Peer Review File

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Reviewer A

Comment 1. Some of the rationale for using the main proteins must come in introduction for readers.

Reply 1: The three target proteins, HSP10, ZC3H8, and UNC45A come through an antibody array (AAH-BLM-2000B, RayBiotec Inc., Norcross, GA, USA) and confirmative evaluation by Enzyme-linked immunosorbent assays (ELISAs).

Changes in the text: In this study, we used an antibody array as a screening tool and identified three proteins HSP10, ZC3H8, and UNC45A, that exhibited significantly different levels when compared between patients with POP and healthy controls.

Comment 2. Authors must share results of other proteins which were evaluated but not significant. Presenting all research data is very critical to scientific community.

Reply 2: Thanks for your suggestion. The result of the antibody array (AAH-BLM-2000B) for all proteins is presented as Figure 1-reply to reviewer. The L-Series human antibody array 2000 membrane kit, which is a combination of human L507, L-493, L-3 and L-4 arrays, used in this study could simultaneously detect 2000 cytokines and related proteins in a given sample. The results were semi-quantitative when assessed by the density of each analyte spot. Multiple analytes were detected positively in the mixed sample created from each group. Among the positive analytes, the plasma levels of HSP10, ZC3H8, UTRN, UNC45A, and UNC5H4, were significantly decreased in the samples from patients with POP through visual observation and the program named RayBio Analysis Tool.

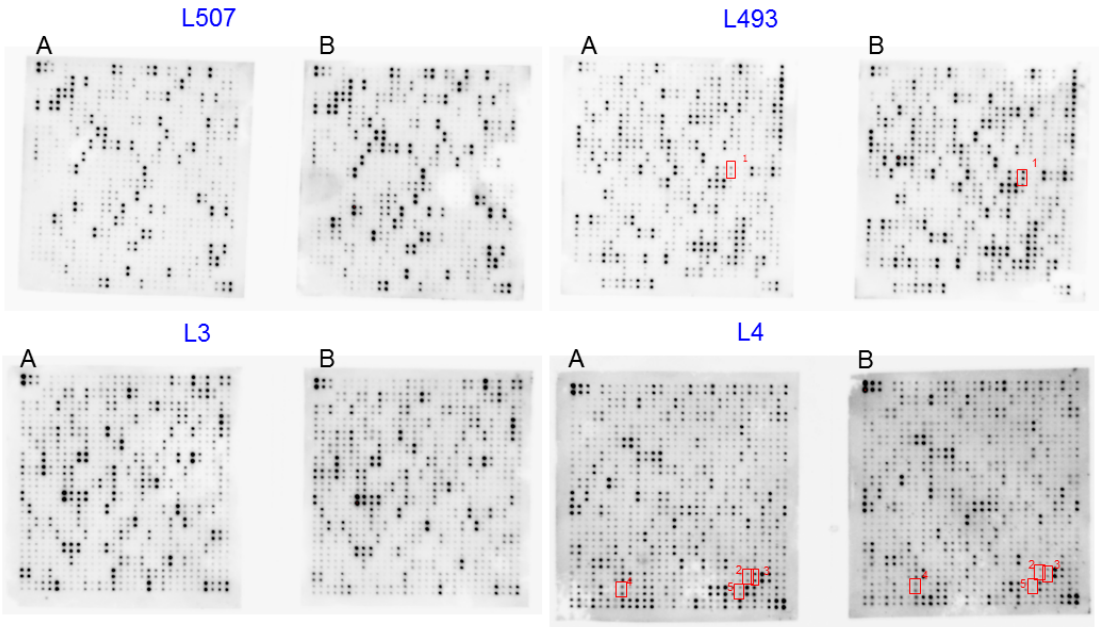

Figure 1-reply to reviewer

Changes in the text: Three plasma samples from 76 patients with POP and three samples from 56 healthy controls were screened using the L-Series human antibody array 2000 membrane kit, which is a combination of human L507, L-493, L-3 and L-4 arrays (AAH-BLM-2000B, RayBiotec Inc., Norcross, GA, USA).

