenopausal). Only 14 of the 89 women in the LEEP group were 46 years or more. In the follow up analysis each woman is her own reference. We have now added analyses that compare the change in mean number of non-Lactobacillus after LEEP between women below 46 years of age to women 46 years and above -
and we found no significant difference (page 11, line 7-10). When comparing the results to the reference group we adjusted for age groups (<46-assumed premenopausal, 46-55-assumed perimenopausal, >55 years assumed postmenopausal).

Use of hormonal or barrier contraceptive products and use of hormone replacement therapy:

We have included information about hormonal contraceptives and barrier contraceptives at the first visit in Table 1. Page 9-10. While we in the first version of the manuscript presented a category of “other contraceptive”, we now present further categories of hormonal injection, vaginal hormonal ring and sterilisation to provide the reader with more detailed information about the participants contraceptive use. None of the women reported a change of contraceptive use at follow up. This information is now included in the paper, page 9, line 2-3. Unfortunately, information about local or systemic hormonal therapy in the postmenopausal women was not registered in the study other than three of the 14 women over 46 years in the LEEP group having a hormonal IUD.

In the follow up analysis each woman is her own reference. We have now added analyses that compare the change in mean number of non-Lactobacillus after LEEP between women with hormonal contraceptive and women without hormonal contraceptive and we found no significant difference, page 11, line 7-10.

When comparing the LEEP group to the control group, adjustment for hormonal contraceptives/therapy did not alter the results.

The time of menstruation: Unfortunately, the date of the last menstrual period was not registered. However, no samples were taken during menstruation since women were told not to come for examination during menstruation, and this information has been included in the manuscript, page 17, line 14-15.

Menstrual hygiene practices and use of vaginal douching: This information has not been registered but vaginal douching is very uncommon in Norway. In the comparison of before and after LEEP status, each woman is her own reference.

Sexual behaviour and numbers of sexual partners; The only variable registered for the study participants was marital status, We have now added analyses that compare the change in mean number of non-Lactobacillus after LEEP between married/cohabiting women and single women showing no significant difference, page 11, line 7-10.

History of recent sexual activity: Unfortunately, these data are not available for the study population.

Antibiotics/probiotics (oral or topical): All women on long term antibiotics were excluded and women treated with antibiotics after their inclusion were excluded in the follow up analysis.
We agree with the reviewer that it would have been optimal to have all of these background data registered. However, in the analysis comparing before and after LEEP status each woman is her own reference and this decreases the risk for bias by any background factors. We clearly state these limitations in the discussion to give the reader the possibility to interpret the results cautiously.

Even with the limitations in study design we believe it is valuable to present our results to the scientific community. We think it is interesting that we found a less diverse microbiota and a tendency towards an increase of Lactobacillus after LEEP confirming the findings by Zhang et al – to the best of our knowledge the only study published comparing microbiota before and after LEEP so far. Zhang et al did however not present data on postmenopausal hormone replacement therapy, exact time of last menstrual period, menstrual hygiene nor number of sexual partners either, similar to our study. We believe that our results, together with Zhang’s, will add further support and valuable information for future studies to be performed in this field.

2) The authors should mention the time of the sample collection. If the participants had menstruation or sexual activity in the previous days which may influence the cervical microbiota.

Thank you, we agree. See our answer under 1: Unfortunately the exact date for the last menstrual period or sexual activity was not registered. However, no samples were collected at time of menstruation.

3) The participants in LEEP group were younger and more of them were single, whether their sexual behavior patterns and contraceptive use have changed after the diagnosis of CIN and receiving LEEP were not followed and recorded. Without the above information, the results are not convincing

Thank you for pointing out the importance of the womens’ sexual behaviour, please see even our answer under 1. No women reported changed contraceptive use.

Women were not asked for number and possible changes of sexual partners, only marital status was registered.

Every woman in the LEEP group was her own reference/control decreasing the possible bias by changed sexual behaviour. We agree though that exact information on sexual activity would have been a strength. However, some women might have withdrawn participation as this type of information might be seen as very sensitive.
4) The discussion about the risk of preterm delivery (PTD) is not reasonable. PTD may result from other factors and pregnant women were excluded in the follow-up analysis. Meta-analyses published a few years ago in the Lancet and the BMJ showed an increased risk of adverse obstetric outcomes after treatment for CIN. The cause remains unclear, and potential mechanisms include anatomical changes, cicatrisation of the cervix, immunological factors, and alterations of the cervicovaginal flora.

