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Identification of Novel Non-invasive Biomarkers of Urinary Chronic Pelvic Pain Syndrome (UCPPS): Findings from the Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network

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Introduction

The idiopathic nature of Urological Chronic Pelvic Pain Syndrome (UCPPS), which encompasses interstitial cystitis/bladder pain syndrome (IC/BPS) and chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), has prompted an intense search for clinical biomarkers. Identification of clinically relevant, validated biologic markers of UCPPS has great potential to inform our understanding of UCPPS pathophysiology, development, and progression, and ultimately clinical management. To date, the cause of this syndrome is unknown and there is no ability to determine who is at risk of progression or who will

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Conflicts of Interest

The authors have nothing to declare.

respond to specific therapy. Accurate noninvasive tests, based on objective, specific, and definable levels of validated biomarkers, are essential to improve and standardized clinical guidelines and for more targeted management strategies based on patient profiles.

The MAPP Research Network was established by the NIDDK of the NIH and represents a comprehensive approach to the study of UCPPS^{1, 2}. A major goal of this initiative is to better understand pathophysiology and identify potential therapeutic targets. In this MAPP Network study, we have utilized a biologically-driven, candidate biomarker discovery strategy, grounded in the basic biochemistry and physiology of this syndrome, to evaluate candidate non-invasive biomarkers of UCPPS.

Reviews of the literature have revealed a number of candidate protein biomarker targets. Increased VEGF levels have been detected in bladder biopsy samples from patients with IC compared to controls, with levels correlating with pain severity^{3, 4}. Prior studies also suggest an important connection between neovascularization and IC, in that new blood vessels in biopsy samples of IC patients showed significantly lower levels of pericyte coverage of the nascent endothelium, evidence consistent with the known association between high levels of VEGF and immature vessel formation³. Moreover, VEGF originally discovered as VPF (Vascular Permeability Factor),^{5, 6} is the most potent endogenous vascular permeability factor and may mediate the dysregulated vascular permeability postulated to be important in UCPPS⁷⁻⁹. Given these associations, we chose to include VEGF and its receptor VEGFR1, as biomarker candidates.

Inflammation has been suggested to be an underlying pathophysiologic mechanism in UCPPS¹⁰ and increased concentrations of pro-inflammatory cytokines (IL-1 β , TNF- α , IL-8) have been reported in patients with UCPPS as compared with controls^{11, 12}. In this respect, MMPs are of particular interest. Originally thought to be associated exclusively with tissue remodeling and destruction, it is now widely appreciated that MMPs play a role in a variety of biologic processes including cytokine and growth factor release, tumor growth and progression, angiogenesis and a number of inflammatory conditions¹³⁻¹⁷. During the inflammatory response, MMPs and their complexes are released from connective tissue cells in response to pro-inflammatory cytokines. Upregulation of MMP activity results in the recruitment of pro-inflammatory cells to the site of tissue injury. MMPs have also been implicated in the regulation of the immune response because of their ability to cleave inflammatory mediators and stimulate the clearance of inflammatory cells¹⁸⁻²¹. Given that MMPs may play an important role in UCPPS as a function of the inflammatory phenotype, abnormal vasculature and the dysregulation of ECM (extracellular matrix) turnover present in this and other potentially related diseases we included these proteins in our study.

NGAL, also known as Lipocalin-2 (Lcn2), is upregulated in numerous chronic and acute inflammatory conditions, including chronic kidney disease, ulcerative colitis and myocardial infarction^{22, 23}. There exists an intricate interplay between NGAL and other inflammatory cytokines as well, such that NGAL upregulation can be triggered by multiple inflammatory cytokines, including TNF- α , IL-1 β and IL-17^{22, 23}. Moreover, NGAL itself is a modulator of the levels of other inflammatory cytokines, as well as of the behavior of inflammatory leukocytes²⁴⁻²⁶. In our laboratory, we have demonstrated that NGAL is a significant

stimulator of VEGF levels in breast tumor cells and can also independently induce the epithelial to mesenchymal transition in breast cancer, as well as being a noninvasive biomarker of this disease^{27–29}. NGAL can also form a complex with MMP-9, representing a distinct protein complex referred to as MMP-9/NGAL complex. We have previously shown that when NGAL complexes with MMP-9, it protects MMP-9 from degradation preserving its enzyme activity^{16, 30}. The MMP-9/NGAL complex has also been detected in urine samples from patients with various diseases. Based on these findings, we chose to measure NGAL as well as MMP-9/NGAL complex in the urine of individuals with UCPPS and correlate them with clinical symptoms.

Here, we report sex-specific comparisons of candidate biomarkers among UCPPS and control cohorts to gain new insights into underlying pathophysiology. In addition, associations of biomarkers with symptom severity within the UCPPS cohort are examined. We anticipate that the identified biological markers may serve as potential candidates for further clinical evaluation, including in the development of new treatment strategies directed toward novel targets, as objective measures for patient classification schemes, and evaluation of clinical outcomes, as well as further characterization of underlying mechanism.

Methods and Materials

Urine collection, storage, shipping

Biologic specimens collected in the Trans-MAPP Epidemiology/Phenotyping Study (EPS)² included urine collected at baseline visits. Clean-catch midstream urine was collected using alcohol-free Triad Medical-Benzalkonium chloride antiseptic towelettes (Allegro Medical)². After collection, the urine was immediately frozen at -80°C and shipped to the MAPP Network Tissue Analysis and Technology Core for centralized processing, where it was then divided into 3 mL aliquots and stored at -80°C until further analysis. All specimens were de-identified to allow for blinded testing². Documentation of sample origin, acquisition, transportation, processing, and storage was tracked and recorded for each sample for quality assurance and quality control purposes.

Protein assays

Urine samples were thawed, aliquoted, and assayed as below. Remaining samples at the time of aliquoting were then stored at -80°C . Total protein concentration was determined using the Bradford method with bovine serum albumin as the standard. Samples were read on a DU 640 spectrophotometer (Beckman Coulter).

Enzyme-linked immunosorbent assay (ELISA)

Monospecific ELISAs (Quantikine, R&D Systems, Inc.) for human MMP-2, MMP-9, MMP-9/NGAL complex, NGAL, VEGF and VEGFR1 were performed according to manufacturer's instructions. All analyses were conducted in duplicate in a double-blinded manner as previously reported by us^{27, 29, 31–33}.

