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Quantification of senescence-associated secretory phenotype proteins in the vaginal secretions of pre- and postmenopausal women with and without prolapse

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Abstract

Objectives: Cellular senescence has been proposed as a pathophysiologic driver in the development of pelvic organ prolapse (POP), especially during aging. In this study, we aimed to determine if markers of cell senescence can be quantified from vaginal secretions collected from pre- and postmenopausal women with and without POP.

Methods: Vaginal swabs were collected from 81 women in four groups: premenopausal with (pre-P) and without prolapse (pre-NP), and postmenopausal with (post-P) and without prolapse (post-NP). Multiplex immunoassays (MagPix) were then used to detect and quantify the presence of 10 SASP proteins in vaginal secretions.

Results: The total protein concentration of vaginal secretions differed significantly among the four groups ($P=0.003$) with highest mean concentrations in pre-P [16, interquartile range (IQR) = 4.6, 38.3 μg/μL] and lowest mean concentrations in post-P (4.4, IQR = 2.6, 7 μg/μL). The normalized concentrations of several SASP markers differed significantly among groups, with the highest concentrations being seen in the post-P group, and the lowest concentrations being in the pre-NP group. Using these key markers, we then constructed receiver-operator curves to determine the relative sensitivity and specificity of these markers in predicting prolapse.

Conclusions: In this study, we found that SASP proteins can be detected and quantified in vaginal secretions. Several of these markers were differentially expressed among the four groups studied, with the highest normalized concentrations of SASP markers found among postmenopausal women with prolapse. Overall, the data support the theory that senescence is associated with prolapse during aging but that other factors may be important in younger women who develop POP before menopause.

KEYWORDS
aging, cellular senescence, inflammation, menopause, pelvic organ prolapse

1 | INTRODUCTION

Pelvic organ prolapse (POP) is a common pelvic floor disorder that affects the lives of millions of women worldwide.[1–3] It is characterized by the loss of support to the uterus, bladder, and bowels, leading to their descent from a normal anatomic position down toward, or through, the vaginal opening.[4] Although this condition is not life-threatening, it can have significant negative effects on quality of life, with impacts on psychological distress, self-perceived body image, and sexual function. Consequently, the economic burden of POP is enormous and translates to over 300,000 surgical procedures performed for this condition annually in the United States.[5]

Factors which are most consistently linked to the development of POP include vaginal parity, body mass index (BMI), and age.[5] However, because the pathophysiology of POP is not completely understood, little progress has been made in developing methods to reliably predict or prevent POP. Multiple studies have supported the notion that childbirth is among the most important risk factors for the development of POP; however, most women do not develop prolapse symptoms until decades after the birth of their first child.[6–9] Accordingly, the peak incidence of POP in the United States occurs among individuals aged 60–69 years old. This has led some authors to argue that POP is a degenerative disease, which can be best understood by first considering the effect that aging cells have on the extracellular matrix and support systems of the pelvic floor.[10,11]

Support of the pelvic organs is dependent on a complex and inter-related hierarchy of anatomic structures, including the levator ani muscles, uterosacral and cardinal ligaments, and structures of the pudenda, including the perineal membrane and perineal body.[9] These structures are functionally connected by a seamless and interdependent web of connective tissue which is formed and maintained in part by fibroblastic cells. The fibroblasts of the pelvic floor are renewable cells that produce extracellular matrix (ECM) proteins.[12] These proteins include type I and type III collagen as well as elastic fibers, which are necessary in maintaining the biomechanical properties (ie, the tensile strength and elasticity) of pelvic floor tissues.[13,14]

As cells within renewable tissues in the body age, these cells may enter a metabolically active, but non-replicative state, called cellular senescence. Cellular senescence is a potent tumor-suppressive mechanism that arrests the growth of cells at risk for malignant transformation.[15] Common triggers of senescence include physical or chemical insults, such as oxidative stress, telomeric shortening, radiation, and exposure to chemotherapeutic drugs, or other stressors which have the potential to cause genomic instability. Senescent cells have been shown to accumulate over the life span of rodents, nonhuman primates, and humans.[11,16] Therefore, cells locked in the senescent phenotype have been proposed as markers of "cellular aging" and have been specifically demonstrated in human fibroblast and epithelial cells.

