Peer Review File

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Reviewer A

The paper titled “The Role of Vaginal Microbiome in Distinguishing Female Chronic Pelvic Pain due to Endometriosis/Adenomyosis” is interesting. Vaginal microbiome from EM/AM patients has significantly higher alpha (phylogenetic) diversity than the other two groups, and higher counts of Clostridium_butyricum, Clostridium_disporicum, Alloscardovia_omnicolens, and Veillonella_montpellierensis resulting in predicted perturbations of functional pathways which could suggest metabolite-specific targeted treatment. The combination of vaginal biomarkers and serum CA125 may provide an original method to differentiate EM/AM-associated-CPP. However, there are several minor issues that if addressed would significantly improve the manuscript.

Comment 1: If women without chronic pelvic pain have inflammation such as vaginitis, will it affect the results? How to exclude the interference and influence of other factors on the flora?

Reply 1: Thanks for your question. All the enrolled patients may suffer from some of the most common vaginal dysbacteriosis, including Bacterial Vaginosis, Candida vaginitis, Trichomonal vaginitis. As this may affect the results, we excluded the patients with acute genital tract inflammation. This has been stated in the part of materials (see Page 8, Line 306-307). In order to eliminate the influence of other factors, all the patients enrolled had been matched, and those with certain disease were also excluded. The enrolled patients for the three groups were age-matched, and there was no significant differencein the three groups referring to the parity, gravidity, phase of menstrual and method of contraception. Besides, postmenopausal women, and those with autoimmune disorders or diabetes mellitus were excluded (see Page 8, Line 307-

308).

Comment 2: Which one occurs first, chronic pelvic pain or changes in vaginal microbiota? What are the different results in the order of occurrence? Please consult the literature and answer this question.

Reply 2: This study is a cross-section observational study, which tries to reveal the association between vaginal microbiota and CPP. Cause and effect cannot be discerned from the association yet (See in Page 19, Line 831-833). Studies on the association between CPP and vaginal microbiome are scarce, and most related papers were retrospective. As far as we know, the sequence of the occurrence of CPP and the changes in vaginal microbiome has not been clarified yet. To figure out this question, prospective research should be performed in future study under strict inclusion criteria and standardized protocol. There are high-quality papers published on nature communications, which revealed that the vaginal or cervical microbiota might be useful for the detection of common disease in the upper reproductive tract. [Cited: Chen C, Song X, Wei W, Zhong H, Dai J, Lan Z, Li F, Yu X, Feng Q, Wang Z et al: The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. Nat Commun 2017, 8(1):875.]

Comment 3: In this study, better analysis software should be used to analyze the flora data, and it may be more meaningful to add analysis of functions and signal pathways.

Reply 3: We would like to extend our profound gratitude for your invaluable suggestion. We have adopted a variety of analysis methods to explore the potential microbiome biomarkers, including Alpha Diversity, LEfSe analysis, T-test analysis, Metastat analysis and Beta Diversity. Furthermore, PiCRUSt was used to analyze the function of the microbiome. As aonther part of study, in our following consecutive research, we have performed metabonomics analysis combined with sequencing of barcoded 16S rRNA V4 gene fragments to better reveal the functions and signal pathways.

Comment 4: What is the significance of the differentially enriched taxa of potential

vaginal microbiome biomarkers identified in this study for the differential diagnosis or treatment of gynecological diseases?

Reply 4: Thanks for your question. As can be seen in this manuscript, *Clostridium_butyricum*, *Clostridium_disporicum*, *Alloscardovia_omnicolens*, and *Veillonella_montpellierensis* are being isolated in high numbers. *Clostridium_disporicum*, a saccharolytic species, was known as an ursodeoxicholic acid producer, yet the functional reports of this opportunistic bacterium are limited. *C. disporicum* is a gram positive obligately anaerobic bacillus which can contain two subterminal spores. Isolation of *C. disporicum* in human specimens is extremely poorly reported, it was previously isolated from a bacteremia patient following a ring pessary insertion for uterine prolapse in a 75-year-old diabetic female. Then the first case of intra-abdominal infection caused by *C. disporicum* was reported in 2013 (See Page 17 Line 700 to Page 18 Line 778). Meanwhile, the remaining three taxa were scarcely reported. Thus, the function of these discrepancy species should be further investigated in future study combing the metabonomics analysis.

