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Urinary Chemokines CXCL9 and CXCL10 are Non-invasive Markers of Renal Allograft Rejection and BK Viral Infection

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Abstract
Renal transplant recipients require periodic surveillance for immune-based complications such as rejection and infection. Noninvasive monitoring methods are preferred, particularly for children, for whom invasive testing is problematic. We performed a cross-sectional analysis of adult and pediatric transplant recipients to determine whether a urine-based chemokine assay could noninvasively identify patients with rejection amongst other common clinical diagnoses. Urine was collected from 110 adults and 46 children with defined clinical conditions: healthy volunteers, stable renal transplant recipients, and recipients with clinical or subclinical acute rejection (AR) or BK infection (BKI), calcineurin inhibitor (CNI) toxicity, or interstitial fibrosis (IFTA). Urine was analyzed using a solid-phase bead-array assay for the interferon gamma-induced chemokines CXCL9 and CXCL10. We found that urine CXCL9 and CXCL10 were markedly elevated in adults and children experiencing either AR or BKI (p=0.0002), but not in stable allograft recipients, or recipients with CNI toxicity or IFTA. The sensitivity and specificity of these chemokine assays exceeded that of serum creatinine. Neither chemokine distinguished between AR and BKI. These data show that urine chemokine monitoring identifies patients with renal allograft inflammation. This assay may be useful for non-invasively distinguishing those allograft recipients requiring more intensive surveillance from those with benign clinical courses.

Keywords
Kidney Transplantation; Pediatric; Immune Monitoring; Chemokines

Introduction
Renal allograft recipients are at continuous risk for numerous adverse conditions including alloimmune rejection, viral infection, and non-inflammatory processes that threaten the long-term survival of the allograft. As such, they require periodic, life-long surveillance for conditions necessitating altered immunosuppressive management in order to avoid permanent allograft injury and/or opportunistic infection. Many methods for monitoring exist. Renal biopsy is generally considered the most sensitive and specific means of diagnosing allograft pathology and guiding corrective therapy. However, biopsies are invasive, costly, and impractical for frequent serial monitoring, particularly in children who require sedation for the procedure (1). Serum creatinine is used widely to monitor graft...
function, but is non-specific in segregating inflammatory causes of graft dysfunction, such as rejection or viral nephropathy, from non-inflammatory conditions such as drug toxicity and dehydration. Furthermore it requires phlebotomy, a benign procedure that nevertheless dissuades some patients, particularly children, from frequent monitoring. Serum creatinine is particularly less sensitive in child recipients of adult allografts due to the substantial mismatch between nephron and body mass in this population (2, 3).

Given the insensitivity of serum creatinine, subclinical rejection is detected more frequently in children than in adults when surveillance biopsies are performed (4, 5). Indeed, this indolent inflammation is consequential, as subclinical rejection in children is associated with subsequent allograft fibrosis (6, 7), and early treatment with corticosteroids reduces the number of subsequent clinical acute rejection (AR) episodes (8). A noninvasive monitoring method that could be used serially without incurring risk would aid in the routine management of transplant recipients, particularly children, potentially distinguishing patients requiring specific diagnostic attention from the patients with stable courses. Such a test would not necessarily need to be definitively diagnostic, but rather would need to be sensitive enough to distinguish those patients who were deviating from a stable course.

Many groups have studied urine biomarkers as non-invasive diagnostic tools. Urinalysis with culture is both sensitive and specific for diagnosing bacterial and fungal urinary tract infections. However, for the transplant specific condition of AR, most studies have focused on establishing associations between the singular diagnosis of AR and a particular urine analyte. In particular, the interferon gamma (IFNG) dependent, CXCR3-binding chemokines CXCL9 (formerly monokine-induced by IFNG, or Mig) and CXCL10 (formerly IFNG induced protein of 10k Da, or IP-10) have shown promise. Hu, et al, screened urine samples for 23 chemokines and cytokines and found CXCL9 and CXCL10 to associate with acute renal injury, including that related to acute rejection (9). Schaub, et al, similarly identified CXCL9 and CXCL10 to be elevated in the setting of subclinical tubulitis (10). Urinary cells from patients with histopathologic tubulitis have been shown to contain elevated levels of CXCL10 mRNA and CXCR3, the ligand for CXCL9 and 10, when compared with urinary cells from patients without acute rejection (11). Other groups, including our own, have shown that the levels of CXCL9 and CXCL10 transcripts are elevated in the allograft (12) and urine (13–15) of patients with clinically evident, biopsy proven acute rejection.