Study Population

The Trans-MAPP EPS enrolled male and female participants in three cohorts: 1) UCPPS participants, 2) positive controls (PC) and 3) healthy controls (HC) ^{1,2}. UCPPS participants were defined by a nonzero response on a pelvic pain scale. PC were defined by the presence of a non-urolological associated syndrome (NUAS) (fibromyalgia, chronic fatigue syndrome, and/or irritable bowel syndrome), and healthy controls were defined by the absence of both UCPPS and other chronic pain conditions. Detailed inclusion and exclusion criteria are described ². Stratified random sampling was used to select participants for this substudy. UCPPS participants with the most severe urologic pain and urinary symptoms and HCs and PCs with the least severe symptoms were more likely to be selected to maximize the likelihood of detecting differences between UCPPS and control cohorts. Sampling was conducted within sex, and severity was determined by the sum of the Genitourinary Pain Index (GUPI) ³⁴ pain and urinary subscores, which was categorized as mild (less than 13), moderate (greater than or equal to 13 and less than 23), or severe (greater than or equal to 23 for a maximum of 33) for selection. Demographic data were collected, and additional clinical characteristics were assessed by the RAND Interstitial Cystitis Epidemiology Study criteria ³⁵, Brief Pain Inventory (BPI) short form ³⁶, and Interstitial Cystitis Symptom Index (ICSI) ³⁷. For analyses of associations of symptom severity with candidate proteins, GUPI Pain and Urinary subscores and items of the ICSI symptom index are reflected in two primary symptom constructs, pain severity and urinary severity ³⁸.

Statistical analyses

Duplicate values of candidate proteins were evaluated for quality prior to analysis. Reliability analyses were conducted using Bland-Altman plots, Coefficients of Variation (CVs) and Intraclass Correlation Coefficients (ICCs). Undetectable concentration values reported as zero were imputed with half the limit of detection (LOD), and values above the maximum detectable limit (MDL) were removed (n=11). Unlike values below the LOD, which we know lie between 0 and the LOD, it cannot be assumed that values above the MDL are indeed that large. Values below the LOD but greater than 0 remained as measured. Results of reliability analyses, LODs and MDLs are provided in Tables S1 and S2.

Imputed concentration values were divided by total urinary protein per sample to normalize concentration to total urinary protein to correct for various degrees of hydration and protein excretion. Concentrations (“classic concentration”, ng/mL or pg/mL) and concentration normalized to total protein (“normalized concentration”, pg/ μ g) were then log-transformed. Candidate biomarker levels were compared within sex using Student’s t-test. We compare UCPPS and HC to assess markers of illness and pain and UCPPS and PC to identify markers unique to UCPPS. Markers that differed significantly between UCPPS and respective controls in univariable analysis after accounting for multiple testing were entered into a multivariable logistic regression with UCPPS versus HC or UCPPS versus PC as the outcome. Corresponding ROC curves were generated. Models did not include any variables beyond the indicated proteins. We accounted for multiple testing by controlling the False Discovery Rate ³⁹ (FDR), a more powerful alternative to the standard Bonferroni correction. The FDR is a generalization of type I error under a single hypothesis test to multiple hypothesis tests. This method of adjusting for multiple testing considers variable thresholds

for the significance of ranked p-values that result from a set of hypothesis tests instead of a single, stringent threshold applied to all tests. The FDR was set to 0.05, and the reference thresholds for p-values for each test were determined by a formula based on the number of tests³⁹. FDR adjustment for six tests of classic concentration was applied separately for tests of UCPPS versus HC and UCPPS versus PC, and similarly to seven tests of normalized concentration for each marker and additionally total protein. Locally weighted scatterplot smoothing was performed to visualize associations of classic and normalized concentration with pain and urinary severity³⁸ in all three cohorts. Among UCPPS patients only, linear regression was used to quantify the association of pain and urinary severity with classic and normalized concentrations of candidate biomarkers, and associated markers in univariable analyses were entered into separate multivariable models for each symptom. Multivariable models again included markers significant after adjustment for multiple comparisons and did not adjust for other factors. Pearson correlation coefficients among markers were generated to evaluate the potential for multicollinearity in regression models.

Results

Table 1 summarizes selected demographic data, prevalence of non-urolological associated syndromes (NUAS), and the primary urologic pain and urinary severity levels of the Trans-MAPP EPS participants included and not included in these analyses (see Materials and Methods). The evaluated subset included 259 UCPPS (139 female, 120 male) participants, 125 healthy controls (75 female, 50 male), and 107 positive controls (75 female, 32 male). UCPPS participants selected for this biomarker study reported longer duration of symptoms (mean (SD) 9.7 (11.6) vs. 6.6 (8.6), $p=0.002$) and were more likely to have a NUAS than those not in the study (42.9% vs. 30.9%, $p=0.014$), and selected healthy and positive controls had less severe urinary ($p<0.001$ HC, $p<0.001$ PC) and pain ($p=0.003$ HC, $p<0.001$ PC) symptoms than those not included. These results were expected given the sampling strategy. Further, males were overrepresented among PC relative to the prevalence of males among all MAPP PC participants, also by design.

Quality control analyses utilizing the duplicate values for each of these 6 biomarkers and total protein, resulted in coefficients of variation (CVs) of 118.2% (MMP-2), 25.9% (MMP-9), 74.4% (MMP-9/NGAL complex), 26.2% (NGAL), 23.5% (VEGF), 57.3% (VEGF-R1), and 7.2% (total protein). ICCs resulting from these duplicate values were 0.837 (MMP-2), 0.990 (MMP-9), 0.972 (MMP-9/NGAL complex), 0.971 (NGAL), 0.980 (VEGF), 0.793 (VEGF-R1), and 0.997 (total protein).

Figure 1a and Figure 1b display biomarker concentration (ng/mL or pg/mL) and normalized concentrations (pg/ μ g), respectively, by cohort and sex. Although boxplots suggest differences in median levels for some proteins among cohorts, large variability in measurements largely prevented such differences from reaching statistical significance. Nonetheless, urinary VEGF classic concentration was significantly higher in female UCPPS than HC (mean UCPPS=3.72, mean HC=3.46; $p=0.0076$). ROC analysis suggested, however, that VEGF did not distinguish UCPPS participants from HC (AUC=0.58, Figure 2a). Males with UCPPS had significantly higher levels of normalized VEGF (mean UCPPS = 1.78, mean HC = 0.98, $p<0.0001$), VEGF-R1 (mean UCPPS=-0.33, mean HC=-1.25,

p=0.004), and MMP-9 (mean UCPPS=2.16, mean HC=1.22, p=0.001) than male HC. In multivariable analysis these three markers fairly distinguished UCPPS from HC (AUC=0.68, Figure 2b).

