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FOXP3 mRNA Profile Prognostic of Acute T-cell–mediated Rejection and Human Kidney Allograft Survival

Danny Luan, MPH[1], **Darshana M. Dadhania, MD**[1,2], **Ruchuang Ding, MD**[1], **Thangamani Muthukumar, MD**[1,2], **Michelle Lubetzky, MD**[1,2], **John R. Lee, MD**[1,2], **Vijay K. Sharma, PhD**[1], **Phyllis August, MD**[1,2], **Franco B. Mueller, PhD**[1], **Joseph E. Schwartz, PhD**[1,3], **Manikkam Suthanthiran, MD**[1,2,*]

[1]Division of Nephrology and Hypertension, Department of Medicine, New York Presbyterian Hospital-Weill Cornell Medical College, New York, NY, USA

[2]Department of Transplantation Medicine, New York Presbyterian Hospital-Weill Cornell Medical College, New York, NY, USA

[3]Department of Psychiatry and Behavioral Health, Renaissance School of Medicine at Stony Brook University, Stony Brook, NY, USA

Abstract

Background.—T-cell–mediated rejection (TCMR) is the most frequent type of acute rejection and is associated with kidney allograft failure. Almost 40% of TCMR episodes are nonresponsive to therapy and molecular mechanisms for the nonresponsiveness are unknown. Our single-center study identified that urinary cell FOXP3 mRNA abundance predicts TCMR reversibility and allograft survival.

Methods.—We developed PCR assays and measured absolute copy numbers of transcripts for FOXP3, CD25, CD3E, perforin, and 18S rRNA in 3559 urines from 480 kidney allograft recipients prospectively enrolled in the multicenter Clinical Trials in Organ Transplantation-04. In this replication study, we investigated the association between mRNA profile and TCMR diagnosis, TCMR reversibility and allograft survival.

Results.—18S rRNA normalized levels of mRNA for FOXP3 (P=0.01, Kruskal-Wallis test), CD25 (P=0.01), CD3E (P<0.0001), and perforin (P<0.0001) were diagnostic of TCMR, but only FOXP3 mRNA level predicted TCMR reversibility (ROC AUC=0.764; 95% confidence interval, 0.611 to 0.917; P=0.008). Multivariable logistic regression analyses showed that urinary cell FOXP3 mRNA level predicted reversal, independent of clinical variables. A composite model of clinical variables and FOXP3 mRNA (AUC = 0.889; 95% CI, 0.781 to 0.997; P<0.001)

*To whom correspondence should be addressed: Manikkam Suthanthiran, MD, 1300 York Avenue, Box 3, New York, NY 10021, USA. Phone: 212-746-4498; Fax: 212-746-6594; msuthan@med.cornell.edu.

Author Roles: M.S. conceived and coordinated the study. D.L., P.A., J.E.S., and M.S. wrote the manuscript. D.M.D. made critical contributions to the preparation of the manuscript including data collection and kidney allograft survival analysis. R.D. designed the oligonucleotide primers and TaqMan probes, and developed and supervised the performance of preamplification-enhanced real-time quantitative PCR assays. T.M., M.L., J.R.L., and F.B.M. made significant contributions to data analysis. V.K.S. coordinated data acquisition and management. D.L. performed the statistical analyses. J.E.S. provided critical statistical guidance to D.L. and assisted in revising the manuscript.

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outperformed FOXP3 mRNA or clinical variables in predicting TCMR reversibility (P=0.01, likelihood ratio test). Multivariable Cox proportional hazards regression analyses showed that FOXP3 mRNA level predicts kidney allograft survival (P=0.047), but not after controlling for TCMR reversal (P=0.477).

Conclusions.—Urinary cell level of FOXP3 mRNA is diagnostic of TCMR, predicts TCMR reversibility, and is prognostic of kidney allograft survival via a mechanism involving TCMR reversal.

INTRODUCTION

T-cell–mediated rejection (TCMR) is the most frequent type of acute rejection.[1–4] Antirejection therapy has evolved over the years, but in a comprehensive study of 256 kidney allograft recipients with kidney allograft biopsies showing TCMR, Bouatou et al. found that 40% of TCMR episodes fail to respond to therapy.[5] Moreover, Banff tubulitis and interstitial inflammation scores at the time of TCMR biopsy diagnosis were not determinants of kidney allograft loss, whereas Banff inflammation and peritubular capillaritis scores observed in the biopsy performed 3 months after antirejection treatment were independent predictors of kidney allograft failure.[5] The need for posttreatment parameters to improve prognostic accuracy is problematic from the perspective of clinical decision making at the time of TCMR diagnosis. Also, the need for multiple biopsies – one to diagnose TCMR and a second one to better prognosticate TCMR outcome – is challenging in view of complications related to the invasive biopsy procedure. The well-documented interobserver variability in the grading of biopsies is yet another challenge.[6,7]

A deficiency in FOXP+ regulatory T cells (Tregs) is a potential mechanism for recalcitrant TCMR. To test this hypothesis, we examined whether urinary cell FOXP3 mRNA profiles predict TCMR responsiveness to antirejection treatment. The focus on FOXP3 was informed by: (i) our earlier single-center study of 83 kidney allograft recipients demonstrating that urinary cell level of FOXP3 mRNA predicts TCMR reversal and identifies patients at risk for allograft failure[8]; (ii) naturally occurring CD4+FOXP3+ regulatory T cells (Tregs) playing a nonredundant role in immune homeostasis and self-tolerance[9,10]; (iii) preclinical data showing that Tregs prevent rejection and promote transplant tolerance[11–13]; and (iv) ongoing clinical evaluation of adoptive Treg therapy to control autoimmunity or promote allograft tolerance.[14,15] We also considered it important to replicate our earlier findings[8] in view of the existing crisis in reproducing scientific observations.[16,17]

In the current investigation, we measured levels of mRNAs in 3505 urine specimens collected from an independent cohort of 480 kidney transplant recipients enrolled in the multicenter Clinical Trials in Organ Transplantation-04 (CTOT-04) and determined whether urinary cell level of FOXP3 mRNA predicts functional reversal of TCMR and predicts kidney allograft survival following an episode of TCMR.

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MATERIALS AND METHODS

Kidney Allograft Recipients

In the parent CTOT-04 study, 485 kidney allograft recipients were prospectively enrolled at 5 transplant sites. Urine was prospectively collected on days 3, 7, 15, and 30 and in months 2, 3, 4, 5, 6, 9, and 12 posttransplantation and at the time of each kidney allograft biopsy and 2 weeks thereafter. Urine cell pellets, prepared at each clinical site, were shipped to the Gene Expression Monitoring core at Weill Cornell Medicine. RNA was isolated from the urinary cell pellets, reverse transcribed to cDNA, and checked for transcript quality thresholds – at least 100 copies of TGFB1 mRNA and $5x10^7$ copies of 18S rRNA per 1 microgram of total RNA – prior to downstream data analysis. Absolute levels of mRNA for CD3E, perforin, granzyme B, proteinase inhibitor-9, CD103, interferon inducible protein-10 (IP-10), CXCR3, TGFB1, and 18S rRNA (CTOT-04 Prespecified mRNA Panel) were measured. A total of 3559 urine specimens from 485 kidney allograft recipients passed the RNA quality thresholds. The primary objectives of the parent CTOT-04 study were to determine whether the urinary cell mRNA levels, measured at the time of biopsy, is diagnostic of TCMR and whether the levels in sequential samples predict future development of TCMR.[4] The parent CTOT-04 study did not investigate whether mRNA levels predict TCMR reversal or are associated with kidney allograft survival. Urinary cell levels of FOXP3 mRNA and CD25 mRNA were not measured in the parent CTOT-04 study.

We obtained independent funding (RO1 AI072790, PI, M. Suthanthiran) to perform this ancillary study. In the current investigation, cDNA prepared from the 3559 urine cell pellets from the parent CTOT-04 were retrieved for the measurement of FOXP3 mRNA and CD25 mRNA. Prior to measurement of mRNAs, the cDNAs were assessed for RNA quality thresholds and 3505 of the 3559 cDNAs (98.5%) prepared from the urine specimens from 480 of 485 kidney allograft recipients from the CTOT-04 study met the quality thresholds. The validated cDNAs were used to measure urinary cell level of mRNAs using customized preamplification-enhanced real-time quantitative (customized) PCR assays.[4] Supplementary Table S1, provided as Supplemental Digital Content, lists the sequences of the oligonucleotide primers and TaqMan probes we designed for the absolute quantification of mRNAs. Additional details of the customized PCR assays have been published.[4]

In this replication study, we applied the same functional criterion we used in our discovery study[8] to classify an episode of TCMR as reversible or nonreversible.

Statistical Methods

Copy number for each mRNA was analyzed before and after normalization with 18S rRNA copies ($x10^{-6}$) and both with and without $\log_{10}$-transformation. Kruskal-Wallis and Mann-Whitney statistical tests were used to compare mRNA levels across diagnoses. Receiver-operating-characteristic (ROC) curves were used to determine the predictive accuracy of each $\log_{10}$-transformed 18S rRNA normalized mRNA and the sensitivity and specificity were determined for the threshold that maximized Youden's index.[18] Multivariable logistic regression was used to evaluate whether urinary cell FOXP3 mRNA level predicts TCMR reversal after controlling for potential confounders.

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Kaplan-Meier survival curves were used to analyze graft survival rates stratified by biopsy status, TCMR reversibility, mRNA levels and serum creatinine measured at the time of TCMR biopsy, and antirejection therapy with antithymocyte globulin (ATG). Time to event was calculated from time of TCMR biopsy until graft failure or censoring. Patients were censored if they died prior to experiencing graft failure or were lost to follow-up. Log-rank tests were used to compare survival curves across strata. Long-term follow-up information on graft outcomes, beyond the 3-year duration of the CTOT-04 study, was obtained from the Organ Procurement and Transplantation Network (OPTN). Multivariable Cox proportional hazards regression was used to evaluate urinary cell mRNA as a predictor of graft failure after controlling for potential confounders.

