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Development and validation of an optimized integrative model using urinary chemokines for noninvasive diagnosis of acute allograft rejection

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Abbreviations

ABMR: antibody-mediated rejection

AIC: Akaike Information Criterion

AR: acute rejection

AUC: area under the curve

BIOMARGIN: Biomarker of renal graft injuries in kidney allograft recipients

BKVN: BK-virus nephropathy

CI: confidence interval

Cr: urinary creatinine

CFU: colony-forming units

CNI: calcineurin inhibitors

CTOT: Clinical Trial in Organ Transplantation

CV: coefficient of variability

CXCL9: chemokines C-X-C motif ligand 9

CXCL10: chemokines C-X-C motif ligand 10

DGF: delayed graft function

DSAs: donor-specific antibodies

ECD: expanded-criteria donor

eGFR: estimated glomerular filtration rate

ELISA: enzyme-linked immunosorbent assay

ESRD: end-stage renal disease

F: female

FN: false negative

FP: false positive

GN: glomerulonephritis

HLA: human leukocyte antigen

IF/TA: interstitial fibrosis and tubular atrophy

IQR: interquartile range

KTRs: kidney transplant recipients

Ln: natural logarithm

MDRD: modification of diet in renal disease

MFI: mean fluorescence intensity

NA: not available

NPV: negative predictive value

NR: no rejection

OR: odds ratio

PBS: phosphate-buffered saline

PPV: positive predictive value

qPCR: quantitative polymerase chain reaction

ROC: receiver operating characteristic

RT: room temperature

SCD: standard-criteria donor

SD: standard deviation

Sen: sensitivity

Spe: specificity

TCMR: T-cell mediated rejection

Th: Threshold

TN: true negative

TP: true positive

UTI: urinary tract infection

VIF: variance inflation factor

WB: wash buffer

ABSTRACT

The urinary chemokines CXCL9 and CXCL10 are promising noninvasive diagnostic markers of acute rejection (AR) in kidney recipients, but their levels might be confounded by urinary tract infection (UTI) and BK-virus reactivation. Multiparametric model development and validation addressed these confounding factors in a training set of 391 samples, optimizing the diagnostic performance of urinary chemokines. CXCL9/creatinine increased in UTI and BKV viremia with or without nephropathy (BKVN) (no UTI/leukocyturia/UTI: -0.10/1.61/2.09, $P=0.0001$ and no BKV/viremia/BKVN: -0.10/1.90/2.29, $P<0.001$) as well as CXCL10/creatinine (1.17/2.09/1.98, $P<0.0001$ and 1.13/2.21/2.51, $P<0.001$, respectively). An optimized 8-parameter model (recipient age, sex, eGFR, DSAs, UTI, BKV blood viral load, CXCL9 and CXCL10) diagnosed AR with high accuracy (AUC: 0.85, 95% CI: 0.80-0.89) and remained highly accurate at the time of screening (AUC: 0.81, 95% CI: 0.48-1) or indication biopsies (AUC: 0.85, 95% CI: 0.81-0.90) and within the first year (AUC: 0.86, 95% CI: 0.80-0.91) or later (AUC: 0.90, 95% CI: 0.84-0.96), achieving AR diagnosis with an AUC of 0.85 and 0.92 ($P<0.0001$) in two external validation cohorts. Decision curve analyses demonstrated the clinical utility of the model. Considering confounding factors rather than excluding them, we optimized a noninvasive multiparametric diagnostic model for AR of kidney allografts with unprecedented accuracy.

INTRODUCTION

The use of the urinary chemokines C-X-C motif ligand (uCXCL) 9 and uCXCL10 in the daily follow-up of kidney transplant recipients (KTRs) has never seemed so close^{1,2} given the growing evidence of, and interest in, its potential for noninvasive diagnosis and prediction of renal allograft rejection. Positive and negative predictive values (PPVs and NPVs, respectively) vary from 55% to 90%³ suggesting that confounding factors might influence the levels of these proteins and thus impact their diagnostic accuracy and limit their value in clinical practice.

Urinary tract infection (UTI) and BK-virus nephropathy (BKVN)⁴⁻⁶ are two conditions associated with inflammation of the urinary tract and thus with potential increases in the levels of urinary chemokines. However, data on the influence of these confounding factors on uCXCL9 and uCXCL10 are scarce. Moreover, BKV-associated pathology has been described as a progressively damaging infection starting with viruria (a sign of BKV reactivation), followed by BKV viremia (when the immune system fails to control the infection) and ultimately biopsy-proven BKVN (when BKV damage can be detected within the allograft)⁷. To date, very few data exist on the influence of BKV viremia without BKVN on the urinary levels of CXCL9 and CXCL10⁴.

The aims of this work were (i) to identify clinical and biological factors influencing the levels of urinary chemokines in a large unselected cohort of well-phenotyped KTRs and (ii) to refine the use of urinary chemokines as a noninvasive diagnostic tool for AR through the construction of a multiparametric model accounting for all meaningful confounding factors, to be used by clinicians in routine clinical practice.

METHODS

Population

We retrospectively considered all consecutive adult patients who received a kidney transplant at Necker Hospital (Paris, France) between February 2011 and February 2016 and selected those with (i) adequate allograft biopsies, (ii) concomitant urine samples available for research and cytobacterial examination, and (iii) blood BKV viral load measurements obtained within ten days before or after biopsy. The screening process and characteristics of included vs excluded patients/samples is provided in **Supplemental Table 1 and Supplemental Figure 1**.

Extensive protocol for urinary chemokines quantification by enzyme-linked immunosorbent assay (ELISA) is provided in the **Supplemental Materials and Methods**, as well as details on histology grading of biopsies, and donor-specific antibodies (DSAs), BK viremia and UTI assessments.

The study was approved by the Ethics Committee of Ile-de-France XI (#13016), and all participating patients provided written informed consent.