Although senescent cells may be in an arrested phase of the cell cycle, they are not metabolically inert. In fact, these cells undergo widespread changes in protein expression and secretion, which causes them to express soluble signaling factors (such as interleukins, chemokines, and growth factors), secreted proteases, and secreted insoluble proteins/extracellular matrix components.[17,18] Cells demonstrating these metabolic changes are said to be expressing a senescence-associated secretory phenotype (SASP), which may induce changes in the tissue ECM and microenvironment through altered secretory behavior. Prior work by our laboratory has demonstrated ultrastructural evidence of this phenotype in smooth muscle cells and fibroblasts taken from prolapsed vaginal tissue. We found that the expression of SASP-associated genes was upregulated in vaginal wall biopsies of women with prolapse as compared with nonprolapse controls. Likewise, supporting the notion that this phenotype is associated with cellular aging, we found that this phenotype was exaggerated in postmenopausal women.

In this pilot study, our primary aim was to determine if protein markers of cell senescence could be quantified from vaginal secretions collected during routine pelvic examinations. Our secondary aims were to determine if SASP proteins are differentially expressed in pre- and postmenopausal women and in women with and without prolapse. We hypothesized that cellular aging and the development of cellular senescence could induce changes in the cellular microenvironment that would allow us to detect the presence of SASP-associated markers in vaginal secretions. Furthermore, we hypothesized that these markers may be differentially expressed in women with and without prolapse as well as women who were pre- and postmenopausal.

2 | MATERIALS AND METHODS

Women in this study were enrolled from two hospital systems, Parkland Health Hospital System and Clements University Hospital, in Dallas, Texas. Institutional review board approval was obtained at both hospitals for the purposes of this study. Four cohorts of women were identified for enrollment in this observational pilot study. These consisted of premenopausal women with (pre-P) and without prolapse (pre-NP) and postmenopausal with (post-P) and without (post-NP) prolapse. Women were recruited at the time of their initial urogynecologic appointments as well as in the pre-operative area before the patients underwent scheduled gynecologic procedures. Exclusion criteria were women <18 years of age, presence of uncontrolled diabetes (hemoglobin A1C >8.0), use of immunosuppressive medications within the last 6 months, BMI >40, presence of a foreign body, such as pessary, previous surgery for pelvic organ prolapse or urinary incontinence, current nonphysiological vaginal discharge, known endometriosis, gynecologic malignancy that may affect the vaginal wall, or presence of cervical infection/vaginitis. Prior hysterectomy did not exclude women from participation in this study. Women with diminished capacity to provide informed consent were excluded from participation.

After obtaining written informed consent, vaginal secretions were collected by the woman's physician at the time of her initial pelvic examination in the clinic or was done prior to betadine

preparation in the operating room. Secretions were collected in a standardized fashion using sterilized endocervical brushes with care taken to specifically sample the vaginal walls. These were collected after swabbing the vaginal walls for about 30 seconds. For each patient, demographic information, surgical and medical histories, presence of urinary incontinence, use of vaginal estrogen, subjective assessment of vulvovaginal atrophy, and standard pelvic organ prolapse quantification (POPQ) examinations were recorded. Data were stored using a secure RedCap database.

Swabs containing vaginal secretions were placed in 2 mL of sterile phosphate-buffered saline and stored at 4°C until protein extraction. Cells were then lysed using a lysate buffer containing protease inhibitor and total protein was extracted using a strongly chaotropic extraction solution containing the zwitterionic detergent ASB-14. Total protein concentration was determined using photo spectrometry at an absorption of 562 nm after treatment bicinchoninic acid (BCA), which allowed for the colorimetric detection and quantitation of total protein. Finally, Multiplex enzyme-linked immunosorbent assay (ELISA; MagPIX; Luminex) was used to quantify the presence of senescent markers IL-6, IFN-gamma, GM CSF, MIF, TFN-a, MCP/CCL2, IL8/CXCL8, MIP-1a/CCL3, Gro-a/CXCL1, and MIP3a/CCL20 from the purified sample.