All analyses were performed with SAS software, version 9.4 (SAS Institute, Cary, NC), except for the creation of ROC curves using the *pROC* R package.[19] The datasets and programming code generated for the current analyses are available from the corresponding author on request.

Study Approval

The institutional review board at Weill Cornell Medicine approved the study, “Multicenter Study: Use of PCR to Evaluate Immune Regulatory Molecules”, Protocol Number 9608002317.

RESULTS

Patients and Biopsies

Figure 1 shows the distribution of the 3505 urine samples with urinary cell mRNA data from the 480 kidney allograft recipients enrolled in CTOT-04. Among the 480 patients, 218 underwent kidney allograft biopsies, which were classified by the on-site pathologist using the updated Banff 97 classification schema.[20] Supplementary Table S2 is the Biopsy Form developed by the NIAID Statistical and Clinical Coordinating Center (SACCC) for the CTOT studies and used by the pathologists to record the biopsy findings.

The TCMR group consisted of 43 biopsies from 34 patients. Among the 43 TCMR biopsies, 38 were for-cause biopsies and 5 were surveillance biopsies. Nineteen of the 43 TCMR biopsies were graded as Banff IA, 10 as grade IB, 11 as IIA, 2 as IIB, and 1 as grade III.

The Supplemental Digital Content (SDC) provides information regarding the 10 AMR biopsies, 19 Borderline Changes biopsies, and 9 biopsies classified as Other Findings; 7 of 9 Other Findings were diagnosed as BK virus nephropathy.

The No Rejection group consisted of 162 biopsies from 126 patients. Among the 162 biopsies, 107 were for-cause biopsies and 55 were surveillance biopsies. This group was designated as “No Rejection” group because none of the biopsies displayed features characteristic of TCMR, AMR, or Borderline Changes. However, several of the No Rejection biopsies displayed features that were recorded in the NIAID SACCC Biopsy Form using Banff 97 Other nonrejection diagnoses.[21] The Banff Other changes included acute tubular necrosis (n=66), tubular atrophy (n=61), interstitial fibrosis (n=51),

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glomerulosclerosis (n=23), vascular narrowing (n=17), calcineurin toxicity (n=11), and/or recurrent disease (n=2). Several of the No Rejection biopsies displayed more than 1 abnormality such as the presence of both interstitial fibrosis and tubular atrophy (n=47).

Among the 480 kidney allograft recipients, 262 did not have a recorded biopsy. Among these 262 patients, 199 patients were classified as having Stable Graft Function by virtue of meeting the following criteria: (i) average of serum creatinine assessed at 6, 9, and 12 months posttransplantation less than or equal to 2.0 mg/dl; (ii) no graft loss or death during the first 12 months following transplantation; (iii) no treatment for acute rejection; (iv) no cytomegalovirus or BK virus infection; and (v) no clinical indication for a biopsy. An additional 63 patients also did not have a biopsy; information regarding these patients who did not meet the criteria for a Stable Graft Function is provided as SDC.

Urinary Cell mRNAs Diagnostic of TCMR

We compared urinary cell levels of mRNAs in (i) 43 urines matched to TCMR biopsies from 34 patients, (ii) 162 urines matched to No Rejection biopsies from 126 patients, and (iii) 1524 urines from 199 patients with stable graft function (Stable). The biopsy matched urine samples were collected within minus 3 days to plus 1 day of biopsy. The 1524 urines from the Stable group were prospectively collected on days 3, 7, 15, and 30 and in months 2, 3, 4, 5, 6, 9, and 12 posttransplantation. Table 1 summarizes the baseline characteristics of the kidney allograft recipients included in this analysis.

Violin plots with in-laid box-and-whisker plots in Figure 2 portray the distribution of $\log_{10}$-transformed ratios of mRNA to 18S rRNA copies ($x10^{-6}$). Urinary cell levels of all 4 mRNAs were significantly higher in urines matched to TCMR biopsies than in urines matched to No Rejection biopsies or in urines collected prospectively from the Stable group. Table S3 shows the median and lower and upper quartiles of the $\log_{10}$-transformed 18S rRNA normalized ratios of mRNAs in urines matched to TCMR biopsies, No Rejection biopsies, and urines from the Stable group. Table S3 also shows the $\log_{10}$-transformed 18S rRNA normalized ratios of mRNAs in urines matched to Borderline Changes biopsies, AMR biopsies, and BKVN biopsies. Table S4 shows the median and lower and upper quartiles of the absolute copy number of mRNAs in urines for the same diagnostic categories.

Supplementary Figure S1 shows ROC curves comparing levels of mRNA for FOXP3, CD3E, CD25, and perforin in urines matched to TCMR biopsies vs. urine matched to No Rejection biopsies; urines matched to TCMR vs. urines from the Stable group; and urines matched to No Rejection biopsies vs. urines from the Stable group. All 4 mRNAs distinguished patients with TCMR biopsies from those with No Rejection biopsies and patients with TCMR biopsies from the Stable patients. Levels of all 4 mRNAs in urines were not different between the No Rejection biopsy group and the Stable group.

Urinary Cell mRNA Levels Stratified by Reversibility of TCMR

In our earlier discovery study,[8] an episode of TCMR was classified as reversible if the serum creatinine level returned to within 15% of the prerejection level within 4 weeks after initiation of antirejection therapy. In this replication study, we used the same functional criterion to classify an episode of TCMR as reversible. With the use of prespecified

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criterion, we classified 39 of 43 TCMR episodes as reversible (n=24) or nonreversible (n=15). Four of 43 TCMR episodes were not classified due to missing creatinine values (n=2), proximity to an earlier TCMR biopsy (n=1), or proximity to BKV nephropathy diagnosis (n=1).

Table 2 summarizes recipient and donor characteristics by TCMR reversibility status. Recipient age was higher in those with reversible TCMR compared to those with nonreversible TCMR (P=0.033, Mann-Whitney) and deceased donor organ was less common among those with reversible TCMR compared to those with nonreversible TCMR (P=0.055). Additional characteristics analyzed are provided as SDC. Table 3 summarizes biopsy-associated characteristics by TCMR reversibility status. The median time from transplantation to biopsy was 117 days in those with reversible TCMR versus 269 days in those with nonreversible TCMR (P=0.015). Additional characteristics analyzed, including Banff biopsy grade and serum creatinine at the time of TCMR biopsy, were not significantly different between the 2 groups and are provided as SDC.

We compared mRNA levels in urines matched to reversible TCMR to levels in urines matched to nonreversible TCMR (Table 4). FOXP3 mRNA level was higher in urines matched to reversible TCMR than in urines matched to nonreversible TCMR (P=0.0096, Mann-Whitney Test). In contrast, levels of mRNA for CD25 (P=0.1531), CD3E (P=0.1887), and perforin (P=0.4322) were not different between the 2 groups.

We compared levels of mRNAs in urines matched to reversible or nonreversible TCMR to levels in urines matched to No Rejection biopsies (Table 4). Levels of mRNA for FOXP3 (P<0.0001), CD25 (P=0.0066), CD3E (P<0.0001), and perforin (P<0.0001) were higher in urines matched to reversible TCMR than in urines matched to No Rejection biopsies. Levels of FOXP3 mRNA (P=0.7212) and CD25 mRNA (P=0.7623) were not different between urines matched to nonreversible TCMR and urines matched to No Rejection biopsies, whereas levels of mRNA for CD3E (P=0.0026) and perforin (P<0.0017) were significantly higher (Table 4).

ROC Curve Analysis of TCMR Reversal

We performed ROC curve analysis to determine the predictive performance of urinary cell mRNA levels. ROC curve analysis yielded an AUC of 0.764 (95% CI, 0.611 to 0.917; P=0.008) for the 18S rRNA normalized values of FOXP3 mRNA (Figure 3A). The cut-point that maximized Youden's index[18] was −1.33; at this threshold, FOXP3 mRNA predicted TCMR reversal with 75% (95% CI, 53% to 90%) sensitivity and 67% (95% CI, 45% to 77%) specificity; the positive and negative predictive values were 78% (95% CI, 63% to 88%) and 62% (43% to 78%), respectively. Levels of mRNA for CD25 (P=0.100) (Figure 3B), CD3E (P=0.173) (Figure 3C), and perforin (P=0.399) (Figure 3D) were not predictive. The CTOT-04 urinary cell 3-gene signature of CD3E mRNA, IP-10 mRNA, and 18S rRNA, previously shown to be diagnostic and anticipatory of TCMR,[4] did not predict TCMR reversal (P=0.253) (Figure 3E). Serum creatinine, measured at the time biopsy, did not predict TCMR reversal (P=0.308) (Figure 3F).

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A Composite Model for Predicting TCMR Reversibility

We examined whether a combination of clinical variables and urinary cell mRNAs predict TCMR reversal better than clinical variables or mRNAs alone. A stepwise logistic regression analysis using a combination of urinary cell levels of mRNA for FOXP3, CD3, CD25, and perforin and kidney allograft function reflected by serum creatinine level measured at time of TCMR biopsy showed that the most parsimonious and best fitting model is the model containing only FOXP3 mRNA. Recipient age (P=0.033, Mann-Whitney test), time from transplantation to biopsy (P=0.015), and type of donor graft (P=0.055) differed significantly between reversible and nonreversible TCMR groups by univariable analysis (Tables 2 and 3). Multivariable logistic regression analyses showed that FOXP3 mRNA level continued to significantly predict TCMR reversal after controlling for recipient age (P=0.0090), time from transplantation to biopsy (P=0.0130), and type of donor graft (P=0.0170) (Table 5). We examined whether a composite model that included the clinical variables and urinary cell FOXP3 mRNA level yields a higher AUC than the AUC of FOXP3 mRNA alone or the AUC of all 3 clinical variables without FOXP3 mRNA. The prediction model that included clinical variables and FOXP3 mRNA yielded an AUC of 0.889 (95% CI, 0.781–0.997, P<0.001). By likelihood ratio test, the composite model was significantly better than (i) the model with FOXP3 mRNA alone (P=0.012) and (ii) the model that included the 3 clinical variables (P=0.006). The regression equations for the models are provided as SDC.

