Author’s response to reviews

Title: Association between cigarette smoking and the vaginal microbiota: A Pilot study

Authors:

Rebecca M Brotman (rbrotman@som.umaryland.edu)
Xin He (xinhe@umd.edu)
Pawel Gajer (pgajer@som.umaryland.edu)
Doug Fadrosh (dougfadrosh@gmail.com)
Eva Sharma (EvaSharma@westat.com)
Emmanuel F Mongodin (EMongodin@som.umaryland.edu)
Jacques Ravel (jravel@som.umaryland.edu)
Elbert D Glover (eglover1@umd.edu)
Jessica M Rath (jrath@legacyforhealth.org)

Version: 6 Date: 27 July 2014

Author’s response to reviews: see over
We thank the reviewers for their helpful comments. Reviewer comments are in plain text and responses to reviewer comments are in bold below.

REVIEWER #1

This manuscript introduces itself to shed more light on two important issues regarding the vaginal bacterial ecosystem: i) how closely is smoking related to community structure and ii) how valid is the theory that bacteriophage induction could disrupt a protective bacterial environment, potentially leading to conditions such as Bacterial Vaginosis. While this paper gives the first pilot data examining the changes smoking cessation has upon the vaginal bacterial community, the data is perhaps too limited and biased to address Phase A, and only gives a single case that shows a net positive effect from smoking cessation in Phase B, and indeed brings to question whether smoking is a confounding factor in previous studies.

Major points:
1. Although the authors did state that the smokers in this pilot study were more sexually active and reported a greater frequency of douching than non-smokers and took this into account, the small sample sizes are worrisome for the conclusions made, in particular when oral contraception usage was biased towards the non-smoking group. It is difficult to tell whether smoking was responsible for the higher low-Lactobacillus community structures or whether oral contraception was promoting a L. crispatus-dominated environment (and perhaps this paper should be based on this). Therefore for Phase A, albeit interesting, cannot elude to smoking being responsible for a change in community structure, and may simply be a confounding factor.

   Phase A is a cross-sectional study and therefore, it does have all the inherent biases associated with observational research. However, we were comprehensive in our behavioral and demographic surveys and did construct a multivariable model to control for important confounders as best as we could given the sample size. Table 1 lists factors that were different between smokers and non-smokers. We did make a comment about our inability to control for hormonal contraception in the discussion.

   It should also be noted that other papers have similarly found a relationship between smoking and BV and some have also reported a dose-response relationship.

   Ultimately, we hope our future studies will be able to quantify the causal relationship between smoking and BV. The current effort was designed to collect preliminary data on the association between the vaginal microbiome and smoking and also to collect pilot data on response of the vaginal microbiome following smoking cessation.

2. Phase B is potentially quite informative as a methods-based paper and perhaps should be made the main focus of this paper, however will be limited based on descriptive data detailing one possible case of a response due to smoking cessation.
We are unclear regarding what the reviewer is trying to communicate to us. Is the reviewer requesting that we detail sample size or statistical modeling of the longitudinal cessation study? We did not conduct formal statistics on Phase B because the sample size was too limited. The data for Phase B are reflected in Figure 2. The purpose of this manuscript was to provide preliminary data on the association between smoking and BV. The pilot data are useful for launching future studies on this topic.

3. I am finding it difficult to see how the discussion leads with the data showing the effect smoking has on the vaginal bacterial environment when those followed in Phase B comprise of each of the described community types. Given the potential longitudinal variability of the vaginal bacterial community, would these cases be possible in those who did not smoke?

Again, we are unclear on what the reviewer is trying to ask in this comment. The vaginal microbiome was evaluated daily in ID#1 and approximately twice-weekly in ID #2, ID #3 and ID#6. The vaginal microbiome does fluctuate (see Gajer and Brotman, *Sci Trans Med* 2012) and therefore it is important to sample frequently. We hypothesize that if smoking does affect the vaginal microbome, overall a woman’s microbiome would transition from a largely *Lactobacillus*-deficient (CST IV) state to a *Lactobacillus*-dominated state over time with fewer and fewer emergences of CST IV. We are in the process of planning future studies which will recruit large numbers of women to a smoking cessation trial, however, this preliminary pilot work was necessary to establish proof of principle and ability to conduct the studies.

4. Do the bacterial genera Peptostreptococcus and Veillonella remain associated with smoking taking into account all factors including contraception usage?

   The *CART* analysis does not take into account confounding factors.

Minor points:

5. Although phage induction is a plausible theory in the development of conditions such as Bacterial Vaginosis in certain cases, this paper does not directly address this, and perhaps should only be commented on in the discussion, in relation to the limited work that has been carried out in this area, and the need for further research. Indeed the perhaps “narrow” focus on bacteria should be changed to acknowledge other microbial elements such as bacteriophage that are most likely very much involved in the vaginal microbial environment in both health and disease states.

   The mention of phage induction in the introduction was to introduce the reader to the biological plausibility of our hypothesis.

6. When describing Nugent scoring, it is perhaps time to start including other bacterial types more readily identified in high Nugent scoring samples with morphologies similar to Mobiluncus spp.

   We do mention other bacterial types in our description of Nugent scoring:
The Nugent score reflects the relative abundance of large Gram-positive rods (lactobacilli), Gram-negative and Gram-variable rods and cocci (i.e., *G. vaginalis*, *Prevotella*, *Porphyromonas*, and peptostreptococci), and curved Gram-negative rods (i.e., *Mobiluncus*).