2.1 | Statistics

2.1.1 | Sample size consideration

Prior to this work, there was no documented evidence that SASP markers could be detected from vaginal secretions. Therefore, we approached the design of this study with the goal of the demonstrating scientific feasibility. Whitehead et al.[19] previously published general guidelines for sample size calculations for randomized controlled trials aimed at detecting effect sizes that are extra small, small, medium, or large. From our experience in studying the molecular composition of

vaginal tissue in vivo and in culture, we proposed that the prolapse and menopause would confer a medium to large effect size on SASP expression. Therefore, assuming a 90% power and a two-sided 5% significance, we chose a goal of 20 samples per cohort group (total n = 80).

2.2 | Data analysis

Descriptive statistics were used to summarize the baseline characteristics of the four cohort groups. Participant characteristics were then compared across the four groups using one-way analysis of variance (ANOVA) for parametric data and Kruskal-Wallis for nonparametric data. SASP marker concentrations were normalized to total protein concentration in each sample. The Shapiro–Wilk test for normality was used to determine the distribution of SASP marker concentrations. Because we found that the concentrations observed were non-normally distributed, Kruskal-Wallis was used to compare SASP marker concentrations among the four groups. Post hoc testing was performed using the Dunn-test and Wilcox test to avoid over-estimation of significant P values. Finally, we performed unilateral logistic regressions in an attempt to determine the relative sensitivity and specificities of specific SASP markers in predicting the outcome of POP. We did this by building receiver operating characteristic (ROC) curves for each of the SASP markers which were significantly different between the four groups.

3 | RESULTS

After accounting for samples which did not yield sufficient protein for analysis and after exclusion criteria, a total of 81 samples (n = 81) were included in the final analysis. This consisted of premenopausal with (pre-P, n = 17) and without prolapse (pre-NP, n = 22) and postmenopausal without (post-NP, n = 18) and with (post-P, n = 24) prolapse (Table 1).

TABLE 1 Patient demographics among the four cohort groups.

Variable	Description	Post-P (n = 24)	Post-NP (n = 18)	Pre-P (n = 17)	Pre-NP (n = 22)	P value[a]
Age, median [IQR]		71 [63, 74]	70 [57, 79]	45 [42, 50]	39 [35, 44]	**<0.01**
Body Mass Index, median [IQR]		26 [24, 31]	28 [21, 31]	29 [25, 36]	31 [25, 34]	0.19
Race, no. (%)						**<0.01**
	Black/African American	4 (17)	2 (11)	2 (12)	6 (27)	
	White	19 (79)	13 (72)	5 (29)	5 (23)	
	Other	0 (0)	0 (0)	0 (0)	3 (14)	
Ethnicity, no. (%)	Hispanic	1 (4)	3 (16)	10 (59)	8 (36)	**0.03**
Smoker, no. (%)		2 (8)	4 (22)	1 (6)	0 (0)	0.09
Prior hysterectomy, no. (%)		4 (17)	7 (39)	4 (24)	2 (9)	0.13
Vaginal delivery, median [IQR]		2 [2, 3]	2 [1, 2]	3 [2, 4]	1 [0, 3]	**<0.01**

Abbreviations: IQR, interquartile range; Post-NP, postmenopausal without prolapse; Post-P, postmenopausal with prolapse; Pre-NP, premenopausal without prolapse; Pre-P, premenopausal with prolapse.

Bold values represent statistically significant difference.

[a]Denotes comparison among the four groups.

As expected, ages differed between pre- and postmenopausal cohorts (38.9 ± 6.8 vs. 70.2 ± 13.4) and between women with and without prolapse (45.8 ± 4.8 vs. 69.5 ± 8.2). Vaginal births were highest in the pre-P group, three [interquartile range (IQR) $=2$, 4] and were lowest in the pre-NP group, one (IQR$=0$, 3). Additionally, women in the pre-P group were more likely to be Hispanic ($P=0.033$). BMI, prior cesarean, and prior hysterectomy were not statistically different among the four groups (Table 1).

