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Original Article

Prospective observational study to validate a next-generation sequencing blood RNA signature to predict early kidney transplant rejection

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Abbreviations: ABMR, antibody-mediated rejection; AR, acute rejection; AUC, area under the curve; AUROC, area under the receiver operating characteristic curve; AUSCAD, Australian Chronic Allograft Dysfunction; CI, confidence interval; GoCAR, Genomics of Chronic Allograft Rejection; HLA, human leukocyte antigen; HR, high risk for rejection; LR, low risk for rejection; NGS, next-gene sequencing; NonAR, nonacute rejection; NPV, negative predictive value; PPV, positive predictive value; PRA, panel reactive antibodies; PVAN, polyomavirus-associated nephropathy; QC, quality control; TCMR, T cell-mediated rejection.

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ABSTRACT

The objective of this study was to validate the performance of Tutivia, a peripheral blood gene expression signature, in predicting early acute rejection (AR) post–kidney transplant. Recipients of living or deceased donor kidney transplants were enrolled in a nonrandomized, prospective, global, and observational study (NCT04727788). The main outcome was validation of the area under the curve (AUC) of Tutivia vs serum creatinine at biopsy alone, or Tutivia + serum creatinine at biopsy. Of the 151 kidney transplant recipients, the mean cohort age was 53 years old, and 64% were male. There were 71% (107/151) surveillance/protocol biopsies and 29% (44/151) for-cause biopsies, with a 31% (47/151) overall rejection rate. Tutivia (AUC 0.69 [95% CI: 0.59-0.77]) and AUC of Tutivia + creatinine at biopsy (0.68 [95% CI: 0.59-0.77]) were greater than the AUC of creatinine at biopsy alone (0.51.4 [95% CI: 0.43-0.60]). Applying a model cut-off of 50 (scale 0-100) generated a high- and low-risk category for AR with a negative predictive value of 0.79 (95% CI: 0.71-0.86), a positive predictive value of 0.60 (95% CI: 0.45-0.74), and an odds ratio of 5.74 (95% CI: 2.63-12.54). Tutivia represents a validated noninvasive approach for clinicians to accurately predict early AR, beyond the current standard of care.

1. Introduction

Improvements in kidney transplantation can be attributed to several key discoveries in surgical procedures, procurement solutions, and technical advancements, such as human leukocyte antigen (HLA) matching and the generation of novel immunosuppressants.[1,2] These developments have had a positive impact on short-term outcomes at 1- and 3-years post–kidney transplant.[3] Moreover, patient survival after kidney transplantation has surpassed survival when staying on dialysis.[4]

However, despite these efforts, the long-term kidney transplant outcomes have not improved to the expected level,[3,5] which represents additional challenges as the number of patients on the transplant list far surpasses the number of available donors; 18% of patients on the US waiting list have already had at least one failed transplant.[6] This shortfall necessitates an expansion of the donor pool to include higher-risk donors such as older living donors and deceased donor kidneys, donors after cardiac death, and high kidney donor profile index donors, which have mixed results in long-term outcomes.[7-9] Serum creatinine, proteinuria, and HLA donor-specific antibody titers are routinely utilized to identify patients with potential acute rejection (AR).[10] Although more invasive compared with other approaches and fraught with sampling and operator differences, histologic evidence from a kidney biopsy remains the gold standard for the diagnosis of rejection. There are differences in how borderline and subclinical AR are treated among transplant programs, which may contribute to a decrease in successful long-term outcomes.[11,12] Finally, diagnoses from local pathologists can be very inconsistent, resulting in untreated or undertreated T cell-mediated rejection (TCMR), subclinical antibody-mediated rejection (ABMR), interstitial fibrosis, and tubular atrophy with early graft loss.[13,14]

Biomarkers to assist clinicians in identifying an individual patient's level of risk for AR and improve long-term outcomes after kidney transplantation are needed. Current biomarkers provide a noninvasive assessment of AR risk but have difficulty identifying early manifestations of rejection. They often do not distinguish TCMR from ABMR or BK nephropathy and are much more accurate in their negative predictive value (NPV) as opposed to their positive predictive value (PPV).[13-16] More recently, biomarkers are being utilized to influence the choice and degree of immunosuppression, while balancing the AR risk, to improve long-term outcomes. However, most have not been readily adopted by the transplant community and only modestly affect clinical decision-making.[17-22]

Tutivia utilizes a peripheral blood next-generation sequencing assay to evaluate a 17-gene mRNA signature in combination with a proprietary artificial intelligence algorithm to categorize kidney transplant patients as at low risk or high risk of AR. Clinical samples from the Genomics of Chronic Allograft Rejection (GoCAR) Study[22] served as the training set for the current machine learning algorithm. The primary objective of the current study is to validate the prognostic performance of the derived RNA signature test to predict the risk of acute clinical or subclinical allograft rejection (for future transplant patients) through correlation with the study subject's kidney biopsy histopathology using Banff 2019 criteria.