Prospective Trajectory

We examined the impact of the antirejection therapy on the postbiopsy prospective trajectories of urinary cell FOXP3 mRNA level and on the CTOT-04 3-gene diagnostic signature score. The prospective trajectory of FOXP3 mRNA for the reversible TCMR group started above the $\log_{10}$-transformed 18S normalized FOXP3 mRNA threshold of −1.33 for TCMR reversal (represented by the red dashed line) at the time of biopsy and remained close to the threshold throughout the subsequent 30 days (Figure 4A). In contrast, the prospective trajectory of FOXP3 mRNA for nonreversible TCMR group started below the threshold at the time of biopsy and remained below the threshold throughout the next 30 days (Figure 4B). The prospective trajectory of CTOT-04 urinary cell 3-gene signature decreased from the time of biopsy and crossed the diagnostic threshold of −1.213 (represented by the red dashed line) within 15 days of initiation of antirejection therapy in those with reversible TCMR (Figure 4C), but remained consistently above the threshold in those with nonreversible TCMR (Figure 4D).

Survival Analyses

Survival probabilities for the entire cohort of 480 kidney allograft recipients at 1, 3, 5, and 10 years were 99%, 95.6%, 91.2%, and 81.5%, respectively (Figure 5A). Survival probabilities, at the same time points, were 100%, 100%, 99%, and 93.1%, respectively, for the Stable group; 97.7%, 93%, 86.7%, and 75.2%, respectively, for the No Rejection biopsy group; and 100%, 83.1%, 77.3%, and 58.8%, respectively, for the TCMR group (P<0.0001) (Figure 5B). The Kaplan-Meier survival curve for the TCMR group was significantly different than the curves for Stable group (P<0.0001, by log-rank test) and No Rejection group (P=0.0510).

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Patients with reversible TCMR had significantly better survival than those with nonreversible TCMR (Figure 5C, P<0.0001). Patients with FOXP3 mRNA levels above −1.33 (the Youden index threshold for TCMR reversal) at the time of a TCMR biopsy had significantly better graft survival compared to those with levels below the threshold (Figure 5D, *P*=0.0325). Multivariable Cox proportional hazards regression analysis showed that FOXP3 mRNA level remains significantly predictive of kidney allograft outcomes after adjustment for age and serum creatinine measured at time of biopsy, but not after adjustment for time from transplant to biopsy, type of transplant, or TCMR reversibility (Table 6).

The relationship between FOXP3 mRNA level and graft outcome may be a direct effect of FOXP3+ Tregs (reflected by FOXP3 mRNA abundance) on graft survival and/or by an indirect effect through the association between FOXP3+ Tregs and TCMR reversibility (Figure 6). Our analysis showed that: (i) FOXP3 mRNA level is significantly associated with TCMR reversibility after adjustment for covariates (a-path; OR=3.88; 95% CI, 1.28 to 11.8, *P*=0.0168, Table 5); (ii) TCMR reversible status is significantly associated with graft survival (*P*<0.0001, Figure 5C) and the association of reversible status with graft failure (HR=0.16; 95% CI, 0.05-0.51; *P*=0.0017) remains statistically significant after adjustment for FOXP3 mRNA level (b-path; HR=0.21; 95% CI, 0.06-0.73; *P*=0.0139); and (iii) the significant association between FOXP3 mRNA level and graft survival (HR=0.55, 95% CI, 0.30-0.99, *P*=0.0474, Table 6) is no longer statistically significantly associated with graft survival after adjustment for TCMR reversibility (c′-path; HR=0.78; 95% CI, 0.39-1.56; P=0.4771, Table 6). Altogether, our data support the hypothesis that the association of FOXP3 mRNA level with graft outcome is mediated through TCMR reversal.

Figure S2 shows that antithymocyte globulin as antirejection therapy (*P*=0.6903, Figure S2) or serum creatinine level, measured at time of biopsy, is not associated with kidney allograft survival *(P*=0.7084). Figure S2 also shows that urinary cell levels of mRNA for CD25 (*P*=0.2915), CD3E (*P*=0.3826), and perforin (*P*=0.8542), or the CTOT-04 3-gene TCMR diagnostic signature score (*P*=0.2138), all measured at the time of TCMR biopsy, are not associated with kidney allograft survival.

DISCUSSION

FOXP3+ Tregs play a pivotal role in preventing autoimmunity and maintaining immune homeostasis.[9,10] Preclinical studies suggest that Tregs prevent or delay the onset of allograft rejection and may induce tolerance.[11–13] In our earlier single-center clinical study,[8] we found that urinary cell FOXP3 mRNA level is diagnostic of TCMR, predicts TCMR reversal, and is associated with kidney allograft survival. In the current investigation, we have replicated these findings using urine samples collected from an external cohort of 480 kidney allograft recipients enrolled in the multicenter CTOT-04 study. Our replication of earlier observations is significant from a biological perspective regarding the potential role of FOXP3+ Tregs in regulating kidney allograft rejection, and is reassuring in the context of the existing crisis in replicating published data.[16,17]

We found that a composite signature of clinical variables and urinary cell FOXP3 mRNA level is a better predictor of TCMR reversal than either the clinical variables alone or

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FOXP3 mRNA level alone. In this regard, whereas the finding that urinary cell FOXP3 level alone predicts TCMR reversal represents replication of our earlier finding,[8] the new composite signature requires validation in a future study.

The prospective trajectory of FOXP3 mRNA started above the threshold for TCMR reversal at the time of biopsy in the reversible TCMR group and remained close to this threshold throughout the subsequent 30 days whereas the prospective trajectory started below the threshold at the time of biopsy in the nonreversible TCMR group and remained below the threshold throughout the next 30 days. Strikingly, the prospective trajectory of CTOT-04 urinary cell 3-gene signature decreased from the time of biopsy and crossed the TCMR diagnostic threshold of −1.213 within 15 days of initiation of antirejection therapy in those with reversible TCMR, but remained consistently above the TCMR rejection threshold among those with nonreversible TCMR. These differential trajectories suggest that the balance between Tregs (reflected in this study by FOXP3 mRNA abundance) and T effectors (reflected in this study by CTOT-04 urinary cell 3-gene signature score) may impact TCMR responsiveness to therapy.

The overall survival of kidney allografts in our multicenter CTOT-04 study cohort was similar to the US kidney graft survival rates[22] suggesting that our study participants are representative of the US kidney transplant population. Kidney graft survival was significantly inferior in patients with biopsy confirmed TCMR, with most graft failures occurring in those with nonreversible TCMR. In the current study, we validate prior findings that urinary cell FOXP3 mRNA level, measured at the time of TCMR, is associated with kidney allograft survival. The association between FOXP3 mRNA level and graft outcome is likely to be mediated by an indirect effect through the association between FOXP3 mRNA level and TCMR reversibility since the significant association between FOXP3 mRNA level and graft survival was no longer statistically significant after adjustment for TCMR reversibility.

Our study has limitations. We characterized TCMR reversal based on functional recovery rather than by histological confirmation with follow-up biopsy. This may not be a significant limitation since graft survival in our study was strongly associated with the functional criterion used to classify an episode of TCMR. We did not assess the functional activity of Tregs, and we inferred Treg deficiency based on FOXP3 mRNA abundance and this could be considered a limitation as well. We note that mRNA expression patterns in themselves have helped inform therapeutic decisions.[23] Another limitation of our study is that the donor specific antibody status was unknown at the time of TCMR biopsy.

We measured FOXP3 mRNA level in urine as a surrogate for intragraft FOXP3 expression. Our whole genome RNA sequencing of urinary cells and kidney allograft biopsies demonstrating that kidney allograft gene signatures are enriched in urinary cells supports the idea that urine is excellent surrogated for the kidney allograft biopsy.[24] In this study, we did not assess intragraft FOXP3 protein level. However, the existing literature suggests a positive correlation between intragraft FOXP3 mRNA level and protein expression.[25–27] It would be important to investigate the relationship between intragraft FOXP3+ cells and urinary cell FOXP3 mRNA levels especially in the context of TCMR reversibility and graft survival.

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The current study focused on TCMR reversal. In a comprehensive study of biopsies diagnosed as Borderline TCMR, Nankievell et al. identified differential outcomes ranging from minimal impact to deleterious consequences including progressive tubular injury and fibrosis, an increased risk for acute rejection, allograft failure and even death.[28] It would be important to investigate the association between urinary cell mRNA profiles and the outcome of biopsies classified as Borderline TCMR.