Total protein concentrations differed significantly among groups ($P=0.003$) with highest mean concentrations in the pre-P group [16 (IQR$=4.6$, 38.3) µg/µL] and lowest median concentrations in the post-P group [4.4 (IQR$=2.6$, 7) µg/µL]. Because we found that protein concentrations of SASP markers were non-normally distributed, the median protein concentrations are displayed in Figure 1 and are likewise shown in Table 2. Median differences were significantly different among the four cohorts ($P=0.016$), with the greatest difference between the pre-P and post-P groups ($P=0.0072$). Due in part to this finding, we normalized all SASP concentrations to the purified protein concentration for each sample. We found that the normalized concentrations of several SASP markers differed significantly among the four groups. Specifically, the pPost-P group had increased concentrations of IFNG 0.3 pg (IQR$=0$, 1.31, $P=0.001$), GM CSF 3.37 pg (IQR$=0$, 7.07, $P=0.003$), MIF 25,244 pg (IQR$=0$, 93,307, $P=0.002$), and GROaCXCL3 232 pg (IQR$=0$, 330, $P=0.031$). Post hoc pairwise comparisons showed that this difference was greatest between the pre-NP and post-P cohorts (Figure 2).

Finally, we constructed ROC curves to model the sensitivity and specificity of each of the markers found to be significantly different among the four groups in predicting the presence or absence of pelvic organ prolapse. Of these, GROaCXCL1 demonstrated the highest area under the curve (AUC) of 62.7%. The MIF marker had an AUC of 61.7%, IFNG had an AUC of 59.7%, and GM CSF had an AUC of 57.7% (Figure 3).

4 | DISCUSSION

Because the development of POP is multifactorial and incompletely understood, little progress has been made in developing strategies to reliably predict or prevent the development of prolapse. In this pilot study, we first demonstrated the feasibility of detecting SASP-associated proteins in vaginal secretions. Specifically, we found that vaginal secretions taken from pre-P women had the highest overall total protein concentrations and post-P women had the lowest total protein concentrations. In large part, postmenopausal women with prolapse are more likely to have thin, atrophic vaginal tissues and therefore low overall protein concentrations were expected in this group. It was interesting, however, that premenopausal women without prolapse had lower vaginal protein concentrations than premenopausal women with prolapse. One theory to explain this finding is that women with prolapse may have altered secretory phenotypes with a larger number of cells displaying the SASP and therefore leading to an increase in extracellular signaling, inflammation, and ECM remodeling in these patients.[18,20–22]

We chose to compare normalized SASP marker concentration in this study because of this finding of different total protein concentration among the four groups. Likewise, we found that the normalized concentrations of SASP-associated markers were present in highest concentrations among postmenopausal women with prolapse, despite having the lowest overall total protein concentration in their vaginal secretions. This leads us to postulate that those proteins that are being expressed in this group are more likely to be SASP-associated and therefore that these patients may have proportionally higher numbers of senescent cells.

Our finding that SASP-associated proteins are present in different normalized concentrations among the four groups and present in the highest number among postmenopausal women with prolapse, bolsters the possibility that these markers may be involved in the pathophysiology of prolapse. Although possible, these increases are