2. Materials and methods

2.1. Participants and study design

The current study is an ongoing nonrandomized, prospective, observational international study (NCT04727788) to validate the ability of Verici Dx genomic tests to predict the risk of kidney clinical and subclinical AR and chronic allograft damage. Thirteen study sites adhering to the Declaration of Helsinki were included in the Tutivia validation set (the list of study sites is in

Supplementary Materials and Supplementary Table S1). Participants were enrolled in this study from March 2021 to January 2023. The study was approved by the Advarra Institutional Review Board, Pro00049177. Subjects from the Australian Chronic Allograft Dysfunction study were also included. Participants were included when they were a living or deceased donor kidney transplant recipient, between 18 and ≤80 years of age, and able to provide signed informed consent. A Consolidated Standards of Reporting Trials diagram is provided in Figure 1. Recipients of multiple organ transplants, excluding kidney-pancreas or patients who were participating in a therapeutic clinical trial for transplant rejection, with active human immunodeficiency virus positive or Hepatitis C positive, or pregnant, were excluded. This observational study adhered to the Strengthening the Reporting of Observational Studies in Epidemiology reporting guidelines.

2.2. Study procedures and specimen collection

Study participants were evaluated at a pretransplant visit, where a detailed demographic, medical, and transplant history was obtained, including the clinical characteristics of the donor. Following a transplant, participants were asked to return at 1, 3, 6, 12, and 24 months posttransplant to provide medication updates and have laboratory, clinical, and pathologic data collected. At 3 and 12 months, a core biopsy of the allograft was obtained either from a protocol-mandated or standard surveillance procedure according to site protocol. Additionally, unscheduled visits for clinically indicated biopsies according to local site procedures were included. Blood samples were collected during all posttransplant visits. Peripheral blood was collected in 2 RNA PAXgene tubes at all protocol and unscheduled visits. At the time of protocol biopsy visits, blood was collected within a median of 0 days from the corresponding biopsy date. Twenty-three patients, in part due to coronavirus diseases and related visit restrictions, along with site-directed procedures requiring research blood work to be obtained postbiopsy, had their blood taken within 31 days postbiopsy.

Given that the intent was to use the blood RNA signature to predict biopsy histology and the risk of rejection, the timing of blood collection with a kidney biopsy is important for the current correlational study and subsequent clinical utility evaluation. Blood samples collected from allograft recipients at corresponding time points for biopsy were sent to the Verici Dx CLIA laboratory for testing.

All diagnostic kidney biopsies were first evaluated by the respective local site pathologist and then sent digitally (including all hematoxylin and eosin-stained biopsies and any special stains, along with C4D immunohistochemistry, when available) for a central pathology review. Approximately 5% of patients had glass slides sent for central pathology review. A secondary central pathology data review was independently obtained from approximately 15% of patients. The use of a secondary review was part of the initial study plan to adjudicate discrepant cases that were challenging, including borderline histology, C4D interpretation, or use of non-2019 Banff criteria. Given the subjective semiquantitative nature of histologic (immune cell/morphologic) phenotyping, particularly for borderline classification, both local and central pathology diagnoses were evaluated. Therefore, when a discrepancy existed, there was a built-in allowance for an additional pathologist to derive a consensus.[16] All biopsies were evaluated using 2019 Banff criteria.[11]

HLA typing was performed according to individual local site and/or organ procurement organization protocols. The results of HLA typing are reported in the study and harmonized for the assessment of the number of relevant mismatches. Hematoxylin and eosin, periodic acid-Schiff, and C4D and SV40 immunohistochemistry for

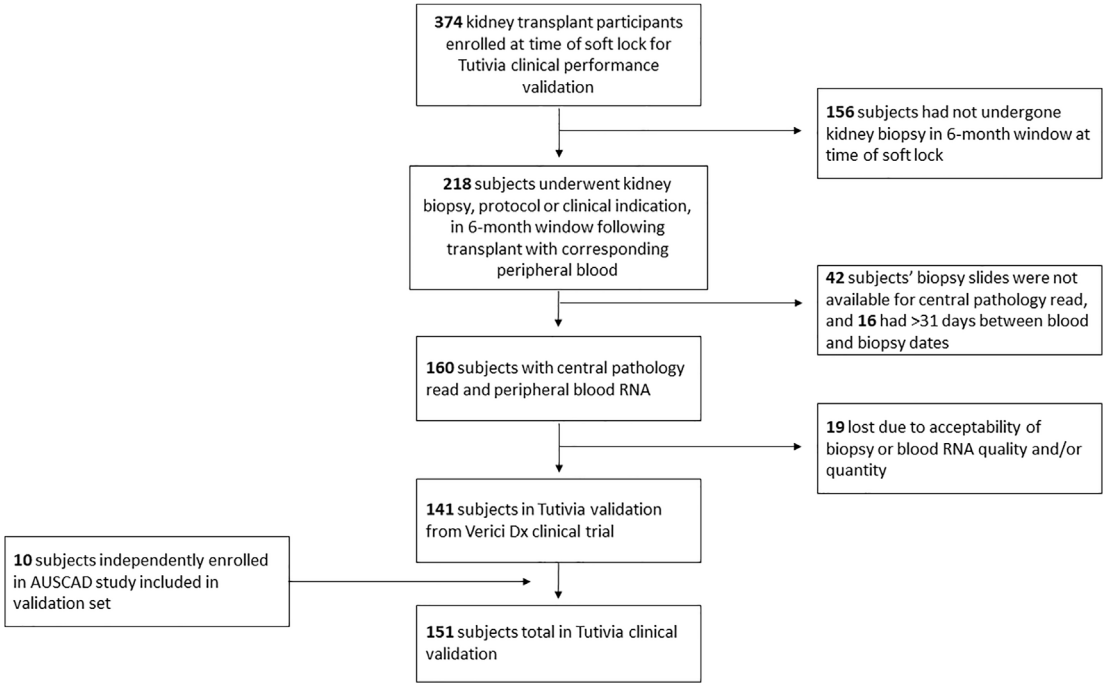

Figure 1. Consolidated Standards of Reporting Trials diagram of study enrollment. AUSCAD, Australian Chronic Allograft Dysfunction.

