

# Elevated Urinary CCL2: Cr at 6 Months Is Associated With Renal Allograft Interstitial Fibrosis and Inflammation at 24 Months

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**Background.** We have demonstrated that 6-month urinary CCL2: Cr is a predictor of interstitial fibrosis and tubular atrophy (IFTA) on 24-month biopsy and death-censored graft loss. However, IFTA is no longer considered prognostically significant, whereas patients with graft loss frequently have interstitial fibrosis and inflammation (IF+i=c>0+i>0). As early CCL2: Cr predicts late graft loss, the goal of this study was to determine if 6-month urinary CCL2: Cr was a predictor of IF+i at 24 months.

**Methods.** Urinary CCL2 at 6 months was measured with ELISA and correlated with IF+i on 24-month surveillance biopsies from a prospective, multicenter adult renal transplant cohort (n=111).

**Results.** Six-month urinary CCL2: Cr was significantly higher in IF+i and transplant glomerulopathy patients compared with normal histology at 24 months. By multivariate analysis, 6-month urinary CCL2: Cr was independently correlated with IF+i at 24 months (OR 2.78, 95% CI 1.38–6.12, AUC 0.695,  $P=0.003$ ). Six-month urinary CCL2: Cr was also an independent correlate of 6-month IF+i (OR 1.99, 95% CI 1.03–4.18, AUC 0.63,  $P=0.04$ ). Six-month urinary CCL2: Cr distinguished noninflamed renal tissue (normal, fibrosis) from IF+i with a sensitivity/specificity of 0.71/0.62 at a cutoff of 15 ng CCL2/mmol Cr (AUC 0.695,  $P=0.003$ , n=91).

**Conclusions.** Urinary CCL2: Cr may be useful for the noninvasive identification of patients with or at risk for IF+i. These patients may benefit from avoidance of drug minimization/withdrawal protocols and more intensive post-transplant surveillance. Furthermore, urinary CCL2: Cr may also identify individuals who may benefit from novel interventional trials targeting IF+i.

**Keywords:** Monocyte chemoattractant protein 1, Chronic allograft injury, Urinary biomarker, Chemokines.

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**R**enal allograft outcomes have improved in terms of both long-term histology (1) and graft survival (2) for living and deceased donor kidney recipients. Despite these

encouraging gains, graft loss remains a clinically significant problem. Indeed, the return to dialysis after graft loss is associated with a three-fold increased risk of death, immunologic

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J.H. designed the study, attained the operating funds, supervised the experimental work and analysis, and wrote the manuscript. C.W. assisted with the statistical analysis and also contributed to writing the manuscript. I.W.G. contributed to evaluating the pathology and writing the manuscript. S.H.K. assisted with critical input on the manuscript. A.G. performed the urine CCL2 experiments. C.R. contributed to the statistical analysis and assisted with the manuscript regarding the statistical methodology section. M.K. contributed to writing the manuscript. L.S. contributed to writing the manuscript. P.W.N. contributed to the collection of the clinical database/urine bio-bank and contributed to the data analysis and writing the manuscript. P.W.N. holds the Flynn Family Chair in Renal Transplantation at the University of Manitoba. D.N.R. contributed to the collection of the clinical database/urine bio-bank and writing of the manuscript.

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sensitization that may impede retransplantation, a lower quality of life and increased costs (3, 4). Recently, the causes of graft loss have been demonstrated to be largely identifiable, primarily immune mediated, and therefore, potentially amenable to intervention (5, 6). Indeed, deconstructing the nonspecific descriptor of “chronic allograft nephropathy” into specific treatable entities has been a key goal of the DeKAF study and other groups to improve long-term graft outcomes (7).

Emerging data indicate that mild interstitial fibrosis and tubular atrophy (IFTA) alone is not prognostically significant (8–12). Instead, multiple groups have now demonstrated that interstitial fibrosis+inflammation (IF+i) is a significant prognostic marker for graft loss (7–13). Even mild degrees of IF+i where the degree of inflammation does not meet diagnostic criteria for Banff borderline rejection are strongly and independently associated with renal allograft functional decline and death-censored graft loss (8, 9, 11). It is noteworthy that IF+i is associated with episodes of acute rejection and increased levels of HLA mismatch (8, 10), as well as a rejection-like gene expression signature (9, 13).

