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Urinary Chemokines as Noninvasive Predictors of Ulcerative Interstitial Cystitis

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Abstract

Purpose—Based on basic research findings an increase in chemokines and cytokines (CXCL-1 and 10, nerve growth factor and interleukin-6) is considered responsible for inflammation and afferent sensitization. In this cross-sectional study we tested the hypothesis that select chemokines are increased in the urine of patients with ulcerative and nonulcerative interstitial cystitis/painful bladder syndrome.

Materials and Methods—Midstream urinary specimens were collected from 10 patients with ulcerative and nonulcerative interstitial cystitis/painful bladder syndrome, respectively, and from 10 asymptomatic controls. Urinary levels of 7 cytokines were measured by a human cytokine/chemokine assay. Nerve growth factor was measured by enzyme-linked immunosorbent assay.

Results—Urinary levels of most chemokines/cytokines were tenfold to 100-fold lower in asymptomatic controls vs patients with ulcerative and nonulcerative interstitial cystitis/painful bladder syndrome. Univariate comparison of 8 tested proteins in the ulcerative vs nonulcerative groups revealed a significant fivefold to twentyfold increase in CXCL-10 and 1, interleukin-6 and nerve growth factor (ANOVA $p < 0.001$).

Conclusions—Differential expression of chemokines in ulcerative and nonulcerative subtypes of interstitial cystitis/painful bladder syndrome suggests differences in paracrine signaling between the 2 entities.

Keywords

urinary bladder; cystitis; interstitial; chemokines; cytokines; ulcer

Interstitial cystitis/PBS is a chronic, painful bladder disease characterized by pelvic pain and irritative voiding symptoms. The subjective perception of the bladder as the source of pain is a necessary criterion for many definitions of IC/PBS. Symptoms associated with IC/PBS can also overlap with those of conditions such as endometriosis, recurrent urinary tract infections, chronic pelvic pain, overactive bladder and vulvodynia.[1] All of these features combine to make IC/PBS a difficult disease to diagnose and treat.

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Objective disease specific information on patients is only available through expensive, invasive procedures such as cystoscopy and tissue biopsy, which can also categorize IC/PBS into the ulcerative or nonulcerative subtype.[2] To avoid the expense and risk of these procedures the development of noninvasive biomarkers to discriminate ulcerative from nonulcerative IC/PBS is a high priority.

Previous studies highlighted important clinical and histopathological distinctions between the 2 subtypes[3,4] and led us to suspect that the 2 subtypes may not be different stages of the same disease. Patients with ulcerative and nonulcerative IC/PBS differ in clinical presentation and in the response to treatment. Those with ulcerative IC/PBS tend to have symptoms for a shorter period, perhaps due to older age at diagnosis. The presumed progression from nonulcerative to ulcerative disease is yet to be documented.[5] Patients with nonulcerative IC/PBS also have significantly more comorbid pain syndromes and other conditions, and usually respond better to systemic therapy while only cautery of the ulcers (localized therapy) tends to significantly improve symptoms in those with the ulcerative type.[5] The 2 subtypes may manifest from different pathophysiological responses to a similar stressor but it is also possible that the 2 subtypes are uniquely different conditions.

To date gene expression studies of bladder tissue from patients have corroborated the findings of histological studies of tissue biopsies from patients with ulcerative IC/PBS.[6,7] Many up-regulated genes were expressed in leukocytes, suggesting that immune and inflammatory responses after leukocyte invasion into the bladder wall is a dominant feature of ulcerative IC.[6] Gene expression studies in nonulcerative IC/PBS cases revealed urothelial abnormalities, including tight junction proteins, neurokinin receptors and acid sensing channels.[8,9] These widely reported findings support the notion that the disease pattern of IC/PBS may be similar to patterns of immune system, lymphatic and autoimmune diseases.

Chemokine expression is a necessary preceding event for the infiltration and cell adhesion into inflamed tissue.[10] This innate feature of chemokines can serve as the basis for their usefulness as biomarkers for the phenotypic characterization of IC/PBS.[7] Increased mRNA expression of CXCR3 receptor binding chemokines (CXCL-9 to 11) was previously detected in bladder biopsy studies.[7] We previously reported an increase in select cytokines and chemokines in the urine of patients with IC/PBS.[11]

Based on key differences already noted between the subtypes and our clinical observations of patients with ulcerative IC/PBS we hypothesized that identifying urinary biomarkers associated with each may provide new insight into the subtypes as well as the IC/PBS condition overall. Identifying condition specific biomarker panels also meets an ongoing need for simple, accurate, noninvasive methods to detect the disease.