FIGURE 1 Median protein concentrations found in vaginal secretions of four patient cohorts. Overall, the four groups were significantly different from one another ($P=0.016$), with the greatest difference seen between the pre-P and post-NP groups ($P=0.007$). The highest overall protein expression was found in the pre-P group with the lowest found in the post-P group. Post-NP, postmenopausal without prolapse; Post-P, postmenopausal with prolapse; Pre-NP, premenopausal without prolapse; Pre-P, premenopausal with prolapse.
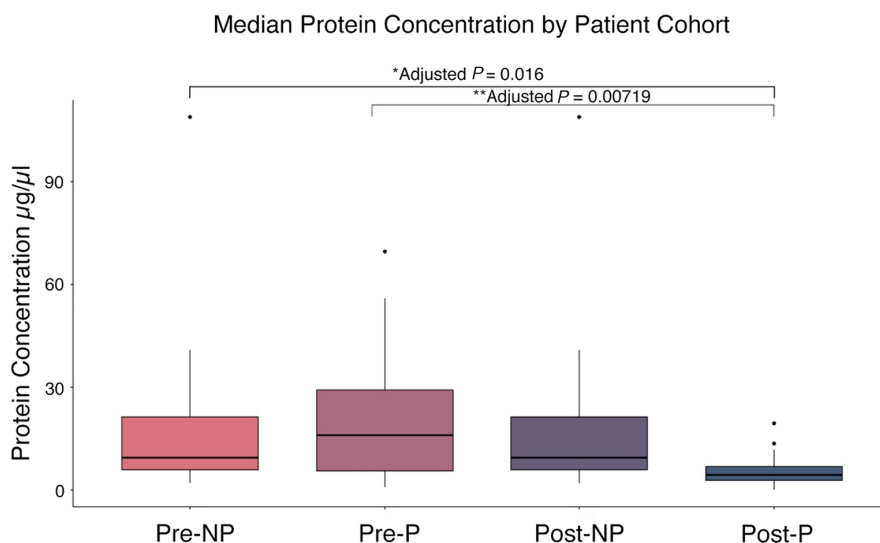
Median Protein Concentration by Patient Cohort

*Adjusted $P = 0.016$
**Adjusted $P = 0.00719$

TABLE 2 Normalized concentrations (µg/µL) of SASP protein markers according to patient cohort group

Variable, median (IQR)	Post-P (n = 24)	Post-NP (n = 18)	Pre-P (n = 17)	Pre-NP (n = 22)	P value[a]
Total protein	4.4 (2.6, 7.0)	9.1 (3.7, 14.4)	16.0 (4.6, 38.3)	9.4 (5.3, 24.4)	**<0.01**
IL-6	0.47 (0.15, 1.84)	0.22 (0.08, 1.31)	0.26 (0.02, 0.40)	0.11 (0.01, 0.93)	0.07
IFNG	0.30 (0.08, 1.43)	0.14 (0.03, 0.29)	0.02 (0.01, 0.15)	0.04 (0.01, 0.20)	**<0.01**
GM CSF	3.37 (0.32, 8.31)	0.52 (0.31, 2.88)	0.19 (0.07, 1.25)	0.28 (0.07, 2.88)	**<0.01**
MIF	25,244 (3754, 99,137)	3544 (1872, 25,734)	2399 (518, 7428)	4348 (564, 6067)	**<0.01**
TNFA	1.66 (0.29, 2.73)	1.17 (0.46, 2.53)	0.15 (0.04, 0.93)	0.13 (0.01, 2.46)	**0.02**
MCP1/CCL2	0.84 (0.28, 2.78)	0.95 (0.52, 2.21)	0.41 (0.10, 2.00)	0.52 (0.13, 1.93)	0.28
IL8/CXCL8	119 (34, 402)	101 (27, 335)	61 (24, 199)	53 (6, 130)	0.33
MIP1a/CCL3	1.69 (0.26, 3.83)	2.68 (0.55, 5.55)	1.20 (0.07, 6.33)	0.50 (0.03, 2.06)	0.18
GROa/CXCL1	232 (74, 421)	60 (36, 199)	67 (10, 269)	32 (3, 179)	**0.02**
MIP3a/CCL20	0.97 (0.20, 3.20)	1.76 (0.26, 8.18)	0.52 (0.05, 2.02)	0.22 (0.07, 2.71)	0.11

Abbreviations: GM CSF, granulocyte macrophage colony-stimulating factor; GROa/CXL1, chemokine ligand 1; IFNG, interferon gamma-1B; IL6, interleukin 6; IL8/CXCL8, interleukin-8/chemokine ligand 8; IQR, interquartile range; MCP1/CCL2, monocyte chemoattractant 1/chemokine ligand 2; MIF, macrophage migration inhibitory factor; MIP1a/CCL3, macrophage inflammatory protein 1a/chemokine ligand 3; MIP3a/CCL20, macrophage inflammatory protein-3/chemokine ligand 20; Post-NP, postmenopausal without prolapse; Post-P, postmenopausal with prolapse; Pre-NP, premenopausal without prolapse; Pre-P, premenopausal with prolapse; TNFA, tumor necrosis factor alpha.