polyomavirus-associated nephropathy (PVAN) detection were examined using digital images or stained slides using standard diagnostic criteria for acute and chronic rejection, calcineurin inhibitor histopathologic features of toxicity, and other conditions that might affect the allograft. Acute cellular and antibody-mediated kidney rejection was determined using 2019 Banff criteria,[11] whereas chronic damage was diagnosed as inflammation within areas of interstitial fibrosis, and tubular atrophy and scored using the chronic allograft damage index and Banff 2019 guidance. Chronic active ABMR was defined according to Banff system criteria.[11] Study personnel, laboratory, central pathology, and clinician investigators were blinded to results to reduce inherent bias.

2.3. Primary objective and study endpoints

The primary objective was to validate the prognostic performance of peripheral blood gene expression signatures to predict the risk of AR through correlation with the histopathology of surveillance or for-cause kidney biopsies. The primary outcome was evidence of clinical or subclinical rejection on histopathology of a kidney biopsy within 6 months posttransplant.

2.4. Seventeen-gene signature analysis

Total RNA was extracted from peripheral blood using a Promega Maxwell simplyRNA kit. Indexed transcriptome copy DNA libraries were generated with an Illumina Stranded messenger RNA Library Prep Ligation Kit following the manufacturer's instructions. The indexed libraries were sequenced on an Illumina NextSeq 2000. Good-quality reads were first trimmed, with rRNA and *HBB* reads removed before being aligned to the human reference genome database. Resultant counts were normalized before calculating the AR risk score using the predefined 17-gene algorithm. All data processing was conducted using validated data processing and prediction pipelines. Results passing prespecified quality control (QC) criteria were transformed to A scale of 0 to 100 and reported as final AR risk scores. The process from sample receipt to Tutivia risk score generation is detailed in Figure 2.

The Tutivia algorithm incorporates quantitative measures of normalized individual gene transcripts, which are differentially weighted and assigned a value toward the computation of the final risk score. The final Tutivia 17-gene algorithm originated from the GoCAR[22] cohort blood samples, which served as the

