Received: 13 February 2021 | Revised: 9 August 2021 | Accepted: 10 August 2021

DOI: 10.1111/ctr.14457

Clinical TRANSPLANTATION
The Journal of Clinical and Translational Research
WILEY

ORIGINAL ARTICLE

Urine marker analysis identifies evidence for persistent glomerular podocyte injury across allograft lifespan

Abhijit S. Naik[1] | Jawad Aqeel[1] | Su Q Wang[1] | Mahboob Chowdhury[1] | Kevin He[2] | Roger C. Wiggins[1]

[1] Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan, USA

[2] Department of Biostatistics, University of Michigan, Ann Arbor, Michigan, USA

Correspondence
Abhijit S. Naik, F6676UHS, 1500 E Medical Center Dr, Ann Arbor, MI 48109-5676, USA.
Email: abhinaik@umich.edu
Roger C. Wiggins, 1570B MSRB2, 1150 W Medical Center Dr, Ann Arbor, MI 48109-5676, USA.
Email: rwiggins@umich.edu

Funding information
Michigan O'Brien Kidney Center, Grant/Award Number: P30 DK08194; National Institute of Diabetes and Digestive and Kidney Diseases, Grant/Award Numbers: K23 DK125529, R01 DK 102643, R01 DK 46073; Michigan Institute for Clinical and Health Research, Grant/Award Number: UL1TR002240

Abstract

Long-term kidney transplant (KT) survival has remained relatively stagnant. Protocol biopsy studies suggest that glomerulosclerosis is a significant contributor to long-term graft failure. We previously demonstrated that podocyte loss in the first year post-transplantation predicted long-term allograft survival. However, whether increased podocyte loss continues over the lifespan of a KT remains unclear. We performed a cross-sectional analysis of 1182 urine samples from 260 KT recipients up to 19-years after transplantation. Urine pellet (UP) mRNAs were assayed for podocyte (NPHS2/podocin and nephrin/NPHS1), distal tubule (aquaporin2), and profibrotic cytokine (TGFbeta1). Multivariable generalized estimating equations were used to obtain "population-averaged" effects for these markers over time post-KT. Consistent with early stresses both podocyte and tubular markers increased immediately post-KT. However, only podocyte markers continued to increase long-term. A role for hypertrophic stresses in driving podocyte loss over time is implied by their association with donor BMI, recipient BMI, and donor-recipient BMI mismatch at transplantation. Furthermore, UP podocin mRNA was associated with urine TGFbeta1, proteinuria, and reduced estimated glomerular filtration rate, thereby linking podocyte injury to allograft fibrosis and survival. In conclusion we observed that podocyte loss continues long-term post-KT suggesting an important role in driving late graft loss.

KEYWORDS
kidney transplantation, podocyte loss, proteinuria

1 | INTRODUCTION

Current protocols that minimize early allograft injuries from ischemia-reperfusion, drug toxicities, acute rejection, infections, recurrent disease, and technical factors have led to remarkable improvements in short and intermediate-term allograft survival. However, these advances have not been accompanied by comparable improvements in long-term graft survival.[1] This raises the question of whether, in addition to chronic microvascular injury, other underappreciated mechanisms drive late graft loss.[2,3] In particular, the question of whether glomerulosclerosis could be playing a role is brought to the forefront by two long-term surveillance biopsy studies. Recently, Stegall and colleagues reported 10-year protocol allograft biopsy data showing that global glomerulosclerosis was a significant pathologic feature. Rejection, advanced interstitial fibrosis, and tubular atrophy were uncommon, leading the authors to conclude that, "By 10-years after

transplantation, much damage appears non-immunological, suggesting that newer approaches are needed to decrease late injury."[4] Similarly, Nankivell and colleagues also found global glomerulosclerosis to be a major pathologic feature in surveillance biopsies 6-10-years after transplantation in diabetics whose diabetes had been cured by simultaneous pancreas transplantation.[5]

As a result of the two-kidneys (2K) to one-kidney (1K) transition at transplantation, allografts hyperfilter to a greater or lesser extent.[6] The degree of hyperfiltration depends on donor age and nephron number determined in part by donor and recipient relative body/kidney sizes. Brenner and colleagues originally suggested this concept of "dose" of the graft needed to sustain long-term graft function.[7,8] Therefore, a key question is whether long-term allograft loss is driven at least in part by hyperfiltration-induced stresses that drive podocyte depletion, thereby contributing to chronic glomerular injury that might also explain the observed relationship of donor and recipient size mismatch on kidney allograft outcomes.[9–11]