The parent CTOT-04 study identified and validated a urinary cell 3-gene signature of CD3E mRNA, IP-10 mRNA, and 18S rRNA that is diagnostic of TCMR and anticipatory of a future episode of TCMR.[4] The current investigation extends the utility of urinary cell mRNA profiling by demonstrating that urinary cell FOXP3 mRNA level predicts functional reversal of TCMR and graft survival following an episode of TCMR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AUC	area under the curve
BMI	body mass index
CI	confidence interval
CTOT-04	Clinical Trials in Organ Transplantation 04
cDNA	complementary DNA
IP-10	interferon inducible protein-10
PI-9	proteinase inhibitor-9
ROC	receiver operating characteristic

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RT-QPCR	real-time quantitative polymerase chain reaction
rRNA	ribosomal RNA
TCMR	T-cell–mediated rejection
Tregs	regulatory T cells
TGFB1	transforming growth factor beta 1

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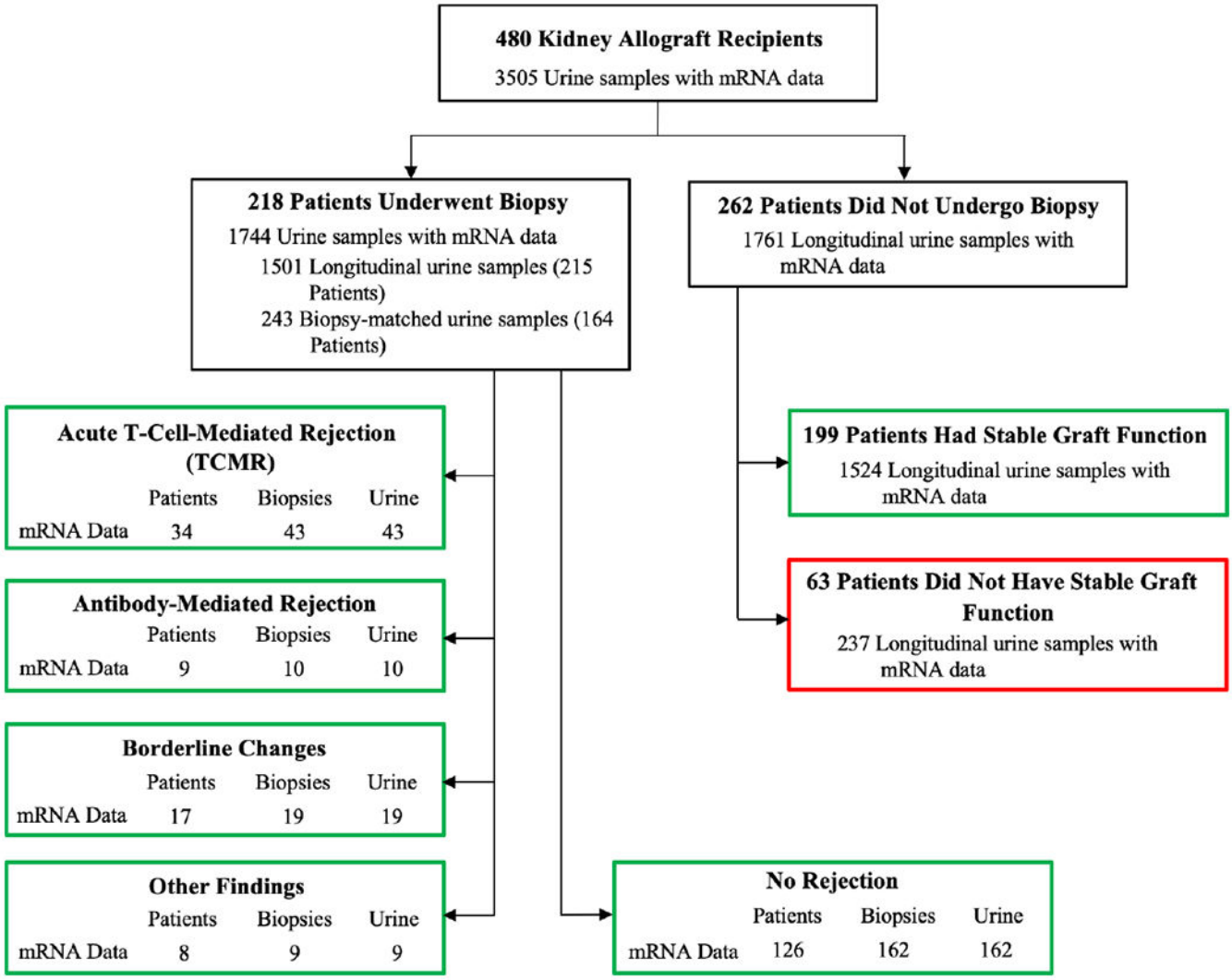

Figure 1.

Patients, biopsy results, and urine samples. The distribution of 3505 urine samples from 480 kidney allograft recipients enrolled in the CTOT-04 study is shown. The number of patients with biopsy-matched urine samples (urine collected from 3 days before to 1 day after biopsy) are shown for patients with Banff TCMR grade 1A or higher, AMR, Borderline Changes, Other Findings, and those without any rejection features in their biopsies (No Rejection). Among patients who did not undergo a biopsy, 199 patients met criteria for stable graft function based on (i) average of recorded serum creatinine values at 6, 9, and 12 months ≤ 2.0 mg/dl, (ii) no graft loss or death, (iii) no treatment for acute rejection, and (iv) no evidence of cytomegalovirus or polyomavirus type BK infection during the first 12 months posttransplantation and contributed 1524 urine samples. Sixty-three patients failed to meet criteria for stable graft function and contributed 237 urine specimens. Green boxes represent samples included in this study, whereas the red box represents samples not included in the data analysis.

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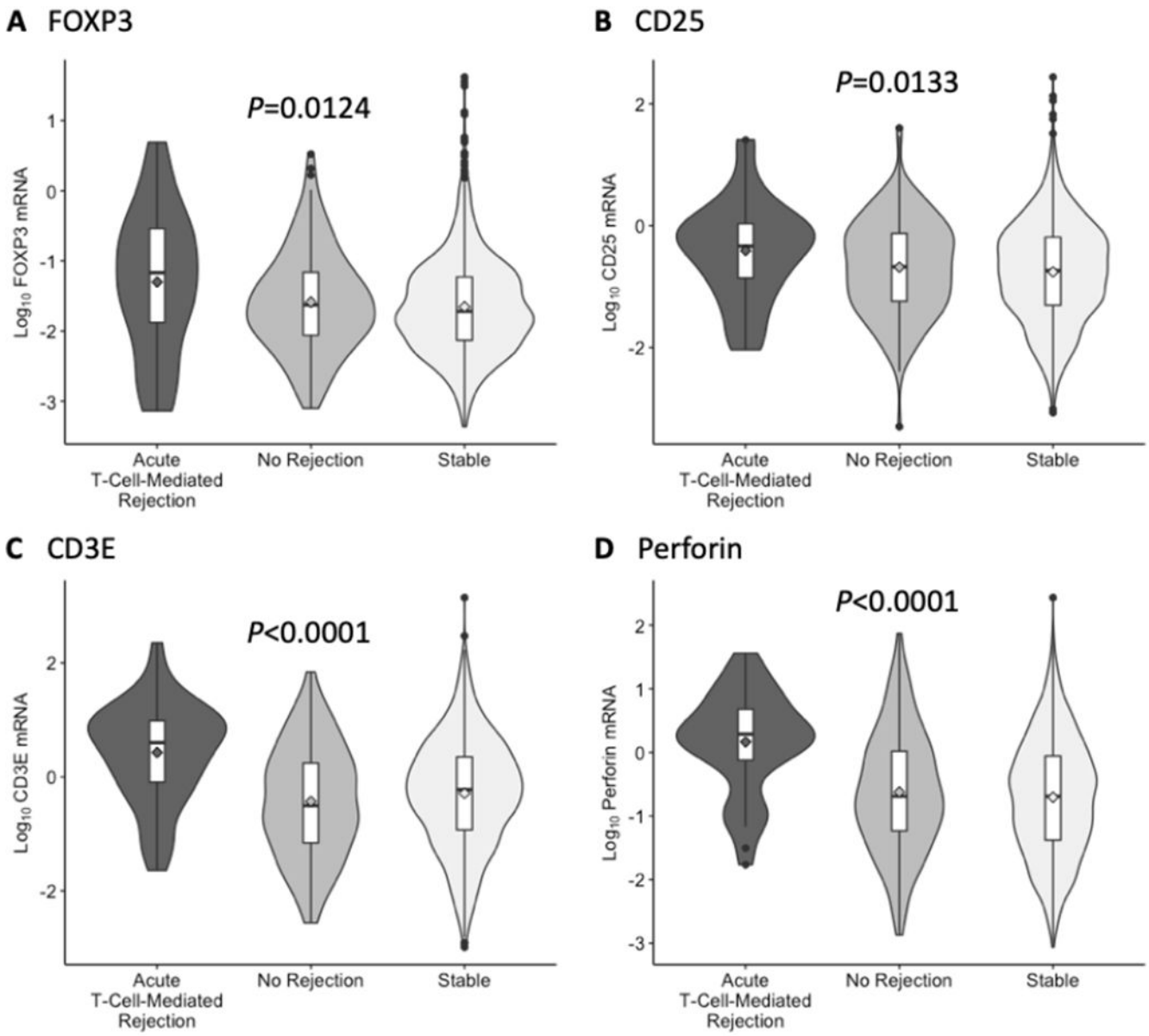

Figure 2.

Levels of mRNA in urinary cells. Violin plots with in-laid box-and-whisker plots show the distribution of $\log_{10}$-transformed ratios of mRNA copies to 18S ribosomal RNA (rRNA) copies ($x10^{-6}$) for FOXP3, CD25, CD3E, and perforin in 43 urine samples matched to 43 biopsy specimens (from 34 subjects) diagnosed as acute T-cell–mediated rejection, 162 urine samples matched to 162 biopsy specimens (from 126 subjects) without any rejection features in the biopsy (No Rejection), and 1524 urine samples collected longitudinally from 199 subjects with stable graft function who did not undergo biopsy (Stable). The in-laid box-and-whisker plots display the 25th, 50th, and 75th percentile values via the bottom, middle, and top lines in the box, respectively, and the 10th and 90th percentile values via the ends of the bottom and top whiskers, respectively; the diamonds represent the mean and circles indicate outliers. The violin plots display the distribution and spread of observations in each

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diagnostic group. The *P*-value from the Kruskal-Wallis test of the null hypothesis of no group differences in the distributions is presented above each set of violin plots.