Statistical analyses

The results are presented as the mean $\pm$ standard deviation (SD) for continuous variables except for the time from transplant to biopsy, viral loads and chemokine levels, which are presented as the median and interquartile range (IQR). Frequencies of categorical variables are presented as numbers and percentages. The distribution of each biomarker exhibited considerable positive skewness, which was substantially reduced through natural logarithm transformation. We compared groups using the Mann-Whitney or Kruskal-Wallis tests, followed by Dunn's posttests when appropriate.

Univariate linear regression analysis was performed to determine the clinical and biological parameters that were significantly associated with urinary chemokine levels. To fulfill the linear regression assumptions, we examined the residuals for normality (both graphically and using normality tests), linearity and homoscedasticity.

Logistic regression analysis was performed to identify parameters independently associated with AR. We tested for clinically and biologically relevant variables. When several modalities of a

single variable were tested (e.g., the presence or absence of DSAs versus the mean fluorescence intensities [MFIs] of the DSAs), the modality associated with the lowest P-value was retained.

Several multivariable logistic regression models were then built that included all variables with a $P < 0.25$ in the univariate analysis. Multicollinearity of variables within models was tested by examining the variance inflation factors (VIFs). Values of $VIF < 2.5$ were considered acceptable⁸. A stepwise forward selection and backward elimination procedure was performed to select the best model according to the Akaike Information Criterion (AIC). For internal validation, the abilities of the different models to discriminate AR cases from non-AR cases were determined through quantification of the area under the curve (AUC) values for receiver operating characteristic (ROC) curves. We applied a bootstrap resampling procedure with 1000 repetitions to compute the 95% confidence intervals (CIs). Sensitivities, specificities, NPVs and PPVs are given at the optimal thresholds given by the Youden index⁹. The AUC values of the different models (paired ROC curves) were compared by generating an estimated covariance matrix¹⁰. We tested the calibration of the prediction model both graphically and with the Hosmer-Lemeshow test for goodness of fit¹¹. We performed sensitivity analyses including the indication for biopsy, time from transplantation and only the first biopsy for patients who provided multiple samples. For external validation, we tested the reproducibility of the model on two independent cohorts. Next, we assessed the additive value of the optimized model descriptively by the reclassification in the AR (event) and no rejection (NR, no event) groups. The Net Reclassification Index or the Integrated Discrimination Improvement were not assessed because they could have raised concerns in the settings of nested logistic regression models. Finally, a decision curve analysis was performed to assess the clinical utility of the model^{12,13}.

Analyses were performed with R software (R Development Core Team, version 1.0.44) and GraphPad PRISM® Software (GraphPad Software, San Diego, USA, version 5.02).

External validation cohorts

For independent validation, we quantified uCXCL9 and uCXCL10 in urinary specimens collected at the time of biopsy in two external cohorts: a French single-center cohort (A) and a European multicenter cohort (B). The screening process and characteristics of included vs

excluded patients/samples of these two validation cohorts is provided in **Supplemental Table 2-3** and **Supplemental Figure 2-3**.

RESULTS

Study cohort

According to the inclusion criteria, 391 triplets of samples (i.e., allograft biopsy/urine/BKV viremia samples) corresponding to 329 individual patients were collected. **Supplemental Table 4** shows the patient and transplant characteristics.

Biopsies were performed at a median time of 8 months post-transplantation (**Table 1**), of which 88.2% were clinically indicated, most frequently (54.7%) for a rise in serum creatinine. At the time of biopsy, the mean estimated glomerular filtration rate (eGFR) was 36.8 ± 15.6 mL/min, and the mean proteinuria-to-creatininuria ratio was 0.8 ± 1.7 g/g. DSAs were detected in 40.6% of cases. BKV viremia was detectable in 15.9% of cases, with a median viral load of 3.5 [2.5-4.3] $\log_{10}$ copies/mL. Urinalysis showed that 10% of cases exhibited UTI according to currently accepted criteria. The most frequent pathologic diagnosis was interstitial fibrosis and tubular atrophy (IF/TA, 39.4%). AR was found in 24.3% of biopsy specimens (N=95), and 5.9% displayed BKVN (N=23).

Non-alloimmune inflammation increases urinary CXCL9 and CXCL10 levels

We first sought to identify which clinical or biological variables might be associated with increased urinary levels of CXCL9 and CXCL10. Univariate linear regression was performed for 18 variables (**Table 2**). No donor or recipient demographic variables were significantly associated with urinary chemokine profiles.

As expected, both chemokines were significantly increased in the presence of DSAs ($P < 0.05$) and histological diagnoses of AR ($P < 0.001$) and were also increased in the presence of leukocyturia, UTI, detectable viremia or BKVN (**Table 2**).

Urinary levels of CXCL9 and CXCL10 are similarly increased in BKV viremia and BKVN

To further address whether different stages of BKV infection might impact urinary chemokine levels, we categorized the samples into three non-overlapping groups according to their BKV status (**Figure 1A**).

In the whole population (N=391), the urinary levels of CXCL9 and CXCL10 (median [IQR], **Figure 1B and 1C**) remained low in the absence of BKV reactivation ($\text{LnCXCL9/cr} = -0.1[-0.8-1.9]$,

LnCXCL10/cr=1.13[-0.3-2.0]), while they were significantly increased in cases of isolated BKV viremia (LnCXCL9/cr=1.9[0.0-3.2], LnCXCL10/cr=2.21[1.9-3.0], $P<0.001$) and BKVN (LnCXCL9/cr=2.29[-0.1-2.9], LnCXCL10/cr=2.51[2.0-3.0], $P<0.001$). Interestingly, there were no significant differences in urinary chemokine levels between the BKV viremia-only group and the BKVN group ($P>0.99$, **Figure 1B and 1C**).

As BKV reactivation may occur concomitantly with UTI or AR, a sensitivity analysis was performed by excluding samples with significant leukocyturia (isolated or with UTI) and those with AR. In this restricted population (N=225), the results remain unchanged (**Figure 1D and 1E**).