In the current study we investigated whether a multimarker panel of 2 or 3 chemokines in urine could noninvasively differentiate ulcerative from nonulcerative IC/PBS.

MATERIALS AND METHODS

Midstream urinary specimens were collected after receiving informed consent from 10 patients with ulcerative IC/PBS, 10 with nonulcerative IC/PBS and 10 gender matched, asymptomatic outpatient controls with no history of IC, recurrent urinary tract infection, bladder cancer, kidney disease or incontinence (see table). The protocol for the cross-sectional prospective study was approved by the institutional review board.

Ulcer in the ulcerative group and its absence in the nonulcerative group of patients with IC/PBS was confirmed by cystoscopy at urine collection. The diagnosis of IC/PBS was based on National Institute of Diabetes and Digestive and Kidney Diseases criteria.[1] Other

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conditions commonly confused with IC/PBS were ruled out. No patient had hydrodistention at least 12 weeks in duration or urinary tract infection when screened before urine collection.

Median age was 61.5 years in the ulcerative IC/PBS group, 48.5 years in the nonulcerative group and 46 years in the control group. Gender distribution was 3 males in the control group, 2 in the ulcerative group and 1 in the nonulcer group. Controls included adults recruited from the community who denied recurrent urinary tract infections, kidney or bladder stones, bladder cancer, kidney disease, urinary incontinence, or IC/PBS diagnosis or symptoms.

All participants completed the validated ICSI problem index at urine collection to assess symptoms of bladder associated pain, abnormal urinary urgency and frequency. Fresh voided urine was immediately centrifuged for 10 minutes at 2,000 rpm at room temperature to remove debris, aliquoted and stored at −80C before chemokine analysis.

Chemokine Assays

On the day of analysis frozen urinary samples were quickly thawed. Seven chemokines/cytokines in urine were measured with the Milliplex® Human Cytokine/Chemokine immunoassay by a previously described procedure.[12] We used an automated Bio-Plex® Luminex® 200 IS System immunoassay analyzer, a continuous, random access instrument that performs automated chemiluminescent immunoassays. We assayed chemokines using commercially available microspheres (Millipore®) according to manufacturer instructions.

Microspheres of defined spectral properties conjugated to antibodies directed against CXCL-1 and 10, IL-6, soluble IL-1ra, MCP-1, RANTES and VEGF were pipetted into 96-well plates. Approximately 2,500 antibody conjugated microspheres per chemokine were added to each well. The protocol for these assays involves mixing 50 μl undiluted urine with 25 μl antibody coated microspheres, overnight incubation in the dark at 4C, washing twice with wash buffer, adding detection antibody, adding 25 μl streptavidin-phycoerythrin solution in the well to form a capture sandwich immunoassay by incubation in the dark for 30 minutes at room temperature, washing twice and then adding 150 μl sheath fluid. Microspheres were aspirated through the flow cell of a dual laser Luminex 200 instrument.

Median fluorescence intensity of microspheres specific for each chemokine was recorded for each well to calculate the chemokine concentration by 5-point logistic regression. Urinary NGF was measured by ELISA using a kit (Promega™) according to manufacturer instructions.

Statistical Analysis

Statistical differences in median chemokine levels between the groups were determined by Kruskal-Wallis non-parametric ANOVA, followed by the Dunn test for multi-group comparisons. The Mann-Whitney U test was used for comparisons of 2 groups. Tests assessed significant differences between groups at 2-tailed $p < 0.05$. Data are shown as the mean ± SEM.

RESULTS

Urine was collected from patients with IC/PBS just before cystoscopy with hydrodistention to evaluate for ulcerative or nonulcerative cystitis. Figure 1 shows representative images from 2 patients per group. Analysis of urine collected from a single void revealed that levels of 5 of the 8 tested chemokines/cytokines in urine were tenfold to 100-fold lower in asymptomatic controls than in patients with IC/PBS. Univariate comparison of 8 tested proteins in the ulcerative and nonulcerative groups revealed a fivefold to twentyfold increase

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in urinary CXCL-1 and 10, NGF and IL-6 in the former group (2-tailed p <0.001, fig. 2). The distribution of all urinary cytokines tested passed the Kolmogorov-Smirnov test for normality.