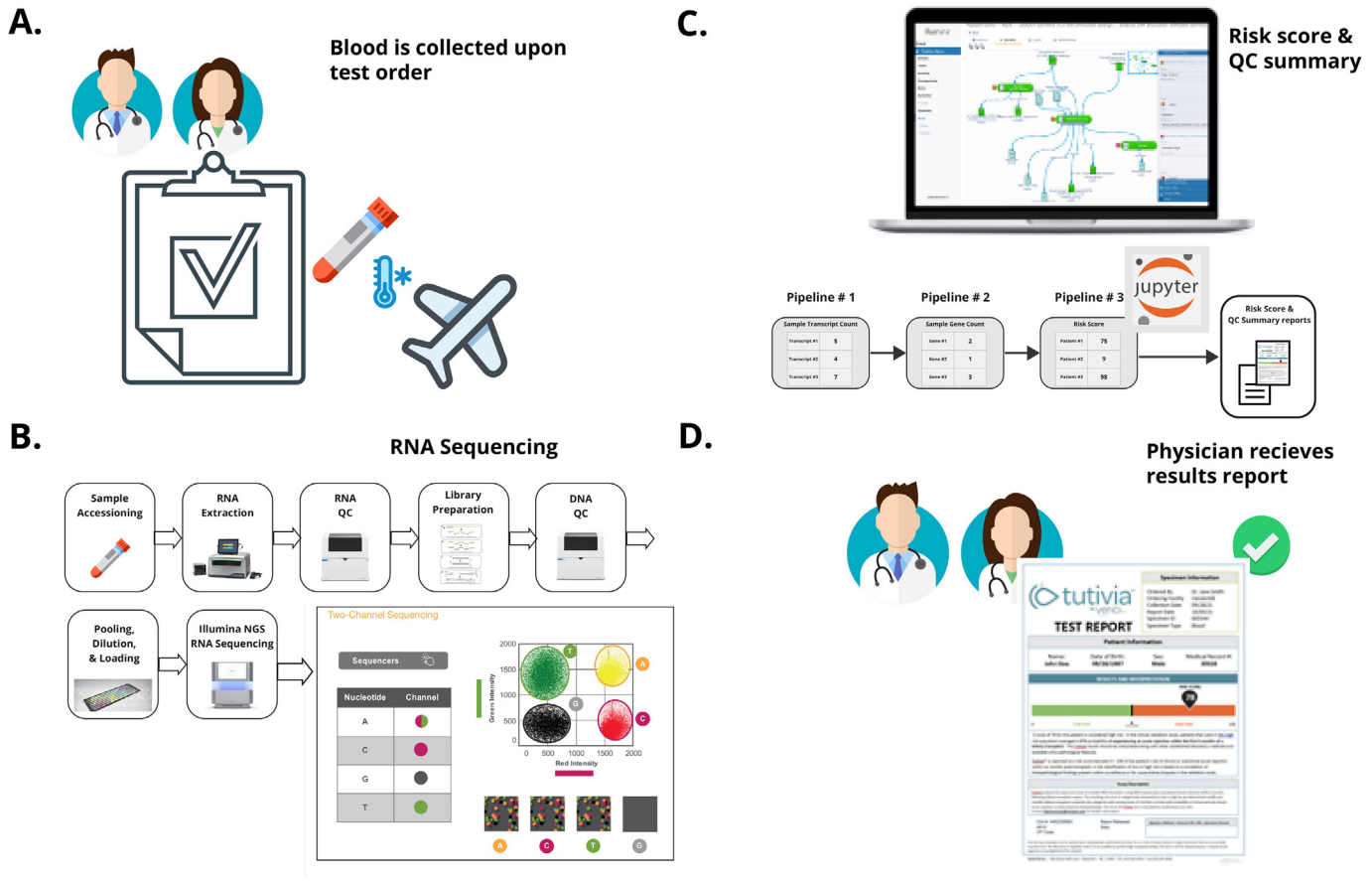

Figure 2. Tutivia process diagram from specimen receipt through risk score generation. Tutivia workflow: (A) The test is ordered, and a blood specimen is collected and sent to the laboratory. (B) RNA is isolated; copy library is prepared, and RNA sequencing is performed. (C) The sequencing data are uploaded and QC is assessed; files enter pipelines and proprietary algorithms that process data to produce test results. (D) The laboratory director reviews and asks patient QC to approve the release of results. QC, quality control.

training set. The GoCAR samples were resequenced as defined in the Methods section above, and the original findings were confirmed (unpublished data). Employing a novel, unbiased, unsupervised bioinformatic discovery interrogation process of >11 000 genes resulted in the current 17-gene signature. During this test development process, only 2 genes from the original signature (Annexin A5 and TSC22D1)[22] were identified, further establishing the uniqueness of the Tutivia gene set and algorithm. The complete list of the 17 genes in the Tutivia signature, including ensemble identification/name, postulated role, and associated references, is in Supplementary Materials and Supplementary Table S2. Of the 17 genes, 7 are associated with maintaining kidney function (including cellular division and metabolism), 4 are linked to immune pathways, such as antigen processing, apoptosis, and activation in T and B cells, and 6 are part of various cytokine cascades that impact macrophage, neutrophil, and natural killer cell activation and both antibody and T cell-based rejection and cell lysis. Some of these genes are directly related to high-level dynamic processing of RNA and protein states within all cell types, which is directly related to immune pathways seen at the "static" level of the biopsy. The final AR risk scores were further stratified into high- or low-AR risk categories based on a predefined score cut-off of 50.

2.5. Statistical analysis

The characteristics of study participants with and without rejection were compared using the Wilcoxon rank sum test (for continuous values) or the chi-square test (for categorical values). The ability of Tutivia to predict rejection was assessed using the area under the receiver operating characteristic curve (AUROC). The primary analysis was a comparison of Tutivia vs a prespecified clinical benchmark model, ie, creatinine at the time of biopsy.[23] The secondary analysis was the combination of the clinical model with Tutivia vs serum creatinine at biopsy with AUROC. It is noteworthy that the current benchmark model of serum creatinine at biopsy was not applied for subjects with specific adverse events (ie, delayed graft function, BK viremia, acute kidney injury, and acute allograft dysfunction) before for-cause biopsies. The consensus was that these subjects did not have a reliable serum creatinine level (ie, missing benchmark values) because of the impact of adverse events on the creatinine value. To reflect this, random values were imputed as the serum creatinine for these subjects to achieve a benchmark AUC of 0.5. All AUROC model measurements were calculated using bootstrapping, utilizing 500 replicates to correct for optimism. Given that AUROC reflects discrimination, all covariates were simply modeled as linear, and no variable selection procedures were performed. With the primary analysis being the comparison of 2 statistical prediction models, the study was powered using the methods developed previously.[24] A sample size of 151 subjects provided 90% power to detect a 5% improvement in Nagelkerke's R-square for the Tutivia test over the benchmark model in predicting clinical and subclinical rejection.

We used the powering method described by Riley et al.[24] This method has been shown to be more accurate than the traditional 10 events per predictor rule.[25] The online calculator for this calculation is available here: https://riskcalc.org/samplesize/.

All statistical analyses were conducted using R Studio, version 4.1.3 (R Foundation for Statistical Computing). A 2-sided $P < .05$ was considered statistically significant.

3. Results

3.1. Validation cohort transcript profile and kidney biopsy characteristics

The current study is part of an ongoing global, nonrandomized, observational trial for the validation of Verici Dx genomic tests to predict the risk of kidney allograft clinical and subclinical AR. As identified in Table 1, there were 151 participants from 5 countries (US, France, Italy, Spain, and Australia). The median age of this cohort at the time of transplant was 53 years, predominantly male (64%), and 79% were first-time transplant patients. The mean time to AR of the biopsy was 57 days, with an overall rejection rate of 31% (n = 47). Race within the population was self-identified, with 72% being White and nearly 21% being Black individuals.[20] The individual patient characteristics were also assessed for their significance in predicting the risk of rejection. There were no restrictions on site immunosuppressive regimens (Supplementary Materials and Supplementary Table S3 provide a complete listing of therapies). The majority of the 151 patients (n = 128, 85%) had their blood collected within 1 month before the day of biopsy, and 15% (n = 23 patients) had blood collected between 1 and 31 days post-biopsy. Importantly, all 151 patients had received some form of induction therapy, including 48% (n = 73) with antithymocyte globulin/thymoglobulin with steroids, 33% (n =4 9) with interleukin 2 receptor antagonists with steroids, and 19% (n = 29) with other combinations.