**REVIEWER #2**

**Strengths:**
The paper has a number of strengths in that it provides pilot data for looking at the effects of smoking on the vaginal microbiota and demonstrates in a very small number that smoking cessation may lead to a reverse of this. Although this is a pilot study of a relatively small group of women, the authors describe in great detail the methodologies and outcomes for each individual participant.

**Revisions:**
The authors may wish to consider some of the following points:

Comment 1: Methods pg 7, ln 138
The authors describe that the region which they applied pyrosequencing to was the V1-V3 hypervariable region that has been previously described. As this results in a long PCR product, is it possible that the PCR was not as efficient as it may be if the region was shorter? Furthermore, are there any known primer biases using this particular set – i.e. could some bacteria be over/under represented?

Primer bias is universal and there is no perfect primer. However, the amplicon is representative and the coverage is comprehensive enough to give a fair view of the community. The 27F* and 534R 16S PCR primer set is specially formulated to eliminate known biases associated with primer 27F and has been validated previously [See Reference 45 -- Frank JA, Reich CI, Sharma S, Weisbaum JS, Wilson BA, Olsen GJ: Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Appl Environ Microbiol* 2008, 74(8):2461-2470]. As a result, *Gardnerella vaginalis* is well-represented as well as all known vaginal *Lactobacillus* species.

**Minor revisions:**
1: Introduction pg 3, ln 42
Before introducing the intervention of smoking cessation, further explanation of other interventions and their inability to decrease the risk of BV recurrence may strengthen the argument that smoking cessation is an important intervention option.

**We have added a few sentences to the introduction.**

Conventional therapy consists of nitroimidazoles or clindamycin administered orally or topically.[15] Unfortunately, BV can be highly recurrent [18] with over 50% of women experiencing a symptomatic relapse within 3-12 months following antibiotic therapy.[19] An unexplored intervention for BV is smoking cessation.
2: Methods Pg 5, In 94-97
Please consider revising the sentence describing the additional criteria, which was slightly confusing.

We have clarified the exclusion criteria in the revised manuscript.

3: Results Pg 9, In 180
The authors could consider expanding the description of the distribution of all CST groups and their differences in smokers vs non-smokers (i.e. fewer smokers had CSTI dominated flora).

We have made this edit. The following text was added: Similarly, fewer smokers had a *L. crispatus*-dominated CST I (30% in smokers versus 65% in non-smokers).

4: Results Pg 9, In 183
Although smokers were significantly more likely to have increased Nugent Scores compared with non-smokers, their vaginal pH was not statistically different to non-smokers so the authors should consider reporting specific percentage values where differences exist and state that these were not significant.

The pH data is listed in Table 1 with the statistical test results and we believe this is sufficient. If the editor would like us to expand the pH discussion, additional text could be added.

5: Results Pg 9, In 187
The authors could include results on the differences in number of sexual partners between smokers and non-smokers considering they then adjust for this variable in the multivariate analysis. Number of lifetime partners has often been associated with increased risk of BV so this is potentially an important observation.

We have made these edits. We added a sentence on the differences in self-reported sexual exposures between smokers and non-smokers and also clarified that we adjusted for these variables because they were associated with BV status and smoking status.

6: Discussion, Pg 11, In 228-231
To help distinguish between the initial pilot and the smoking cessation pilot, consider re-wording the second sentence in the Discussion.

This has been clarified. The sentence now reads: The cross-sectional study (Phase A) suggests that women who smoke cigarettes are significantly more likely to have a vaginal microbiota characterized by low proportions of *Lactobacillus* spp.

7: Discussion Pg 12, In 237-239
It is a little unclear in this sentence what the authors mean about heterogeneity and if it is in any way related to the increased risk to STIs and/or BV.
This paragraph has been edited for clarity.

8: Discussion Pg 12, ln 248
Consider using the word ‘future’ study instead of ‘a’ study.

This edit has been made.

9: Discussion, Pg 13, ln 258
The authors comment that racial and ethnic differences may affect biomarkers of smoking exposure. How do their pilot data relate to this comment?

The self-reported data are well correlated with the biomarker data in this study.

10: Discussion, Pg 13, ln 270
The authors correctly state that smokers may have reduced usage of hormonal contraception due to WHO guidelines in which it is recommended that women who smoke and are >35 are not prescribed the OCP. In this pilot study however, the smokers were also more likely to be >40 and therefore are less likely to use the pill anyway if they are peri-menopausal. The relevance of the overlap of these findings could be discussed further.

We agree with the reviewer and have added this statement: Further, smokers tended to be older (over age 40 and possibly peri-menopausal) and therefore less likely to use HCs.

Similarly, reduced HC-use in smokers may also be attributed to a higher rate of Nugent scores indicative of BV described in line 274. Further discussion could be included.

We agree as well with this statement and have clarified the line: Another important factor which may be driving the inverse association between Lactobacillus-dominated CSTs (and also Nugent scores) with smoking status is hormonal contraception (HC).

11: Table 1
Please consider including the statistical test used in the footnote as well as bolding significant values.

P-values were determined by Fisher’s exact test. This has been added to the footnote of the table. It does not appear that bolding significant findings in the table is the Journal’s style but we would be happy to make that change if the editor requests.

12: Table 2
Please consider including the number of women who fell into each variable category and rearrange so that the 95% confidence intervals are reported before the p-values. The authors could also include the statistical test used and bold the significant p-values in the footnote.
The number of women in each category is listed in Table 1. We have re-arranged the table so that the 95% CI are reported before the p-values. P-values were not bolded as in the response above.