Urinary levels of CXCL9 and CXCL10 are increased in UTI cases but not in isolated leukocyturia cases

Next, we investigated the association between UTI and urinary chemokine levels. In the whole population, urinalysis identified 56 samples with isolated leukocyturia and 39 with UTI (**Figure 2A**). Compared to the group of 257 samples with no leukocyturia, the groups with isolated leukocyturia or UTI exhibited significant increases in CXCL9 (median [IQR], LnCXCL9/cr: 1.61[-0.4-3.2] and 2.09[-0.6-2.8] vs -0.10[-0.7-1.5], $P<0.01$ for both, **Figure 2B**) and CXCL10 (LnCXCL10/cr: 2.09[0.4-3.1] and 1.98[1.1-3.6] vs 1.17[-0.2-2.1], $P=0.001$ and $P<0.001$, respectively, **Figure 2C**).

As previously described, a sensitivity analysis was performed by excluding cases of AR and cases of BKV viremia (with or without BKVN). In this restricted population (N=243, **Figure 2D and 2E**), compared to the samples with no leukocyturia, the samples with UTI (N=27) had significantly increased CXCL9 ($P<0.01$) and CXCL10 ($P<0.001$) urinary levels, contrary to samples with isolated leukocyturia ($P=0.14$ and $P=0.80$, respectively).

Construction of an optimized model for noninvasive diagnosis of acute rejection

Then, uCXCL9 and uCXCL10 were compared between samples with or without AR. In the total population (**Supplemental Figure 4A and 4B**), chemokines were indeed significantly higher in AR than non-AR cases (LnCXCL9/cr: 1.92[-0.3-3.2] vs -0.10[-0.8-1.6], LnCXCL10/cr: 2[1.2-2.9] vs 1.12[-0.5-2.1], $P<0.0001$). In a sensitivity analysis after exclusion of BKV viremia (with or without BKVN) and leukocyturia (with or without UTI), CXCL9 and CXCL10 remained significantly increased in AR cases, as depicted in **Supplemental Figure 4C and 4D**.

Next, we performed a logistic regression analysis to build several models for noninvasive diagnosis of AR. Seventeen candidate variables were considered in the univariate analysis, and all variables with a P-value ≤ 0.25 (N=14) were entered into multivariable regression (**Supplemental Table 5**). **Supplemental Figure 5A** shows that the two chemokines are highly correlated (Spearman $r=0.60$, 95% CI: 0.53-0.66, $P<0.0001$) raising the methodological issue of multicollinearity. However, with a VIF well below the most conservative upper limit of 2.5, collinearity can be regarded as low, thus allowing us to keep both chemokines for further analysis (**Supplemental Figure 5B**).

Figures 3A and 3B illustrate the poor diagnostic performance of the three parameters used in clinical practice to assess the risk of AR (i.e., eGFR, proteinuria and DSAs) eventually leading to graft biopsy. In comparison, combining eight variables among four categories (clinical variables [recipient age and sex], biological variables [eGFR and DSA score], confounding factors [BKV viremia and UTI]) and urinary chemokines (CXCL9/cr and CXCL10/cr) into an optimized mathematical model dramatically improved the assessment of AR risk ($P<0.0001$). Interestingly, though usually regarded as a relevant biological item in the context of AR, once adjusted for urinary chemokine levels in the multivariable analysis, proteinuria was not found to be independently significant ($P=0.126$).

The results of the bootstrapped logistic regression analysis of the final model are given in **Table 3**. The final multiparametric model strongly discriminated AR from non-AR samples (-0.02 vs -2.21 , $P<0.0001$, **Figure 3C**) with an AUC of 0.85 (95% CI: 0.80-0.89, $P=2.43E-23$), and the calibration curve showed good concordance between observed and predicted AR, as confirmed by the Hosmer-Lemeshow goodness-of-fit test ($\chi^2=7.96$, $P=0.44$, **Figure 3D**).

Ultimately, eGFR, proteinuria and DSAs were entered into a statistical model to compute a decision-making model based on these 3 usual parameters. This clinical model, though far more advanced than current clinical practice, only slightly improved diagnostic accuracy (AUC: 0.77, 95% CI: 0.71-0.82, $P<0.001$, **Figure 3E**). We assessed the value of the optimized model to reclassify the risk of AR, compared to this clinical model (**Figure 3F**). In the non-AR group, the optimized model correctly reclassified 69% (N=194/283) of patients to a lower risk. In the AR group, appropriate reclassification to a higher risk was found in 68% (61/90) of AR patients. Overall, the optimized model improved classification both by correct net downward

reclassification in patients without AR events (37%) and correct net upward risk reclassification in patients with AR events (36%).

Validation of the optimized model for noninvasive diagnosis of acute rejection

Internal validation of the model included several sensitivity analyses (**Figure 4A-C**). The optimized model remained highly accurate when only the first biopsy of each patient was retained (AUC: 0.85, 95% CI: 0.80-0.90; $P=1.4E-19$), in cases of stable graft function (AUC: 0.81, 95% CI: 0.48-1; $P=1.3E-02$), at the time of graft dysfunction (AUC: 0.85, 95% CI: 0.81-0.90; $P=1.2E-22$), and within the first year (AUC: 0.86, 95% CI: 0.80-0.91; $P=9E-16$) or later (AUC: 0.90; 95% CI: 0.84-0.96; $P=5.9E-13$) after transplantation. Moreover, a subset analysis was performed in KTR with pre-existing or *de novo* DSA, to assess the benefit of the model to detect antibody-mediated rejection (ABMR). In comparison to the DSA score alone, the optimized 8-variable model significantly improved ABMR diagnosis accuracy (0.79 vs 0.65, $P<0.01$, **Supplemental Figure 6**).