As noted in Table 1, the median donor age was 46 years, with 51 living donors and 100 deceased, of which 52 deceased donors were identified as standard criteria, 17 as expanded criteria, and 31 as donors following cardiac death. Four (2.6%) participants were blood type ABO incompatible, and 16 (11%) had positive (>30%) panel reactive antibodies to both HLA class I and II at the time of enrollment. Seventy-three (48%) patients had >4 HLA mismatches at A, B, DRB1, and DQB1.

Within 6 months posttransplant, all subjects had at least 1 surveillance or for-cause kidney biopsy, which was histologically evaluated for rejection by a central pathologist using the Banff 2019 criteria;[11] 107 (71%) surveillance (protocol) and 44 (29%) for-cause (clinically indicated) biopsies were included. The comparison of central vs local pathology diagnoses is provided in Supplementary Materials and Supplementary Table S4. As demonstrated in this table, the central pathologist classified approximately 50% more biopsies as exhibiting evidence of rejection than the local pathologists. Given the subjectivity of the diagnostic process, utilizing a single expert pathologist to review all cases provides a level of diagnostic interpretive consistency,

Table 1
Patient Characteristics.

	Total cohort N = 151	No reject (N = 104)	Reject (N = 47)	*P*-value
Recipient age, y, median (range)	53.0 (18.0, 79.3)	53.0 (18.0, 79.3)	52.0 (22.0, 74.0)	.384
Recipient sex, N (%)				.855
Male	97 (64)	66 (64)	31 (66)	
Female	54 (36)	38 (37)	16 (34)	
Recipient race, N (%)				<.001
Asian	5 (3)	4 (4)	1 (2)	
Black	31 (21)	29 (28)	2 (4)	
Native American	0 (0)	0 (0)	0 (0)	
Pacific Islander	3 (2)	1 (1)	2 (4)	
White	108 (72)	67 (64)	41 (87)	
Not Answered	4 (3)	3 (3)	1 (2)	
Recipient participation location, N (%)				
USA	84 (56)			
Europe (Italy, France, and Spain)	57 (38)			
Australia,	10 (7)			
Donor age in y, median (range)	46.0 (5.0, 81.0)	45.0 (5.0, 81.0)	47.0 (19.0, 79.0)	.214
Donor sex, N (%)				.856
Male	76 (50)	52 (50)	24 (51)	
Female	62 (41) mMi	41 (39)	21 (45)	
Missing	13 (9)	11 (11)	2 (4)	
Donor race, N (%)				.007
Asian	1 (1)	1 (1)	0 (0)	
Black	12 (8)	12 (12)	0 (0)	
Native American	0 (0)	0 (0)	0 (0)	
Pacific Islander	1 (1)	0	1 (2)	
White	111 (73)	72 (69)	39 (83)	
Not answered	26 (17)	19 (18)	7 (15)	
PRA class I, N (%)				1.0
0%	92 (61)			
1%-30%	19 (13)			
>30%	20 (13)			
Not performed	20 (13)			
PRA class II, N (%)				.615
0%	93 (62)			
1%-30%	5 (3)			
>30%	33 (22)			
Not performed	20 (13)			
Living donor recipient, N (%)	51			.397
Living related donor	28	22 (21.2%)	6 (12.8%)	
Living unrelated donor	23	14 (13.5%)	9 (19.1%)	

(*continued on next page*)

Table 1 (*continued*)

	Total cohort N = 151	No reject (N = 104)	Reject (N = 47)	*P*-value
Deceased donor recipient, N (%)	100			
Standard criteria donor	52	37 (35.6%)	15 (31.9%)	
Expanded criteria donor	17	9 (8.7%)	8 (17.0%)	
Donors after cardiac death	31	22 (21.2%)	9 (19.1%)	
Previous kidney transplant recipient, N (%)				.815
0	120 (79)	83 (80)	37 (79)	
1	18 (12)	11 (11)	7 (15)	
2	3 (2)	2 (2)	1 (2)	
Missing	10 (7)	8 (8)	2 (4)	
ABO incompatibility, N (%)				.589
No	147 (97)	102 (98)	45 (96)	
Yes	4 (3)	2 (2)	2 (4)	
Cold ischemia time for diseased donor		N = 68	N = 32	.003
Mean (±SD)		15.8 (6.90)	11.8 (5.39)	
Median (range)		14.5 (4.00, 39.9)	9.00 (3.00, 25.5)	
Missing, N (%)		4 (6)	1 (3)	
HLA mismatches (A, B, DRB1, and DQB1), N				.265
0-4	53	35	18	
5-8	82	55	27	
Missing data	16	14	2	

HLA, human leukocyte antigen; PRA, panel reactive antibody.

which is crucial for a correlative trial design as described in this report. Of the 47 allograft rejections, 20 (42%) were in the surveillance group with a median time to rejection of 97.5 (78-133) days, and 27 (58%) in the for-cause group displayed a median time to rejection of 21 (6-175) days. The median time to rejection for any biopsy was 58 (6-175) days (Supplementary Material and Supplementary Table S5). Of the 47 AR, 11 were borderline TCMR, 13 were TCMR-IA or higher, 12 were ABMR, and 11 were classified as mixed rejections. Of the 23 patients whose blood was drawn after the biopsy, 18 (78%) were for-cause and 5 (22%) were surveillance biopsies. Of these, 8 were classified as rejection by local pathology, with 7 for-cause (4 TCMR, 1 ABMR, and 1 mixed) and 1 surveillance biopsy (mixed).