We have modeled hypertrophic podocyte stress induced by uninephrectomy in rats to prove that hypertrophy per se can drive long-term kidney failure and that prevention of glomerular enlargement prevents progressive glomerulosclerosis.[12–14] Because kidney transplantation (KT) is essentially a form of "reverse nephrectomy," we expected similar mechanisms to be operating in humans. We first performed an observational study to document that accelerated podocyte loss starts immediately after transplantation and persists long-term as would be expected if glomerular injury was triggered by hypertrophic stress associated with the transplantation process itself.[15] The increased rate of podocyte loss was then modeled to demonstrate that this level of increased podocyte loss could quantitatively account for the shorter-than-expected allograft half-life at 15-years and the effect of donor age on allograft lifespan.[16] Third, we performed a prospective study where we observed that podocyte loss in urine in the first year after transplantation is related to loss of allograft function over the subsequent 4.5-years, thereby linking the amount of podocyte detachment in the first year to a loss of allograft function. In that study, we also noted that body size difference between the donor and recipient (a surrogate for kidney size and hypertrophic stress) strongly predicted the rate of podocyte loss, thereby supporting the concept that hypertrophic stress might play a role.[17] Taken together, these reports are consistent with hypertrophic podocyte stress incurred at the time of transplantation being a significant driver of allograft loss. However, they do not address the question of whether podocyte detachment persists long-term to account for global glomerulosclerosis observed in long-term protocol kidney biopsies.[4,5]

Podocytes are resident on the urinary space side of the glomerular basement membrane so that as they detach, they and their products can be identified in the urine, where they can serve as markers for monitoring glomerular disease activity and response to treatment in real-time.[18,19] To further test the hypothesis that accelerated podocyte loss in allografts can persist long-term as a hypothetical driver of progressive glomerulosclerosis in allografts, we performed a cross-sectional analysis of KT recipients up to 19-years post-transplantation.

2 | MATERIAL AND METHODS

The studies described were approved by the University of Michigan under IRB (HUM00118077 and HUM0055525). The cross-sectional study design included all-comers immediately post-transplantation and longer-term in the transplant clinic. In total, we collected 1182 urine samples from 260 kidney allograft recipients between day 0 and 19-years after transplantation from January 1, 2016 to December 31, 2018. A previously reported healthy living donor cohort ($n = 87$) was used to establish control values.[20]

2.1 | Demographic data

Donor and recipient data were abstracted from the electronic medical records. Donor information collected included age, race, body mass index (BMI), gender, and type of the organ (living vs. deceased). Recipient data included age, self-identified race, gender, body mass index, serum creatinine, and estimated glomerular filtration rate (eGFR) using the modified diet in renal disease formula at the time of urine sample collection.[21]

2.2 | Urine sample processing and mRNA assay

Surplus urine sample produced by clinic attendees was stored at 4°C in a plastic container for up to 8 h. The urine container was gently swirled to re-suspend all contents and then the available urine (10–50 ml) was poured into a 50 ml plastic centrifuge tube for centrifugation at 4000 rpm at 4°C for 15 min. A total of 1.8-ml of urine supernatant was transferred to a 2 ml plastic tube and stored at −20°C for urine protein and creatinine assay. After pouring off the remaining supernatant, the pellet was suspended in 750 µl diethylpyrocarbonate-treated PBS at 4°C (DEPC-PBS) and then transferred to a 1.7 ml plastic centrifuge tube. A second 750 µl aliquot of DEPC-PBS was then used to wash the bottom of the 50 ml centrifuge tube to recover any remaining pellet material, which was then added to the 1.7-ml tube. The urine pellet (UP) in 1.5 ml DEPC-PBS was centrifuged at 12 000 rpm 5 min at 4°C. The supernatant was discarded. To the remaining pelleted material was added 350 µl of RLT buffer containing β-mercaptoethanol at 10 µl/ml according to the RNeasy Qiagen protocol (Germantown, MD, USA). The pellet was suspended in the RLT/β-mercaptoethanol buffer and then frozen at −80°C. The total UP RNA was isolated using the RNeasy Mini Kit protocol (catalog no. 74106; Qiagen). RNA purity was assessed using the A260/A280 ratio with a value of 1.8 used as a cut-off. Samples were reverse transcribed to cDNA using High-Capacity cDNA reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA) and stored at −20°C until use. Quantitation of the absolute nephrin, podocin, TGF-beta1, and aquaporin-2 cDNA abundance was performed using the 7900 HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan Fast Universal PCR MasterMix, with sample cDNA in a final volume of 25 µl per reaction. TaqMan Probes