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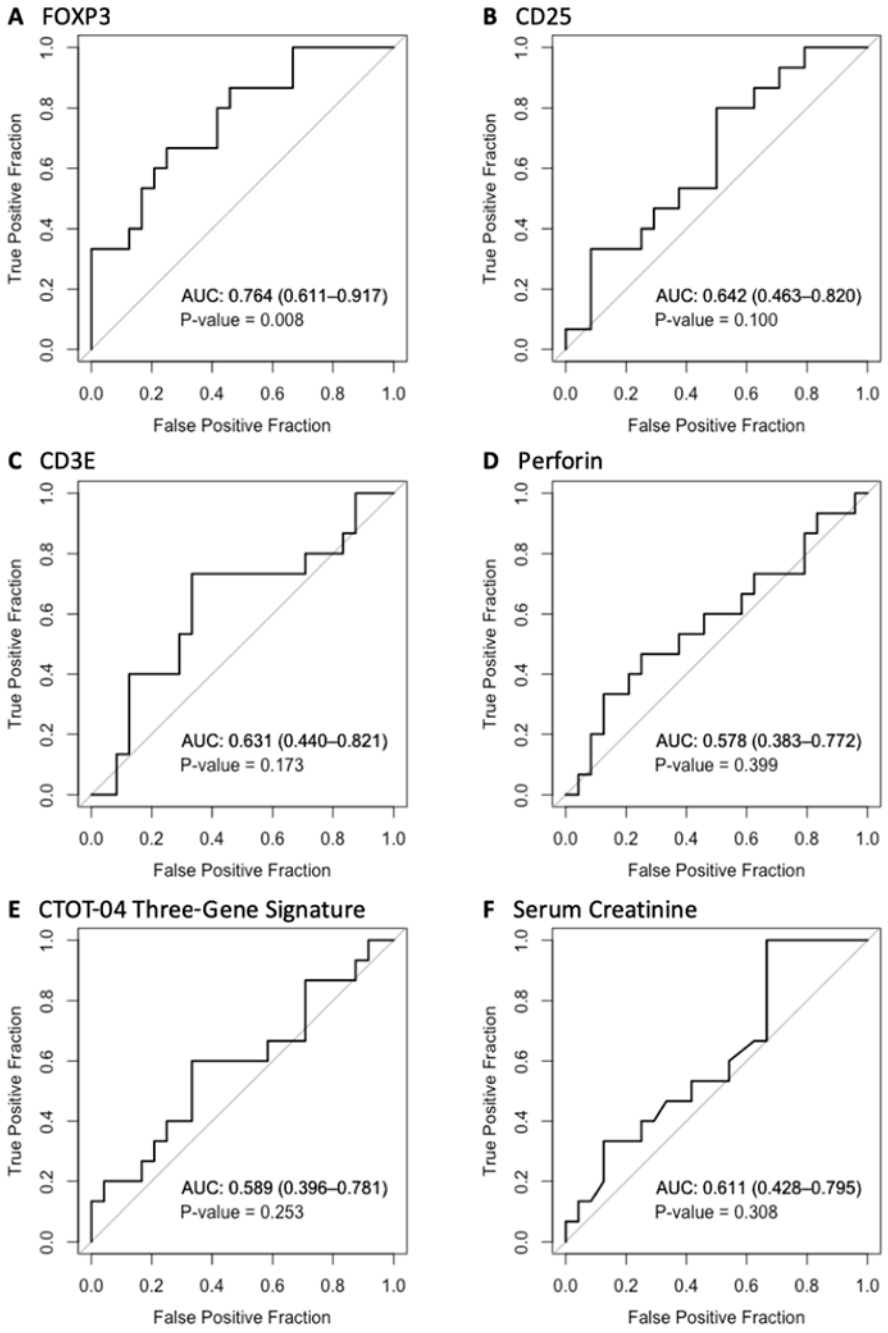

Figure 3.

Receiver-operating-characteristic (ROC) curve analyses for TCMR reversal. ROC curves for (A) 18S ribosomal RNA (rRNA) normalized FOXP3 mRNA; (B) 18S rRNA normalized CD25 mRNA; (C) 18S rRNA normalized CD3E mRNA; (D) 18S rRNA normalized perforin mRNA; (E) the CTOT-04 3-gene TCMR diagnostic signature (calculated from 18S rRNA normalized CD3E and IP-10 mRNAs and 18S rRNA); and (F) serum creatinine level measured at time of TCMR biopsy. In addition to the ROC curve (a plot of the fraction of true positive results [sensitivity] and the fraction of false positive results [1- specificity] for

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discriminating reversal versus nonreversal of an episode of TCMR using different thresholds of a predictor), each panel gives the area under the receiver operating characteristic curve (AUC) with its 95% confidence interval and the *P*-value for the test of the null hypothesis that the AUC=0.5. An AUC value of 0.5 is no better than that expected by chance (the null hypothesis) whereas a value of 1.0 reflects a perfect discriminator. P-values are obtained from Wald tests from logistic regression analyses predicting reversible status from the measure of interest. Among the variables tested, only urinary cell FOXP3 mRNA level, measured at the time of biopsy, predicted TCMR reversal (ROC AUC: 0.764, 95%CI, 0.611 to 0.917, P=0.008).

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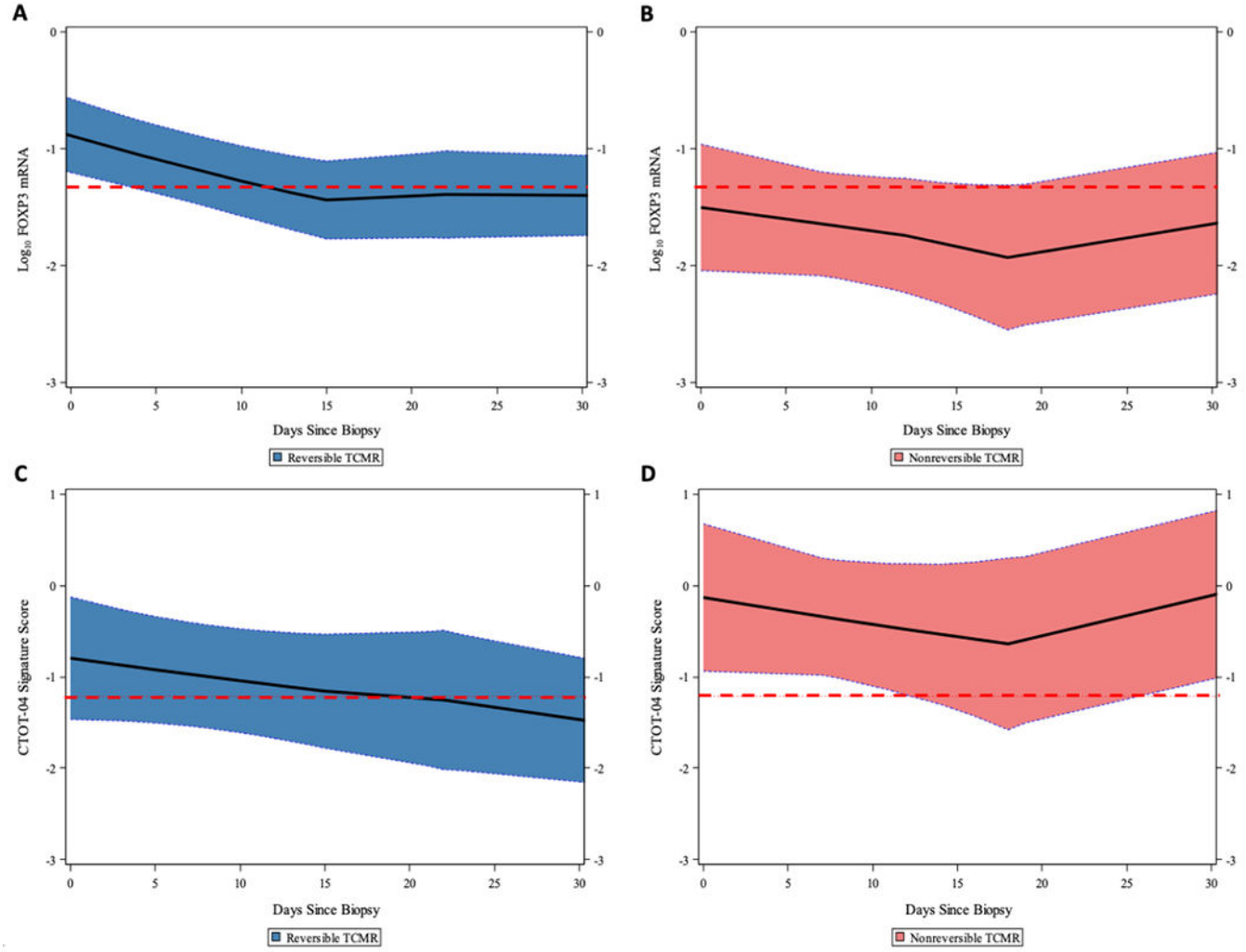

Figure 4.

Prospective trajectories of 18S rRNA normalized FOXP3 mRNA level and CTOT-04 3-gene TCMR diagnostic signature score as a function of time since TCMR biopsy. The loess-smoothed average within-person trajectories and 95% confidence bands of the urinary cell $\log_{10}$-transformed, 18S rRNA normalized FOXP3 mRNA level and the median score of CTOT-04 3-gene TCMR diagnostic signature are shown for the reversible TCMR group (A, C) and the nonreversible TCMR group (B, D). Levels of mRNA in 81 urines from 21 patients with reversible TCMR and 43 urines from 12 patients with nonreversible TCMR were used to generate the prospective trajectories. (A) The median level of urinary cell FOXP3 mRNA at the time of TCMR biopsy was significantly higher in patients with reversible TCMR than in patients with nonreversible TCMR. The prospective trajectory in the reversible TCMR group started above the −1.33 threshold (for discriminating reversible from nonreversible TCMR) at time of TCMR biopsy and remained close to the threshold throughout the 30 days after the biopsy. (B) The prospective trajectory in patients with nonreversible TCMR started below the threshold at time of TCMR biopsy and remained well below the threshold through 30 days after the biopsy. (C) The prospective trajectory of the CTOT-04 3-gene TCMR diagnostic signature at time of TCMR biopsy did not differ

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significantly between those with reversible TCMR or nonreversible TCMR. Among the patients with reversible TCMR, the average score decreased from time of TCMR biopsy and crossed the diagnostic threshold of −1.213 within 15 days of initiation of antirejection therapy. (D) The CTOT-04 3-gene TCMR diagnostic signature prospective trajectory remained consistently above the threshold among the patients with nonreversible TCMR.