We found a significant tenfold increase in ulcerative vs nonulcerative cases in CXCL-1 (mean 170 ± 60.55 vs 12.55 ± 3.38 pg/ml) and a fivefold increase in CXCL-10 (684.7 ± 68.10 vs 123.3 ± 45.35 pg/ml) (Kruskal-Wallis ANOVA p <0.001). There was a twentyfold increase in NGF (60.82 ± 25.38 vs 3.5 ± 1.6) and a fivefold increase in IL-6 (16.06 ± 6.99 vs 2.42 ± 1.94 pg/ml). NGF was below the range of detection in 7 of 10 controls and 7 of 10 nonulcerative cases. IL-6 was below the range of detection in 9 controls and 6 nonulcerative cases.

Urinary levels of the remaining proteins, namely MCP-1, sIL-1Ra, RANTES and VEGF, were not significantly increased in the ulcerative IC/PBS group relative to the other groups (see table and fig. 3). Even among these 4 chemokines MCP-1 and IL-1ra were quantitatively higher than RANTES and VEGF. Control levels were also high, which may explain the lack of a significant difference. Two-group comparison of nonulcerative IC/PBS cases and controls revealed a significant increase in CXCL-1 and RANTES in the nonulcerative IC/PBS group (Mann-Whitney U test p <0.01). CXCL-10, sIL-1Ra and MCP-1 levels trending toward significance. The high MCP-1, sIL-1Ra and VEGF levels in the control group agreed with their established role of paracrine messengers under healthy conditions.[13]

Differences in the IC/PBS subtypes were reflected in the patient urinary chemokine profiles. These results lend support to our hypothesis that an increase in chemokines is linked to inflammatory cells, of which the role was previously confirmed in IC/PBS cases.[2]

DISCUSSION

The urinary proteome receives contributions from filtered plasma proteome and from approximately 20% to 25% of all cellular proteins secreted from the kidney and bladder.[14] The quantity of some of these secreted proteins is usually low to merit classification as a biomarker for upper or lower urinary tract disease. In contrast, the level of chemokines secreted in urine from the bladder or kidney is sufficiently high to be detectable by standard laboratory assays. The ability of chemokines to contribute to inflammatory induced changes by inducing chemotaxis, and the activation of mast cells and monocytes in rheumatoid arthritis and Crohn disease was responsible for their qualification as biomarkers.[15]

Chemokines belong to a family of small secreted glycoproteins with a molecular weight of 7 to 10 kDa and with more than 50 ligands.[15] Chemokines are subdivided into 2 major families (CXC and CC) based on the position and number of conserved cysteines, and by the presence of intervening amino acid(s) between the first 2 conserved cysteine residues. The CC class of chemokines, which is distinguished by an adjacent position of the first 2 cysteines, eg MCP-1/CCL2 or RANTES/CCL5, causes chemotactic migration of monocytes, eosinophils, basophils, lymphocytes and mast cells in general but does not act on neutrophils.[16] CXC chemokines can be divided into members that contain the ELR motif in the primary structure motif, such as CXCL-1. They are chemoattractants for neutrophils and potent promoters of angiogenesis. In contrast, members that are inducible by interferons, such as CXCL-10, lack the ELR motif (ELR−) and are potent inhibitors of angiogenesis and chemoattractants for mononuclear cells.[17]

Basic research has revealed that urothelial lesions associated with ulcers result from the infiltration of mononuclear cells and neovascularization (angiogenesis), which is facilitated by growth factors and chemokines.[18] Attracting neutrophils and mononuclear cells to the

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bladder ulcer site requires close interplay of the inflammatory signals presented via chemokines and 7-transmembrane spanning G protein-coupled receptors present on the glycosaminoglycans linked to endothelial cell layers.[15] Angiogenesis is a key component of ulcerative inflammation. The disparate impact on angiogenesis of different chemokines measured in urine warrants further investigation.