Scatterplots suggested a positive association of concentration and normalized concentration with pain severity and urinary severity for several candidate proteins (eg. MMP9/NGAL and pain severity (Figure 3); urinary severity and other candidate proteins not shown). Tables 2a and 2b show the complete results of univariable and multivariable linear regression, respectively, of the two primary measures of UCPPS symptom severity³⁸ with respect to concentrations of each of the six candidate biomarkers within the UCPPS patient cohort, stratified by sex. In UCPPS females, pain severity was significantly positively associated with concentrations of all biomarkers except NGAL (MMP-2 p=0.0113, MMP-9 p=0.0094, MMP-9/NGAL complex p<.0001, VEGF p <.0001, VEGF-R1 p=0.0012), and urinary severity was significantly positively associated with concentrations of all biomarkers except VEGF-R1 in univariable analysis (MMP-2 p=0.0004, MMP-9 p=0.0012, MMP9/NGAL p <.0001, NGAL p=0.0149, VEGF p <.0001). After adjustment for other markers in multivariable analysis, concentrations of MMP-9/NGAL complex, VEGF, and VEGF-R1 remained significantly positively associated with pain severity (MMP-9/NGAL complex p=0.0013, VEGF p=0.0169, VEGF-R1 p=0.0092), and only MMP-9/NGAL complex was significantly positively associated with urinary severity in females (p=0.0211). In males pain severity was significantly positively associated with the classic concentration of MMP-9 (p=0.0006) and MMP-9/NGAL complex (p=0.0007), and urinary severity was significantly positively associated with the classic concentrations of MMP-9 (p=0.0001), MMP-9/NGAL complex (p=0.0001), and VEGF-R1 (p=0.0107) (Table 2a). In multivariable analysis none of these markers were associated with pain or urinary severity in males, likely due in part to the apparent collinearity among markers (Table 2b, $\rho=0.68$ MMP9 and MMP9/NGAL complex; $\rho=0.16$ MMP9 and VEGF-R1; $\rho=0.22$ MMP9/NGAL complex and VEGF-R1 among male UCPPS).

Tables 3a and 3b show analogous regression analyses for normalized concentrations (pg/ μ g). In females, pain and urinary symptom severity at baseline were each significantly positively associated with MMP-9/NGAL complex (p<0.0001). Further, increased MMP-2 and VEGF were significantly associated with greater urinary (p=0.0074) and pain severity (p=0.0127), respectively. In multivariable analysis MMP-9/NGAL complex remained significantly positively associated with pain severity in females (p=0.0001). Similarly, in the multivariable model for urinary severity in females, MMP-9/NGAL complex was significantly associated with symptom severity (p=0.0004). In male UCPPS participants, no significant correlates of pain and urinary severity were found among normalized concentration levels of candidate biomarkers in univariable analysis, however total urinary protein was positively associated with pain severity (p=0.0042).

Conclusion

There is a lack of understanding of the etiology and risk factors of UCPPS and diagnosis still relies primarily on patient reported symptoms.¹ It was previously believed that the symptoms of UCPPS were primarily seen in women. However, emerging data from the

MAPP Network has shown that a large percentage of men with pelvic pain also have similar bladder symptoms⁴⁰. Unfortunately, to date, patient therapies are generally ineffective¹.

To identify non-invasive protein biomarkers for UCPPS that may inform underlying pathophysiology and provide targets for further clinical evaluation and hypothesis testing, the extensive resources and expertise of the MAPP Network have been leveraged to perform the largest UCPPS non-invasive biomarker analysis to date involving biological samples from over 491 individuals. Ultimately, identification and subsequent validation of such non-invasive, unbiased and accurate biomarkers may aid in identifying the presence of this syndrome, reveal clinically relevant patient sub-groups, monitor disease progression and therapeutic efficacy; predict flares, and objectively assess hallmarks of this syndrome, such as pain severity, urinary severity and other clinical manifestations. The results of this MAPP Network biomarker study provide important insights for addressing such long term goals through demonstrating that males with UCPPS had significantly higher normalized protein concentration of VEGF, VEGF-R1, and MMP-9 than healthy male controls. Furthermore, males with UCPPS had significantly higher concentrations of VEGF than female healthy controls. Additionally, pain and urinary symptoms were found to be positively associated with the MMP9/NGAL complex in women.

While the underlying mechanisms of UCPPS are not understood, inflammation, abnormal vascular activation, fibrosis and extracellular matrix remodeling have all been suggested to be contributing factors. Increased levels of urinary VEGF, as well as MMP-9, detected in UCPPS may reflect the upregulation of these pathways in patients. For instance, VEGF is known to regulate endothelial activation via upregulation of MMP-2 and MMP-9 in several cell types. MMP-2, in turn, may regulate VEGF expression on a transcriptional level via an integrin/PI3-kinase-dependent pathway⁴¹. Increased VEGF has been reported in IC/BPS and VEGF levels were reduced after onabotulinumtoxinA injections and hydrodistention suggesting that VEGF may contribute to the pathogenesis⁴². During angiogenesis, endothelial cells are guided into a vascular area by macromolecules such as VEGF. VEGF binds and activates 2 tyrosine kinase receptors (VEGF-R1 and VEGF-R2) and plays a role in migration of inflammatory cells and inflammation-induced lymphangiogenesis and angiogenesis⁴³. Of importance in UCPPS may be the relationship of VEGF to hypoxia and ischemia, a stimulus for angiogenesis^{44, 45}. A relative ischemia in the bladder of IC/BPS patients during filling compared to controls has been reported⁴⁶ and the density of blood vessels in the bladder subepithelium of IC/BPS patients is decreased⁴⁷. In parallel to UCPPS in humans, animal models of chronic bladder ischemia from partial bladder outlet obstruction results in increased VEGF expression⁴⁸. Bladder epithelial cells taken from patients with overactive bladder symptoms (urinary frequency and urgency), as opposed to asymptomatic patients, respond to stretch stimuli by a 1.5 fold increase in hypoxia-inducible factors 1 α and 2 α and a greater than 3-fold increase in VEGF⁴⁹. Similarly, expression of HIF-1 α and VEGF are increased in the umbrella cells of IC/BPS patients. Finally, macrophages stimulated in low oxygen environment also secrete soluble VEGFR1⁵⁰. Identifying the factors that contribute to hypoxia/ischemia in UCPPS could reveal potential therapeutic targets.

In a murine stress model, in addition to VEGF release, bladder vascular permeability was also increased⁵¹. This relationship may explain the association of worsening symptoms by stress in patients with IC/BPS^{52, 53}. Corticotropin releasing hormone stimulates mast cells to release newly synthesized VEGF which causes vasodilation⁵⁴. The effect is mediated through CRH receptor 2 (CRH-R2)⁵¹ and elevated VEGF levels may explain this association. In men, receptors for VEGF are present not only on endothelial cells, but also in prostate cancer and glandular and basal cells in BPH tissue specimens⁵³⁻⁵⁶. In prostate cancer cell lines, inflammation results in increased levels of MMP-9⁵⁷. VEGF is also an immunosuppressive cytokine^{58, 59}.

In addition to roles in inflammation and angiogenesis, VEGFR1 and MMP-9 are implicated in pain regulation. VEGFR1 contributes to modulating the response to VEGFR2⁵⁸ and has recently been described to influence pain. In mouse models, injection of VEGFA produces no spontaneous pain response, but animals develop significant hypersensitivity to graded von Frey filaments and noxious heat stimuli. This response was blocked by neutralizing antibodies to VEGFR1 but not by antibodies against VEGFR2. Recently, it has been reported that ligands of the VEGF family can augment pain sensitivity through selective activation of VEGFR1 expressed in sensory neurons in human cancer and mouse models⁶⁰. In these studies, systemic blockade of VEGFR1 prevented tumor-induced nerve remodeling and reduced cancer pain, suggesting that the VEGF/VEGFR1 targeting by antiangiogenic therapies could provide a palliative effect in these models⁶⁰.