Taken together, these findings expand upon earlier work, which demonstrate that subclinical rejection is associated with poor graft outcomes (14–16) and suggest that IF+i may represent an ongoing, low-grade cellular rejection state that is not recognized in the current Banff schema (8, 17). Indeed, recurrent subclinical inflammation has been shown to result in worsening IFTA, declining renal allograft function and increased graft loss (12, 18).

The goal of this study was to identify early, noninvasive predictors for the development of IF+i. CCL2 [Chemokine (C-C ligand) motif ligand 2] is a CCR2 receptor chemokine that is a chemoattractant for monocytes/macrophages, T cells, and natural killer cells. We have demonstrated that 6-month urinary CCL2: Cr is associated with the development of IFTA at 24 months in a multicenter (FKC008) cohort (19, 20). Furthermore, in an independent renal transplant cohort, we demonstrated that 6-month urinary CCL2: Cr is a predictor for death-censored renal allograft loss (21). Therefore, we hypothesized that reanalysis of the FKC008 cohort would demonstrate that 6-month urinary CCL2: Cr is an early correlate for 24-month IF+i.

**TABLE 1.** Patient characteristics, n=111

Characteristics	Overall n=111	Normal n=30	Inflammation n=5	Fibrosis n=33	IF+i n=28	TG n=15	P
Recipient:							
Recipient age (yr)	48±12	50±11	42±17	48±12	45±12	47±12	0.5
Recipient gender (male)	72.1%	80%	100%	78.8%	64.3%	46.7%	0.04
Recipient race							0.6
Aboriginal	2.7%	3.3%	0	0	7.1%	0	
Asian	11.7%	16.7%	20%	12.1%	7.1%	6.7%	
Black and Hispanic	2.7%	0	0	3.0%	7.1%	0	
White	76.6%	76.7%	80%	75.8%	71.4%	86.7%	
Unknown	6.3%	3.3%	0	9.1%	7.1%	6.7%	
Primary reason for transplant (%)							0.5
Diabetic nephropathy	20.7%	16.7%	0	27.3%	25%	13.3%	
Glomerulonephritis and glomerulosclerosis	37.8%	36.7%	60%	30.3%	42.9%	40%	
PCKD and other hereditary disease	18.9%	20%	0	27.3%	10.7%	20%	
Other	22.5%	26.7%	40%	15.1%	21.4%	26.7%	
Donor:							
Donor age (yr)	38±14	33±12	17±8	41±14	43±12	40±16	0.0003
Donor (deceased)	71.2%	56.7%	80%	75.6%	71.4%	86.7%	0.24
Terminal creatinine (μmol/L)	68±22	68±23	72±37	72±20	64±17	65±26	0.7
Immunologic:							
PRA	1±4	0.5±2	0.5±1	0.9±2	1±4	5±9	0.1
Total HLA mismatch	4±1	4±1	4±1	3±2	4±1	4±1	0.7
Post-transplant:							
Delayed graft function, dialysis	9.0%	3.3%	20%	9.1%	7.1%	20%	0.4
Acute rejection episode	4.5%	0	0	9.1%	0	13.3%	0.06
Urine CCL2: Cr at 6 mo	22±20	15±10	13±10	19±15	27±17	36±36	0.016
CrCl (mL/min) at 6 mo	75±19	80±17	89±10	73±24	73±17	72±19	0.3
CrCl (mL/min) at 12 mo	75±20	81±19	85±25	71±20	72±18	73±24	0.3
CrCl (mL/min) at 24 mo	78±22	87±22	87±20	77±24	69±17	75±23	0.046

RESULTS

Study Population

The inclusion criteria of a 6-month urine with a matched 24-month surveillance biopsy were met in 111 patients (Figure S1, SDC, <http://links.lww.com/TP/A931>), most of whom also had a 6-month surveillance biopsy (n=94, 85%). Using Banff criteria nomenclature (17), we classified graft histology as did the Mayo Clinic group into the following (8–10):