In addition, robustness of the optimized model was assessed in two external validation cohorts. First of all, Cohort A included 147 urine specimens collected at time of mainly indication biopsies (75.5%) in 109 single-center KTRs. In addition, Cohort B included 295 urine specimens collected at time of mainly screening biopsies (73.2%) in 282 KTRs from four European centers. The same 8 parameters diagnosed AR with an AUC of 0.92 (95% CI: 0.88-0.97; $P<0.0001$) in cohort A, and 0.85 (95% CI: 0.78-0.91; $P<0.0001$) in cohort B (**Figure 4D**).

Accuracy metrics of the optimized model for noninvasive diagnosis of acute rejection

We next examined the cut-off value of the optimized multiparametric model. **Figure 5A and 5B** shows the accuracy metrics of the model. The optimal cut-off (-1.48) had a sensitivity of 84.4%, a specificity of 69.3%, PPV of 46.6%, and NPV of 93.3%. To adapt to various contexts of use, we defined two arbitrary thresholds: a “low risk” cut-off of -3, which optimized sensitivity and NPV by reducing the fraction of false negative results and a “high-risk” cut-off of +1, which maximized specificity and PPV by reducing the fraction of false positive results.

This optimized multiparametric non-invasive model provides a risk of AR at an individual level. Probability (p) of acute rejection can be computed from the following equation (with each β coefficient of each variable (x) as defined in **Supplemental Table 6**):

$$p = \frac{1}{1 + \exp(-(\beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_4x_4 + \beta_5x_5 + \beta_6x_6 + \beta_7x_7 + \beta_8x_8))}.$$

As an example, a 43-year-old woman with a eGFR of 20 mL/min, the presence of DSAs with an MFI of 1811, no UTI, no detectable BK viremia, uCXCL9/cr level of 3.04 ng/mmol, and uCXCL10/cr level of 20.65 ng/mmol had a value of 1.45, which corresponds to a predicted risk of AR of 81% (**Figure 5C**).

Finally, practical or financial considerations could stimulate the use of a slightly degraded model with only one of the two chemokines. As shown above, collinearity is low (**Supplemental Figure 5**) and both chemokines were selected in the multivariable logistic regression (**Table 3**) indicating that both provide specific information. **Supplemental Table 7** shows the performances of CXCL9 and CXCL10 alone, in combination, or included in multiparametric models for the diagnosis of AR, T-cell mediated and antibody-mediated rejections.

Clinical utility of the urinary chemokine model in optimizing the cost/benefit ratio of biopsies

Traditional statistical measures for the evaluation of prediction models (accuracy metrics, discrimination, calibration) do not provide an answer as to whether the model should be used in clinical practice. To put benefits (identifying AR) and harms (biopsy complications and/or costs) on the same scale¹⁴, we performed a decision curve analysis, which calculates a clinical “net benefit” for one or more prediction models in comparison to default strategies of performing a biopsy to all or no patients. **Figure 6A** shows the net benefit (identifying true positive cases) of the optimized model compared to the clinical model (eGFR, proteinuria, DSAs) according to the threshold probability. The threshold probability varies according to clinicians and patients’ preferences and can be better understood if considered as “biopsies performed to find one rejection” (see secondary x-axis). The blue line, corresponding to the optimized model, has the highest benefit across a wide range of reasonable threshold probabilities. **Figure 6B** shows the “net benefit” expressed as biopsies avoided (secondary y-axis), corresponding to true negative cases. Again, the optimized model has a high benefit across a wide range of risks. As an example, at the threshold risk of 10% (“I would not want to do more than 10 biopsies to find one acute rejection”), 13 biopsies would be avoided per 100 patients if using the optimized model compared to the clinical model.

Figure 7 suggests how the urinary chemokine model could be used across an individual patient scenario. Routine graft status work-up (serum creatinine, proteinuria, DSAs, sonography, calcineurin inhibitors trough level) defines the context of use. In unstable patients, the net benefit of the model was assessed compared to a default strategy of performing a biopsy on all patients (**Figure 7, upper panel**). At the threshold risk of 10%, the number of unnecessary biopsies avoided was 17 per 100 unstable patients. In stable patients, in a transplant center where no screening biopsy is performed, the model would help to identify 6 out 100 apparently stable patients who would benefit from a biopsy to diagnose a subclinical rejection. In a transplant center with screening biopsy as part of routine work-up, the model could help to avoid 58 unnecessary biopsies out of 100 stable patients (**Figure 7, lower panel**).

DISCUSSION

Urinary chemokines have been consistently demonstrated as promising biomarkers for the noninvasive diagnosis of AR of renal allografts. However, insufficient consideration of confounding factors has led to inconsistencies in their reliability in terms of sensitivity and specificity, and has limited their adoption in clinical practice. In addition, if assessed in the clinics, urinary chemokines should be considered as additional parameters to be examined with other important markers (e.g., graft function, DSAs, and proteinuria) during the decision-making process. In this study, using a fully phenotyped cohort of KTRs, we have clearly established the clinical conditions that should be considered when using urinary chemokine levels to noninvasively identify patients with high probability of AR, and we have developed and validated (in two external validation cohorts) an optimized multiparametric model that predicts individual risk of AR with high accuracy. Finally, by using decision curve analyses in different clinical scenarios and contexts of use, we demonstrated and quantified the clinical utility of the model compared to conventional approaches.