Comment 3. What are some of the other advances in this area related to biomarkers. Authors must update on that in the introduction.
Reply 3: Thanks for your suggestion, the advances of biomarkers were updated already in the introduction. Ouping Huang, et. Al. utilized UHPLC-Q-TOF-MS to comprehensively investigate serum and urine 17 metabolomes of POP patients and controls and found that 6 metabolites including GPC, 1-methyladenosine, maleic acid, L-pyroglutamic acid, inosine, and citrate are significantly changed (VIP $\geq$ 1 and p $\leq$0.05) in both serum and urine samples from patients with POP. Stefano Salvatore, et. Al. reported that the higher levels of MMP-2 and -9 and lower levels of TIMP-2 in the plasma of Caucasian women may indicate a greater tendency for collagenolysis and weaker connective tissue with increased risk of developing pelvic floor dysfunctions, and they may potentially serve as biomarkers for pelvic floor tissue integrity.
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Comment 4. In the figures, age of patients need to be described, at least in figure legends.
Reply 4: Thanks for your suggestion. Age of patients was described in the figure legends.
Changes in the text: Women with malignant tumors, fibromyoma, pelvic masses, and mental abnormalities were excluded. Of the women included, 76 were diagnosed with POP and 56 women without POP were recruited as healthy controls (Table 1). As presented in Table 1, age of healthy controls was between 45 and 75 years, with a mean age of 57 years. Age of patients with POP was between 41 and 83 years, with a mean age of 63 years.
Changes in the text: Figure 1. Differences in the plasma levels of HSP10, ZC3H8, and UNC45A, between patients with POP and healthy controls, were determined by ELISA. The three analytes were significantly reduced in POP samples when compared to control samples. Age of healthy controls was between 45 and 75 years, with a mean age of 57 years. Age of patients with POP was between 41 and 83 years, with a mean age of 63 years.

Comment 5. What is the benefit of a biomarker test when POP can be determined by vaginal examination? how will this benefit the treatment?
Reply 5: Since the pathogenesis of POP is a chronic cumulative process involving many factors, we try to find the plasma biomarker at the early stage when POP cannot be determined by vaginal examination which is effective in the latter phases of POP. In this study, we collect the plasma of women companied with POP and controls which were clinically confirmed to screen the potential biomarkers. In further study, we will test the concentration of the markers in the follow-up cohort samples to evaluate its ability of early screening. Applying easy methods depended on the plasma biomarker at early screening and community health care will help us to identify high-risk individuals, and provide scientific evidence for early diagnosis, intervention, and treatment.

Reviewer B

Major comments:
Comment 1. Introduction: Introduction needs to be more informative in terms of POP development, etiology, treatments etc…
Reply 1: Thanks for your suggestion, informative in terms of POP development, etiology, treatments were updated already in the introduction.
Changes in the text: The global incidence of POP is approximately 20% and is increasing on an annual basis (1). Multiple factors including age, menopause, parity, obesity, hysterectomy, and connective tissue disorders have been reported to be in association with POP development (2). Age more than 55 years old and natural childbirth history were reported as two independent risk factors for pelvic organ prolapse, suggesting the existence of long-term pathology. However, the underlying molecular mechanisms of POP are still unclear.

Comment 2. The authors need to discuss the rationale for choosing selected serum proteins HSP10, ZC3H8 and UNC45A. previous literature on plasma protein detection needs to be cited in this area. There is only one citation in the Introduction!!
Reply2: Thanks for your suggestion, the advances of biomarkers were updated already in the introduction. The three target proteins, HSP10, ZC3H8, and UNC45A come through an antibody array (AAH-BLM-2000B, RayBiotec Inc., Norcross, GA, USA) and confirmative evaluation by Enzyme-linked immunosorbent assays (ELISAs). The three proteins HSP10, ZC3H8, and UNC45A exhibited significantly different levels when compared between patients with POP and healthy controls.

Comment 3. What was the age range of the selected patient? How was their parity status in POP and non-POP patient? These need to be mentioned in Methods.
Reply 3: Thanks for your suggestion. As presented in Table 1, age of healthy controls was between 45 and 75 years, with a mean age of 57 years. Age of patients with POP was between 41 and 83 years, with a mean age of 63 years. Also presented in Table 1, 32 women with POP were parity with 1 and 44 women with POP were parities with

equal and more than 2,while 22 non-POP women were parity with 1 and 33 non-POP women were parities with equal and more than 2.
Changes in the text: Of the women included, 76 were diagnosed with POP and 56 women without POP were recruited as healthy controls (Table 1). As presented in Table 1, age of healthy controls was between 45 and 75 years, with a mean age of 57 years. Age of patients with POP was between 41 and 83 years, with a mean age of 63 years. Thirty-two women with POP were parity with 1 and 44 women with POP were parities with equal and more than 2,while 22 non-POP women were parity with 1 and 33 non-POP women were parities with equal and more than 2.