The chemokines and cytokines that we measured in urine are produced by detrusor smooth muscles.[16,19] CXCL-10 is produced as a result of the cascading effect induced by interferon-γ on fibro-blasts, epithelia, monocytes and T cells.[20] It is involved in afferent sensitization,[21] which may be involved in the mediation of painful bladder symptoms. Urinary CXCL-10 measured in patients with ulcerative IC/PBS was several fold higher than previously reported in the serum of patients with IC/PBS.[22] This further corroborates bladder tissue as the primary source of CXCL-10. Higher urinary CXCL-10 also agrees with the higher levels of its transcript detected in bladder tissue.[7]

As stated earlier, CXCL-10 is a potent inhibitor of angiogenesis[17] while VEGF has a key stimulatory role in angiogenesis.[23,24] These factors function in autocrine/paracrine pathways to maintain homeostasis, which has been noted in various tissues, including the lung and breast.[23,24] VEGF also has an established role as a paracrine messenger in healthy conditions to maintain vascular homeostasis, including the maintenance and function of mature vascular beds.[23,24] Presumably this also happens in the bladder. The increase in VEGF noted in controls may be explained by an offsetting homeostatic response to the increase in CXCL-10 (fig. 2, *B* and *C*).

The usefulness of CXCL-10 as a reliable drug target for IC/PBS was supported in a preclinical study, in which its blockade by a specific antibody ameliorated chemically induced cystitis.[22] Pain is a shared symptom of ulcerative and nonulcerative IC/PBS. Based on our findings the chemokines that mediate pain may be different in ulcerative and nonulcerative IC/PBS cases. CXCL-10 seems to predominate in mediating the pain symptom of ulcerative IC/PBS while MCP-1 and other chemokines may mediate the pain symptom of nonulcerative IC/PBS by influencing nociceptive fibers.[25]

Study limitations include our few patients and urinary measurement made at only 1 time point. The increased urinary levels in patients with ulcerative IC/PBS reflect the ongoing bladder inflammation noted at the bladder ulcer site. Passive secretion of chemokines from bladder to stored urine is independent of the urinary flow rate downstream of the kidney. Thus, no additional benefit would have accrued from adjusting chemokine levels to creatinine. Adjusting protein to creatinine did not alter the outcome in previous studies in which bladder epithelium derived growth factors were measured in the urine of patients with IC/PBS and controls.[26] Similar results were obtained in series of bladder malignancy and a urinary biomarker.[27]

Higher NGF levels in the ulcerative IC/PBS group agree with previous reports on this topic.[28] The urinary levels of IL-6 that we noted in patients with IC/PBS agreed with reports from other groups.[29] IL-6 levels were comparatively lower than levels of other chemokines (CXCL-1 and 10) (fig. 2). Basic research revealed that chemokines and cytokines (CXCL-1 and 10, NGF and IL-6) have key responsibility for inflammation and afferent sensitization.

The Luminex xMAP technology used in our series applies a sandwich format, as used in traditional ELISA exploiting antibody specificity.[30] This provides higher specificity for chemokines and minimizes cross-reactivity with other urinary proteins. Multiplexing analytes in solution phase through flow cytometry and equilibrium in antibody-antigen reactions is attained more quickly in the solution phase to increase assay efficiency.

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Biomarker proteins detected in urine and other biological fluids are generally assumed to reflect the authentic biochemistry of the disease site with which they remain in intimate contact. They are not generally as subject to imperfect human perceptions or to opinions as the ICSI score. Identifying urinary biomarkers by proteome-wide analysis may provide an unbiased, noninvasive, expeditious discrimination of IC/PBS into ulcerative and nonulcerative groups. In conclusion, differential expression of chemokines in association with ulcerative and non-ulcerative IC/PBS suggests differences in paracrine signaling between the 2 entities.

Acknowledgments

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Abbreviations and Acronyms

CXCL	CXC chemokine ligand
ELR	Glu-Leu-Arg
IC	interstitial cystitis
ICSI	IC Symptom Index
IL	interleukin
IL-1ra	IL-1 receptor antagonist
MCP-1	monocyte chemoattractant protein-1
NGF	nerve growth factor
PBS	painful bladder syndrome
RANTES	regulated on activation, normal T-cell expressed and secreted
sIL-1Ra	soluble IL-1ra
VEGF	vascular endothelial growth factor