We hypothesized that the 31% rejection rate was most likely the result of the addition of surveillance biopsies with for-cause biopsies and the inclusion of borderline within the rejection group. In support of our observation, a high rejection rate ranging from 29% to 46% in surveillance biopsies within 6 months of transplantation had previously been reported in several studies, including one publication (Shapiro et al[26]) with a protocol biopsy at 8 days posttransplant.[26-30]

3.2. Performance of the 17-gene Tutivia assay

The 17-gene Tutivia assay was evaluated using the receiver operating curve with an AUC of 0.69 (95% CI: 59.7-78.3) vs. the baseline clinical model of creatinine at the time of biopsy with an AUC of 0.51 (95% CI: 42.9-60.0), *P*-value = .009. This demonstrated Tutivia as a continuous predictor for differentiating rejection from nonrejection (Fig. 3A). Moreover, noteworthy is that even when combined with the baseline clinical model of creatinine at the time of biopsy[23] (AUC = 0.68 [95% CI: 59.2-77.2]), the 17-gene assay remained an independent predictor of transplant risk. Applying a predetermined cut-off of ≤50 as low risk and >50 as high risk for rejection classified 40 (26.5%) patients as high risk and 111 (73.5%) as low risk. Eighty-eight of the 111 low-risk patients had no AR, 7 had borderline AR, and 24 of the 40 high-risk patients with Banff 2019-characteristics confirmed ARs, translating to an NPV of 79% and a PPV of 60% with an odds ratio of 5.74 (Fig. 3B and Table 2). Only 8 of the 23 patients whose blood was drawn after the biopsy (median 15 days) exhibited AR by local pathology, and 50% (4/8) had their blood taken within 10 days. The Tutivia assay correctly classified all 8 as rejected, although 7 out of the 8 patients had received some form of immunosuppressive therapy either on the day of biopsy or soon thereafter, suggesting no significant impact (ie, no change in risk category) on the Tutivia signature.

3.3. Clinical subgroup analyses

In the 151 patients, 35 (23%) clinically indicated (for-cause) biopsies were performed before 60 days posttransplant. Of these

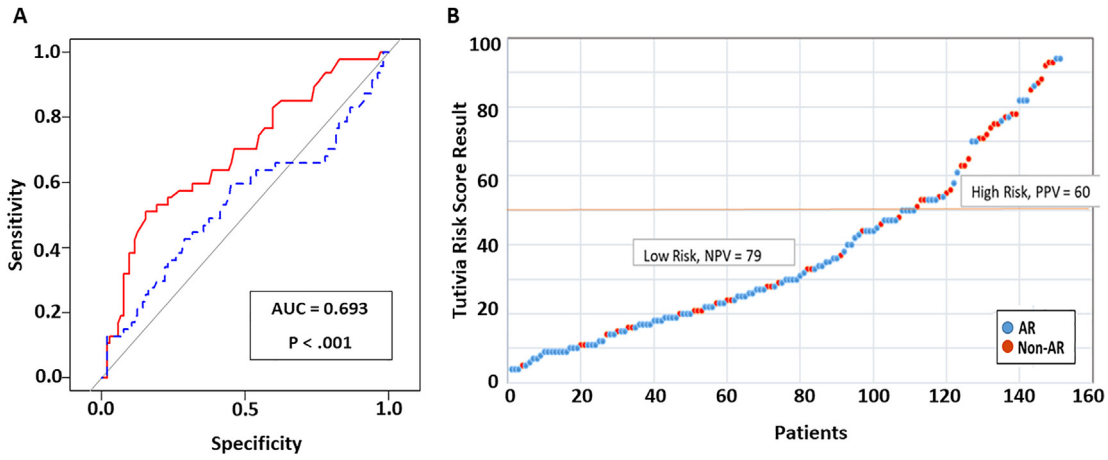

Figure 3. The clinical performance of the next-generation sequencing (NGS) 17-gene test Tutivia vs clinical model. (A) The clinical performance of the NGS test Tutivia (red line) was superior to the clinical model (creatinine at the time of biopsy, blue line), as demonstrated using the AUC and (B) an applied threshold of 50 identified patients most likely to have a transplant rejection within 6 months. AR, acute rejection; AUC, area under the curve; NonAR, nonacute rejection; NPV, negative predictive value; PPV, positive predictive value.

Table 2
Performance of Tutivia with a model cut-off to stratify patients into high- and low-risk groups utilizing correlation to either surveillance or for-cause kidney biopsy.

Validation model	Rejection	No rejection	Total
Risk score $>$ 50	24	16	40
Risk score $\leq$ 50	23	88	111
Validation performance metric		**Performance (95% CI)**	
Sensitivity		0.51 (0.37-0.65)	
Specificity		0.85 (0.76-0.90)	
PPV		0.60 (0.45-0.74)	
NPV		0.79 (0.71-0.86)	
Odds ratio		5.74 (2.63-12.54)	

CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value.