Comment 5: If chronic pelvic pain is caused by the microbiome of neighboring organs, how to discuss their relationship with CPPS patients?

Reply 5: It is becoming increasingly clear that there is an interplay and symbiotic relationship between our bodies and microorganisms. The relationship has been widely investigated and preliminary results have been achieved. Gut microbiome dysbiosis has been discovered in a variety of diseases and conditions. Urine microbiota or expressed prostatic secretion microbiome is associated with chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS). The aim of our study was to reveal the inherent relationship between vaginal microbiome and CPP, while the relationship between CPP and microbiome of neighboring organs should be further investigated in the future (See in Page 20, Line 874-877).

Comment 6: What are the characteristics of chronic pelvic pain caused by endometriosis? What is the best treatment currently? What is the correlation between

pelvic adhesions and pain in patients with endometriosis?

Reply 6: CPP is defined as an intermittent or continuous pain in the lower abdomen or pelvis of at least 6 months in duration and is associated with negative cognitive, behavioral, sexual and emotional consequences (See Page 5 Line 117-120). And the CPP caused by endometriosis is not so specific from the symptoms alone, and this is the aim of our study, to investigate the potential microbiota biomarker to distinct EM/AM-associated CPP from other types of CPP. Only an accurate diagnosis can lead to better treatment. Once endometriosis-associated CPP is considered, individualized treatment should be made. Continual or periodical Compound oral contraceptives can be prescribed to young patients without fertility requirements temporarily, while progesterone can be prescribed for those young patients with a plan of fertility. What's more, pain killer, GnRHa, physiotherapy and some other treatment regimens are also available. Surgery should be considered as well when drug treatment is proved to be ineffective. The adhesions caused by endometriosis may be one of the multiple influencing factors, while the relationship is not so definite and there is no direct proportion.

Comment 7: In this study, only 37 patients were enrolled in EM/AM-associated-CPP, 25 patients were enrolled in CPPS without EM/AM, and 66 women without CPPS. The samples were too small. How to handle with the limitation. Such limitations should be addressed in the discussion.

Reply 7: The participants from the three groups were age-matched, and there were no significant difference referring to their parity, gravidity, phase of menstrual and method of contraception (See in Page 9, Line 349-354). Our study is also limited within a rather small sample size in spite of the long timespan and the equivalent result in the validation trial. The patients were strictly screened and samples were collected under consistent conditions. Even with a small sample size, we were still able to identify significant microbiome differences within the three groups and identify differential abundant taxa of potential vaginal microbiome biomarkers in the differential diagnosis of CPPS. A large sample study may be needed to disentangle these confounding effects with

confidence. Extending this study to a larger number of patients will allow for further verification of the findings and increase the statistical power. (See in Page 19, Line 836-874).

Comment 8: Does gonadotropin releasing hormone agonist affect the microorganisms in patients with endometriosis and adenomyosis? If so, what impact will it have?
Reply 8: Our samples were collected before treatments including drug use and surgery. Thus, the data analysis was not affected by the usage of GnRHa. We didn't explore the effect of GnRHa on vaginal microbiome, and this can be explored in future research.

Reviewer B

Interesting manuscript, well written and informative. My only 2 comments.

Comment 1: How was the diagnosis od Adenomyosis made?
Reply 1: Patients with CPPS confirmed with EM/AM by exploratory laparoscopy or surgical pathology were defined as group A (Page 7, Line 264-265). And all patients with a diagnosis of adenomyosis were confirmed by the surgical pathology, including biopsy and partial resection of the lesion.

Comment 2: Why was Ca125 used as a marker of Chronic Pelvic pain.
Reply 2: Our previous study revealed that an elevation of preoperative CA125 was found in 72.4% of those adenomyosis patients with or without coexisting endometriosis [Cited: Li YW, Liu YT, Wang S, Shi HH, Fan QB, Zhu L, Leng JH, Sun DW, Sun J, Lang JH: Clinical Manifestations of Adenomyosis Patients with or without Coexisting Endometriosis. Chin Med J (Engl) 2018, 131(20):2495-2498]. Thus, we adopt the level of serum CA125 as a combination biomarker to differentiate EM/AM-associated CPP from other types of CPP.