Thank you, we totally agree that the pathophysiology for the increased PTD risk after LEEP is still poorly understood and could be due to anatomical changes, cicatrisation of the cervix, immunological factors, and alterations of the cervicovaginal flora. We clearly state in the paper that the underlying mechanism of how excisional treatment increases the risk of PTD remains unclear and that lack of mechanical support may be one explanation, page 4, line 8-11 and page 16, line 6-8. However, a changed genital microbiota after LEEP could be one further explanation that has not been studied in detail yet. Zhang et al is the only study published on this topic thus far. Even if we are studying a non pregnant population, we think it is interesting to discuss our findings in the microbiota in regard to PTD risk. We want to argue for the importance of more studies in this field including pregnant women in the future, page 18, line 10-11.

As the author said in the article: "The results were not significant possibly due to small sample size."(P.15, line12)

Thank you for addressing the topic of power. When this study was planned no other study on the effect of LEEP on the cervical microbiota had been published, therefore it was impossible to calculate a solid power estimation. See also the answer to Reviewer 2, under 7.

For specific bacterial species the material was small and we have deleted the analysis of individual bacterial species in relation to HPV status and cone depth from this manuscript. In this revised version we have tried to focus more on the number of non-Lactobacillus than specific species, this also after suggestions from Reviewer 2. We mention the sample size in the manuscript as a limitation of the study for the reader to have that in mind when interpreting the findings. We believe that the results of this study will help other researchers when planning future studies in this field.

5) In addition, the study couldn't control the interference factors, so the authors couldn't determine the reason why the study result was different from the previous research results, and couldn't explained the relation between LEEP therapy, microbial colonization and preterm delivery.

The earlier mentioned study on microbiota after LEEP - Svare et al, 1992, studied pregnant women between 26 and 34 weeks of gestational length. Lactobacillus was cultured in only 2/11 of women with earlier conization and 74/135 without earlier conization (p<0.05). We found this
study too different from our study to discuss it in relation to our results. We merely mentioned it to point out the shortage of studies on this subject. Since the article by Svare et al is old (other conization methods were used during this period), is written in Danish and we don’t discuss it further we have now decided to remove it from the article. We have added a larger more recent study in pregnant women (Stout et al, 2015) and the article by Zhang et al. Now we focus on discussing our results in relation to the article by Zhang et al which was not published when writing the first draft of this manuscript.

Our results confirm the results of Zhang et al as we found less non-Lactobacillus bacteria and a tendency towards the presence of more Lactobacillus after LEEP page 15, line 7-14.

Our intention was not to explain the relationship between LEEP therapy, microbial colonization and PTD since we are studying a non pregnant population. Although, we think it is interesting to discuss our findings in the microbiota with PTD in mind since LEEP is a known risk factor for PTD. Studies of the microbiota before and after LEEP has not been done in a pregnant population. Please see even our answer under 4).

6) Other minor issues:

1. The Table 1 should be moved to the "Results."

We agree and table 1 has been moved accordingly.

7) 2. In Table 4: the authors should explain in more detail about why authors group the women with the bacterial findings "Pos-pos, Neg-neg, Neg->pos" into one category.

Thank you for pointing out that these analyses would need further description. Considering even the comments of Reviewer 2, we decided to omit these analyses from the paper, please see our answer to Reviewer 2, under R2-10.

Reviewer 2, James Malone-Lee

Major Essential Revision

1) Page 8 Line 26/27 "included" should be included

Thank you, we have changed accordingly.
2) Page 8 Lines 24 to 34

I am not too keen of derived variables or categories, as in this case, because the involve the risk of additional error. They are dealing with biological continua that are not really suitable to categorisation and the categories are inevitably arbitrary.

Thank you, we agree and have omitted the category “Disturbed Microbiota”. However, we have kept the category “any non-Lactobacillus” for some of the analyses, which we do not think is arbitrary. Since the material is too small to study some of the individual bacteria species we want to keep this category. It also shows that in some of the women we couldn´t detect any non-Lactobacillus. However, the category is now excluded from the analyses before and after LEEP with McNemar test

Page 9

Statistical tests

3) I do not think that they should be using paired t-tests for the between group analyses.

This must be a misunderstanding. For the in the between group analyses we did not use paired t-tests but

- independent t-test for continuous variables and

- Fishers exact t-test for categorial variables.

4) I doubt that the data are compliant with the parametric criteria so it would be better for them to stick to non-parametric analysis typically Kruskall Wallis for three group comparison and Mann-Whitney U test for two groups and the Wilcoxon signed-rank test for paired samples.

We would like to keep the parametric analyses. No real life variables are perfectly normally distributed. The mean will however be approximately normally distributed also for moderate sample sizes like in this study. The nonparametric tests suggested are less powerful and their main merit is robustness against severe outliers, which we do not have.