We have demonstrated that UTI (but not isolated leukocyturia) and BKV viremia (with or without BKVN) are clinical conditions associated with increased urinary levels of CXCL9 and CXCL10. UTI is the most frequent infectious complication after kidney transplantation, with an estimated incidence of cystitis per 100 recipient-years of 13.84 and an estimated incidence of acute pyelonephritis per 100 recipient-years of 3.66¹⁵. However, very few data are available on

the impact of UTI on the levels of urinary CXCL9 and CXCL10. The Clinical Trial in Organ Transplantation-01 (CTOT-01), in which 280 adult and pediatric KTRs were prospectively followed, found that the mean urine concentrations of the CXCL10 protein were similar in patients with AR or infection, but the values for CXCL9 were higher in patients with AR¹⁶. However, in CTOT-01 the number of patients with urinary infection was very low (N=7), and this small group included different diagnoses (bacterial and BK infections). Very recently, Handschin et al. addressed the problem of the impact of leukocyturia on urinary CXCL10 levels in a large cohort¹⁷ and demonstrated leukocyturia as a major confounding factor. However, as no blood BKV viral load nor histological data were available, one cannot rule out that other conditions accounted for part of CXCL10 increased levels. In our study, isolated leukocyturia tended to be associated with increased chemokine levels, but in the absence of rejection or BKV reactivation, the differences failed to reach significance. However, our study clearly indicates that significant bacterial UTI independently increases the urinary concentrations of CXCL9 and CXCL10, with the most robust impact on CXCL10. Overall, as suggested by others^{16,17}, UTI must be addressed before urinary CXCL9 and CXCL10 levels can be used as noninvasive diagnostic markers of AR in KTRs.

BKV reactivation is another frequent inflammatory condition in KTRs. BKV viruria occurs in 30-50% of KTRs with potential progression to BKV viremia in 10-30% of recipients¹⁸⁻²¹ and to biopsy-proven BKVN in 1-10% of patients^{22,23}. Few data are available on the impact of BKV reactivation on the levels of urinary biomarkers. Hu et al. showed in two studies^{5,6} that the urinary concentrations of the CXCL10 protein were increased in the few patients with BKVN (6 and 4 in the first and second studies, respectively), compared to the stable patients. Jackson et al. also found increased urinary levels of CXCL9 and CXCL10 protein in a cohort of 24 KTRs with biopsy-proven BKVN⁴. Interestingly, the authors conducted a subset analysis of patients with subclinical BKV infection (defined as BKV viremia without biopsy-proven BKVN). Although the sample size was very small (N=5), the researchers found urinary levels of CXCL9 and CXCL10 similar to those of patients with BKVN. Karminik et al. also published two studies showing that BKVN increases serum levels of CXCL10 and CXCL11^{24,25}. However, no study on large datasets regarding the influence of BKV viremia without BKVN on the levels of urinary biomarkers has been published so far. Recently, Weseslindtner et al. interestingly demonstrated that the urine CXCL10 level was

gradually increasing with BKV infection development, from isolated BKV viruria to full-blown BKVN²⁶. However, biopsies were performed only in BKVN cases, suggesting a potential selection bias with the most serious cases (highest viral load) leading to performing a biopsy. Moreover, histological lesions of BKVN can be deep and focal within the graft and can be missed by allograft biopsy^{7,27}, and thus it cannot be ruled out that BKV viremia, even in the absence of biopsy-proven BKVN, could be associated with intragraft inflammation that increases the urinary concentrations of chemokines. Our study provides insights regarding this issue obtained from a large population of patients with different stages of BKV reactivation. BKV viremia and BKVN increased the levels of urinary CXCL9 and CXCL10. Interestingly, the urinary CXCL9 and CXCL10 concentrations from patients with BKV viremia and BKVN were similar, confirming earlier observations made by Jackson et al.²⁷. These findings suggest that once detectable in blood, BKV leads to inflammation in the tubular epithelium substantially increasing urinary concentrations of CXCL9 and CXCL10, even if histological lesions are not seen on allograft biopsy. Moreover, the results suggest that urinary biomarker levels might better estimate whole-graft inflammation than the allograft biopsy, whose risk of sampling error and false negativity is particularly high in the context of BKV reactivation.

Previous studies focusing on innovative biomarkers of AR have often been hampered by intrinsic limitations. For example, when urinary cell messenger RNA profiling is used to diagnose AR, approximately 15% of samples do not pass quality control²⁸. Similarly, when assessing the risk of AR using KSORT® blood assays, approximately 15% of results are reported as 'indeterminate'²⁹. An intrinsic limitation of the use of urinary chemokine levels to noninvasively detect AR that was already mentioned¹⁶ is that these chemokines are also increased in other inflammatory conditions. To circumvent this limitation, previous studies have excluded samples with UTI or BKV reactivation³⁰. Another strategy that has been used by the CTOT9 investigators is to confirm any increased values of CXCL9 through repeated analysis in the absence of infection before mandating allograft biopsy³¹. UTI and BKV reactivation both frequently occur after kidney transplantation, particularly in early stages after transplantation, thus may hamper the use of urinary chemokines as a noninvasive strategy to monitor graft status when the risk of AR is also at its highest, therefore when monitoring is of particular interest. In the present study, we reasoned that instead of excluding samples with confounding factors, a simple multiparametric

model accounting for all factors influencing urinary chemokine levels could be used as a more accurate and easily applicable strategy. We have thus developed a multiparametric model that is not only broadly applicable but also achieves unprecedented diagnostic accuracy for noninvasive diagnosis of allograft rejection. We independently validated this model in two validation cohorts, including a multicenter cohort with primarily screening biopsies (73.2%), specifically aimed at characterizing the model's performance in detecting subclinical rejection. In clinical practice, the decision-making process relies on graft function parameters. Our study illustrates the great potential of more mathematic approaches to kidney transplant patients, to calculate disease probability instead of relying on "graft functional impairment" alone.

Another important aspect of our study was our motivation to advance the use of urinary chemokines in clinical settings. The CTOT-01 trial¹⁶ and other studies that addressed the use of urinary chemokines as non-invasive markers of acute rejection provided invaluable results. These studies suggested the great potential of this approach to personalize patient care; yet, this approach is not embraced by the community in daily clinical practice. In addition to the lack of controlled interventional trial results, we believe that several intrinsic limitations probably participate in the reluctance of using them. One limitation is the fact that all the available studies evaluated urinary chemokines on their own, with no consideration of other relevant parameters that are used in daily clinical practice. Here, we show how incorporating all relevant parameters in a single score improves risk assessment, a strategy that was never applied before. Another limitation is the fact that urinary chemokines are usually considered as a means to avoid biopsies but without clear description of the intended use in the decision-making process. Our analysis addresses this limitation and illustrates how a biomarker-based risk stratification could be used in clinical practice with practical consequences in decision-making processes including the number of biopsies to be avoided or performed.