MMP-9 plays a role in regulating the acute phase of neuropathic pain. MMP2 is constitutively expressed in many tissues whereas MMP-9 is inducible⁶¹. After sciatic nerve ligation, MMP-9 is induced in less than one day and inhibition of MMP-9 attenuated neuropathic pain at day 1⁶². The mechanism of MMP-9 sensitization is through activation of IL-1 β and microglia⁶¹. Increased MMP-9 levels could reflect ongoing acute neurogenic inflammation.

Another possible contributing factor is the presence of microorganisms. The NIH sponsored CPCRN found that significant bacteria were present in men with CP/CPSP but also in asymptomatic controls⁶³. Although prior history of urinary tract infection is more common in women with IC/PBS from childhood⁶⁴, bacteria are not usually found in the bladder⁶⁵. In the MAPP study, in women with IC/PBS, it was shown that gram negative bacteria bind the TLR-4 receptor and the response to TLR-4 stimulation correlated with pain⁶⁶. Exposure of prostate epithelial cells to LPS, on the outer membrane of gram negative bacteria, resulted in TLR-4-mediated release of both TGF β ₁ and VEGF through activation of NF- κ B⁶⁷. Taken together, specific bacterial stimulation in the proper setting could contribute to increased levels of VEGF regardless of gender.

In the present study, females had increased urinary MMP-9/NGAL complex in correlation with pain and urinary symptoms. The finding of elevated MMP-9/NGAL complex suggests a possible link to bacterial infection. NGAL is rapidly and locally produced in the urinary tract by TLR receptors in response to bacterial exposure⁶⁸. NGAL is involved in protection from bacterial infection by competing for iron with the bacteria and selecting for bacteria with alternative chemistry of siderophores used for iron acquisition⁶⁹. A recurring theme in

patients with IC/BPS is a history of urinary tract infection that was treated, but had persistence of symptoms including pain. Elevated MMP-9/NGAL may signal bacterial involvement in pain and urinary symptoms development. NGAL may also be a marker for ongoing inflammation and perhaps apoptosis.

Interestingly, our analyses demonstrated variability in the utility of particular protein markers depending on whether concentration values or concentration normalized to total protein values were used. Both concentration and normalized concentration values were analyzed because particular proteins values may be independent of overall total protein concentration and, therefore, when normalized, their ability to discriminate is lost. A limitation of our study is the variation of duplicate measurements. CVs of $\leq 20\%$ are desirable for duplicate laboratory values, and for MMP-2, MMP-9/NGAL complex, and VEGF-R1 CVs exceeded 50% while MMP-9, NGAL, and VEGF had CVs of 25.9%, 26.2%, and 23.5%, respectively. Normalized concentrations of MMP-9, VEGF and VEGF-R1 distinguished UCPPS men from healthy controls, but only total protein in the univariate analysis of normalized concentrations and symptom severity was associated with pain severity in men with UCPPS (Table 3a). Elevated levels of total urinary protein may be a reflection of more concentrated urine samples possibly due to decreased fluid intake to limit painful and frequent voiding. Specifically in men, total urinary protein may also reflect additional prostatic secretions that contribute to overall urinary protein. What remains unclear in most urinary biomarker studies, is whether a particular marker is a reflection of the local (bladder or prostate) versus overall systemic changes, or a combination. This lack of specificity may contribute to the variability associated with total urinary protein normalization as total urinary protein can also change based on local injury or inflammation or hydration status. While not all UCPPS patients demonstrate inflammation, these results support the convergence of inflammation, neuropathic pain and infection in UCPPS. Indeed, prior findings from the MAPP Network on the role of the TLR4 receptor response support these relationships⁷⁰.

The non-invasive nature of these biomarkers would allow for frequent and economical testing in a variety of settings and research studies and ultimately in a clinical setting if warranted. In addition to their potential to provide insights into underlying disease mechanisms, these biomarkers may potentially prove useful as targets for development of novel interventions, pending future evaluation. Indeed, FDA-approved drugs that target some of these proteins, such as VEGF, are currently in clinical use⁵⁸. Such markers, once sufficiently validated, and may provide objective correlates to patient-reported symptoms and enhance strategies for multiplexing diverse phenotypic information to predict symptom progression and response to therapy for UCPPS patient sub-groups. Ongoing characterizations of these and other biomarkers are focused on validation and further assessment of relationships with clinical symptoms in a longitudinal study design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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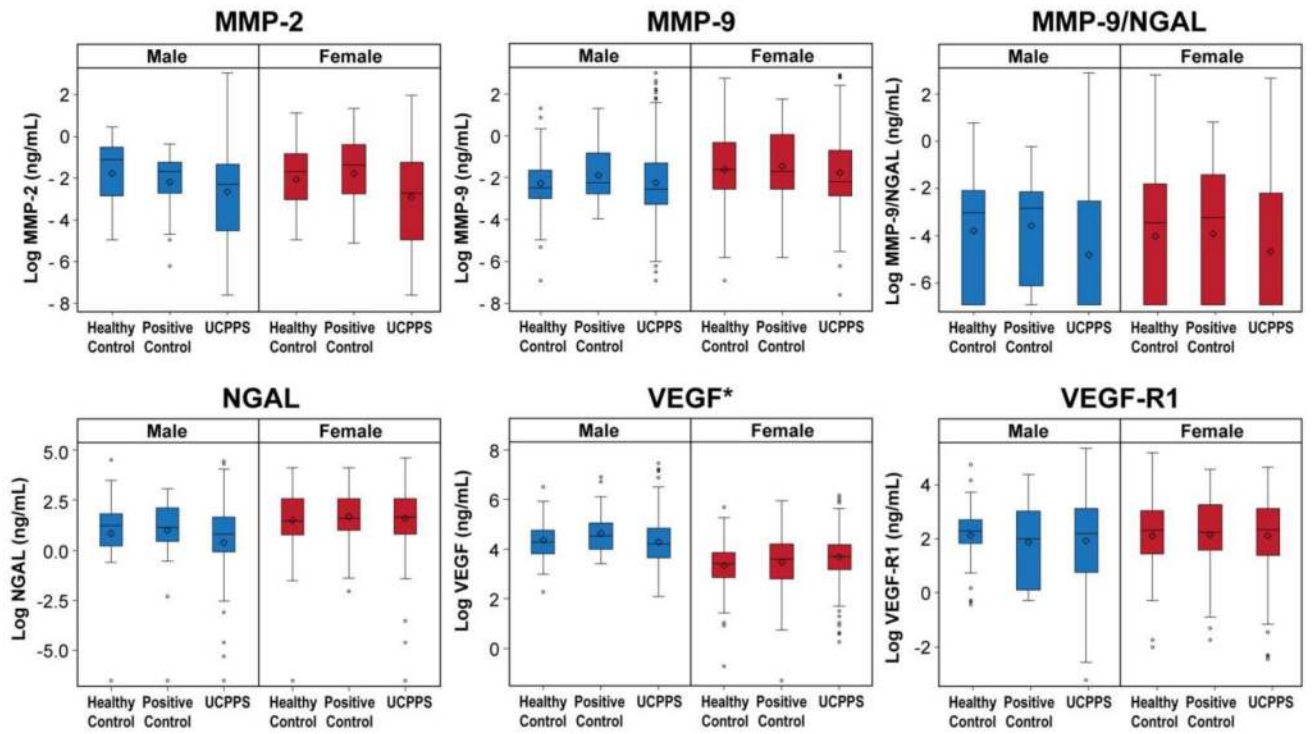
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a