Table 3
Accuracy of Tutivia risk categorization according to biopsy type and rejection status.

Clinically indicated (for-cause biopsies)	Rejection	No rejection	Total
Risk categorization			
Tutivia HR	21	7	28
Tutivia LR	6	10	16
Total	27	17	44
Point estimates and 95% CIs:			
Sensitivity*		0.78 (0.59, 0.89)	
Specificity*		0.59 (0.36, 0.78)	
Positive predictive value*		0.75 (0.57, 0.87)	
Negative predictive value*		0.63 (0.39, 0.82)	
Protocol (surveillance biopsies)	**Rejection**	**No rejection**	**Total**
Tutivia HR	3	9	12
Tutivia LR	17	78	95
Total	20	87	107
Point estimates and 95% CIs:			
Sensitivity*		0.15 (0.05, 0.36)	
Specificity*		0.90 (0.81, 0.94)	
Positive predictive value *		0.25 (0.09, 0.53)	
Negative predictive value *		0.82 (0.73, 0.89)	
*Wilson Cis			

CI, confidence interval; HR, high risk for rejection; LR, low risk for rejection.

35 early biopsies, 24 (69%) exhibited AR, and 20 (83%) had a high-risk Tutivia score, suggesting a role for Tutivia as an early predictor of AR. The evaluation of Tutivia's performance according to the type of clinical rejection identified in the for-cause biopsies has a PPV of 0.75 (95% CI: 0.57-0.87) and an NPV of 0.63 (95% CI: 0.39-0.82) (Table 3). Supplementary Table S6 provides additional performance metrics, including sensitivity and specificity for both for-cause and surveillance biopsies.[16,31-35] Although it is challenging to compare Tutivia with other commercially available tests predicting allograft rejection (due to assay type, trial design, Banff endpoints, and prevalence), the Tutivia PPV of 0.75 and sensitivity of 0.78 were the highest for predicting for-cause rejection across all tests listed in Supplementary Table S6. It is worth noting that the only other commercially available gene expression test listed in Supplementary Table S6 is TruGraf,[16] which is contraindicated in the first 90 days. TruGraf was designed and validated to rule out a need for biopsy in quiescent patients, which is quite different from Tutivia. Moreover, the current version of TruGraf has 120 genes in the algorithm, and none overlap with

Tutivia. This is not unexpected, given that TruGraf was developed using microarray techniques on surveillance-only biopsies from quiescent kidneys with stable kidney function as a rule-out test. In contrast, Tutivia uses RNA sequencing and was developed as an "all-comers" test regardless of a clinical state. Gene discovery in biomarker development is highly influenced by the design, training cohort, and clinical definition of rejection. For example, in TruGraf, a tubulitis score of t2 or t3 with i0 was classified as borderline.[14] This is divergent from Banff criteria, whereas Tutivia is aligned with Banff 2019. Meanwhile, in subclinical AR (ie, surveillance biopsy), Tutivia had a PPV of 0.25 (95% CI: 0.09-0.53), sensitivity of 0.15 (0.05, 0.36), NPV of 0.82 (95% CI: 0.73-0.89), and specificity of 0.90 (0.81, 0.94). Additional efforts are underway to improve the prediction of rejection for Tutivia in the surveillance biopsy setting; however, in its current form, the assay performs quite well for ruling out rejection in a subclinical setting. Similar challenges for other tests have also been reported (see Supplementary Table S6).[16,32-34,36]

Finally, we evaluated kidney biopsies for PVAN in those with SV40 staining; BK virus can be difficult to differentiate from rejection with current biomarker testing. We identified 6 (4%) patient biopsies as positive for PVAN. Compared with the group with negative SV40 (which included both patients with rejection and nonrejection), SV40+ staining was highly correlated with low-risk Tutivia results (C = 0.78, Supplementary Material and Supplementary Fig.).

4. Discussion

In this multicenter, international prospective study, we validated the prognostic performance of Tutivia to predict the risk of AR using correlation with the histopathology of surveillance or clinically indicated kidney biopsies as determined using Banff 2019 guidelines.[12,36] The results identified that 83% of the early indicated (clinical for cause) biopsies diagnosed with Banff 2019-characterized rejection had a high-risk Tutivia score, highlighting a particular discrimination in predicting early clinical AR with a PPV of 75% and an NPV of 63%. For surveillance biopsies, Tutivia performed quite well for ruling out rejection with an NPV of 82% and a specificity of 90%, compared with a suboptimal performance for identifying AR with a PPV of 25% and a sensitivity of 15%, as shown in Table 3. Furthermore, the generalized AR prediction, with an NPV of 79% and a PPV of 60%, regardless of biopsy type, does support a broader clinical use of the Tutivia assay. This is especially important as the signature was validated in an all-comers prospective, correlational real-world evidence study where clinically efficacious information is provided at both ends of the rejection spectrum.