While multiple groups have studied IFNG-induced chemokines, they have yet to be applied specifically to pediatric transplant recipients – a population who would benefit most from noninvasive monitoring. In addition, prior studies have not evaluated a single platform across a broad array of ages and non-alloimmune conditions, such as calcineurin inhibitor (CNI) toxicity and BK viral infection, conditions that clearly pose a diagnostic dilemma in clinical practice. Nor have prior studies assessed the relative sensitivity of these urinary chemokines to serum creatinine, the current benchmark for diagnostic surveillance. In this clinical study, we have evaluated urinary CXCL9 and CXCL10 in pediatric and adult renal allograft recipients across multiple diagnoses to include inflammatory and non-inflammatory conditions. We find that these urinary chemokines can sensitively distinguish adult and pediatric patients with clinical and sub-clinical acute rejection and BK nephropathy from non-inflammatory conditions, but cannot distinguish BK nephropathy from AR. Nevertheless, they may serve as a non-invasive screening tool to identify patients warranting additional scrutiny and potential altered immune management.
Materials and Methods

Patient Data

Children (n=46) and adult (n=79) patients who had received renal transplants from either living or deceased donors, and healthy volunteers (n=31), were enrolled in a tissue acquisition protocol approved by the Children’s Healthcare of Atlanta and Emory University Institutional Review Boards after informed consent, and when appropriate assent, was obtained. Non-transplanted healthy volunteers were required to have a normal urinalysis and no known diagnosis of renal disease.

Demographic and clinical information was collected on all patients undergoing transplant during the study period, including: maintenance and induction immunosuppression, functional data, and Banff biopsy score. Serum creatinine values were obtained at the time of the urine sample collection; baseline creatinine was determined as an average of 5 serum creatinine values from a time of graft stability prior to the urine sample, or if never stable, time of best function. For clinically stable patients (those without rejection or with subclinical rejection), urine was collected at the time of standard-of-care surveillance biopsies, which were performed between three and six months post-transplant in pediatric patients, and between five and six months post-transplant in adult patients. In addition, enrollment was offered to all patients requiring renal biopsy for cause. All urine specimens were obtained prior to biopsy. For patients who received rescue immunosuppressive therapy, urine samples were obtained prior to rescue therapy initiation. Although patients may have had multiple urine specimens collected, only the first diagnostic specimen per patient was included in the comparisons between groups. To avoid the confounding effects of reperfusion injury and delayed graft function, particularly anuria which would be a contraindication for this urine-based assay, all samples were collected at least 25 days after transplant.

Immunosuppressive therapy

Adult patients typically received induction immunosuppression with rabbit anti-thymocyte globulin and maintenance immunosuppression with tacrolimus, mycophenolate mofetil, and prednisone. Two patients received induction with alemtuzumab and one patient with OKT3. Pediatric patients received immunosuppression with a CD25-specific monoclonal antibody, basiliximab or daclizumab, and maintenance immunosuppression with tacrolimus or sirolimus, mycophenolate mofetil, and prednisone.