* VEGF concentration was higher in female UCPPS participants than female Healthy Controls ($p=0.0076$)

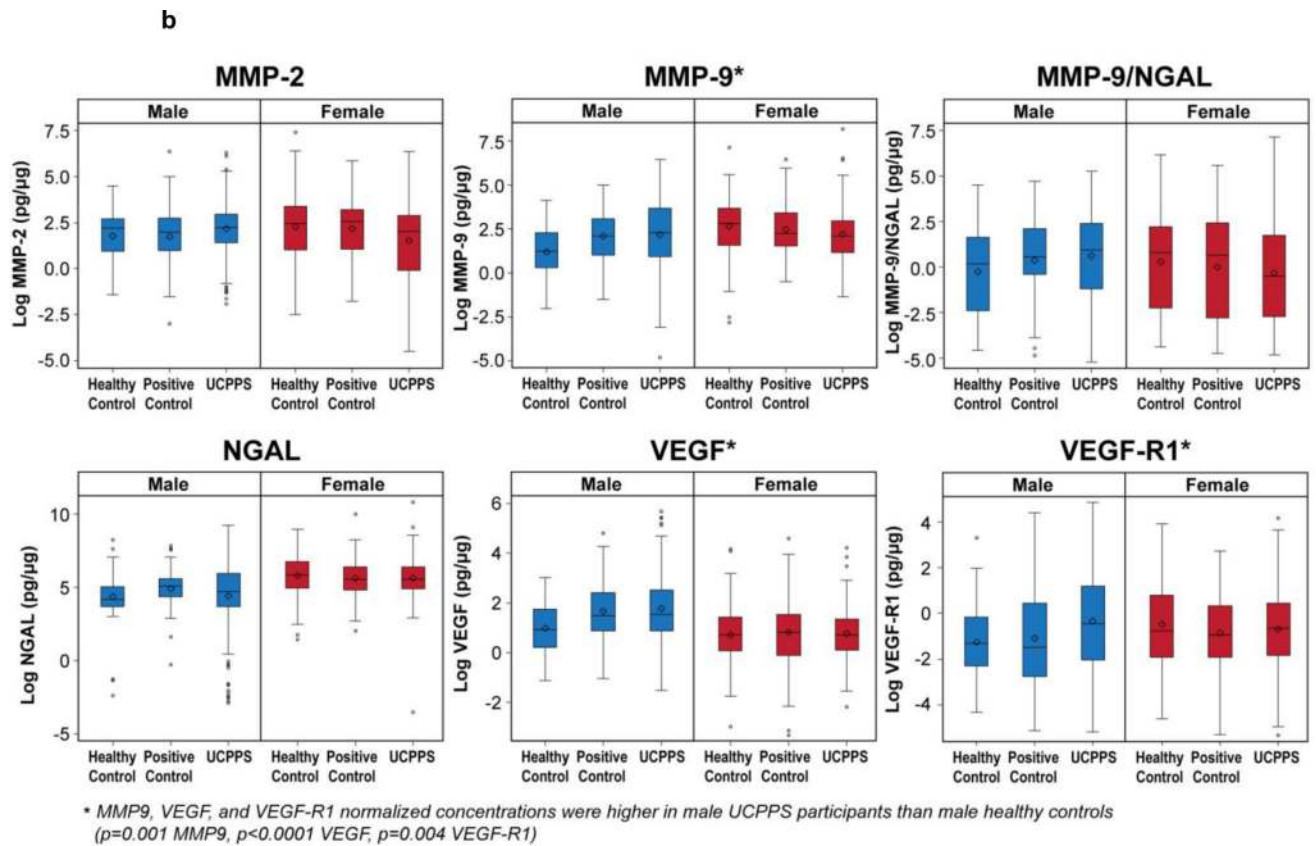


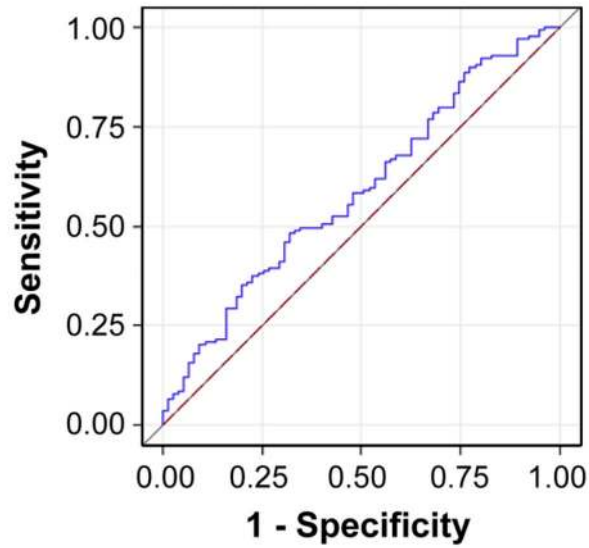
Figure 1.

Log biomarker concentrations (ng/mL or pg/mL, subfigure 1A) or normalized concentrations (pg/μg) are presented for male (blue) and female (red) participants by patient cohort (Healthy Control, Positive Control, or UCPPS). Candidate proteins that differed significantly by cohort within sex are indicated by '*'.

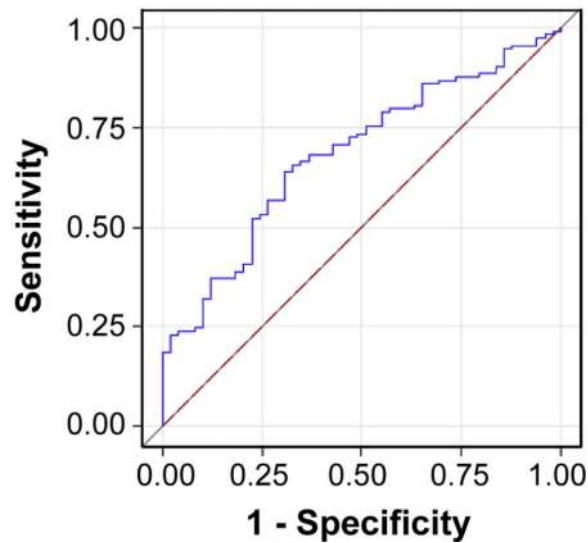
a Biomarker Concentration (ng/mL or pg/mL) by Cohort and Sex

b Biomarker Concentration Normalized to Total Protein (pg/μg) by Cohort and Sex

a.



b.