This multiparametric approach may have great utility in modifying current approaches to screening biopsies. The use of this model might optimize the use of screening biopsies in apparently stable patients (in centers that do not routinely perform protocol biopsies) or, more importantly, might justify the use of fewer screening biopsies (in centers that routinely perform them). Our multiparametric approach providing the risk of AR at the individual level may serve as the platform for a prospective study. The next step to assess the benefit of a chemokine-guided

patient follow-up is indeed a prospective randomized controlled trial in unselected *de novo* KTRs. In an experimental arm, allograft biopsy would be driven by the individual risk of AR as assessed by the optimized chemokine model, compared to current standard of care in the control arm (including or not screening biopsies as per local policies). The primary endpoint would be the proportion of allograft biopsies identifying AR (e.g. optimizing the number of biopsies showing AR while avoiding undue protocol and/or indication biopsies). Secondary endpoints would address cost/benefit ratio of such clinical management including adverse events associated with biopsies, graft function, cost-effectiveness evaluation, and quality of life assessment.

Our study has some limitations. First, our inclusion criteria required blood BKV viral load screening at the time of biopsy. Therefore, we did not include samples collected very early or very late after transplantation, two time periods when the diagnosis of BKV reactivation is very unlikely and so was not assessed. This possible selection bias is addressed in **Supplemental Table 1-3** showing with full transparency comparisons between the included and excluded samples. For the discovery cohort and validation cohort B, the excluded samples were indeed collected later post-transplantation. As a result, eGFR was significantly lower in the excluded samples. As a counterpart, BKVN was found less frequently in the excluded samples from the discovery cohort. Another limitation was the absence of a perfect correlation between our model and the histological diagnosis of AR. However, this was expected because the gold standard diagnostic tool (i.e., histology) is itself imperfect, with cases of false negative and even false positive results³². Only longitudinal assessment of graft status will yield information on the potential benefits of a noninvasive, chemokine-based strategy to correctly reclassify patients with high probability of AR compared to conventional monitoring. However, regardless of the quality of the gold standard, our alternative noninvasive multiparametric model performs very well to estimate the individual risk of AR. A third limitation was the cross-sectional design of our study. Indeed, we did not record clinical information before or after the biopsy, which was the starting point of our inclusion criteria. Therefore, we cannot exclude that we missed clinical features that could have influenced the urinary concentrations of chemokines we obtained at the time of biopsy.

In conclusion, UTI and BKV viremia (with or without BKVN) are associated with increased urinary concentrations of CXCL9 and CXCL10. Rather than excluding these confounding factors, we incorporated them in an optimized multiparametric diagnostic model for AR of kidney allografts. This model, which includes easily available clinical and laboratory data and results from simple ELISA tests for CXCL9 and CXCL10, achieves unprecedented accuracy for a noninvasive diagnostic tool.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

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

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FIGURE LEGENDS

Figure 1: BKV infection analysis: sample distribution and chemokine levels. (A.) Euler diagram illustrating the sample distribution according to detection of viremia and histological diagnosis of BKVN and classification into three non-overlapping subgroups according to BKV status: no BKV infection, viremia without BKVN and BKVN. (B. and C.) Urinary CXCL9 and CXCL10 levels in the different subgroups of the total population. (D. and E.) Urinary CXCL9 and CXCL10 levels in the different subgroups after exclusion of cases with significant leukocyturia $\geq 10^4$ /mL (including isolated leukocyturia and UTI) and/or acute rejection. The P-values were obtained using the Kruskal-Wallis test followed by the Dunn's multiple comparisons test. Abbreviations: BKVN, BK-virus nephropathy; Cr, urinary creatinine; UTI, urinary tract infection.

Figure 2: Urinary tract infection analysis: sample distribution and chemokine levels. (A.) Euler diagram illustrating the sample distribution according to the urinalysis results. The samples were classified into three non-overlapping subgroups according to bacteriological status: no UTI, isolated leukocyturia ($\geq 10^4$ /mL) and UTI (bacteriuria $\geq 10^3$ CFU/mL and leukocyturia $\geq 10^4$ /mL). (B. and C.) Urinary CXCL9 and CXCL10 levels in the different subgroups of the total population. (D. and E.) Urinary CXCL9 and CXCL10 levels in the different subgroups in a restricted population after exclusion of cases with BKV viremia (including isolated viremia and BKVN) and/or acute rejection. The P-values were obtained using the Kruskal-Wallis test followed by the Dunn's multiple comparisons test. Abbreviations: BKVN, BK-virus nephropathy; CFU, colony-forming units; Cr, urinary creatinine; UTI, urinary tract infection.

Figure 3: Construction, discrimination, performance and calibration of a multiparametric chemokine model. (A. and B.) Diagnostic accuracy of usual biological biomarkers used alone or within the 8-parameter chemokine model ("optimized model"). ROC curves (A.) illustrating the diagnostic performance and Box plots (B.) illustrating the distribution of AUCs generated by 1000

bootstrap replicates to compute 95% CIs. The P-values were obtained from AUC comparisons using the DeLong test. **(C.)** Box plots comparing the values obtained from the multiparametric chemokine model for the different rejection groups. The P-values were obtained from a Mann-Whitney test. **(D.)** Calibration curve for the multiparametric chemokine model illustrating the correlations between the observed and predicted values. Goodness of fit was tested by Hosmer-Lemeshow statistics (chi-square values and P-values). **(E.)** ROC curves illustrating the diagnostic performance of the optimized model for acute rejection diagnosis as compared to a clinical model (based on kidney dysfunction [K], proteinuria [P] and DSAs [D]). **(F.)** Reclassification analysis between the clinical model (K+P+D) and the optimized model (K+P+D+I+B+C), among patients with and without rejection. Blue histograms indicate the appropriate reclassification to a lower risk in the NR group and to a higher risk in the AR group. Gray histograms indicate a wrong upward reclassification in the NR group, or wrong downward reclassification in the AR group. Net reclassification is given as: (%correct – %wrong) in each group. Abbreviations: AR, acute rejection; AUC, area under the curve; CI, confidence interval; DSAs, donor-specific antibodies; eGFR, estimated glomerular filtration rate; MDRD, modification of diet in renal disease; NR, no rejection; ROC, receiver operating characteristic.