(Applied Biosystems) used are as follows: human NPHS1 (nephrin; catalog no. s00190446_m1), human NPHS2 (podocin; catalog no. Hs00922492_m1), human aquaporin2 (cat. no. Hs00166640_m1), and human TGF-beta1 (cat. no. Hs99999918_m1). The primer sets used span one or more introns to minimize DNA replication. All data were from 2 μl samples of the reverse-transcribed cDNA measured in duplicate. Standard curves using were constructed for each assay using serially diluted cDNA standards. Assays were accepted only if the R^2 was ≥.97 for standard curves using SDS 2.2.2 software (Applied Biosystems). cDNAs of known sequence and concentration are used as standards for all assays. Following RT-PCR quantitation all values were first corrected for the volume of urine used by expressing the measured cDNA amount per ml of initial urine used. As a second step to correct for variation in 24 h urine volume, the cDNA per ml was divided by the creatinine concentration and expressed as cDNA per g creatinine. We have previously shown that the quality and quantity of total RNA recovered from the UP is quite variable and does not provide useful additional information in relation to interpretation of the amount of podocyte transcript detected.[22] We therefore do not routinely measure RIN values for urine samples and we do not provide this information for the current dataset. The coefficient of variation of the assay is 35%.[18]

TABLE 1 Relationship of eGFR with urine podocin mRNA across eGFR strata that takes within patient variation and different sampling numbers into account

	Coef.	Robust std. err.	*p*-value	95% confidence intervals	
Entire eGFR spectrum					
Log2 UP PodCR	−.12	.22	.54	−.54	.29
eGFR ≥ 45 ml/min/1.73 m^2					
	−.22	.25	.37	−.71	.27
eGFR < 45 ml/min/1.73 m^2					
Log2 UP PodCR	−.39	.20	.05	−.79	−.00
eGFR < 30 ml/min/1.73 m^2					
Log2 UP PodCR	−.61	.27	.02	−1.14	−.09

TABLE 2 Relationship of urine podocin mRNA with proteinuria that takes within patient variation and different sampling numbers into account

Log2 UPCR	Coef.	Robust std. err.	*p*-value	95% confidence intervals	
Log2 UP PodCR	.23	.022	<.0001	.190	.277

2.3 | Urine mRNA assay interpretation

We utilized the two quantitated podocyte markers (podocin and nephrin mRNA) and a tubular marker (aquaporin2 mRNA) normalized to urine creatinine (all as continuous variables) to assess nephron segment specific patterns of injury. Urine TGFbeta1 was used as a non-specific marker of innate immune activation and profibrotic activity. Podocyte hypertrophic stress leads to relative downregulation of nephrin versus podocin mRNA expression and thus an increasing UPod:Neph ratio is suggestive of podocyte stress.[23] The urine podocin mRNA to aquaporin mRNA ratio (UPod:Aqp2) was used to assess evidence of preferential glomerular versus tubular injury.[20] Spot urine protein to creatinine ratio was used (UProt:CR) as a measure of urinary protein loss rate.

2.4 | Statistics

Descriptive statistics were computed for the study cohort. Continuous variables were summarized by the mean and standard deviation. Categorical variables were reported as proportions of the total for that variable. Since our *focus was on estimating the "population-averaged" effect*, we used a generalized estimating equation (GEE). This statistical model also allows us to account for correlation of values of urine markers in individuals with one or more samples.[24,25] Given the nonlinearity of urine markers in the long-term, as noted in Figures 1 and 2, we included restricted cubic splines using four knots based on the data's distribution. Knots were placed at 14 days (2 weeks), 216 days (~6 months), 630 days (~2 years), and 3716 days (~10 years) post-transplant. The placement of knots was driven by the distribution of the data and the best fit. For the multivariable GEE analysis to determine factors associated with long-term changes in the urine mRNA markers, we used a priori defined variables that included donor BMI, recipient BMI, the interaction of donor and recipient BMI, and the time post-transplantation. The relationship between podocin mRNA with proteinuria and eGFR at the time of sample collection given the different sampling numbers was assessed using linear mixed models (Tables 1 and 2).

2.5 | Plots

Figures 1 and 2 were constructed using fractional polynomial prediction plots to obtain the predicted mean level of urine markers (y variable) from the estimation of a fractional polynomial (second degree) of the time post-transplant (x variable) along with its 95% confidence interval. Contour plots were used in Figure 3 to understand the effect of interaction of donor and recipient BMI on individual urine mRNA markers.