**Figure 2.**

Receiver Operating Characteristic (ROC) curves are presented for markers that differed significantly between cohorts within sex to demonstrate the capacity for candidate proteins to distinguish Urological Chronic Pelvic Pain Syndrome (UCPPS) participants from Healthy Controls (HC). Satisfactory performance is indicated by an area under the ROC curve (AUC) of 0.70. Subfigure 2A shows the potential of candidate protein VEGF concentration to distinguish between female UCPPS and HC. Results are based on a logistic regression model with UCPPS vs HC as the outcome and VEGF concentration as the predictor, fit among female participants only. Subfigure 2B shows the potential of normalized

concentrations of MMP9, VEGF, and VEGF-R1 to distribution between male UCPPS and male HC. Results are based on a multivariable logistic regression model with UCPPS vs. HC as the outcome and normalized MMP9, VEGF, and VEGFR1 as predictors, fit among male participants only.

a. ROC Analysis of VEGF (ng/mL) for Discriminating UCPPS versus Healthy Control Females, AUC=0.58

b. ROC Analysis of MMP-9, VEGF, and VEGF-R1 (pg/ μ g) for Discriminating UCPPS versus Healthy Control Males, AUC=0.68

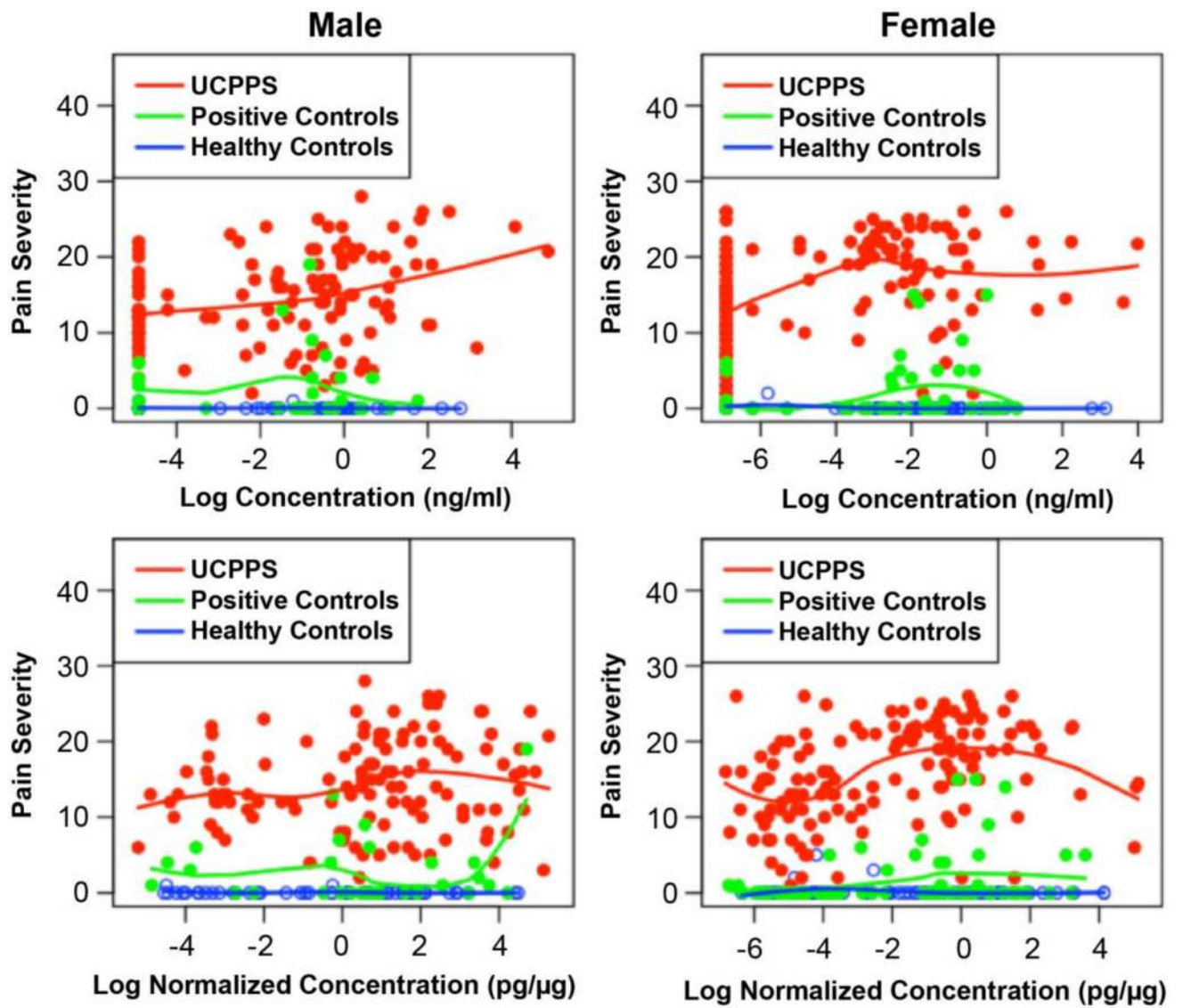


Figure 3.

Pain severity is plotted against MMP9/NGAL log concentration (ng/ml, top) or log normalized concentration (pg/μg, bottom) separately for male (left) and female (right) Urological Chronic Pelvic Pain Syndrome participants (UCPPS, red), Positive Controls (PC, green), and Healthy Controls (HC, blue). Trend lines were determined by locally weighted scatterplot smoothing.