Patient groups

All patients were assigned to a diagnostic category based on an aggregate of all available diagnostic data: healthy volunteers (n=31), clinically stable renal transplant recipients (n=50), and recipients with acute rejection (AR, n=25), BK infection (BKI, n=24), CNI toxicity (n=17), or isolated interstitial fibrosis and tubular atrophy (IFTA, n=9). Patients classified in the AR group had histologic changes consistent with the Banff criteria for rejection with or without renal dysfunction, and/or met clinical criteria with renal dysfunction (serum creatinine elevation >30% above baseline), which improved with subsequent rescue immunosuppression. Patients were considered to have sub-clinical rejection or BK infection if their biopsy was obtained at the time of clinical stability (serum creatinine within 15% of baseline), and showed acute rejection (a Banff 1A or greater rejection on histology) or BK viremia and/or SV40 staining on histology, respectively. Calcineurin inhibitor toxicity patients had a trough tacrolimus level greater than 20 ng/mL, a cyclosporine A level greater than 200 ng/mL, and/or had reversible renal dysfunction that improved with reduction of CNI therapy. All patients classified as having IFTA had Banff grade 2 or 3 IFTA on biopsy without superimposed AR or BKI. BK viral infection was
diagnosed by detection of the viral genome in serum by PCR+/− histological confirmation of SV-40 staining on renal biopsy, and included patients with subclinical and clinical BK nephropathy. Stable transplant patients had no evidence of subclinical pathology. Patients included in this study had exclusive diagnoses; those with multiple diagnoses or in whom no definitive diagnosis could be established were excluded from the study.

Urine Collection and Processing

Urine samples were collected and refrigerated within 30 minutes of collection. Specimens were centrifuged at 2000g for 30 minutes to remove sediment. Urine supernatants were frozen in 1 mL aliquots at −80°C. Dipstick urinalysis was performed on all specimens {Seimens (Bayer) Multistix, Deerfield, IL}. The urine samples were then investigated for CXCL9 and CXCL10 using the PlexMark 3 kit (Invitrogen, Carlsbad, CA), a solid phase fluorophore-based assay for Luminex with a pH buffer specifically designed for analyzing urine samples. The assay can be performed with a same day turnaround, making it logistically feasible for clinical testing.

Statistical Analysis

Statistical analyses were performed using SAS statistical analysis software (SAS v.9.2, Cary, NC) and SPSS (version 18, Chicago, IL), and a p value less than 0.05 was considered significant. Continuous variables were compared with t tests or ANOVA, and categorical with chi-squared analysis. Skewed variables such as CXCL9 and CXCL10 were analyzed with nonparametric tests (i.e. Kruskal-Wallis, Mann-Whitney U) to determine differences among patient groups. To evaluate urine chemokine levels and serum creatinine as diagnostic tools, logistic regression models were run to create Receiver Operating Characteristic (ROC) curves. ROC curves were developed using data from transplant recipients only; no healthy control patients were included in the ROC determinations.

Results

Analysis across all patients

A total of 46 pediatric and 110 adult patients were studied (Table 1). Combining pediatric and adult populations, 31 healthy controls were identified, along with 50 stable transplanted patients, 25 undergoing AR (4 also with antibody mediated rejection), 9 with IFTA, 24 with BKI, and 17 with CNI toxicity. The median age of pediatric patients was 11.6 years and for adults 45.6 years. Considering all patients, approximately half were male; 40% were Caucasian, 40% black, 13% Hispanic, and 7% other. Fifty-nine percent of pediatric patients underwent deceased-donor kidney transplantation compared to 74% in adults.

In order to determine whether the findings at the time of urine collection and biopsy were functionally significant, renal function was assessed in each transplant group comparing function at the time of biopsy to the pre-established baseline (Figure 1a). Stable transplant patients demonstrated no increase in their creatinine from baseline (p=0.86). Both AR and BKI patients had a significant percent increase from baseline creatinine (103% and 12.8% respectively; p<0.002). Acute rejection and IFTA patients had higher creatinine levels than all other groups (p<0.0001).