- 1. Normal histology (i0 ci0 cg0), n=30
- 2. Inflammation (i>0 ci0 cg0), n=5
- 3. Fibrosis (i0 ci>0 cg0), n=33
- 4. IF+i (i>0 ci>0 cg0), n=28
- 5. Transplant glomerulopathy (TG) (i>0 ci>0 cg>0), n=15

The patient demographics are listed in Table 1, in aggregate and by histologic outcome. The donors were significantly younger in the inflammation group, and there was also a trend to more female subjects and biopsy-proven acute rejection episodes in the TG group. Notably, 24-month renal function in the IF+i group was lower relative to the other histologic classifications.

Six-Month Urinary CCL2: Cr Is Associated With IF+i and TG

Elevated urinary CCL2: Cr at 6 months was significantly associated with IF+i and TG on the 24-month biopsy and was able to differentiate both normal and pure fibrosis from IF+i and TG (Fig. 1A). In contrast, elevated urinary CCL2: Cr at 6 months only distinguished IF+i from normal histology at 6 months, which could be due to the limited number of TG (n=8) cases at this early period (Fig. 1B).

Histologic Progression Between 6 and 24 Months

Ninety-four patients (85%) of the total study population (n=111) underwent a 6-month surveillance biopsy. IF+i was present in 23% (22/94) and TG in 8.5% (8/94) of these patients. The 24-month biopsy in the patients with IF+i at 6 months showed persistent IF+i in almost half (9/22), and TG had developed in 4 of 22 patients. The 24-month biopsy in those patients with TG at 6 months showed persistent TG

in 5 of 8. Interestingly, 7 of 17 patients with inflammation at 6 months had developed IF+i by 24 months (Fig. 2A).

In the cohort with IF+i at 24 months, a 6-month biopsy was available in 24 of 28 patients. The 6-month histology in these patients demonstrated inflammation alone in 7 of 24 and IF+i in 9 of 24. In the cohort with TG at 24 months, 12 of 15 had a 6-month biopsy, which showed IF+i in 4 of 12 and preexisting TG in 5 of 12 patients (Fig. 2B).

Predictors of Histology and Graft Function

Univariate analysis demonstrated that 6-month urinary CCL2: Cr and 6-month i-scores were the only significant predictors for the development of 24-month IF+i (Table 2a), and both 6-month urinary CCL2: Cr and i-scores remained independently associated with 24-month IF+i on multivariate analysis (Table 2b). Furthermore, 6-month Banff i-scores and urinary CCL2: Cr were significantly correlated ( $R^2$  0.0574,  $P=0.02$ ), suggesting that CCL2: Cr may be a noninvasive correlate of concomitant i-scores. Six-month i-scores were significant for distinguishing “noninflamed” (normal histology and pure fibrosis) from “inflamed” renal tissue (inflammation, IF+i, and TG) at 24-months (OR 4.08, 95% CI 1.87–9.75,  $P=0.0003$ , n=94). However, as the primary goal of this study was to characterize early noninvasive markers for 24-month IF+i, 6-month histology was censored from further analyses.

Stepwise logistic regression analysis of potential noninvasive variables demonstrated that 6-month urinary CCL2: Cr remained the only independent correlate of 24-month IF+i. The complete model (n=111) demonstrates that 6-month urinary CCL2: Cr differentiates “noninflamed” (normal histology and pure fibrosis) from “inflamed” (inflammation, IF+i, and TG) renal tissue at 24 months [OR 1.88, 95% CI 1.14–3.24, AUC 0.641,  $P=0.01$ ]. Furthermore, 6-month urinary CCL2: Cr differentiates “noninflamed” (normal histology and pure fibrosis) renal tissue from IF+i (OR 2.78, 95% CI 1.38–6.12, AUC 0.695,  $P=0.003$ ) (Table 2c). In this model, the sensitivity and specificity for 6-month urinary CCL2: Cr to identify IF+i from “noninflamed” renal tissue (AUC 0.695), with a cutoff value of 15 ng/mmol, was 71% and 62%, respectively (Table S1, SDC, <http://links.lww.com/TP/A932>). Interestingly, 6-month urinary CCL2: Cr was also independently