Bold values represent statistically significant difference.

[a]Denotes comparison among the four groups.

not likely to be a consequence of prolapse alone, since premenopausal women with prolapse did not show similar increases in SASP protein concentrations. Therefore, there may be additional pathophysiological mechanisms involved in the development of symptomatic prolapse among a younger cohort. Clinically, we also see that younger women tend to present slightly differently than postmenopausal women. Specifically, we incidentally observe a higher proportion of premenopausal women with findings of cervical elongation with fewer numbers of women presenting with post-hysterectomy prolapse or symptomatic isolated anterior or posterior vaginal wall prolapse.[8,20] Therefore, our data support the theory that senescence is associated with prolapse during aging but that other factors may be responsible for the development of prolapse in younger women. Furthermore, the markers that were found to be significantly higher in the postmenopausal prolapse group, GROaCXCL1, MIF, IFNG, and GM CSF, deserve further exploration despite their relatively low sensitivity and specificity. These markers are all related to immune cell trafficking, which is an important process in ECM remodeling. Research focusing on immune pathways regulation and the effect on tissue remodeling could provide further insights into the pathophysiology of prolapse.

Our study design had several strengths and limitations. In this study, we recruited four distinct cohorts of pre- and postmenopausal women from an ethnically and socioeconomically diverse patient population. Our choice to look at pre and postmenopausal women allowed us to better appreciate how SASP markers (which may be independent markers of age) are expressed in increased concentrations among women with prolapse. As this was a pilot study, we are confident that our work establishes the feasibility of assessing SASP marker concentrations in vaginal secretions. Nevertheless, this study has significant limitations which restrict our ability to draw any firm

conclusions from this work. Beyond small sample sizes, our study was limited by slightly unequal sample sizes among the four groups, with slight over-representation by postmenopausal women with prolapse. Additionally, our study groups were largely recruited from the urogynecology clinics, and therefore many of the women included in the study had complaints of urinary incontinence or another pelvic floor disorder in addition to pelvic organ prolapse. Furthermore, these data need to be validated within patients as well as in larger study groups. For example, it would bolster the validity of our findings if it could be demonstrated that SASP markers once elevated, remain elevated within one patient. This could be done by either repeating sampling in the same patient several weeks apart, or by further attempting to correlate relative SASP expression in vaginal secretions to blood samples. Additionally, in our study, the cells collected within each vaginal swab were lysed prior to protein extraction and analysis. However, it may be beneficial to explore the relative concentrations of different cell types within each sample. One theory is that women with prolapse may have higher relative concentrations of immunologic cells within their samples, as compared to women without prolapse. Finally, as senescence triggers an inflammatory reaction with activation of matrix metalloproteases, it could be useful to specifically quantify the relative concentrations of these markers in secretions as well. This would support our previous findings and further help to establish the validity of the results presented here.[21–24]

Due to the novel nature of our findings, our work lends itself to several future studies. For one, this work should be repeated in a much larger patient cohort. Larger sample sizes may demonstrate the relationship among prolapse, age, and SASP marker secretion more clearly. Larger sample sizes may also reveal statistically significant differences in the expression of markers which were not found to be significantly different in our study. This work could additionally

FIGURE 2 Median Normalized SASP marker concentrations among four patient cohorts. *Denotes a statistically significant difference among groups with *P* value of <0.05. Post-NP, postmenopausal without prolapse; Post-P, postmenopausal with prolapse; Pre-NP, premenopausal without prolapse; Pre-P, premenopausal with prolapse.