Urinary CXCL9 (Figure 1b) and CXCL10 (Figure 1c) levels were assessed for each group. No statistical difference in urine chemokine signature was found between healthy controls and stable transplant patients (p=0.57). Urine CXCL9 and CXCL10 levels were elevated for AR and BKI (p<0.0001) but were not different between the two groups (p=0.6). ROC analysis was performed (Figure 2), which showed that CXCL9 was a modestly better predictor of category, with a C statistic of 0.873 compared to 0.831 for CXCL10. Based on
the ROC curve (figure 2a), a CXCL9 value of 37.8 had 86% sensitivity and 80% specificity in detecting a patient with either AR or BKI. In comparison, a CXCL10 value of 28 had a sensitivity of 80% and specificity of 76% (figure 2b). An algorithm combining the two chemokines did not significantly improve sensitivity or specificity, with a C statistic of 0.848 (data not shown). Both urine chemokines were more sensitive and specific for detecting BKI and AR than serum creatinine. Even when sensitivity was set at a trivial increase in creatinine of 0.1mg/dl over baseline, the maximum sensitivity for creatinine was 75% (figure 2c) with a72% specificity in detecting the same conditions.

As our acute rejection and BK infection groups included patients with subclinical disease, we conducted a subset analysis of subclinical rejection and BK viremia. When comparing chemokines in subclinical rejection (n=10) with those of stable patients, we found a significant elevation for CXCL9 and CXCL10 (p<0.001). Subclinical rejection patients had equivalent levels with rejecting patients with impaired renal function (CXCL9 p=0.556, CXCL10 p=0.983). Similarly, subclinical BK infection (n=5) was also associated with elevated chemokines compared to stable patients (CXCL9 p=0.002, CXCL10 p=0.003), but equivalent to BK infection with nephropathy (CXCL9 p=0.871, CXCL10 p=1.0). These findings are in accordance with Schaub et al(10), and further support that urine chemokines can herald inflammatory conditions earlier than serum creatinine.

**Analysis of the pediatric subset**

Given the different creatinine dynamics in children, we performed a subset analysis in the pediatric patients. Of the 46 pediatric patients studied, 7 were healthy controls. The remaining patients were distributed across study groups as depicted in Table 1. Pediatric patients had a higher percent increase from their baseline creatinine than adult patients during episodes of AR (p=0.02). Seventeen patients underwent renal biopsies; based on histology and immunohistochemical staining, their diagnoses included 1 antibody-mediated rejection, 7 acute cellular rejections (3 were Banff grade 1a, 4 were 1b), and 2 IFTA (1 grade 2, 1 grade 3). Six children had BKI and 10 had CNI toxicity. There was no quantitative difference in BK virus PCR values between infected children and adults (p=0.51).

As with the analysis for all patients, we found that a significant difference among study groups for both CXCL9 and CXCL10 (p<0.0001) persisted in the pediatric subset (data not shown). Elevated urinary CXCL9 and CXCL10 correctly identified AR and BKI against all other patients (p<0.0001) but did not distinguish between the two diagnostic groups (p=0.17 and 0.44 for CXCL9 and CXCL10, respectively). All children with BK virus infection had positive BK viremia compared to no children with AR, thus, combining an elevated urine chemokine level with BK PCR identified the cause of acute inflammation in all cases.

Unlike urine chemokines, an elevated value for serum creatinine did not identify patients with BK infection, and was only significantly elevated for AR (p<0.0002).

Median urine chemokine levels were higher for pediatric than adult patients, with CXCL10 showing a significant difference (p<0.03). No difference was found between children and adults for stable and BKI groups (p>0.13). Urine chemokine expression during AR, however, did show a difference between children and adults (p=0.003 for CXCL9, p=0.002 for CXCL10).