Figure 4: Internal and external validation of the multiparametric chemokine model. **(A-C.)** The ROC curves for the multiparametric chemokine model from various sensitivity analyses are shown: when considering only the first biopsy of each patient (N=317 samples; **Panel A**), according to the indication for biopsy (screening biopsy (N=45) and for-cause biopsy (N=228); **Panel B**), and according to the time elapsed since transplantation (before 12 months; N=233, and after 12 months; N=140); **Panel C**). **(D.)** External validation of the model in two independent cohorts. ROC curves illustrating the diagnostic performances of the same 8 parameters in cohort A (monocentric cohort, 147 urine samples) and cohort B (multicentric cohort, 295 urines samples). Abbreviations: AUC, area under the curve. ROC, receiver operating characteristic.

Figure 5: Accuracy metrics of the multiparametric model. **(A.)** Sensitivity, specificity and Youden index $[(\text{Sen} + \text{Spe}) - 1]$ are shown according to the model value. NPV, PPV and accuracy (percentage of correctly classified cases: $[\text{TN} + \text{TP}] / [\text{TN} + \text{TP} + \text{FN} + \text{FP}]$) are shown in Panel B.

Optimal cut-off is defined by the maximal Youden index. Two other thresholds were arbitrary chosen: -3 ("low-risk threshold") to optimize sensitivity and NPV or +1 ("high-risk threshold") to optimize specificity and PPV. (C.) Model prediction (*i.e.*, risk of acute rejection) according to the model value. The blue dotted line illustrates the example of a patient with a model value of 1.45 corresponding to an 81% risk of acute rejection. Abbreviations: AR, acute rejection; FN, false negative; FP, false positive; NPV, negative predictive value; NR, no rejection; PPV, positive predictive value; Sen, sensitivity; Spe, specificity; Th, threshold; TN, true negative; TP, true positive.

Figure 6: Clinical utility of the multiparametric chemokine model assessed by a decision curve analysis. (A.) Decision curve analysis showing benefit of performing a biopsy in patients at risk for acute rejection. A reasonable range of threshold probability was chosen and plotted on the x-axis. A secondary x-axis was added to illustrate the number of biopsies to perform to find 1 acute rejection case. The y-axis shows net benefit which is defined as benefit – (harm x threshold probability). Net benefit is given by the following equation: $(TP/N) - (FP/N) \times p_t / (1 - p_t)$, with TP being true positives (rejection), FP being false positives, N being the total sample size, and p_t the threshold probability. Physicians (taking into account patient preferences) may vary in their propensity to perform a biopsy. One can then consider the benefit/harm ratio in performing a biopsy across several individual scenarios. The blue line shows the net benefit (identifying a true positive case) according to the optimized model compared to a clinical model (gray line) including allograft function, proteinuria and DSAs. Dotted lines show the default strategy of performing a biopsy in all patients (red) or none (green). (B.) Decision curve analysis showing the benefit in identifying true negative patients. This net benefit can also be expressed as the number of biopsies avoided per 100 patients (secondary y-axis). Net benefit of the optimized model (blue line) is plotted as well as the clinical model and the 2 default strategies ("biopsy all", "biopsy none"). Abbreviations: DSAs, donor-specific antibodies.

Figure 7: Proposed implementation of the multiparametric chemokine model for individual risk assessment in various clinical scenarios. Routine work-up, including serum creatinine,

proteinuria, DSA, sonography and CNI trough level assessment is performed to specify the context of use. In the upper panel, unstable patients can be classified according to the model in a high-risk group ("perform a biopsy"), or a low-risk group. Therefore, low-risk patients may benefit from not performing a biopsy compared to a strategy of performing a biopsy on all unstable patients. In the lower panel, the strategy depends on the local policy regarding screening biopsies. In a low-risk group of stable patients, the true benefit of the model is expressed as the number of futile biopsies, compared to a strategy of performing screening biopsies in all stable patients. In a high-risk group, the model helps to identify apparently stable patients who would benefit from a biopsy to demonstrate their subclinical rejection. Abbreviations: CNI, calcineurin inhibitors; DSAs, donor-specific antibodies.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table 1: Clinical, histological and biological characteristics at the time of allograft biopsy

Variables	N=391
Time after transplantation (mo). <i>median (IQR)</i>	8 (33)
Indication of biopsy	
Screening biopsy. <i>n (%)</i>	46 (11.8)
Clinically indicated biopsy. <i>n (%)</i>	345 (88.2)
Rise in serum creatinine. <i>n (%)</i>	214 (54.7)
Proteinuria. <i>n (%)</i>	32 (8.2)
<i>De novo</i> DSAs. <i>n (%)</i>	10 (2.6)
Control after rejection. <i>n (%)</i>	46 (11.8)
BKV viremia. <i>n (%)</i>	43 (11.0)
Other. <i>n (%)</i>	1 (0.3)
Pathologic primary diagnosis	
ABMR. <i>n (%)</i>	64 (16.4)
TCMR. <i>n (%)</i>	17 (4.3)
Mixed rejection. <i>n (%)</i>	14 (3.6)
BKVN. <i>n (%)</i>	23 (5.9)
IF/TA. <i>n (%)</i>	154 (39.4)
Acute tubular injury. <i>n (%)</i>	11 (2.8)
Recurrent disease. <i>n (%)</i>	9 (2.3)
Normal. <i>n (%)</i>	21 (5.4)
Other ^a . <i>n (%)</i>	78 (20.0)
Laboratory test results at the time of biopsy	
Serum	
Creatinine ($\mu\text{mol/L}$). <i>mean$\pm$SD</i>	188 $\pm$ 102
DSAs. <i>n (%)</i> ^b	155 (40.6)
Detectable BKV viremia. <i>n (%)</i>	62 (15.9)
Viral load (Log_{10} copies/mL). <i>median (IQR)</i>	3.5 (1.8)