2.6 | Missing data

Donor BMI associated with 7.5% of the urine values was missing, while recipient BMI was missing for 1.9% of the values. We thus singly imputed missing values of donor BMI to the sample mean donor BMI. A comparison of results was then performed between the imputed dataset, and the original data set (complete case analysis) revealed no differences in the significance of the results. We thus used the imputed

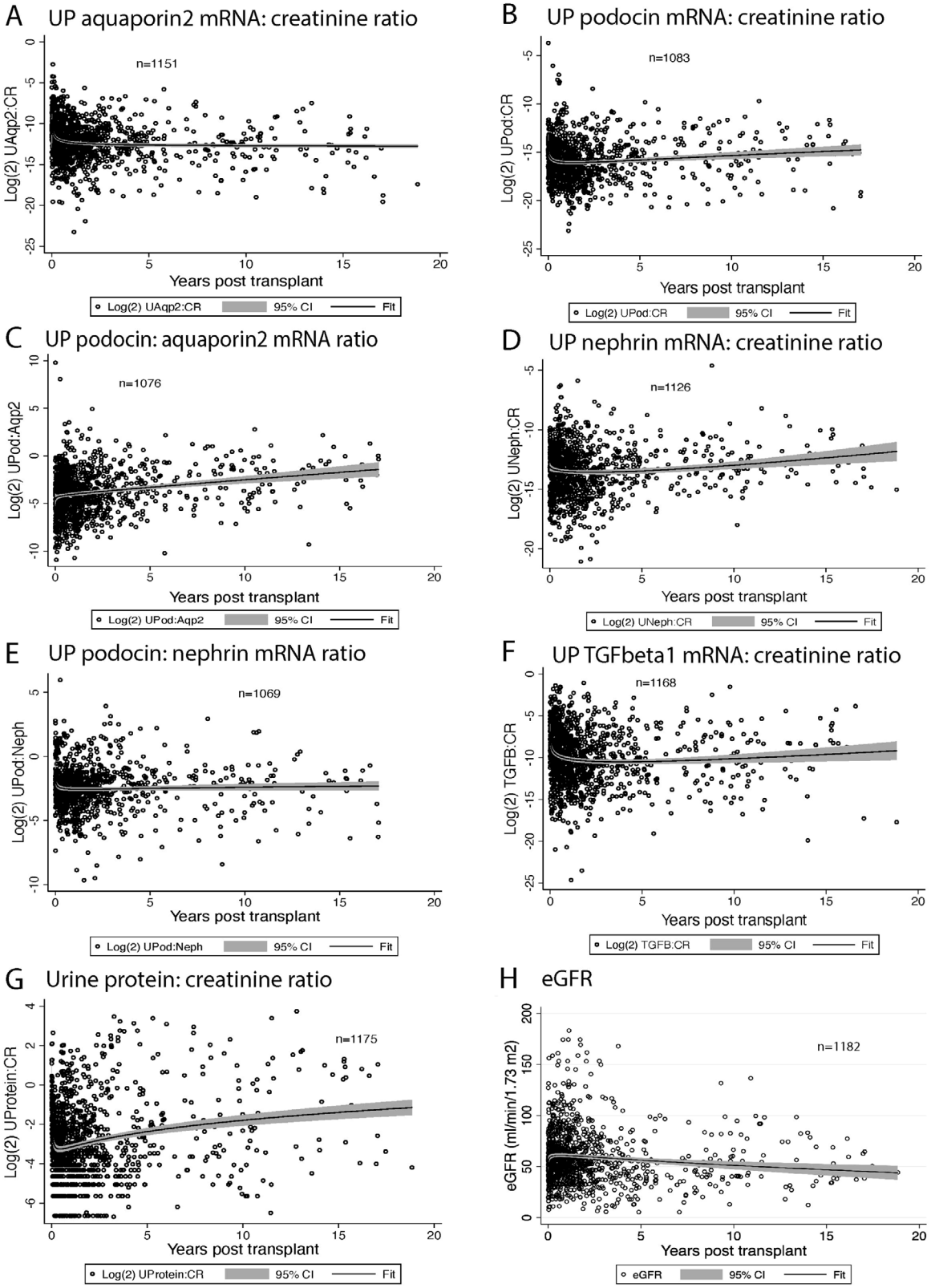

FIGURE 1 Urine pellet mRNA markers in relation to time after transplantation (panels A–F). Panel G shows urine protein to creatinine ratio while panel H shows the spread of eGFRs across all time points. Individual data points are shown. The predicted mean and its 95% confidence interval are shown by the bold line and gray area, respectively