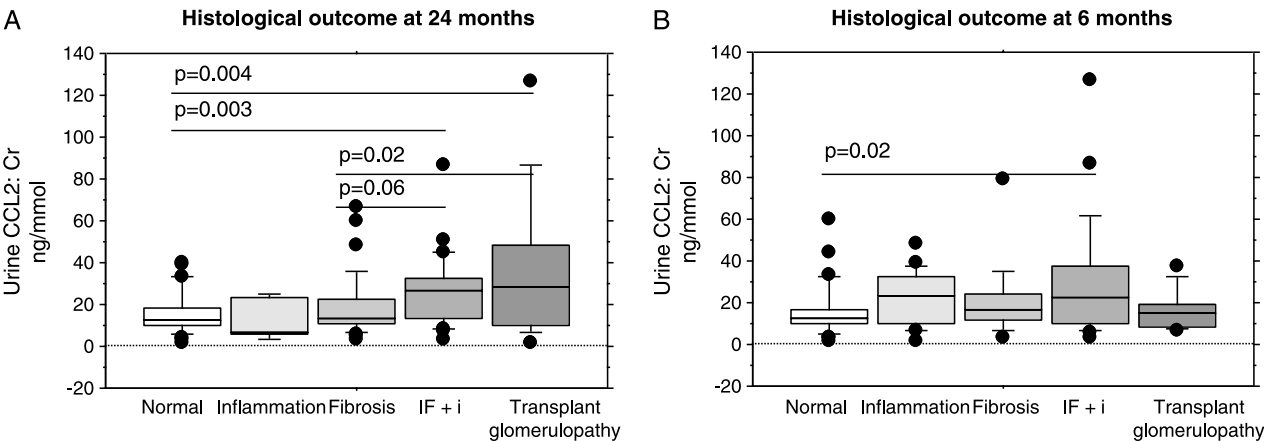
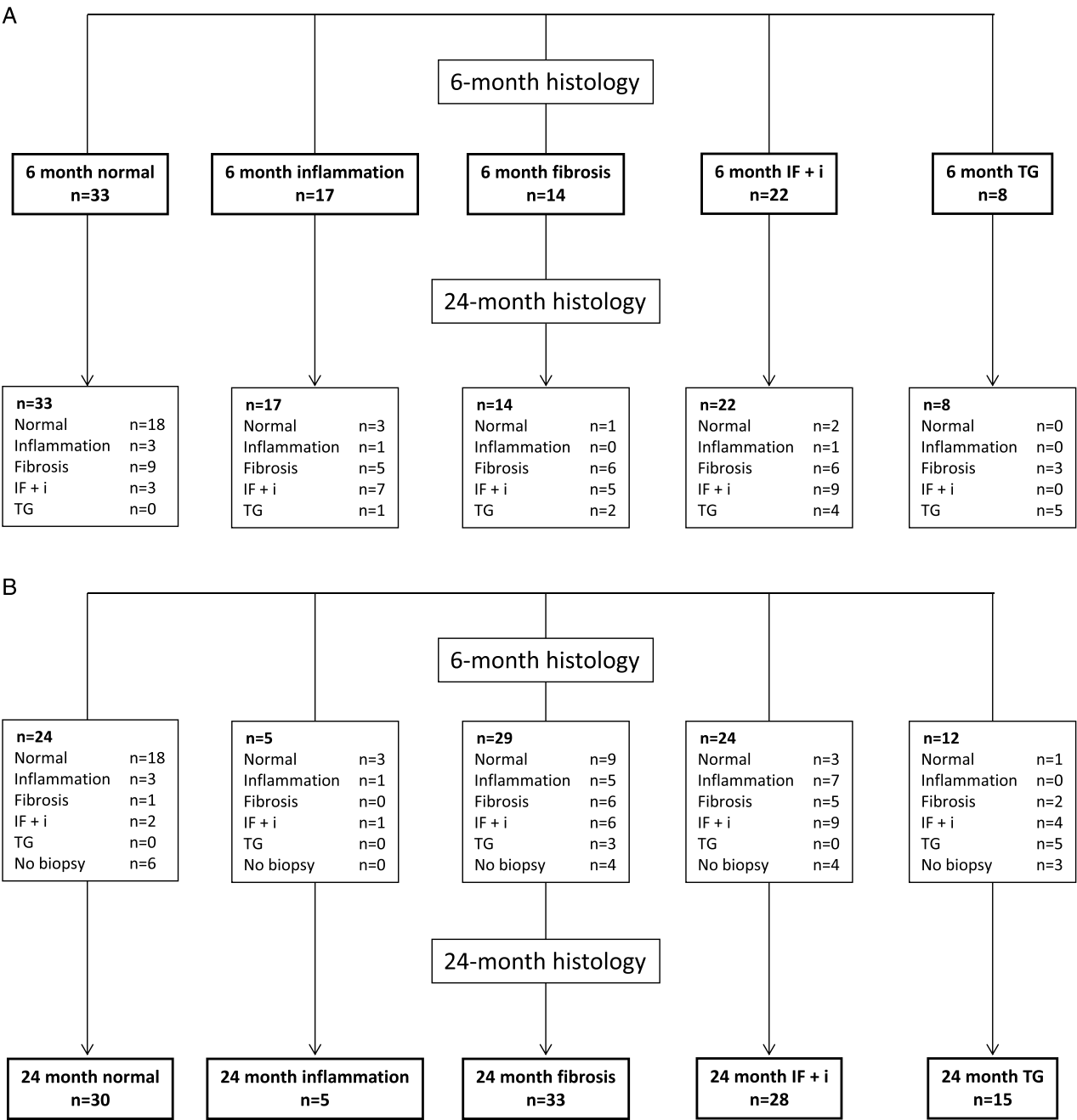