Pain Severity and MMP9/NGAL by Cohort and Sex

Table 1

Characteristics of Sample and Study Groups

Variable	Category	UCPPS				Healthy Controls (HC)				Positive Controls (PC)			
		Included ¹	Not Included	Total	P value	Included ¹	Not Included	Total	P value	Included ¹	Not Included	Total	P value
N		259	165	424		125	290	415		107	92	199	
Age	Mean (SD)	43.3 (14.7)	43.5 (15.8)	43.4 (15.1)	0.918	42.5 (13.3)	39.7 (14.3)	40.5 (14.1)	0.062	41.6 (13.1)	41.3 (14.4)	41.5 (13.7)	0.899
Duration of UCPPS Symptoms*	Mean (SD)	9.7 (11.6)	6.6 (8.6)	8.5 (10.6)	0.002	-	-	-	-	-	-	-	-
Sex*	Female	139 (53.7%)	94 (57.0%)	233 (55.0%)	0.505	75 (60.0%)	158 (54.5%)	233 (56.1%)	0.299	75 (70.1%)	82 (89.1%)	157 (78.9%)	0.001
	Male	120 (46.3%)	71 (43.0%)	191 (45.0%)		50 (40.0%)	132 (45.5%)	182 (43.9%)		32 (29.9%)	10 (10.9%)	42 (21.1%)	
Race	White	229 (88.4%)	145 (87.9%)	374 (88.2%)	0.859 ²	90 (72.0%)	226 (77.9%)	316 (76.1%)	0.810	76 (71.0%)	73 (79.3%)	149 (74.9%)	0.513
	Black	10 (3.9%)	6 (3.6%)	16 (3.8%)		16 (12.8%)	32 (11.0%)	48 (11.6%)		12 (11.2%)	10 (10.9%)	22 (11.1%)	
	Hispanic	19 (7.4%)	9 (5.5%)	28 (6.6%)	0.441	8 (6.4%)	27 (9.3%)	35 (8.4%)	0.328	10 (9.4%)	3 (3.3%)	13 (6.6%)	0.080
Ethnicity	Non-Hispanic	239 (92.6%)	156 (94.5%)	395 (93.4%)		117 (93.6%)	263 (90.7%)	380 (91.6%)		96 (90.6%)	89 (96.7%)	185 (93.4%)	
	Any	111 (42.9%)	51 (30.9%)	162 (38.2%)	0.014	0 (0.0%)	0 (0.0%)	0 (0.0%)		107 (100.0%)	92 (100.0%)	199 (100.0%)	
	None	148 (57.1%)	114 (69.1%)	262 (61.8%)		125 (100.0%)	290 (100.0%)	415 (100.0%)		0 (0.0%)	0 (0.0%)	0 (0.0%)	
Pain* Severity	Mean (SD)	15.2 (6.1)	14.7 (4.7)	15.0 (5.6)	0.353	0.1 (0.6)	0.4 (1.2)	0.3 (1.0)	0.003	1.8 (3.7)	5.8 (5.5)	3.6 (5.0)	0.000
Urinary* Severity	Mean (SD)	12.7 (6.6)	12.5 (5.5)	12.6 (6.2)	0.767	1.6 (2.0)	3.5 (3.1)	2.9 (2.9)	0.000	4.4 (4.4)	8.8 (6.0)	6.4 (5.6)	0.000
BPS⁴ Type	BPS: No	48 (18.5%)	26 (15.8%)	74 (17.5%)	0.463	117 (93.6%)	268 (92.4%)	385 (92.8%)	0.669	83 (77.6%)	58 (63.0%)	141 (70.9%)	0.025
	BPS: Yes	211 (81.5%)	139 (84.2%)	350 (82.5%)		8 (6.4%)	22 (7.6%)	30 (7.2%)		24 (22.4%)	34 (37.0%)	58 (29.1%)	
Pain Region⁵	Pelvic Pain & Beyond	197 (76.1%)	119 (72.1%)	316 (74.5%)	0.364	48 (38.4%)	127 (43.8%)	175 (42.2%)	0.307	85 (79.4%)	77 (83.7%)	162 (81.4%)	0.442
	Pelvic Pain only	62 (23.9%)	46 (27.9%)	108 (25.5%)		77 (61.6%)	163 (56.2%)	240 (57.8%)		22 (20.6%)	15 (16.3%)	37 (18.6%)	

^{**} denotes variables for which there was a significant difference between participants that were selected for the substudy compared to those who were not selected for the substudy in at least one participant cohort (UCPPS, HC, or PC)

¹ Participants were selected for this substudy by stratified random sampling within sex and cohort. UCPPS participants with the most severe urologic pain and urinary symptoms and HC and PC with the least severe symptoms were more likely to be selected to maximize the likelihood of detecting differences between UCPPS and control cohorts.

² The comparison of race between included and not included subjects was based on a 7-level race variable within the categories of white, black, Asian, multi-race, native Hawaiian, other, and unknown. White and black are shown for brevity.

³ NUAS denotes the presence of a nonurological associated syndrome, defined as Chronic Fatigue Syndrome, Irritable Bowel Syndrome, or Fibromyalgia

⁴ BPS Type indicates Bladder Pain Syndrome type. Patients classified as 'yes' reported the presence of painful filling or painful urgency on the RAND Interstitial Cystitis Epidemiology Study criteria³⁸

Pain region indicates the widespreadness of pain. 'Pelvic Pain only' indicates the presence of pain in the pelvic region only, and 'Pelvic Pain and Beyond' indicates pain outside of the pelvic region. All participants were classified as pelvic pain only by default and designated as pelvic pain and beyond by indicating pain outside of the pelvic region on a modified version of the Brief Pain Inventory short form³⁶.

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Table 2

a. Univariable Regression Analyses for Symptoms and Biomarker Concentration by Gender within UCPPS

Outcome	Biomarker	FEMALE				MALE			
		β	95% CI	P value	R-Square	β	95% CI	P value	R-Square
PAIN SEVERITY	MMP-2 (ng/mL)	0.684	(0.16, 1.21)	0.0113	0.0463	0.214	(-0.48, 0.90)	0.5406	0.0032
	MMP-9 (ng/mL)	0.988	(0.25, 1.73)	0.0094	0.0496	0.979	(0.43, 1.53)	0.0006	0.0977
	MMP-9/NGAL (ng/mL)	0.919	(0.56, 1.28)	<.0001	0.1604	0.725	(0.31, 1.14)	0.0007	0.0955
	NGAL (ng/mL)	0.774	(-0.10, 1.65)	0.0818	0.0235	0.157	(-0.20, 0.52)	0.3874	0.0064
	VEGF (pg/mL)	2.392	(1.36, 3.42)	<.0001	0.1334	0.633	(-0.39, 1.65)	0.2211	0.0129
	VEGF-R1 (pg/mL)	1.452	(0.58, 2.32)	0.0012	0.0741	0.447	(-0.28, 1.18)	0.2280	0.0123
URINARY SEVERITY	MMP-2 (ng/mL)	0.995	(0.46, 1.53)	0.0004	0.0910	0.641	(-0.15, 1.43)	0.1099	0.0217
	MMP-9 (ng/mL)	1.275	(0.51, 2.04)	0.0012	0.0763	1.281	(0.65, 1.91)	0.0001	0.1248
	MMP-9/NGAL (ng/mL)	0.917	(0.53, 1.30)	<.0001	0.1449	0.938	(0.48, 1.40)	0.0001	0.1233
	NGAL (ng/mL)	1.143	(0.23, 2.06)	0.0149	0.0462	0.301	(-0.11, 0.71)	0.1515	0.0176
	VEGF (pg/mL)	2.251	(1.15, 3.35)	<.0001	0.1086	-0.217	(-1.41, 0.97)	0.7179	0.0011
	VEGF-R1 (pg/mL)	0.910	(-0.02, 1.84)	0.0563	0.0269	1.080	(0.26, 1.90)	0.0107	0.0544

b. Multivariable Regression Analyses for Symptoms and Biomarker Concentration by Gender within UCPPS