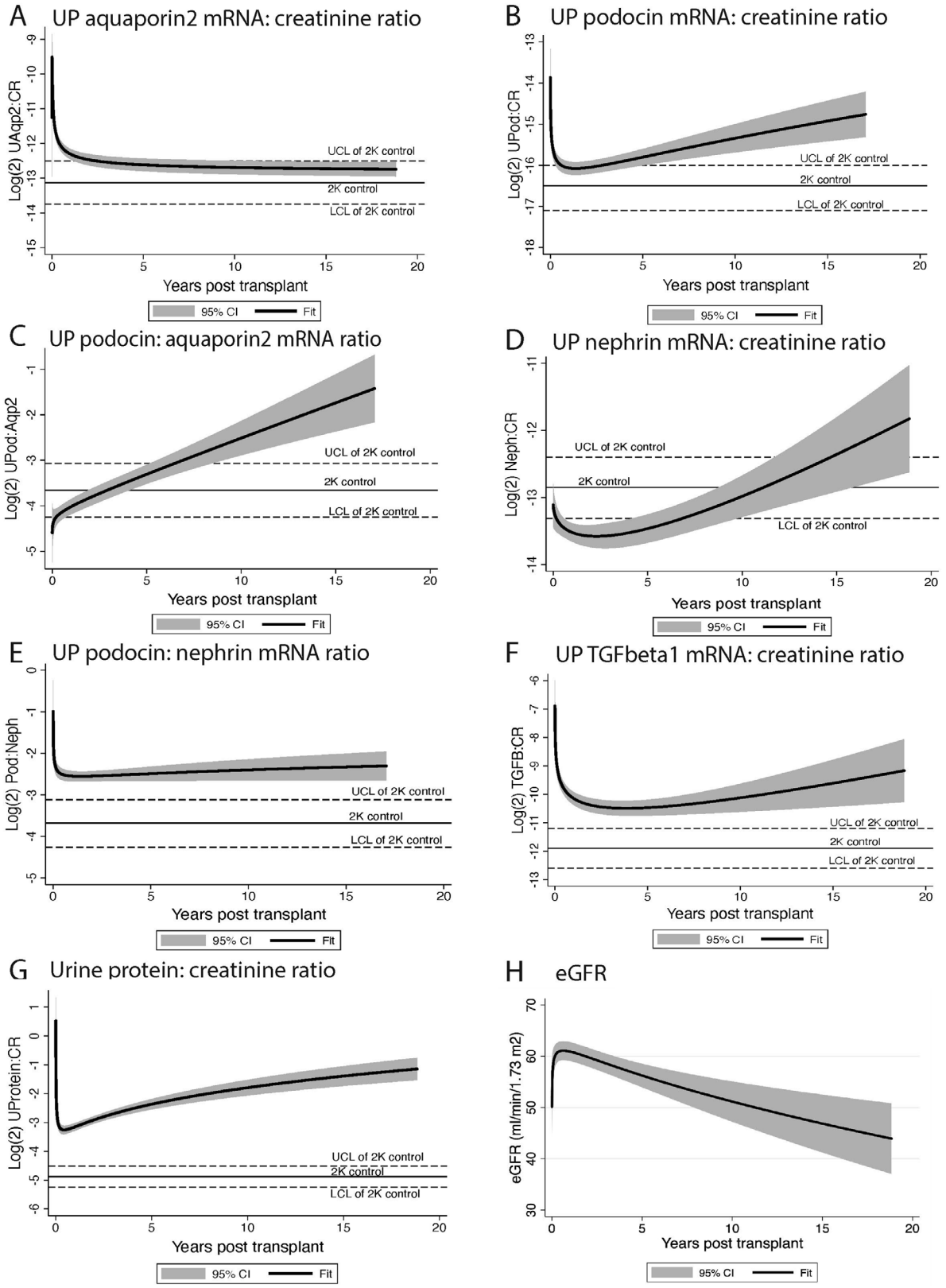

FIGURE 2 Predicted mean values and 95% confidence intervals for urine markers from allografts by time after transplantation up to 17–19 years derived from Figure 1 and compared to the normal range obtained from kidney donors. Panel G shows urine protein to creatinine ratio while panel H shows eGFRs across all time points. LCL, lower confidence limit; UCL, upper confidence limit; UP Aqp2:CR, urine pellet aquaporin2 mRNA to creatinine ratio; UP NepCR, urine pellet nephrin mRNA to creatinine ratio; UP PodAqp2 mRNA ratio, urine pellet podocin to aquaporin2 transcript ratio; UP PodCR, urine pellet podocin mRNA:creatinine ratio; UP TGFbeta1CR, urine pellet transforming growth factor beta1 mRNA to creatinine ratio; UP PodNep, urine pellet podocin mRNA to nephrin mRNA transcript ratio; UProtCR, urine protein to creatinine ratio.