FIGURE 1. Increased urinary CCL2: Cr is associated with interstitial fibrosis+inflammation at 6 and 24 months.



**FIGURE 2.** A, histologic progression from 6 to 24 months in the study population with 6-month biopsies, n=94. B, histologic progression from 6 to 24 months in the total study population, n=111.

associated with 24-month TG (OR 2.15, 95% CI 1.03–4.78,  $P=0.002$ ).

The “noninflamed” group (normal histology and pure fibrosis) was used as the control group because IF alone is prognostically similar to normal histology (8–12). However, when normal histology alone was used as the control group, 6-month urinary CCL2: Cr better differentiated “inflamed” renal tissue (inflammation, IF+i, and TG) at 24 months (OR 2.05, 95% CI 1.15–3.92, AUC 0.66,  $P=0.01$ ) and remained an independent correlate of 24-month IF+i (OR 3.42, 95% CI 1.50–9.11, AUC 0.72  $P=0.003$ ) (Table 2d). This slight

improvement in diagnostic performance may be because the pure fibrosis group may have included patients with inflammation in areas of IFTA that are not scored in the current Banff schema.

In a secondary analysis, 6-month urinary CCL2: Cr was only borderline significant ( $P=0.06$ ) for differentiating “non-inflamed” from “inflamed” 6-month histology but remained an independent correlate of 6-month IF+i ( $P=0.04$ ) (Table S2a, <http://links.lww.com/TP/A932>). When normal histology alone was the comparator group (pure fibrosis excluded), urinary CCL2: Cr both differentiated normal from “inflamed” renal tissue

**TABLE 2A.** Univariate predictors of 24-month IF+i

Variable	Odds ratio	95% CI	P	n
6-mo urinary CCL2: Cr <sup>a</sup>	1.96	1.11–3.63	0.026	111
Donor age >50 yr	1.44	0.53–3.75	0.46	111
Delayed graft function	0.72	0.10–3.11	0.69	111
Living donor versus deceased donor	0.98	2.48–3.66	0.97	111
ACEi or ARB exposure	0.80	0.33–1.91	0.63	111
HLA mismatch	1.08	0.80–1.49	0.60	111
PRA	0.98	0.85–1.08	0.72	96
6-mo Banff i score	3.37	1.60–7.52	0.002	94
6-mo Banff t score	1.62	0.87–2.99	0.12	94
6-mo Banff g score	3.83	0.24–68.96	0.31	94
6-mo IF+i	2.63	0.93–7.34	0.06	94

<sup>a</sup> Per natural log change in regressor.

ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; HLA, human leukocyte antigen; PRA, panel reactive antibody; IF+i, interstitial fibrosis and inflammation.

and independently correlated with 6-month IF+i (Table S2b, <http://links.lww.com/TP/A932>).

Finally, because the IF+i group had a borderline significant lower creatinine clearance at 24 months relative to the other groups (Table 1), we sought to determine predictors for decline in renal function. Linear regression analysis demonstrated that 6-month CCL2: Cr was only borderline significant for predicting renal function decline from 12 to 24 months ( $-0.11 \pm 0.055$ ,  $R^2$  0.046,  $P=0.051$ ) in this small cohort ( $n=90$ ) and was not a significant predictor for decline in renal function from 6 to 24 months (data not shown).

## DISCUSSION

The principal finding of this study is that urinary CCL2: Cr at 6 months is independently correlated with IF+i on both 6- and 24-month surveillance biopsies, which may provide an explanation for our previous observation that 6-month urinary CCL2: Cr is an independent predictor of death-censored graft loss (21). Furthermore, we and others have demonstrated that urinary CCL2 is elevated in subclinical and clinical rejection (22–25), both of which have been correlated with graft loss (14, 16, 26). Our group has also validated urinary CXCL10: Cr as a noninvasive marker for subclinical and clinical rejection (27, 28); however, urinary CXCL10: Cr was unable to distinguish patients with pure IFTA from IFTA+borderline rejection (27). These findings suggest that a panel

**TABLE 2C.** Multivariate analysis of non-invasive predictors for the development of 24-month IF+i<sup>a</sup>

Model	Outcome	n	Urine CCL2: Cr odds ratio <sup>b</sup> (95% CI)	AUC	P
1	Normal, fibrosis versus inflammation, IF+i, transplant glomerulopathy	111	1.88 (1.14–3.24)	0.641	0.01
2	Normal, fibrosis versus inflammation and IF+i	96	1.96 (1.06–3.82)	0.645	0.03
3	Normal, fibrosis versus IF+i	91	2.78 (1.38–6.12)	0.695	0.003

Stepwise logistic regression was performed with the covariates: donor age >50 years; delayed graft function; living versus deceased donor, ACEi/ARB exposure, PRA and HLA match. Only 6-month urinary CCL2: Cr was significant and included in the final analysis.

<sup>a</sup> Control group=normal histology and pure fibrosis.

<sup>b</sup> Per natural log change in regressor.

of urinary markers, such as CCL2 and CXCL10, may be most useful to distinguish underlying graft histology.

We have previously shown that 6-month urinary CCL2: Cr predicted death-censored graft loss independent of both pretransplant and de novo donor-specific antibody (DSA). Indeed, only 7 of 20 graft losses from that cohort were potentially attributable to DSA and chronic antibody-mediated rejection (21). Similarly, the DeKAF study demonstrated that inflammation in areas of scarring predicted graft loss independently of DSA (11), and more recently, El Ters et al. have reported that persistent graft inflammation can lead to severe interstitial fibrosis and graft loss that is also independent of DSA (26). Several lines of evidence suggest that IF+i is an alloimmune inflammatory response. IF+i is associated with increased HLA mismatch, a higher incidence of acute rejection, retransplants, and a rejection-like gene expression signature (8–10, 13). Taken together, these data suggest that early urinary CCL2: Cr may be linked to graft loss via IF+i-related cellular inflammation independent of DSA (Fig. 3).