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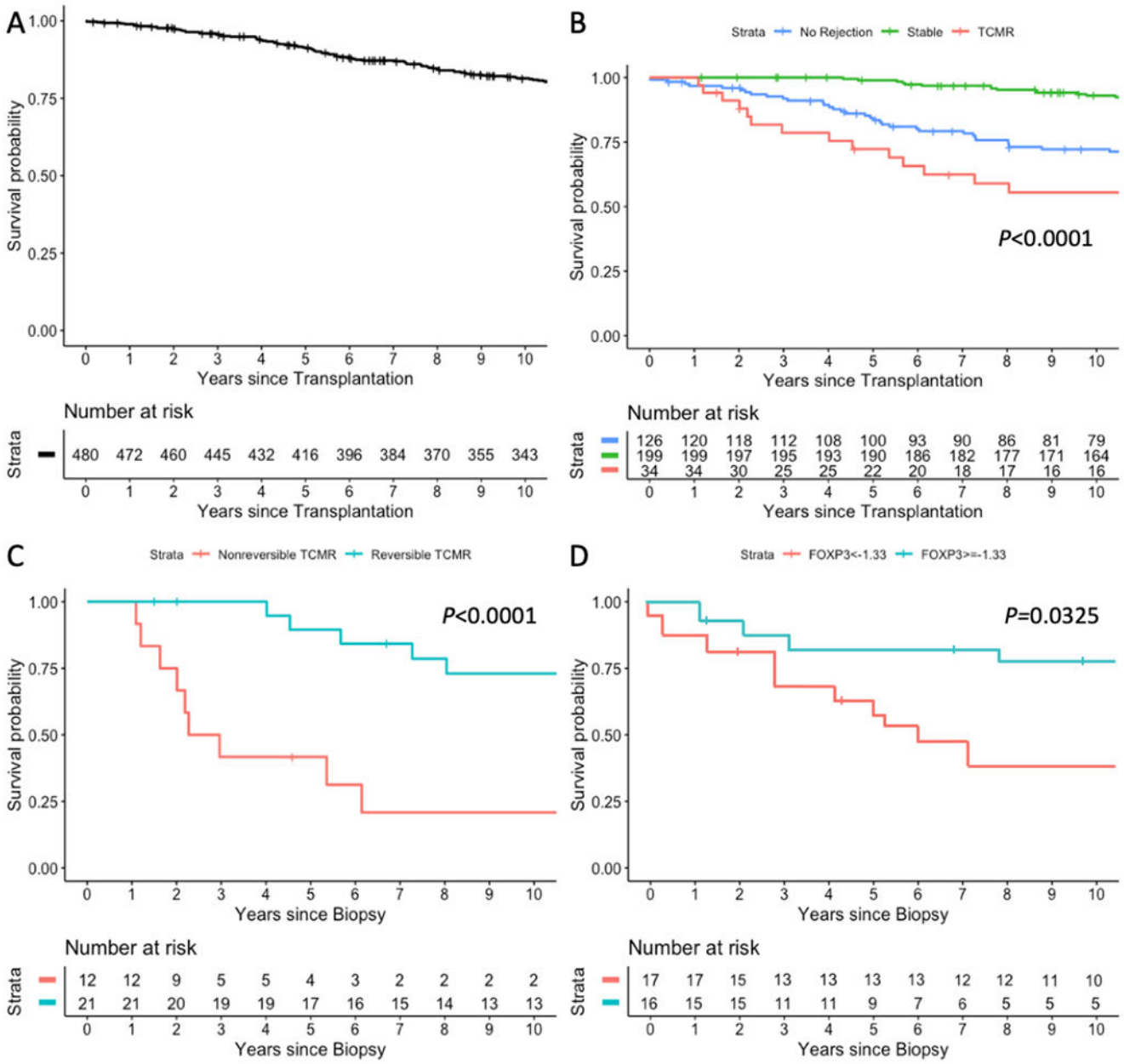

Figure 5.

Kaplan-Meier kidney allograft survival curves. (A) Survival curve, from time of transplantation, for the entire cohort of 480 kidney allograft recipients (patients); (B) Survival curves, from time of transplantation, for the 199 patients with stable graft function (Stable), for the 126 patients with 162 biopsies showing no rejection features in their biopsies (No Rejection) and for the 34 patients with 43 biopsies classified as TCMR Banff grade IA or higher (TCMR); (C) Survival curves of 33 patients, from time of TCMR biopsy, stratified by TCMR reversibility status. One patient with BKVN diagnosis in close proximity to TCMR could not be classified as reversible or nonreversible TCMR and is excluded in this analysis; (D) Survival curves for the 33 patients, from the time of biopsy,

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stratified by $\log_{10}$-transformed 18S rRNA normalized FOXP3 mRNA threshold of −1.33 for TCMR reversal. The patient with BKVN diagnosis in close proximity to TCMR is excluded in this analysis. Time to event was calculated from date of TCMR biopsy (or the date of last TCMR biopsy for the 3 patients with multiple episodes) until graft failure or last follow-up date. Subjects were censored if they experienced death prior to graft failure or were lost to follow-up. *P*-values are based on log-rank tests. At-risk tables are shown in each plot, just above the X-axis.

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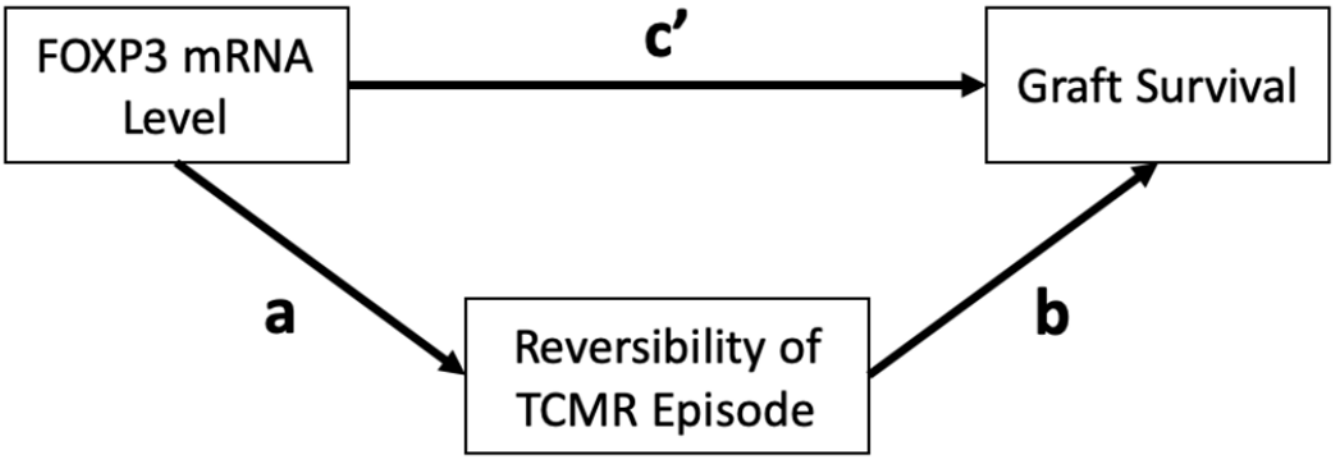

Figure 6.

Proposed mechanism for the association between urinary cell FOXP3 mRNA abundance and kidney allograft outcome. FOXP3 mRNA level may impact graft outcome via TCMR reversal (path a), through a direct effect that is independent of its effect on TCMR reversal (path c') or both. Data analysis showing that: i) after adjustment for covariates, FOXP3 mRNA level is significantly associated with TCMR reversibility (a-path; OR=3.88; 95% CI, 1.28 −11.8, *P*=0.0168); (ii) TCMR reversible status is significantly associated with graft survival after adjustment for FOXP3 mRNA level and covariates (path b; HR=0.21; 95% CI, 0.06-0.73; *P*=0.0139) and (iii) the association between FOXP3 mRNA level and graft survival is negligible after adjustment for TCMR reversibility (path $c\bar{Y}$; HR=0.78; 95% CI, 0.39-1.56; P=0.4771) support the hypothesis that the association of FOXP3 mRNA level with graft outcome is primarily mediated through TCMR reversal.

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Table 1.