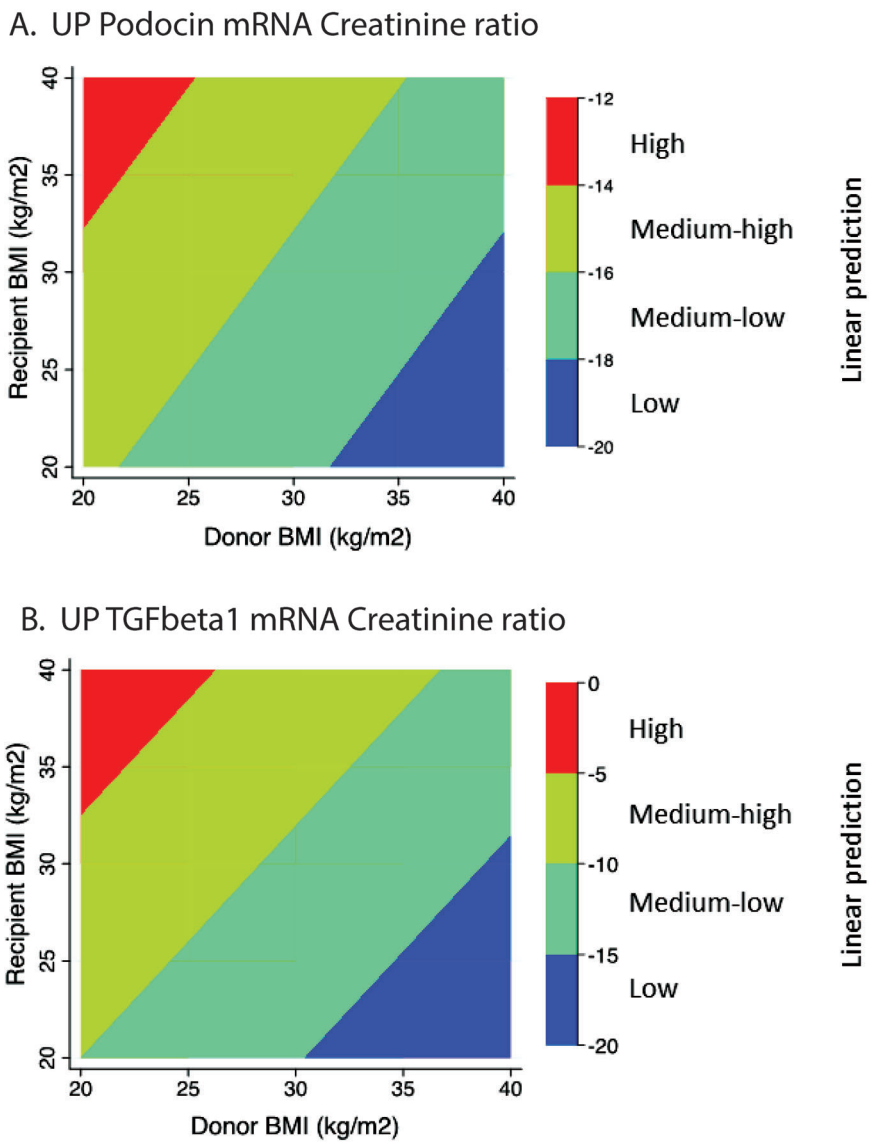

FIGURE 3 The contour plot explores the interaction of recipient and donor body mass index on UP PodCR, urine pellet podocin mRNA:creatinine ratio (panel A), and UP TGFbeta1CR, urine pellet transforming growth factor beta1 mRNA to creatinine ratio (panel B). As an example (panel A), a recipient with BMI > 35 kg/m^2 is likely to have higher levels of podocyte loss (red) if they were to get a kidney from a donor with a BMI of <25 kg/m^2 but relatively lower levels of loss if donor BMI > 32–33 kg/m^2

dataset to maximize urine marker data. Stata 15 I/C (College Station, TX, USA) was used for all analyses.

3 | RESULTS

Table 3 shows the demographic data for the 260 allograft recipients from whom 1182 urine samples were collected. Table 4 shows normal ranges obtained from 87 healthy kidney donors (before donation) previously reported.[20] Timing of urine collections after transplantation was left-skewed, given the higher frequency of patient contact in the first post-transplant year. The median time of follow-up was 390-days with a fifth percentile value of 14-days and 95th percentile value of 3705-days (~10-years) post-transplant and ranged from 0 to 6879 days (~19-years). Samples were collected over 3 years, with the average number of samples from any one allograft recipient being 4, ranging from 1 to 18.