Interestingly, some patients that had IF+i on their 6-month surveillance biopsy developed TG by 24 months. Independent cohorts have demonstrated that early acute cellular rejection episodes increase the risk for developing de novo DSA, suggesting one possible mechanism for this TG development (6, 26, 29). However, TG may also develop in the

**TABLE 2B.** Multivariate analysis of all predictors for the development of 24-month IF+i

Model	Outcome	n	6-mo urinary CCL2: Cr odds ratio <sup>a</sup> (95% CI, P)	6-mo Banff i-score odds ratio <sup>a</sup> (95% CI, P)	AUC
1	Normal, fibrosis versus IF+i	77	2.32 (1.00–5.83, $P=0.049$ )	4.99 (2.00–13.86, $P=0.0004$ )	0.80
2	Normal versus IF+i	48	3.19 (1.11–10.82, $P=0.03$ )	7.37 (2.21–34.07, $P=0.0006$ )	0.85

<sup>a</sup> Per natural log change in regressor.



**TABLE 2D.** Multivariate analysis of noninvasive predictors for the development of 24-month IF+i<sup>a</sup>

Model	Outcome	n	Urine CCL2: Cr Odds ratio <sup>b</sup> (95% CI)	AUC	P
1	Normal versus inflammation, IF+i, transplant glomerulopathy	78	2.05 (1.15–3.92)	0.66	0.01
2	Normal versus inflammation and IF+i	63	2.28 (1.13–5.05)	0.67	0.02
3	Normal versus IF+i	58	3.42 (1.50–9.11)	0.72	0.003

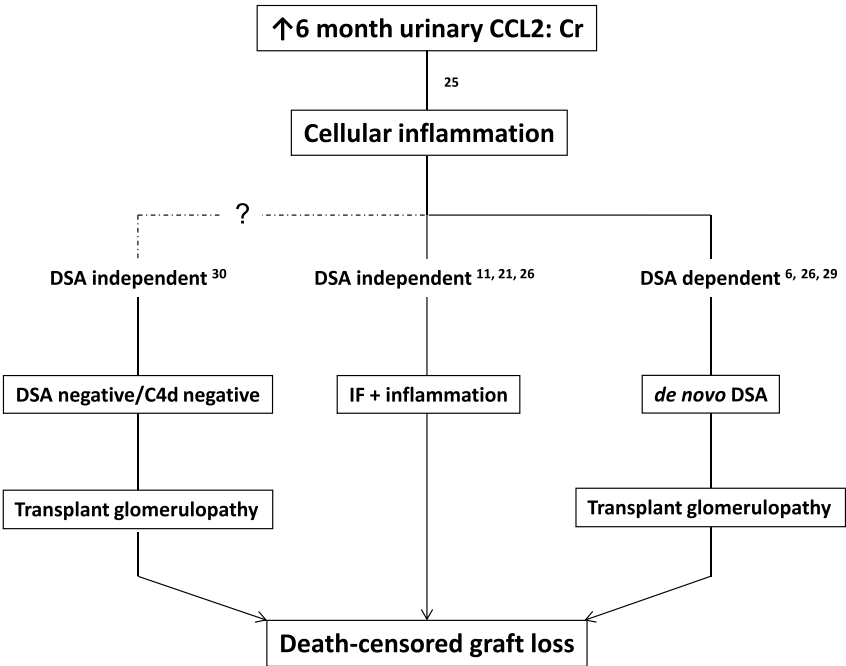
Control group=normal histology alone.  
Stepwise logistic regression was performed with the covariates: donor age >50 years; delayed graft function; living versus deceased donor, ACEi/ARB exposure, PRA and HLA match. Only 6-month urinary CCL2: Cr was significant and included in the final analysis.  
<sup>a</sup> Control group=normal histology alone.  
<sup>b</sup> Per natural log change in regressor.

absence of DSA. Hayde et al. recently demonstrated that a cohort of TG patients (DSA negative/C4d negative) had increased cytotoxic T cell-associated transcripts on microarray analysis, suggesting T cell activation as a mechanism of injury (30). Unfortunately, DSA and C4d were not prospectively evaluated in this cohort, so the pathogenesis of TG in our patients is unknown.

There are few studies that have attempted to characterize noninvasive urine markers for IF+i. In a recent study, Amer et al. demonstrated that urinary IgG and IgM levels are elevated in patients with IF+i on 12-month surveillance biopsy compared with patients with normal histology (31).