5) "We analysed if women that underwent treatment, more often aquired bacteria than lost earlier detected bacteria." This is difficult to understand.
Thank you for pointing out the need of further clarification. We focused on an eventual change in the detection of different bacteria species after LEEP in the individual. We compared the number of women that lost a bacteria species they had before LEEP to the number of women that acquired that bacteria species after LEEP. We did not focus on those women that either kept earlier detected bacteria or did not have bacteria at all, neither before nor after LEEP. We believe this is a more interesting analyses than merely comparing findings at a group level before and after LEEP. We have provided a more detailed explanation of the statistical procedure in the Methods section page 7, line 14-20.

6) I think that the statistics are overdone. There is no point in analysing quantitative differences in species before and after because they are not using reliable quantitative methods. The numbers are also too small for the statistical analysis of the dichotomies presence or absence of individual species. Thus they are limited to analysing the non-lactobacilli species dispersion - How many different species before and after? - it is as simple as that. Then how many had lactobacilli before and after?

Thank you for this comment. We want to clarify that we did not analyse quantitative differences of individual bacterial species but only the presence or not of different bacteria species. See method section page 6, line 5-7. “Growth results were reported as growth or no growth”. We do agree that the material is too small to study some of the individual bacteria species which is why we described the category “any non–Lactobacillus”.

The subanalysis for HPV status and cone depth for different bacterial species was omitted. In accordance with your suggestion we focused on the mean number of non-Lactobacillus and on Lactobacillus.

7) The trouble with using these repeated statistical tests is that they run into troubles with multiplicity and a statistical nightmare. Simplicity is a much better option.

They do not have the statistical power to analyse a subgroup (Aged <46) and should probably limit their contribution to no apparent difference in this small sample.

Thank you. We agree that Mc Nemar test requires at least 6 occurrences to detect a difference but for descriptive purposes we keep the results of different bacterial species. Since age (menopausal status) is an important background factor we think it is important to do this subgroup analysis. The importance to study background factors has also been pointed out by Reviewer 1, see under R1-1. However, following your advice we have now decided not to show the results of women under 46 for specific bacterial species in the comparisons before and after LEEP, page 12, line 5-7.
Results

8) "The bacteria composition in the LEEP group before and after LEEP as well as in the reference group are described in Supplementary Table 1"

It would make much more sense of have a table describing the species as, number of isolates and % of all isolates for each assessment and for the healthy controls. There is not need for all of the complicated sub-group analysis and categorisation it is too confusing.

The comparison before and after LEEP should stick to differences in lactobacilli isolation and non-lactobacilli dispersion (number of different species). There is not point in going on about the presence of individual microbial species because it does not have any known significance.

Thank you for this suggestion. We have moved Supplementary table 1 to the results section instead, becoming Table 2. By doing so we can show the reader which species that are included in the non-Lactobacillus category.

We think it is a good suggestion to study the number of non-Lactobacillus instead of focusing too much on different species. Even so, different non-Lactobacillus species are associated with different problems, - for example M. hominis, Gardnerella vaginalis and Bacteroides and U Parvum seem to be associated with preterm birth (see under Discussion) and the anaerobic bacteria Gardnerella vaginalis is associated with Bacterial Vaginosis. Therefore we still think it is interesting for descriptive purposes to present some of the results for specific bacterial species.

We have now omitted the subanalysis of women under 46 years from the supporting data. According to your suggestion, we removed the analyses of specific bacterial species in relation to HPV and cone depth and focus on the number of non-Lactobacillus instead.

9) "We found no difference in the detection of bacterial species or the occurrence of the constructed broader groups according to HPV DNA positivity after LEEP, neither when analysing all women at six and 12 months (Table 3) nor just women positive for bacteria before treatment "

Adding in these subgroup analyses was not part of the deal and the underlined bit should go.

There was no difference between the HPV DNA group - end of story

Thank you for this suggestion. Following your recommendation, we have omitted the analysis of HPV and specific non-Lactobacillus bacterial species.

10) I think that the cone depth analysis goes too far into another sub group analysis:
"We found no association between the microbiota changes after LEEP and the cone depth" Say no more the subgroup analysis is not statistically sound.

Thank you. We agree and have removed table 4 (Cone depth and change of bacteria after LEEP). Instead we studied the correlation between cone depth and non-Lactobacillus in general, see page 13, line 6-10.

11) "Comparisons of microbiota in women planned for treatment versus healthy references"

This section should also be simplified - There was a greater non-lactobacilli species dispersion in the patients compared to the controls. That is it and it is an important finding because it is commensurate with data from other groups reporting on UTI - Disease is associated with increased species dispersion.[1-3]

We agree and focus now on the difference in mean number of non-Lactobacillus between the groups instead of the individual bacteria species, page 13, line 14-20 and page 14, line 9-14.

We hope that you agree that the manuscript has improved and will find it suitable for publication in BMC Womens Health. We look forward to your response.

On behalf of all the authors,

Sincerely

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