Normalized SASP Marker Concentration by Menopause and Prolapse Status
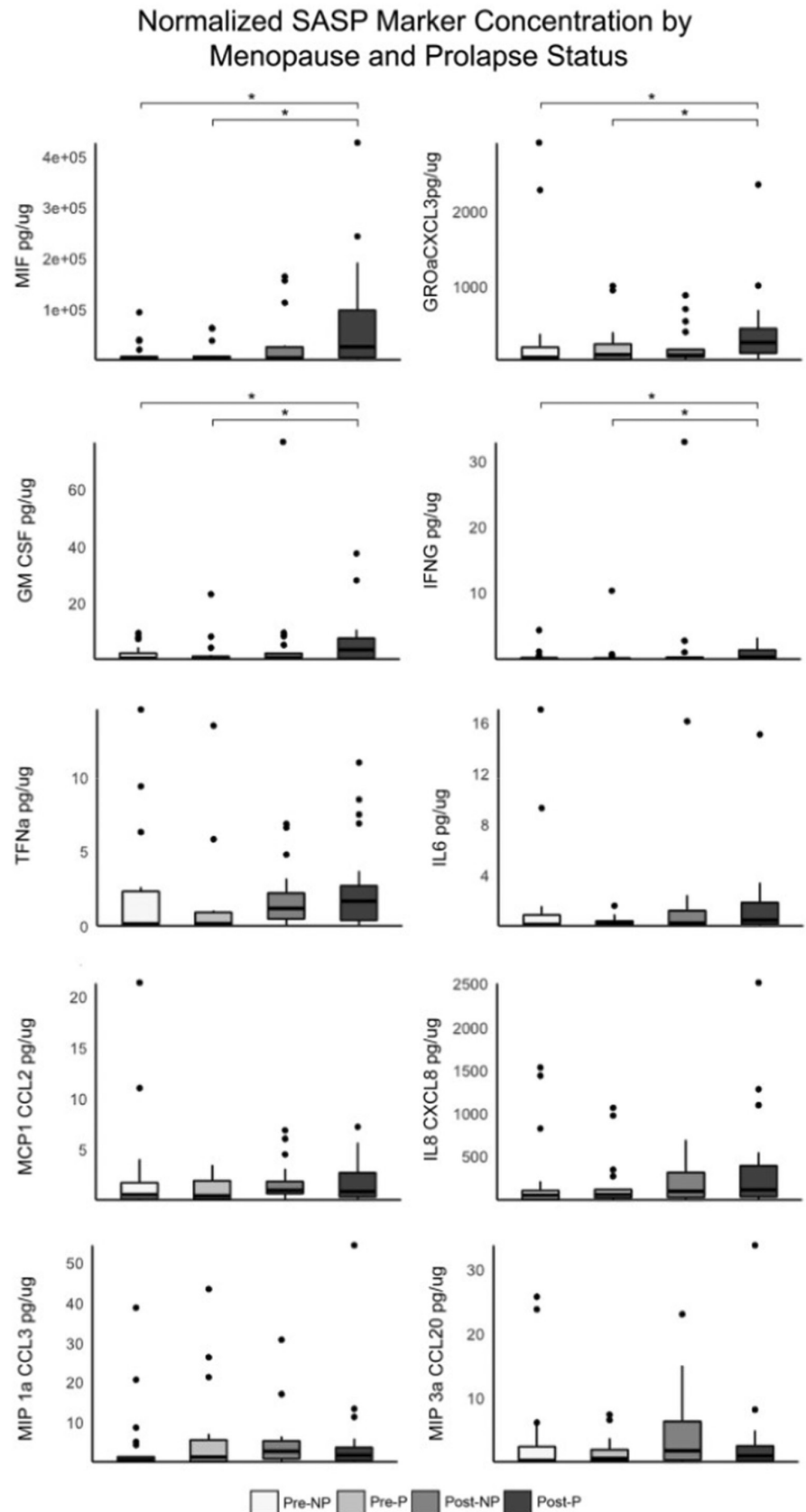
be validated by using vaginal wall biopsies with proteomic analysis to determine if there is relative upregulation in the expression of the SASP phenotype in prolapsed versus non-prolapsed vaginal walls. Finally, as the vaginal swabs used in this study can be collected during routine pelvic examinations, our findings lend themselves to the possibility that SASP markers could be used to make predictions about a patient's risk of developing prolapse, or her risk of recurrence following surgical procedures.
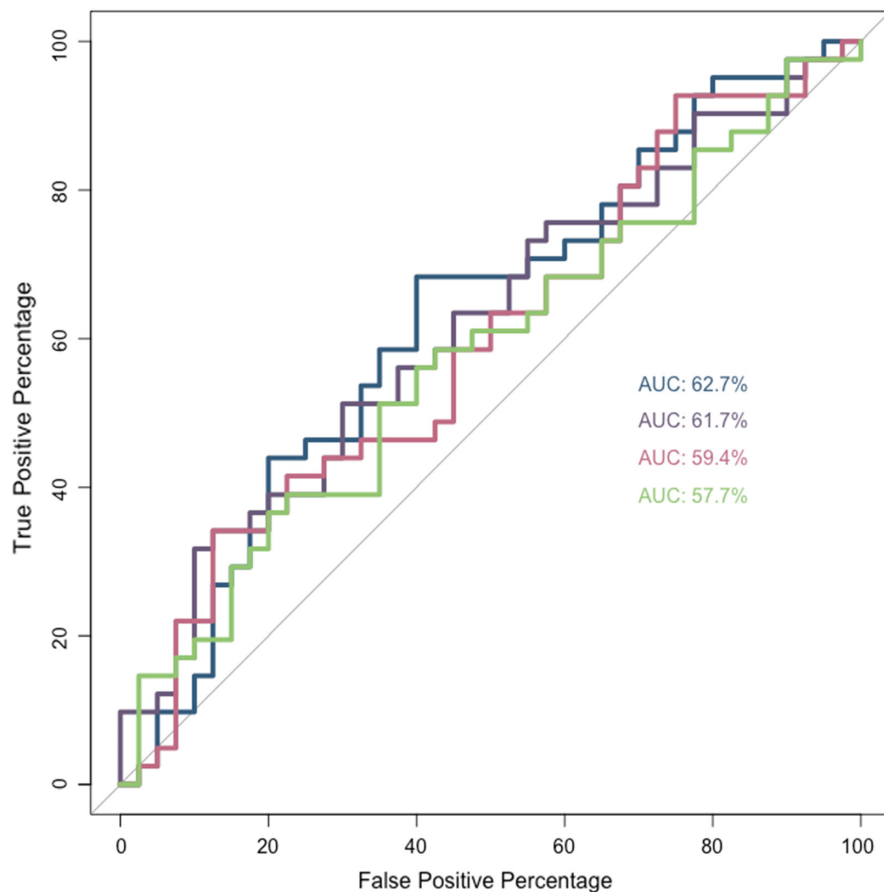
FIGURE 3 Superimposed receiver operator characteristic (ROC) curves for the four statistically significant SASP markers with presence of prolapse as predicted outcome. AUC, area under the curve.