However the diagnostic performance of urinary IgG and IgM to detect IF+i was not reported, nor were these proteins significant for predicting death-censored graft loss (31). Rush et al. evaluated the urinary metabolome by magnetic resonance in patients from the DeKAF study and demonstrated that metabolomics could distinguish patients with IFTA+i versus IFTA alone (32). However, these analyses showed correlations between metabolomic signatures and graft histology but were not used prospectively.

There are some limitations to this study. First, this is strictly an observational study that is able to identify associations but cannot make causal links. Second, these novel findings need to be independently validated in a larger, separate cohort, particularly in a cohort of greater immunologic risk. Indeed our low immunologic risk population experienced a low rate of both clinical and subclinical rejection, and the diagnostic utility of urinary CCL2: Cr may in fact increase in a higher immunologic risk population (19). Finally, there are no universally accepted criteria for scoring IF+i, and this is an ongoing limitation of the literature. In this study, we used the Mayo Clinic criteria (8–10), but we acknowledge that this does not account for inflammation in areas of atrophy and may therefore underrepresent the extent of IF+i. Nevertheless, while the magnitude of effect for urinary CCL2: Cr may have been diluted by this classification schema, it still significantly correlated with both concomitant and late IF+i. The strengths of this study are the prospective, multicenter renal transplant study design, which included serial urines and surveillance biopsies. This allows for the evaluation of early urinary predictors of late histologic outcomes and analysis of histologic progression within individuals. Finally, the urinary CCL2 assay is a straightforward ELISA, which is easily translatable from bench to bedside.



**FIGURE 3.** Possible relationships between cellular inflammation and late graft loss. The numbers denote references describing the different pathways.

In conclusion, we have shown that urinary CCL2 is an early noninvasive marker that detects patients with or at risk of developing IF+i, which could be used to identify patients in whom avoidance of drug minimization/withdrawal protocols and more intensive monitoring may be required. Finally, urinary CCL2: Cr could be used as a tool to help identify patients for inclusion in novel interventional trials evaluating specific therapies for IF+i.

## MATERIALS AND METHODS

### Study Design

The study population for 6-month urinary CCL2: Cr was a cohort derived from an open label, multicenter parallel group trial (2001–2004) that has been described in detail (19, 20). Two-hundred forty patients were enrolled from 11 Canadian centers and 1 in the United States, and all patients underwent serial surveillance biopsies at 0, 6, and 24 months with concurrent urine sample collection. All patients received tacrolimus, mycophenolate mofetil, and prednisone. Based on the inclusion criteria of a 6-month urine and a 24-month surveillance biopsy for analysis, 111 patients were evaluated for 6-month urinary CCL2: Cr (Figure S1, SDC, <http://links.lww.com/TP/A931>). Biopsies were scored centrally according to the Banff '07 criteria (17) by blinded central pathologists and then classified using the central Banff scores according to the Mayo Clinic criteria for IF+i (8–10). Urinary CCL2 was measured with a sandwich ELISA as previously described and corrected for urinary creatinine to account for dilutional factors (20, 21).

### Statistical Analyses

Data were analyzed with SAS version 9.2 (SAS Institute, Cary, NC). Results are presented as mean±SD for continuous variables and compared with ANOVA. Frequencies of categorical variables are presented as counts and percentages and compared with a chi-square or Kruskal-Wallis test for nonparametric data. Urinary CCL2: Cr was highly skewed and was therefore log transformed (ln) for the purposes of analysis. Univariate analysis was performed to identify variables associated with 24-month IF+i. Stepwise logistic regression analysis was then performed to assess the independent predictors for IF+i. Potential covariates considered for inclusion were 6-month urinary CCL2: Cr, donor age older than 50 years, delayed graft function, living versus deceased donor, angiotensin converting enzyme inhibitor or angiotensin receptor blocker exposure, panel reactive antibody, HLA mis-match, and 6-month histology. In the final multivariate models, to identify noninvasive predictors of IF+i, 6-month histology was censored from the analysis. Linear regression analysis was performed to determine if 6-month urinary CCL2: Cr correlates with decline in renal function over time. The Harrell's C-statistic was calculated as the primary measure of model discrimination and is equivalent to the area under the receiver operating characteristic curve (AUC).

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