Comment 4. Methods: It is said "three plasma samples from each group was mixed ". Does this mean that only three blood samples out of 76 patients in the POP patient group were selected? this needs to be clarified in methods.
Reply 4: Yes, three blood samples out of 76 patients in the POP patient group were selected for antibody array. It was recognized that the pooled samples may be more representative for antibody assay. Therefore, a pooled sample of three plasma from each group was tested in the antibody microarray assay instead of one sample from each group in this study.
Changes in the text: Three plasma samples from 76 patients with POP and three samples from 56 healthy controls were screened using the L-Series human antibody array 2000 membrane kit, which is a combination of human L507, L-493, L-3 and L-4 arrays (AAH-BLM-2000B, RayBiotec Inc., Norcross, GA, USA).

Comment 5. Discussion: In this section, only previous studies and findings have been cited whereas the possible relation between other studies and the finding in this paper (related to POP) needs to be discussed. For example, on page 12 line 233, it is said that "ZC3H8 can inhibit the inflammatory response in zebrafish mediated by the NF-κB pathway and causes the degeneration of multiple organs, including the liver, gut, and pancreas". Other studies on the process of inflammation in POP patient in particular the involved signalling pathways (Role of NFKB in inflammatory signalling pathways) can be discussed here.
Reply 5: Thanks for your suggestion! We made revision in this section.
Changes in the text: ZC3H8 has been reported with organ degeneration by inhibiting the inflammatory response in zebrafish mediated by the NF-κB pathway and leading the degeneration of multiple organs, including the liver, gut, and pancreas (9). Whether ZC3H8 is related to the degeneration of muscle cell function in POP wasn't reported in previous research. In the present study, we found that the expression levels of ZC3H8 were significantly lower in patients with POP when compared to healthy controls. The mechanisms underlying this observation need to be elucidated in further study.

Comment 6. On page 12 line 238, the contribution of ZC3H8 in human and mouse breast cancer cell lines, what is the relation between the role of ZC3H8 in cancer and its possible role in POP? Indeed, the authors need to discuss the involved mechanisms

(molecules) in cancer development by ZC3H8 that might have a role in POP as well. These suggestions apply to UNC45R and HSP10 as well.
Reply 6: Thanks for your suggestion! We made revision in those sections.
Changes in the text:

HSP10 is known to be associated with the degeneration of muscle cell function. One of the main characteristics of muscle aging is a reduction in maximal tetanic force (Po). Previous research has shown that the overexpression of HSP10 prevents the age-related decline in Po and can prevent damage caused by muscle contraction, although damage that had already being incurred could not be repaired (7). Both HSP10 and HSP60 have been found in various types of muscle cells and are known to exert anti-apoptotic effects; these proteins are also related to the development of cardiomyopathy and the process of apoptosis in cardiomyocytes (8). However, prior to the present study there was no evidence to suggest that plasma levels of HSP10 could be used as a biochemical biomarker to facilitate the clinical diagnosis of POP.

ZC3H8 has been reported with organ degeneration by inhibiting the inflammatory response in zebrafish mediated by the NF-κB pathway and leading the degeneration of multiple organs, including the liver, gut, and pancreas (9). Whether ZC3H8 is related to the degeneration of muscle cell function in POP wasn't reported in previous research. In the present study, we found that the expression levels of ZC3H8 were significantly lower in patients with POP when compared to healthy controls. The mechanisms underlying this observation need to be elucidated in further study.

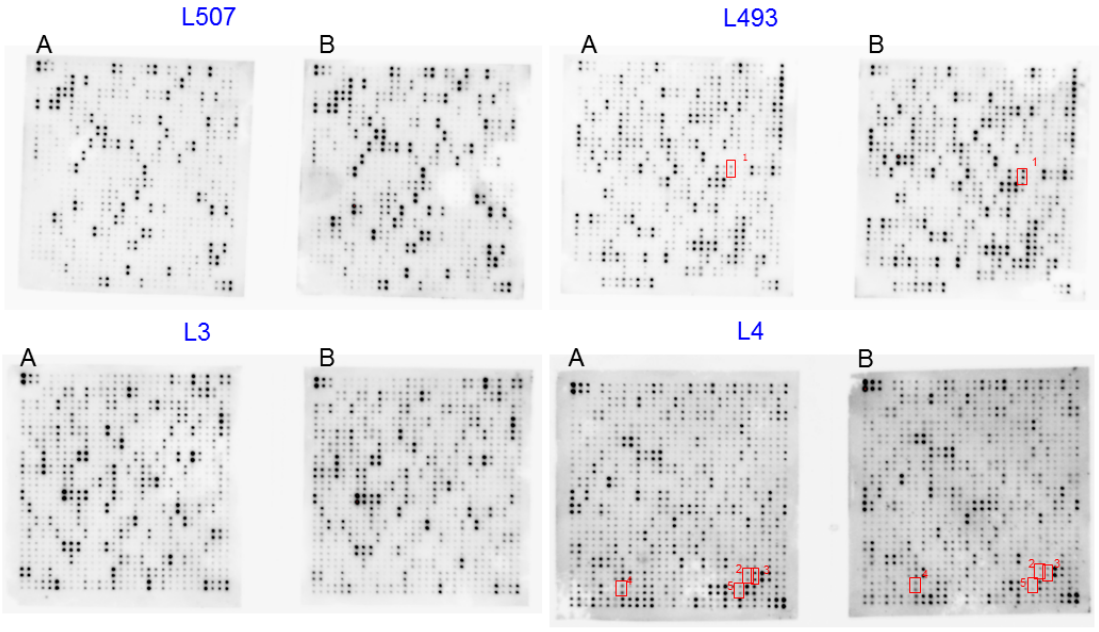
UNC45A is widely expressed in various muscle cells. It is an essential protein for the assembly and function of muscle tissue. Studies have shown that UNC-45A molecule is a dynamic component of actin stress fibers and acts as a myosin chaperone (10). Cells knocked out UNC45A were reported serious defects associated with fiber assembly errors that resulted in changes in cell morphology, abnormal migration, and eventually, symptoms associated with reduced muscle function (11-13). In the present study, we found that the plasma levels of UNC45A were significantly lower in samples from patients with POP than those in healthy controls. Since weaken or decline of muscle cell may be the cause of POP, the role of UNC45A in the pathogenesis of POP should be explored in further research.