**Urine chemokine stability and variability**

The stability of urine chemokine measurements during repeated freeze-thaw cycles was studied. A sample of urine from three different patients (acute rejection with known elevated levels of urine chemokines, stable transplant and normal control) was divided into 6
aliquots. Each aliquot was subjected to repeated freeze thaw cycles ranging from 1–6 cycles. Urinary chemokine measurements for both CXCL9 and CXCL10 were consistent for each sample up to 6 freeze-thaw cycles with a percent coefficient of variation of 13.3% and 11.2% for CXCL9 and CXCL10 respectively (data not shown). To test for day-to-day variability in urine chemokine levels, urine was collected on three consecutive days from three healthy controls. Urine for each subject displayed consistent readings on 3 consecutive days. For all three subjects urine chemokine levels were low and consistent with the readings from the aggregate of healthy controls. The sensitivity and specificity of the assay was validated internally and found to be consistent with that reported by the manufacturer (16). Specifically, standard curves were generated using recombinant CXCL9 and CXCL10 for each assay replicate. The dynamic range of the assay in our hands was between 30 and 10,000 for CXCL9 and 5 and 10,000 pg/ml for CXCL10. Values under 30 are reported as non-detectable for CXCL9, and values under 5 are reported as non-detectable for CXCL10.

Discussion

Post-transplantation immune management requires active navigation between under-and over-immunosuppression to avoid rejection and infection, respectively. Consequently, there is a persistent need for monitoring tools to maintain a satisfactory therapeutic course. While diagnostic precision would be ideal, no diagnostically specific screening tools exist to follow patients. Rather, patients are followed functionally, typically with urinalysis and serum creatinine, and because of the generally recognized incomplete sensitivity of serum creatinine, many programs perform periodic surveillance biopsies to detect occult inflammatory conditions. In this study, we evaluated the utility of urine chemokines in detecting inflammatory events. We found that urine CXCL9 and CXCL10 were universally elevated for children and adults during episodes of AR or BKI, and not elevated in CNI toxicity, stable patients, or patients with isolated IFTA. Serum creatinine, on the other hand, was both less sensitive and less specific for these conditions, as it was elevated in conditions unrelated to allograft inflammation, CNI toxicity and IFTA, while being less consistently elevated in AR and BK nephropathy, particularly in children. These data suggest that urinary chemokine detection could serve as a non-invasive adjunctive test to help identify patients in need of intervention without additional phlebotomy, and provide data supporting the development of a prospective trial evaluating such a strategy.

Certain aspects of the study warrant highlighting. It is clear that this assay is not a specific diagnostic test for rejection. Both CXCL9 and CXCL10 appear to be equivalently elevated in AR and BKI indicating that the assay is perhaps responsive to intragraft lymphocytic inflammation regardless of the etiology. However, as a screening tool, this distinction is less important than its detection, and both analytes appear to be highly sensitive for either condition with or without overt renal dysfunction. Indeed, this data set demonstrates the potential clinical utility and limitations of chemokine monitoring in that it places the elevations seen in rejection in the context of other common diagnoses. Unlike creatinine, these chemokines rise in inflammatory conditions but not in non-inflammatory circumstances such as isolated IFTA and CNI toxicity. It is thus likely that by adding this assay to those already available clinically -creatinine and BK viral PCR - some degree of diagnostic certainty could be established. This would require a prospective, randomized, longitudinal trial, but the data reported herein support the design of such a trial and provide data required for establishing the trial size and power.

These data also speak to the development of this assay for use in children. Children are difficult patients to monitor for several reasons. In very small children, significant rejection can lead to trivial changes in serum creatinine due to the relatively high, and dynamic nephron mass of child recipients of adult kidneys(17–19). In addition, phlebotomy...
represents a logistical challenge in terms of patient adherence, and for very small children, blood volume limitations. Our results suggest that urinary chemokines are, if anything, more sensitive in children than adults, and exist in a more dynamic range, perhaps indicative of the more responsive immune system of young patients. Thus, the most important aspect of a screening tool, sensitivity for the clinical conditions most in need of prompt diagnosis, appears to be satisfied. Similar benefits may be detected in applying this assay to elderly patients, particularly those with low body mass.