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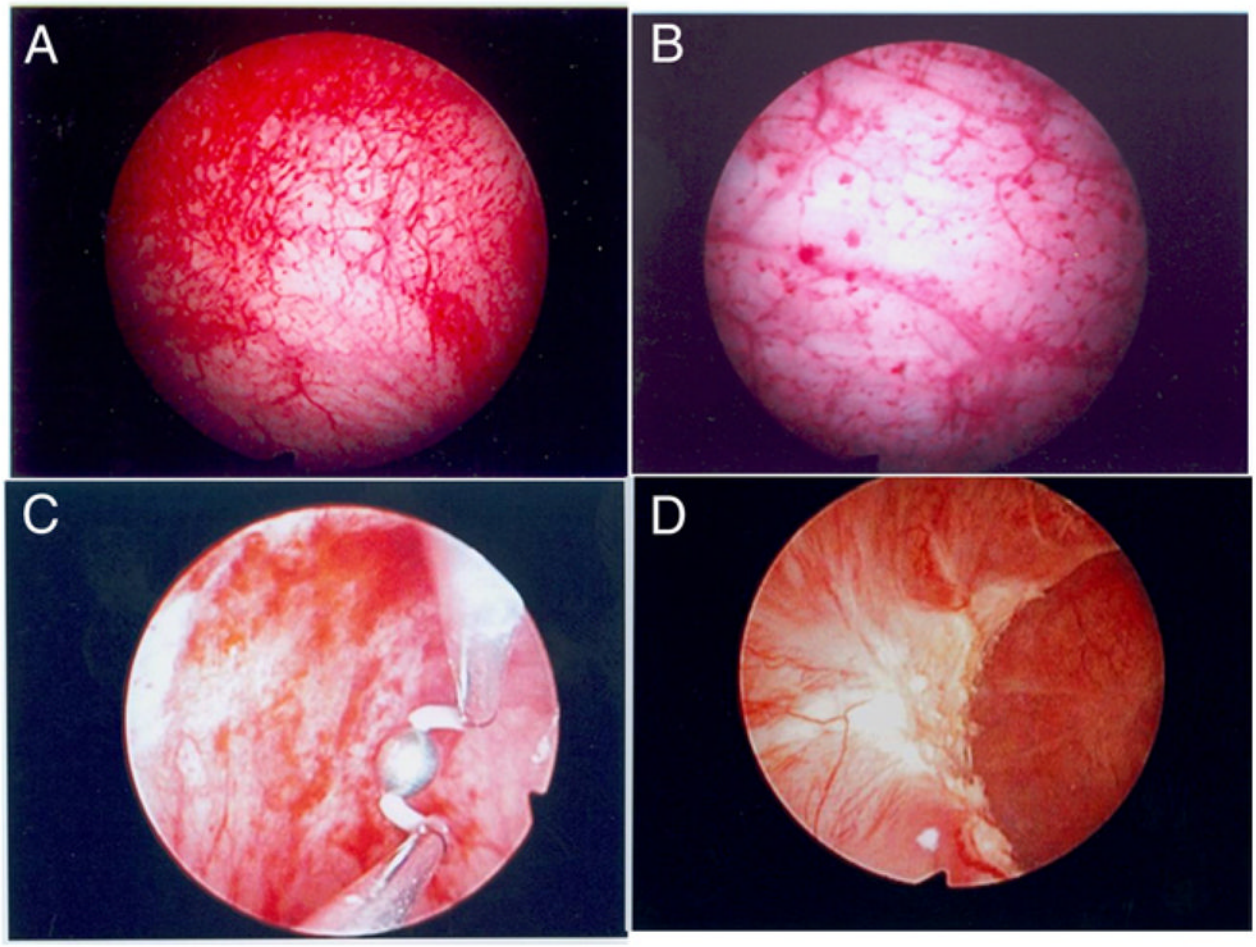

Figure 1.

Representative cystoscopic images from patients with nonulcerative (*A* and *B*) and ulcerative (*C* and *D*) IC/PBS. Urine collected before cystoscopy was used to confirm ulcer, characterized by mucosal bleeding, and inflamed tissue in ulcerative group, and absent ulcer in nonulcerative group. Glomerulations (pinpoint petechial hemorrhages) were noted in nonulcerative group.

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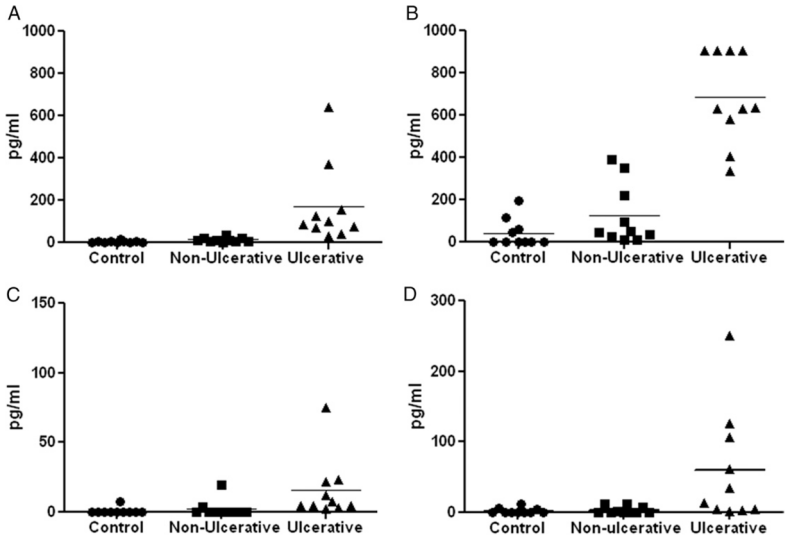