The GoCAR study[22] provided the initial feasibility evidence that peripheral blood transcripts can successfully identify individuals at higher risk of AR and future graft loss at 3 months posttransplant. Although serial surveillance biopsies could provide key information to characterize the current immune response and guide clinical care decisions, they are time-consuming, costly, invasive, and often accompanied by an increased risk of secondary complications. Thus, a noninvasive clinical bioassay that provides an assessment of the graft without the need for a biopsy is highly advantageous.[17,18,37]

Here, we provide evidence that Tutivia is a useful assay to identify and potentially monitor both low- and high-risk kidney transplant recipients in various clinical scenarios. The current study design is prospective and inclusive of all-comer adult kidney transplant recipients with multiple site locations throughout the world, such that the results obtained are not biased by patient selection criteria or the absence of diversity. Moreover, all study investigators and central pathologists were blinded to all study results to remove any bias in evaluating all patient kidney biopsies. Moreover, unique to this study is that the majority of patients underwent a planned surveillance biopsy independent of suspected rejection, rather than only enrolling patients with a clinically indicated (for-cause) biopsy posttransplant.

A recent review article detailed the importance of noninvasive "liquid biopsy" approaches for predicting and monitoring transplant rejection, specifically for patients with kidney transplants.[38] In this article, the authors Benincasa et al[38] introduced the field of "transplantomics," which emphasizes the necessity for a "network" machine learning approach for deciphering and providing clarity when introducing "omics" into clinical rejection. Tutivia has followed the machine learning strategy for gene identification and broad applicability to validate a generalizable signature that equates to a gene expression profile for predicting early AR. The diverse gene panel represents specific cell-based mechanisms of protein processing and receptor biology that are directly aligned with classical immune regulation, supporting the complex "network" referenced previously.

In addition, a recent completely independent post hoc assessment of the Tutivia assay was published from a prospective randomized therapeutic trial on 21 patients to predict either subacute or clinical rejection.[39] The study reported an NPV of 0.92 (95% CI: 0.63-98.60) and a PPV of 0.70 (95% CI: 0.45-0.87) with an AUC of 0.83, further supporting the generalizability of the approach and the promising performance of the Tutivia assay as a tool to predict the likelihood of rejection.

Serum creatinine has thus far been the most utilized test to assess kidney function and remains the gold standard in clinical practice as a predictor of acute kidney injury.[40] Tutivia, acting as a biomarker in the current study, demonstrated significant improvement over the measure of serum creatinine in identifying acute kidney rejection. Moreover, in the event of monitoring and clinical need for surveillance biopsies, Tutivia was effective in ruling out rejection, and investigations are underway to ascertain the molecular drivers behind the diagnosed tissue-based (acute) rejection and the relationship (whether any) to long-term graft survival. Overall, Tutivia provided a more accurate prediction of AR, representing an improvement to current standard clinical care alone. Another important finding was the performance of Tutivia in 8 patient blood samples collected postbiopsy, 7 of which were for cause, and all had received different types and durations of treatment (except for the 1 subclinical biopsy patient). All were identified as high-risk using the Tutivia gene signature. Thus, the limited time window from

biopsy to blood collection combined with the reported treatment variability supports the stability and robustness of the Tutivia gene signature in the acute setting.

Perhaps even more promising is the correlation between patients with BK nephropathy and lower Tutivia scores, allowing for the differentiation between acute graft rejection and viral-related processes. Although clinically interesting and relevant to the field due to the absence of biomarkers to identify BK-associated inflammation, additional patients with BK nephropathy are needed to further confirm these early observations.

4.1. Limitations

One limitation of the study was that, although the sample size was determined using a power calculation, including more patients would have potentially improved overall performance. Secondly, race was based on self-identification rather than relying on ancestral informative markers. Thirdly, this initial study focused on a 6-month post–kidney transplant, and, thus, whether Tutivia as a biomarker will be as effective in predicting more long-term outcomes posttransplant requires future study. Finally, we noted the challenges with Tutivia in effectively identifying subclinical or surveillance rejection. Although the ability to rule out rejection remains strong, additional studies are underway from a transcriptomic level to interrogate gene signatures that are reflective of a process that, at a point in time, does not result in clinical symptoms.

5. Conclusions

Our study provides clinical validation of Tutivia as a noninvasive, accurate predictor of early AR beyond the current standard of care. Implementation of this blood-based transcriptomic signature assay offers a noninvasive baseline and future serial approach for clinicians to monitor a patient's wellbeing post–kidney transplant.

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Author contributions

O.B., R.B.M., P.O.C., M.J.D., and L.G. conceived and designed the study. Data collection: O.B., J.A., A.W., E.P., R.B.M., C.B., D.M., S.B., N.L., G.L., M.S., S.S., B.C., L.R., F.A., P.O.C., F.S., M.J.D., L.G., and A.C. collected the data. Analysis and interpretation of results: M.K., M.J.D., L.G., O.B., and R.B.M. analyzed and interpreted the data. M.J.D., L.G., and O.B. drafted the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Declaration of competing interest

The following authors of this manuscript have conflicts of interest to disclose, as described by the American Journal of Transplantation. R.B. Mannon reported serving as a member of the Scientific Advisory Board, Verici Dx. P.O. Connell reported serving as a member of the Scientific Advisory Board, Verici Dx. F. Salem reported serving as a paid consultant pathologist for Verici Dx. M.W. Kattan reported serving as a paid consultant biostatistician to Verici Dx. L. Gallon reported serving as chairman of the Scientific Advisory Board and member of the Board of Directors of Verici Dx. M.J. Donovan reported serving as a consultant, chief medical officer, and medical laboratory director for Verici Dx. The other authors of this manuscript have no conflicts of interest to disclose, as described by the American Journal of Transplantation.

Data availability

The data sets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajt.2023.09.021.

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