3.1 | Urine markers in relation to time after transplantation

Figure 1 shows UP data from allograft recipients presented as individual data points with the estimated mean and 95% confidence interval. Figure 2 shows these data as the estimated mean and 95% confidence interval up to 19 years post-transplant. The mean and 95% confidence intervals derived from healthy living donors are also shown in Figure 2 to allow for comparison.

A marker of distal tubular loss: UP aquaporin2creatinine ratio (UP Aqp2CR) increases initially post-transplant and then stabilizes over time to a level not significantly different from the 2K controls (Figures 1 and 2, panel A).

Markers of podocyte loss: UP podocin mRNA creatinine ratio (UP PodCR) as an estimate of podocyte loss increases rapidly after transplantation reflecting initial post-transplant-associated hypertrophic stresses on the podocyte. The average UP PodCR then trends towards

TABLE 3 Clinical characteristics of the transplant recipient and donor cohorts

	n	Mean ± 1SD or %	Range
Total *n* for all transplanted subjects	260		
Recipient data			
Recipient age at transplantation (years)	260	47.2 ± 15.1	18–75
Recipient gender (% female)	259	33.7	NA
Recipient race (% white)	259	75.4	NA
Recipient eGFR at time of urine sampling (ml/min/1.73 m^2)	1182	58.8 ± 26.5	5.4–183.2
Serum creatinine at the time of urine sampling (mg/dl)	1182	1.60 ± .83	.6–10.02
Recipient BMI at transplant (kg/m^2)	253	28.7 ± 5.8	17.2–46.1
Class 1 PRA pre-transplant	225	11.2 ± 26.5	0–100
Class 2 PRA pre-transplant	242	13.7 ± 28.9	0–100
Donor data			
Donor age (years)	194	41.2 ± 11.1	7–64
Donor gender (% female)	247	65.9	NA
Donor race (% white)	184	58.1	NA
Donor type (% living donor)	260	67.3	NA
Cold ischemia time (hours)	248	6.8 ± 7.22	.42–34.4
Donor BMI (kg/m^2)	189	26.9 ± 5.4	13.7–47.9

The values for $n < 260$ represent missing data. Donor data was frequently missing due to incomplete data capture for deceased donors. Data is reported as Mean ± SD for continuous variables and % for categorical variables.
Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate; PRA, panel reactive antibodies.

TABLE 4 Clinical characteristics of the control cohort

	Mean ± 1SD
n	87
Age (years)	43.5 ± 12.5
Female gender	59.8%
White race	78%
BMI (kg/m^2)	27.0 ± 4.7
Serum creatinine (mg/dl)	.83 ± .16
eGFR (CKD-EPI) ml/min/1.73 m^2	97.3 ± 15.6

the 2K control but then, in contrast to the tubular marker, steadily increases over the long term (Figures 1 and 2, panel B).

A marker of preferential glomerular versus tubular injury: Immediate after transplantation the UP PodAqp2 mRNA ratio was at the lower limit of the normal range reflecting somewhat greater initial loss of aquaporin2-containing tubular cells versus podocin-containing glomerular cells. However, beyond 3-years post-transplant, the UP PodAqp2 ratio steadily increases, reflecting preferential glomerular versus distal tubular compartment injury over time (Figures 1 and 2, panel C).

A marker of podocyte hypertrophic stress: Early after transplantation UP nephrinCR remains below the normal 2K values (Figures 1 and 2, panel D) while the podocin mRNA signal is increased above normal, reflecting increased podocyte loss. When shown as the ratio of the two podocyte-specific markers this remains very high immediately after transplant but stabilizes to a two-fold higher level than the 2K controls (Figures 1 and 2, panel E).

A marker of innate immune activity and profibrotic activity: UP TGF-beta1CR increased immediately after transplant and continued to remain above baseline in the long-term, reflecting ongoing diverse injury processes (Figures 1 and 2, panel F).

Proteinuria: UProtCR was highest (32-fold above control levels) immediately post-transplant (Figure S1). This likely reflects the increased protein leak from native kidneys present in recipients before transplantation in addition to early post-transplant hypertrophic stresses. The UProtCR rapidly decreases to reach a level about four-fold above 2K control by 1-year post-transplant, reflecting a combination of decreasing residual native kidney urine production and adaptations by the allograft. However, by 1-year post-transplant, the UProtCR (Figure 2G) begins to steadily increase over time, paralleling the UP PodCR (Figures 1 and 2, panel B) that signals progressive glomerular injury.