Figure 2.

Urinary CXCL-1 (*A*), CXCL-10 (*B*), IL-6 (*C*) and NGF (*D*) were fivefold to twentyfold, significantly increased in patients with ulcerative IC/PBS vs those with nonulcerative IC/PBS and asymptomatic controls (Kruskal-Wallis ANOVA $p < 0.01$). CXCL-1 was significantly increased in nonulcerative IC/PBS cases vs controls (Mann-Whitney U test $p < 0.01$). NGF and IL-6 were below detection limit in controls. Horizontal bars represent group mean.

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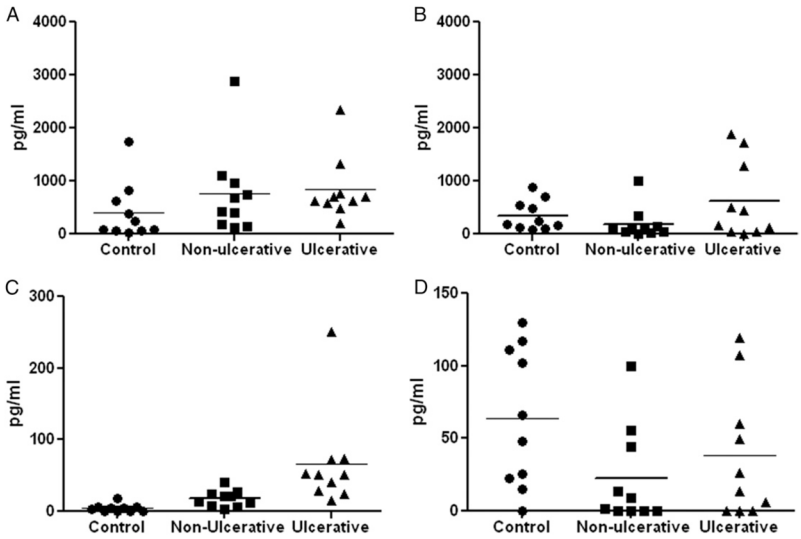

Figure 3.

Urinary MCP-1 (*A*), IL-1ra (*B*), RANTES (*C*) and VEGF (*D*) in patients with ulcerative IC/PBS were not significantly higher than in those with nonulcerative IC/PBS and asymptomatic controls (Kruskal-Wallis ANOVA $p > 0.05$). RANTES was significantly increased in nonulcerative IC/PBS cases vs controls (Mann-Whitney U test $p < 0.01$). Horizontal bars represent group mean.

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Table 1

Table Patient clinical characteristics and chemokine values

	Control	Ulcerative IC/PBS	Nonulcerative IC/PBS
Median ICSI problem index score	0.2	27.5	26.5
Ulcer on cystoscopy	Not applicable	Yes	No
Pain	No	Yes	Yes
Mean ± SEM chemokine (pg/ml):			
CXCL-1	3.54 ± 1.49	170 ± 60.55 *	12.55 ± 3.38
CXCL-10	41.81 ± 20.13	684.7 ± 68.10 *	123.3 ± 45.35
NGF	2.34 ± 1.3	60.82 ± 25.38 *	3.5 ± 1.6
IL-6	0.75 ± 0.71	16.06 ± 6.99 *	2.42 ± 1.94
MCP-1	409.97 ± 162.25	672.7 ± 93.75	527.97 ± 113.96
RANTES	4.53 ± 1.72	66.34 ± 21.42	17.88 ± 3.52
IL-1ra	349.77 ± 85.23	621.96 ± 230.36	191.39 ± 94.38
VEGF	63.67 ± 14.38	42.47 ± 14.3	22.39 ± 10.67

* Kruskal-Wallis nonparametric ANOVA with Dunn test 2-tailed $p < 0.05$.