The primary benefit of this test, its non-invasiveness, opens up an opportunity for frequent, serial monitoring. The current assay can be deployed in the clinic to guide clinical decision-making regarding the need for a biopsy, for example, segregating patients into those that should proceed promptly to biopsy from those that could be followed and advised to hydrate and perhaps reduce their calcineurin inhibitor, or similar maneuvers. Given the ability to sample daily or more frequently if necessary, this assay could be envisioned as one that could be used dynamically to assess response to therapy. Certainly, these uses await prospective study. Although the existing platform requires use of a clinical lab, the technology is such that it could be adapted to a point-of-care test that patients could use at home much like patients with diabetes monitor themselves for hyperglycemia.

Many other groups have studied biomarkers in urine and peripheral blood in the search for noninvasive monitoring tools (9, 20). Elevated urine levels of perforin, granzyme B, PI-9, CD103, granulysin, CXCL10, CXCR3, F as L, FOXP3, Tim-3, and IFNG, and elevated serum levels of granzyme B, perforin, F as L, IL-4, IL-5, IL-6, IFNG, CD40L, Tim3, PI-9 and FOXP3 were identified as diagnostic and sometimes predictive biomarkers of acute rejection (13, 14, 20–25). Despite the predictive potential of many of these biomarkers, they are detectable during other inflammatory states such as urinary tract infection, BK virus or cytomegalovirus infections, and even in diabetic nephropathy (23, 26, 27). While many biomarkers have been able to detect inflammatory conditions, CXCL9 and CXCL10 were selected for this study due to their involvement in the earliest aspects of acute rejection (28).

Previous work from our laboratory and others demonstrate a temporal sequence of the immune response: initial infiltration of monocytes produces IFNG, which induces renal tubular epithelial cells to produce chemokines that attract chemokine receptor (e.g. CXCR3) expressing T cells(29–31). Based on these data, IFNG induced chemokines such as CXCL9 and CXCL10, would appear first in the urine, followed by TH1 derived transcripts, Tbet and CXCR3, and later granzyme B and perforin as destruction ensues with effector T cells accumulating in the tubules(32, 33). The association of IFNG induced chemokines with subclinical rejection(10) is further evidence that these analytes in particular flag early stage changes and this too speaks to their appropriate deployment as screening tools.

We conclude that urine CXCL9 and CXCL10 can sensitively identify patients with rejection earlier in the destructive process than traditional monitoring with serum creatinine. As these analytes are well defined and conducive to ELISA-type detection, the potential for a dip-stick-type home or point-of-care test appears to be feasible. The accessibility of urine testing and the potential for home monitoring is attractive as clinicians and patients look for less obtrusive monitoring regimens post transplant. The ease and flexibility of more frequent monitoring, particularly in the pediatric population, has the potential to improve quality of life and long-term outcomes. These data warrant a randomized, prospective, longitudinal trial evaluating the clinical use of urinary chemokines for post-transplant monitoring.

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**Abbreviations**

- ANOVA: analysis of variance
- AR: acute rejection
- BKI: BK virus infection
- CNI: calcineurin inhibitor
- ELISA: enzyme-linked immunosorbent assay
- IFNG: interferon gamma
- IFTA: interstitial fibrosis and tubular atrophy
- PCR: polymerase chain reaction
- ROC: Receiver Operating Characteristic
- SV40: simian virus 40

**References**


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Figure 1.
Levels of (a) serum creatinine, (b) urine CXCL9, and (c) urine CXCL10 were ranked and compared by nonparametric analysis across study groups. Figure 1a depicts the elevation of creatinine in both inflammatory and non-inflammatory conditions. In contrast, urine CXCL9 and CXCL10 both were elevated for acute rejection and BK virus infection but not in non-inflammatory conditions.
Figure 2.
ROC curves for (a) urine CXCL9, (b) urine CXCL10, and (c) serum creatinine in detecting either acute rejection or BK virus infection. Among these tests, CXCL9 had the highest sensitivity and specificity (86% and 80%, respectively) with a c statistic of 0.873.
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<th>Adult pts</th>
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<td>2.5 (3.8)</td>
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<td>1.7 (1.5)</td>
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<tr>
<td><strong>TOTAL</strong></td>
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* IFTA = interstitial fibrosis and tubular atrophy (≥ grade 2)