Outcome	Biomarker	FEMALE (Models 5 and 7)				MALE (Models 1 and 3)			
		β	95% CI	P value	R-Square	β	95% CI	P value	R-Square
PAIN SEVERITY	MMP-2 (ng/mL)	-0.13	(-0.69, 0.42)	0.6376	-	-	-	-	-
	MMP-9 (ng/mL)	-0.41	(-1.3, 0.53)	0.3909	0.71	(-0.05, 1.47)	0.0660	0.0660	
	MMP-9/NGAL (ng/mL)	0.87	(0.35, 1.39)	0.0013	0.33	(-0.24, 0.91)	0.2488	0.2488	
	NGAL (ng/mL)	-	-	-	-	-	-	-	-
	VEGF (pg/mL)	1.49	(0.27, 2.70)	0.0169	-	-	-	-	
	VEGF-R1 (pg/mL)	1.15	(0.29, 2.02)	0.0092	-	-	-	-	
URINARY SEVERITY	MMP-2 (ng/mL)	0.43	(-0.16, 1.02)	0.1506	-	-	-	-	
	MMP-9 (ng/mL)	-0.10	(-1.2, 0.99)	0.8562	0.69	(-0.16, 1.54)	0.1119	0.1119	
	MMP-9/NGAL (ng/mL)	0.70	(0.11, 1.30)	0.0211	0.52	(-0.13, 1.17)	0.1160	0.1160	
	NGAL (ng/mL)	0.26	(-0.68, 1.21)	0.5804	-	-	-	-	

b. Multivariable Regression Analyses for Symptoms and Biomarker Concentration by Gender within UCPPS							
Outcome	Biomarker	FEMALE (Models 5 and 7)			MALE (Models 1 and 3)		
		β	95% CI	P value	β	P value	R-Square
	VEGF (pg/mL)	1.10	(-22, 2.41)	0.1018	-	-	-
	VEGF-RI (pg/mL)	-	-	-	0.69	(-12, 1.51)	0.0957

β denotes the change in mean symptom severity associated with a 1-unit increase in the log-transformed classic concentration

FDR thresholds out of 6 tests are 0.05, 0.042, 0.033, 0.025, 0.016, 0.0083. Significant values are bolded for emphasis.

β denotes the change in mean symptom severity associated with a 1-unit increase in the log-transformed concentration, holding other markers constant. Significant values are bolded for emphasis.

Table 3

a. Univariable Regression Analyses for Symptoms and Biomarker Normalized Concentration by Gender within UCPPS

Outcome	Biomarker	FEMALE			MALE				
		β	95% CI	P value	R-Square	β	95% CI	P value	R-Square
PAIN SEVERITY	TOTAL PROTEIN	0.572	(-0.30, 1.45)	0.1992	0.0120	0.982	(0.32, 1.65)	0.0042	0.0674
	MMP-2 (pg/ μ g)	0.440	(-0.07, 0.95)	0.0906	0.0209	-0.644	(-1.26, -0.02)	0.0422	0.0345
	MMP-9 (pg/ μ g)	0.508	(-0.17, 1.18)	0.1390	0.0164	0.216	(-0.30, 0.73)	0.4056	0.0060
	MMP-9/NGAL (pg/ μ g)	0.845	(0.48, 1.21)	<.0001	0.1349	0.313	(-0.10, 0.72)	0.1335	0.0193
	NGAL (pg/ μ g)	0.386	(-0.37, 1.15)	0.3174	0.0078	-0.130	(-0.54, 0.28)	0.5318	0.0034
	VEGF (pg/ μ g)	1.280	(0.28, 2.28)	0.0127	0.0445	-0.692	(-1.42, 0.04)	0.0627	0.0295
	VEGF-R1 (pg/ μ g)	0.366	(-0.22, 0.95)	0.2198	0.0111	-0.257	(-0.71, 0.20)	0.2638	0.0106
	TOTAL PROTEIN	0.593	(-0.32, 1.51)	0.2033	0.0120	0.859	(0.08, 1.64)	0.0308	0.0392
	MMP-2 (pg/ μ g)	0.723	(0.20, 1.25)	0.0074	0.0523	-0.183	(-0.91, 0.54)	0.6179	0.0021
	MMP-9 (pg/ μ g)	0.714	(0.01, 1.41)	0.0455	0.0300	0.521	(-0.07, 1.11)	0.0826	0.0262
URINARY SEVERITY	MMP-9/NGAL (pg/ μ g)	0.837	(0.45, 1.23)	<.0001	0.1200	0.545	(0.08, 1.01)	0.0211	0.0454
	NGAL (pg/ μ g)	0.614	(-0.19, 1.42)	0.1325	0.0179	0.091	(-0.38, 0.57)	0.7040	0.0012
	VEGF (pg/ μ g)	1.125	(0.06, 2.19)	0.0378	0.0316	-1.070	(-1.91, -0.23)	0.0126	0.0529
	VEGF-R1 (pg/ μ g)	0.117	(-0.50, 0.74)	0.7092	0.0010	0.038	(-0.49, 0.56)	0.8851	0.0002

b. Multivariable Regression Analyses for Symptoms and Biomarker Normalized Concentration by Gender within UCPPS

Outcome	Independent Variable	FEMALE (Models 13 and 16)		
		β	95% CI	P value
PAIN SEVERITY	MMP-2 (ng/mL)	-	-	-
	MMP-9 (ng/mL)	-	-	-
	MMP-9/NGAL (ng/mL)	0.77	(0.38, 1.16)	0.0001
	NGAL (ng/mL)	-	-	-
	VEGF (pg/mL)	0.59	(-0.42, 1.61)	0.2502
	VEGF-R1 (pg/mL)	-	-	-
URINARY SEVERITY	MMP-2 (ng/mL)	0.35	(-0.18, 0.89)	0.1922
	MMP-9 (ng/mL)	-	-	-

b. Multivariable Regression Analyses for Symptoms and Biomarker Normalized Concentration by Gender within UCPPS

Outcome	Independent Variable	FEMALE (Models 13 and 16)		
		β	95% CI	P value
	MMP-9/NGAL (ng/mL)	0.75	(0.34, 1.17)	0.0004
	NGAL (ng/mL)	-	-	-
	VEGF (pg/mL)	-	-	-
	VEGF-R1 (pg/mL)	-	-	-

β denotes the change in mean symptom severity associated with a 1-unit increase in the log-transformed normalized concentration FDR thresholds out of 7 tests: 0.0500, 0.0429, 0.0357, 0.0286, 0.0214, 0.0143, 0.007. Significant values are bolded for emphasis.

β denotes the change in mean symptom severity associated with a 1-unit increase in the log-transformed normalized concentration, holding other markers constant. Significant values are bolded for emphasis.