Reviewer C

1) General comments
This manuscript examined the potential biomarkers for pelvic organ prolapse (POP). The purpose of this may be useful and contribute to the early diagnosis of POP. However, there are major concerns related to the drawn results and Discussion.

2) Special comments for revision:
a) major;
Comment 1. Initially, the authors screened potential proteins using antibody array, and selected 5 proteins (HSP10, ZC3H8, UTRN, UNC45A, and UNC5H4). This step is much important, but details are not shown, this cannot be accepted.

Reply 1: Thanks for your suggestion. The result of the antibody array (AAH-BLM-2000B) for all proteins is presented as Figure 1-reply to reviewer. The L-Series human antibody array 2000 membrane kit, which is a combination of human L507, L-493, L-3 and L-4 arrays, used in this study could simultaneously detect 2000 cytokines and related proteins in a given sample. The results were semi-quantitative when assessed by the density of each analyte spot. Multiple analytes were detected positively in the mixed sample created from each group. Among the positive analytes, the plasma levels of HSP10, ZC3H8, UTRN, UNC45A, and UNC5H4, were significantly decreased in the samples from patients with POP through visual observation and the program named RayBio Analysis Tool.



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Comment 2. Second, they examined the differences in 5 proteins between POP and healthy controls with a small group, and selected three plasma levels. Then they examined three plasma levels with an increased number. This kind of second steps is not understandable. Why did not they examine the differences in 5 plasma levels between two groups (76 pop vs 56 healthy volunteers) from a first step?

Reply 2: The quantitative screening was conducted by ELISA in two rounds for economical and effective consideration. The first round included UNC45A, UNC5H4, HSP10, ZC3H8, and UTRN (Table 2), and the second round included HSP10, ZC3H8, and UNC45A (Fig. 1). UNC5H4 and UTRN were excluded from a further evaluation because of their much smaller difference than UNC45A, HSP10 and ZC3H8 between the POP patients and the non-POP controls.

Comment 3. Third, Figure2. In differences in plasma levels of HSP10, ZC3H8, and UNC45A, healthy controls aged more than 60 had lower levels of these proteins, suggesting that low plasma levels of three proteins are not pop specific, rather than age specific. In Table, age is different between two groups. They have to compare the change of interest, using an analysis of covariance that incorporated the following variables as covariates: age, BMI (weight). I think these proteins are referred to the muscle reduction, such as frail. They have to also show the physics (height, wight, blood pressure etc) and blood tests (liver, kidney function, albumin atc). It is mandatory.

Reply 3: Age is a well-known factor to participate in POP and other factors may play key roles in the pathogenesis of POP. Our data also showed that aging was associated with POP. Since people older than 60 years of age are considered to be elderly, subjects in this study were grouped by age at 60 years old, as shown as Fig. 2. Obviously, the plasma levels of HSP10, ZC3H8, and UNC45A, were significantly lower in the plasma samples from patients with POP compared with the control within the same age group at aged < 60 years or > 60 years. It indicates that these biomarkers were capable to distinguish between patients or controls even the plasma concentration was related with aging. There were no significant differences in the plasma levels of these three proteins in the controls irrespective of whether the controls were aged < 60 years or > 60 years. A similar trend was evident in the samples acquired from patients with POP.

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