Characteristics of the kidney allograft recipients and their organ donors[a]

Recipient Characteristics	Acute T-Cell–Mediated Rejection (N of Patients = 34)	No Rejection (N of Patients = 126)	Stable (N of Patients = 199)	*P*-value[b]
Biopsy samples	43	162		
Urine samples	43	162	1524	
Age, years				0.2652
Mean (SD)	45.0 (11.8)	48.0 (13.0)	49.0 (13.9)	
Median	43	48	50	
Min, Max	24, 73	<1, 76	<1, 78	
Sex, N (%)				0.0146
Female	8 (23.5)	43 (34.1)	92 (45.8)	
Male	26 (76.5)	83 (65.9)	109 (54.2)	
Ethnicity, N (%)				0.5532
Hispanic or Latino	3 (8.8)	20 (15.9)	31 (15.4)	
Not Hispanic or Latino	30 (88.2)	101 (80.2)	161 (80.1)	
Unknown or Not Reported	1 (2.9)	5 (4.0)	9 (4.5)	
Race, N (%)				0.0497
Black or African American	13 (38.2)	49 (38.9)	45 (22.4)	
White	20 (58.8)	62 (49.2)	119 (59.2)	
Asian	1 (2.9)	9 (7.1)	10 (5.0)	
American Indian or Alaska Native	0 (0)	0 (0)	2 (1.0)	
Other	0 (0)	4 (3.2)	20 (10.0)	
Unknown or Not Reported	0 (0)	2 (1.6)	5 (2.5)	
Induction Therapy, N (%)				<0.0001
IL-2 Receptor Antibody	6 (17.6)	12 (9.5)	20 (10.1)	
CAMPATH-1H	10 (29.4)	58 (46.0)	29 (14.6)	
Thymoglobulin	15 (44.1)	38 (30.2)	135 (67.8)	
More than 1 induction therapy	2 (5.9)	7 (5.6)	6 (3.0)	
No Induction Therapy	1 (2.9)	2 (1.6)	1 (0.5)	
Missing Information	0 (0)	9 (7.1)	10 (5.0)	
BMI				0.0022
Mean (SD)	30.5 (6.2)	28.5 (6.3)	27.0 (5.6)	
Median	29	28	26	
Min, Max	22, 43	17, 45	16, 43	
< 18.5	0 (0)	2 (1.6)	4 (2.0)	
18.5 - 24.9	9 (26.5)	35 (27.8)	58 (28.9)	
25.0 - 29.9	7 (20.6)	41 (32.5)	56 (27.9)	
≥ 30.0	14 (41.2)	40 (31.7)	44 (21.9)	
Missing	4 (11.8)	8 (6.3)	39 (19.6)	
Donor Characteristics				

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Recipient Characteristics	Acute T-Cell–Mediated Rejection (N of Patients = 34)	No Rejection (N of Patients = 126)	Stable (N of Patients = 199)	*P*-value[b]
Age, years				0.5344
Mean (SD)	41.8 (11.5)	39.8 (14.8)	38.9 (14.7)	
Median	40	41	39	
Min, Max[b]	20, 65	5, 66	1, 73	
Missing	0	0	3	
Sex, N (%)				0.3621
Female	17 (50.0)	61 (48.4)	83 (41.3)	
Male	17 (50.0)	65 (51.6)	118 (58.7)	
Ethnicity, N (%)				0.3895
Hispanic or Latino	4 (11.8)	21 (16.7)	36 (17.9)	
Not Hispanic or Latino	29 (85.3)	92 (73.0)	140 (69.7)	
Unknown or Not Reported	1 (2.9)	13 (10.3)	25 (12.4)	
Race, N (%)				0.0013
Black or African American	10 (29.4)	34 (27.0)	19 (9.5)	
White	22 (64.7)	82 (65.1)	154 (76.6)	
Asian	1 (2.9)	3 (2.4)	5 (2.5)	
American Indian or Alaska Native	0	1 (0.8)	1 (0.5)	
Other	0	0 (0)	3 (1.5)	
Unknown or Not Reported	1 (2.9)	6 (4.8)	19 (9.5)	
Source of Donor, N (%)				0.6900
Deceased	15 (44.1)	57 (45.2)	85 (42.3)	
Living/related	9 (26.5)	39 (31.0)	73 (36.3)	
Living/unrelated	10 (29.4)	30 (23.8)	43 (21.4)	
Cause of Death, N (%)				0.0455
Anoxia	4 (26.7))	9 (15.8)	15 (17.6)	
Cerebrovascular Accident/Injury/Stroke	6 (40.0)	27 (47.4)	16 (18.8)	
Head Injury/Trauma	2 (13.3)	8 (14.0)	24 (28.2)	
Intracranial Bleed	1 (6.7))	2 (3.5)	6 (7.1)	
Motor Vehicle Accident	0 (0)	1 (1.8)	2 (2.4)	
Other	1 (6.7)	4 (7.0)	11 (12.9)	
Unknown	0 (0)	3 (5.3)	10 (11.8)	
Missing	1 (6.7)	3 (5.3)	1 (1.2)	

[a]Demographics of recipients and their organ donors are shown. 34 patients underwent 43 kidney allograft biopsies and contributed 43 urine samples matched to acute T-cell–mediated rejection biopsies. 126 patients underwent 162 biopsies and contributed 162 urine samples matched to No Rejection biopsies. 199 patients did not undergo a biopsy and contributed 1524 urine samples. These 199 patients were classified as Stable based on meeting the following criteria: average serum creatinine values at 6, 9 and 12 months ≤ 2.0 mg/dl, no treatment for acute rejection, and no evidence of cytomegalovirus or polyomavirus type BK infection during the first 12 months posttransplantation. The biopsy matched urine samples were collected within minus 3 days to plus 1 day of biopsy. The 1524 urines from the Stable group were prospectively collected on days 3, 7, 15, and 30 and in months 2, 3, 4, 5, 6, 9, and 12 posttransplantation.

[b]P-values are based on 1-way ANOVA for continuous variables and Chi-square tests for categorical variables.

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Table 2.

Characteristics of kidney transplant recipients with acute T-cell–mediated rejection by reversible status[a]

Recipient Characteristics	Total	Reversible TCMR	Nonreversible TCMR	*P*-value[b]
Kidney allograft recipients[c]	33	21	12	
Number of biopsies[d]	39	24	15	
Age, years				0.033
Mean (SD)	45.0 (12.0)	48.0 (10.9)	39.8 (12.5)	
Median	43	46	36	
Min, Max	24, 73	35, 69	24, 73	
Sex, N (%)				0.730
Female	8 (24.2)	6 (28.6)	2 (16.7)	
Male	25 (75.8)	15 (71.4)	10 (83.3)	
Ethnicity, N (%)				0.898
Hispanic or Latino	2 (6.1)	1 (4.8)	1 (8.3)	
Not Hispanic or Latino	30 (90.9)	19 (90.5)	11 (91.7)	
Unknown or Not Reported	1 (3.0)	1 (4.8)	0 (0)	
Race, N (%)				0.455
Black or African American	13 (39.4)	6 (28.6)	7 (58.3)	
White	19 (57.6)	14 (66.7)	5 (41.7)	
Asian	1 (3.0)	1 (4.8)	0 (0)	
American Indian or Alaska Native	0 (0)	0 (0)	0 (0)	
Other	0 (0)	0 (0)	0 (0)	
Unknown or Not Reported	0 (0)	0 (0)	0 (0)	
Induction Therapy, N (%)				0.679
IL-2 Receptor Antibody	6 (18.2)	5 (23.8)	1 (8.3)	
CAMPATH-1H	9 (27.3)	7 (33.3)	2 (16.7)	
Thymoglobulin	15 (45.5)	8 (38.1)	7 (58.3)	
More than 1 induction therapy	2 (6.1)	0 (0)	2 (16.7)	
No Induction Therapy	1 (3.0)	1 (4.8)	0 (0)	
Missing Information	0 (0)	0 (0)	0 (0)	
BMI				0.772
Mean (SD)	30.6 (6.3)	30.8 (6.7)	30.2 (5.6)	
Median	30	29	30	
Min, Max	22, 43	22, 43	24, 38	
< 18.5	0 (0)	0 (0)	0 (0)	
18.5 - 24.9	9 (27.3)	6 (28.6)	3 (25.0)	
25.0 - 29.9	6 (18.2)	4 (19.1)	2 (16.7)	
≥ 30.0	14 (42.4)	10 (47.6)	4 (33.3)	
Missing	4 (12.1)	1 (4.8)	3 (25.0)	
Donor Characteristics				

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Recipient Characteristics	Total	Reversible TCMR	Nonreversible TCMR	*P*-value[b]
Age, years				0.409
Mean (SD)	41.8 (11.7)	40.6 (12.4)	44.0 (10.5)	
Median	40	40	43	
Min, Max	20, 65	20, 65	26, 61	
Missing	0	0	0	
Sex, N (%)				0.554
Female	17 (51.5)	10 (47.6)	7 (58.3)	
Male	16 (48.5)	11 (52.4)	5 (41.7)	
Ethnicity, N (%)				0.852
Hispanic or Latino	3 (9.1)	2 (9.5)	1 (8.3)	
Not Hispanic or Latino	29 (87.9)	19 (90.5)	10 (83.3)	
Unknown or Not Reported	1 (3.0)	0 (0)	1 (8.3)	
Race, N (%)				0.940
Black or African American	10 (30.3)	7 (33.3)	3 (25.0)	
White	21 (63.6)	13 (61.9)	8 (66.7)	
Asian	1 (3.0)	0 (0)	1 (8.3)	
American Indian or Alaska Native	0	0 (0)	0 (0)	
Other	0	0 (0)	0 (0)	
Unknown or Not Reported	1 (3.0)	1 (4.8)	0 (0)	
Source of Donor, N (%)				0.055
Deceased	15 (45.5)	6 (28.6)	9 (75.0)	
Living/related	8 (24.2)	8 (38.1)	0 (0)	
Living/unrelated	10 (30.3)	7 (33.3)	3 (25.0)	
Cause of Death, N (%)				0.932
Anoxia	4 (26.7)	2 (9.5)	2 (15.4)	
Cerebrovascular Accident/Injury/Stroke	7 (46.7)	2 (9.5)	5 (38.5)	
Head Injury/Trauma	2 (13.3)	1 (4.8)	1 (7.7)	
Intracranial Bleed	1 (6.7)	0 (0)	1 (7.7)	
Motor Vehicle Accident	0 (0)	0 (0)	0 (0)	
Other	0 (0)	0 (0)	0 (0)	
Unknown	0 (0)	0 (0)	0 (0)	
Missing	1 (6.7)	1 (4.8)	0 (0)	

[a] An episode of TCMR was classified as reversible if the serum creatinine level returned to within 15% of the prerejection level within 4 weeks after initiation of antirejection therapy. This was the functional criterion used to classify an episode of TCMR as reversible or nonreversible in our earlier discovery study[8]. Using this criterion, 39 of 43 TCMR episodes were classified as reversible (n=24) or nonreversible (n=15). Four of 43 TCMR episodes were not classified due to missing creatinine values (n=2), proximity to an earlier TCMR biopsy (n=1), or proximity to BKV nephropathy diagnosis (n=1).