Urine

Proteinuria/creatininuria ratio (g/g). *mean±SD* 0.8±1.7

Bacteriuria ($\geq 10^3$ /mL) and leukocyturia ($\geq 10^4$ /mL). *n (%)* 39 (10.0)

Abbreviations: ABMR, antibody-mediated rejection; BKVN, BK-virus nephropathy; DSAs, donor-specific antibodies; IF/TA, interstitial fibrosis/tubular atrophy; SD, standard deviation; TCMR, T-cell-mediated rejection. Borderline rejection lesions were classified among normal biopsies (N=14). Blood BKV viral load is expressed as the number of copies ($\log_{10}$) per mL of plasma. ^a Nonspecific lesions including calcineurin inhibitor toxicity. ^b Data not available for 9 cases.

Table 2: Association between urinary chemokines and clinical and laboratory data

	LnCXCL9/cr			LnCXCL10/cr		
	β	95% CI	P	β	95% CI	P
Demographic variables						
Recipient age	0.002	-0.01-0.01	0.695	-0.001	-0.01-0.01	0.906
Recipient sex:						
Male	0.237	-0.14-0.61	0.213	-0.154	-0.56-0.25	0.459
Donor age	0.001	-0.01-0.01	0.789	0.005	-0.01-0.02	0.412
Donor type:						
Living donor	-0.224	-0.63-0.19	0.284	-0.158	-0.61-0.29	0.491
Preformed DSAs	-0.102	-0.47-0.26	0.581	0.201	-0.19-0.61	0.305
At the time of transplantation						
DGF	0.316	-0.05-0.68	0.089	0.148	-0.25-0.55	0.469
Induction therapy:						
Thymoglobuline®	-0.168	-0.54-0.2	0.376	0.102	-0.31-0.51	0.623

Time from transplantation to biopsy	0.003	0-0.01	0.158	0.003	0-0.01	0.227
Laboratory test results at the time of biopsy						
DSAs	0.405	0.04-0.77	0.032	0.575	0.17-0.98	0.006
Serum creatinine	0.004	0-0.01	< 0.001	0.004	0-0.01	< 0.001
Proteinuria/creatininuria ratio	0.014	0.01-0.02	< 0.001	0.156	0.01-0.02	< 0.001
Histological variables						
ABMR	1.006	0.56-1.45	< 0.001	1.138	0.65-1.62	< 0.001
TCMR	2.037	1.40-2.67	< 0.001	1.610	0.90-2.32	< 0.001
Acute rejection (ABMR, TCMR, mixed)	1.194	0.79-1.60	< 0.001	1.199	0.75-1.65	< 0.001
BKV infection						
Detectable BKV viremia	1.215	0.73-1.70	< 0.001	1.620	1.10-2.14	< 0.001
BKVN	1.163	0.40-1.93	0.003	1.477	0.64-2.31	< 0.001
Bacterial UTI						
Leukocyturia ($\geq 10^4$ /mL)	1.116	0.71-1.53	< 0.001	1.142	0.69-1.59	< 0.001
Bacteriuria ($\geq 10^3$ /mL) and leukocyturia ($\geq 10^4$ /mL)	0.946	0.35-1.55	0.002	1.210	0.55-1.86	< 0.001

Abbreviations: ABMR, antibody-mediated rejection; BKVN, BK-virus nephropathy; CFU, colony-forming units; CI, confidence interval; cr, urinary creatinine; DGF, delayed graft function; DSAs, donor-specific antibodies; TCMR, T-cell-mediated rejection; UTI, urinary tract infection (bacteriuria [$\geq 10^3$ CFU/mL] and leukocyturia [$\geq 10^4$ /mL]).

Table 3: Multivariable model for the diagnosis of acute allograft rejection

	Adjusted OR	95% CI	P-value
Clinical variables at the time of transplantation			
Recipient sex (F)	2.43	1.34-4.47	0.004
Recipient age	0.97	0.95-0.99	0.005
Laboratory test results at the time of biopsy			
eGFR (MDRD):			
30-59 mL/min/1.73 m ²	4.34	1.16-22.98	0.049
15-29 mL/min/1.73 m ²	8.17	2.05-45.39	0.007
<15 mL/min/1.73 m ²	12.27	2.29-84.47	0.006
DSA score:			
500 ≤ MFI <1000	2.02	0.83-4.75	0.113
1000 ≤ MFI <3000	4.08	1.86-9.08	4.8E-04
3000 ≤ MFI	4.15	1.81-9.60	0.001
Confounding factors			
Blood BKV viral load (upper quartile, ≥4.3 log)	0.08	0.00-0.51	0.026
UTI	0.27	0.08-0.74	0.016
Chemokines			
LnCXCL9/cr	1.41	1.16-1.73	0.001
LnCXCL10/cr	1.26	1.04-1.55	0.021

Abbreviations: CI, confidence interval; Cr, urinary creatinine; eGFR, estimated glomerular filtration rate; F, female; MDRD, modification of diet in renal disease; MFI, mean fluorescence intensity; OR, odds ratio; UTI, urinary tract infection.

Figure 1

Figure 2

Figure 3

A.

B.

C.

D.

E.

F.

Figure 4

Figure 5

A.

First biopsy of each patient

B.

According to the indication for biopsy

C.

According to the time post-transplantation

D.

External validation cohorts

Figure 6

A.

B.

Figure 7