5 | CONCLUSIONS

Markers of cellular senescence can be extracted and quantified from vaginal secretions of pre and postmenopausal women with and without pelvic organ prolapse. In our cohorts, several of these markers were seen to be significantly elevated in postmenopausal women with prolapse as compared to premenopausal women without prolapse. Further research is needed to validate our findings. First, these findings should be repeated within the same patient to confirm that these elevations are persistent and not simply transiently observed in our study population. Next, additional validation of our findings should be done in larger cohorts, and ideally with a more stringent control group that has no pelvic floor dysfunction. Pending these additional investigations, our findings signal that there may be potential utility in using SASP markers to make predictions about lifetime risk of prolapse or risk of prolapse recurrence in certain patient groups.

AUTHOR CONTRIBUTIONS

Study design: Sawyer and Florian-Rodriguez. *Sample and data collection:* Sawyer, Shi, Keller, and Florian-Rodriguez. *Sample processing:* Sawyer. *Statistical analysis:* Sawyer and Brown. *Manuscript writing:* Sawyer and Florian-Rodriguez. *Manuscript review:* Sawyer, Shi, Keller, Brown, and Florian-Rodriguez.

CONFLICTS OF INTEREST STATEMENT

Dr Florian-Rodriguez is a consultant for Boston Scientific and paid researcher for AbbVie. The other authors have no financial disclosures.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

This study was approved by UT Southwestern's institutional review board under study number STU-2018-0432.

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