[b] *P*-value based on Mann-Whitney test for continuous variables and chi-square tests for categorical variables.

[c] Thirty-three of 34 patients with TCMR were analyzed for TCMR reversible versus nonreversible; a single patient with TCMR biopsy was excluded from analysis because of BKVN diagnosis in proximity to TCMR diagnosis.

[d] Thirty nine of 43 TCMR biopsy matched urine samples were analyzed for TCMR reversible vs. nonreversible; 4 urine samples matched to TCMR biopsies were excluded from data analysis because serum creatinine level was not available after antirejection treatment in 2, 2 episodes of TCMR occurred in proximity in 1 patient and 1 TCMR occurred in close proximity to BKVN.

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Table 3.

Biopsy associated characteristics of kidney transplant recipients with acute T-cell–mediated rejection by reversible status[a]

	Total	Reversible TCMR	Nonreversible TCMR	*P*-value[b]
Study subjects[c]	33	21	12	
Number of biopsies[d]	39	24	15	
Time from Transplant to Biopsy, days				0.015
Mean (SD)	217 (188)	159 (160)	310 (196)	
Median	180	117	269	
Min, Max	3, 701	3, 491	18, 701	
Banff Grade				0.911
Grade IA	16	10	6	
Grade IB	9	5	4	
Grade IIA	11	8	3	
Grade IIB	2	0	2	
Grade III	1	1	0	
Serum Creatinine at Baseline, mg/dL				0.436
Mean (SD)	1.9 (1.2)	2.0 (1.4)	1.7 (0.5)	
Median	1.6	1.6	1.6	
Min, Max	0.9, 8.2	0.9, 8.2	1.1 (3.3)	
Serum Creatinine at Time of Biopsy, mg/dL				0.315
Mean (SD)	3.1 (2.8)	2.8 (2.3)	3.7 (3.4)	
Median	2.3	2.3	2.5	
Min, Max	1.1, 13.3	1.1, 12.2	1.7, 13.3	
Serum Creatinine 4 weeks postbiopsy, mg/dL				0.011
Mean (SD)	2.2 (1.2)	1.8 (1.2)	2.8 (0.9)	
Median	1.9	1.6	2.7	
Min, Max	0.9, 6.6	0.9, 6.6	1.6, 4.5	
Antirejection Regimen[e]				N/A
Glucocorticoids	37	23	14	
Antilymphocyte antibodies	14	9	5	
Other	6	2	4	

[a] An episode of TCMR was classified as reversible if the serum creatinine level returned to within 15% of the prerejection level within 4 weeks after initiation of antirejection therapy. This was the functional criterion used to classify an episode of TCMR as reversible or nonreversible in our earlier discovery study[8]. Using this criterion, 39 of 43 TCMR episodes were classified as reversible (n=24) or nonreversible (n=15). Four of 43 TCMR episodes were not classified due to missing creatinine values (n=2), proximity to an earlier TCMR biopsy (n=1) or proximity to BKV nephropathy diagnosis (n=1).

[b] *P*-value based on Mann-Whitney test for continuous variables and chi-square tests for categorical variables.

[c] Thirty-three of 34 patients with TCMR were analyzed for TCMR reversible versus nonreversible; a single patient with TCMR biopsy was excluded from analysis because of BKVN diagnosis in proximity to TCMR diagnosis.

[d]Thirty nine of 43 TCMR biopsy matched urine samples were analyzed for TCMR reversible vs. nonreversible; 4 urine samples matched to TCMR biopsies were excluded from data analysis because serum creatinine level was not available after antirejection treatment in 2, 2 episodes of TCMR occurred in proximity in 1 patient and 1 TCMR occurred in close proximity to BKVN. Three patients had 3 TCMR biopsies and contributed 3 biopsy-matched urine samples. One of the 3 patients contributed 1 urine sample matched to a nonreversible episode based on absence of improvement in serum creatinine followed by 2 urine samples matched to 2 episodes of reversible TCMR. The 2 remaining patients contributed 1 urine sample matched to a reversible episode of TCMR followed by 2 urine samples matched to 2 episodes of nonreversible TCMR each (5 months apart in 1 patient and 12 months apart in the second patient).

[e]The sum of antirejection treatments for biopsies within a particular column exceeds the total number of biopsies (TCMR diagnoses) because several TCMR episodes were treated with multiple antirejection regimens.

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Table 4.

18S rRNA normalized, log_{10}-transformed levels of mRNA in urinary cells from reversible TCMR group, nonreversible TCMR group and No Rejection group[a]

Type of mRNA	Reversible TCMR Group (N = 24 samples, 21 patients)	Nonreversible TCMR Group (N = 15 samples, 12 patients)	No Rejection Group (N = 162 samples, 126 patients)		*P*-Value[b] Reversible TCMR Vs.	*P*-Value[b] Nonreversible TCMR Vs.
FOXP3	−0.82	−1.637	−1.628	Nonreversible TCMR	0.0096	
	(−1.445, −0.322)	(−2.579, −0.982)	(−2.061, −1.161)	No Rejection	<0.0001	0.7212
CD25	−0.156	−0.385	−0.675	Nonreversible TCMR	0.1531	
	(−0.739, 0.255)	(−1.286, −0.180)	(−1.247, −0.122)	No Rejection	0.0066	0.7623
CD3E	0.858	0.312	−0.504	Nonreversible TCMR	0.1887	
	(0.199, 1.061)	(−0.285, 0.995)	(−1.159, 0.242)	No Rejection	<0.0001	0.0026
Perforin	0.35	0.197	−0.69	Nonreversible TCMR	0.4322	
	(0.116, 0.817)	(−0.219, 0.822)	(−1.232, 0.019)	No Rejection	<0.0001	0.0017

[a]Median (lower, upper quartiles) log-transformed ratio of mRNA copies to 18S rRNA copies (x 10^{-6}) is shown for each mRNA measure. The number of patients with biopsy-matched urine samples (urine collected from 3 days before to 1 day after biopsy) are shown for patients with functional reversal of acute T-cell–mediated rejection (reversible TCMR), nonreversible TCMR, and those without any rejection features in the biopsy (No Rejection). An episode of TCMR was classified as reversible if the serum creatinine level returned to within 15% of prerejection level within 4 weeks after initiation of antirejection therapy.

[b]*P*-values for pairwise differences are based on Mann-Whitney test.

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Table 5.

Standardized odds ratios for TCMR reversal: multivariable logistic regression analyses[a]

Covariates[b]	FOXP3 OR	P-Value
Unadjusted	3.33 (1.36, 8.11)	0.0083
Serum Creatinine[c]	3.32 (1.38, 7.99)	0.0075
Age at Transplant	3.88 (1.40, 10.7)	0.0090
Time from Transplant to Biopsy	3.73 (1.32, 10.5)	0.0130
Type of Transplant	2.97 (1.21, 7.24)	0.0170
Fully Adjusted Model[d]	**3.88 (1.28, 11.8)**	**0.0168**

[a]Standardized odds ratios (per 1-SD difference in $\log_{10}$-transformed, 18S rRNA normalized FOXP3 mRNA) are presented along with estimated 95% confidence intervals in parentheses. Urinary cell level of FOXP3 mRNA remained predictive of TCMR reversal after individual adjustment for serum creatinine measured at time of biopsy, age at transplant, time from transplant to biopsy, and type of transplant (deceased donor, unrelated live donor, related live donor. Data are derived from 39 urine samples matched to 39 TCMR biopsies categorized as reversible (n=24) or nonreversible (n=15) from 33 patients.

[b]For each row, TCMR is regressed on FOXP3 mRNA level plus the covariate listed.

[c]Serum creatinine level (mg/dL) measured at time of biopsy.

[d]Adjusted for serum creatinine, age at transplant, time from transplant to biopsy, and type of transplant.

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Table 6.

Kidney allograft survival: multivariable Cox proportional hazards regression analyses[a]

Covariates[b]	FOXP3 HR	P-Value
Unadjusted	0.55 (0.30, 0.99)	0.0474
Serum Creatinine[c]	0.55 (0.30, 0.99)	0.0475
Age at Transplant	0.53 (0.28, 0.98)	0.0445
Time from Transplant to Biopsy	0.64 (0.30, 1.33)	0.2309
Type of Transplant	0.65 (0.36, 1.18)	0.1584
Reversible Status	0.78 (0.39, 1.56)	0.4771
Fully Adjusted Model[d]	**0.69 (0.30, 1.61)**	**0.3986**

[a]Standardized hazard ratios (per 1-SD change difference in $\log_{10}$-transformed, 18S rRNA normalized FOXP3 mRNA) for kidney allograft survival are presented along with estimated 95% confidence intervals in parentheses. Urinary cell FOXP3 mRNA level remained predictive of graft survival after individual adjustment for serum creatinine measured at time of biopsy and age at transplant, but not after adjustment for time from transplant to biopsy, and type of transplant. Data are derived from 39 urine samples matched to 39 TCMR biopsies categorized as reversible or nonreversible from 33 patients.

[b]For each row, graft survival is regressed on FOXP3 mRNA level plus the covariate listed.

[c]Serum creatinine level (mg/dL) measured at time of biopsy.

[d]Adjusted for serum creatinine, age at transplant, time from transplant to biopsy, and type of transplant.