3.2 | Hypertrophic stress as a potential mechanism responsible for causing persistent podocyte loss in allografts

Based on model systems, we hypothesized that hypertrophic stresses inherent in the single kidney state drives allograft failure and that urine podocyte markers report these stresses. Although all allografts hyperfilter as a result of their transition from the 2K to the 1K state at

TABLE 5 Multivariable generalized estimating equation (GEE) with restricted cubic splines were used to model non-linear time-dependent changes (A–G)

	Coef.	Robust std. err.	*p*-value	95% confidence intervals	
A. Log2 UP aquaporin2 mRNA creatinine ratio					
Donor BMI	−.076	.080	.344	−.233	.081
Recipient BMI	.043	.074	.559	−.101	.187
B. Log2 UP podocin mRNA creatinine ratio					
Donor BMI	−.199	.075	**.008**	−.347	−.051
Recipient BMI	.136	.069	**.050**	.000	.273
C. Log2 UP podocin aquaporin2 mRNA ratio					
Donor BMI	−.112	.081	.165	−.272	.046
Recipient BMI	.078	.079	.306	−.072	.230
D. Log2 UP nephrin mRNA creatinine ratio					
Donor BMI	−.157	.060	**.009**	−.275	−.039
Recipient BMI	.087	.056	.120	−.023	.196
E. Log2 UP podocin nephrin mRNA ratio					
Donor BMI	−.045	.060	.453	−.162	.072
Recipient BMI	.047	.055	.389	−.060	.154
F. Log2 UP transforming growth factor beta1 creatinine ratio					
Donor BMI	−.479	.118	<**.0001**	−.711	−.247
Recipient BMI	.401	.115	<**.0001**	.176	.626
G. Log2 urine protein creatinine ratio					
Donor BMI	−.133	.088	.134	−.307	.041
Recipient BMI	.093	.086	.284	−.077	.262

The regression is adjusted for donor BMI, recipient BMI, and the interaction between donor and recipient BMI and also accounts for the within-patient correlation of values. The order of Table 5A–G is the same as that shown in Figures 1–3. Donor and recipient BMI are represented in kg/m^2. Restricted cubic splines to model for non-linearity were constructed with knots at 14 days (2 weeks), 216 days (~6 months), 630 days (~2 years), and 3716 days (~10 years) days post-transplant. Table 5H shows correlation coefficients for Log2 UP TGFbeta1 in relation to the Log2 UP PodCR and both donor and recipient BMI.

the time of transplantation, the degree of hypertrophic stress experienced will be either amplified or mitigated as a result of the relationship between the body size of the recipient and donor where body mass index is a surrogate for kidney size. Thus, if the hypothesis that hypertrophic stress is a driver of accelerated podocyte loss in allografts is correct, then a larger recipient body size in relation to a smaller donor body size would be expected to be associated with greater podocyte loss, and vice versa. To test this hypothesis, we estimated a "population averaged" effect for each urine marker and tested its relationship to donor and recipient kidney size mismatch.

As shown in Table 5A, the tubular marker (UP Aqp2CR) was not significantly associated with the donor or recipient's BMI. In contrast, Table 5B,F shows that the urine podocyte marker (UP PodCR) and UP TGFbeta1CR were both significantly positively associated with the recipient's BMI and negatively associated with the donor BMI.

Figure 3A shows graphically how the recipient and donor BMI interact to determine the degree of podocyte loss. The higher the recipient BMI (x-axis) and the lower the donor BMI (y-axis), the greater the podocyte loss, and vice versa. Figure 3B shows parallel data for TGF-beta1.

TABLE 6 Relationship between urine pellet transforming growth factor beta 1 mRNA in urine with podocyte loss (UP PodCR)

	Coef.	Robust std. err.	*p*-value	95% confidence intervals	
Predictors of Log2 UP transforming growth factor beta1 creatinine ratio					
Log2 UP PodCR	.588	.052	<**.0001**	.486	.689

The model was adjusted for donor BMI, recipient BMI, and donor and recipient size mismatch.

3.3 | Are podocyte loss and TGFbeta1 related to one another?

To assess the effect of podocyte loss on the UP TGFbeta1 signal, we added UP PodCR as a dependent variable to the BMI variables analysis (Table 6). We observed that UP PodCR (podocyte loss) per se is associated with UP TGFbeta1CR independent of donor BMI, recipient BMI, and recipient/donor mismatch.

3.4 | Relationship of podocyte loss to eGFR and proteinuria

Table 1 shows the relationship between UP PodCR with eGFR accounting for differences in sampling numbers between patients. Given that averaged eGFR was 58 ml/min/1.73 m^2, we tested this relationship at eGFR ≥ 45, <45, and eGFR < 30 respectively and noted a significant association at lower eGFR ranges. Table 2 shows the association between UP PodCR and proteinuria a well-known surrogate of long-term outcomes.[26]

4 | DISCUSSION

The approach taken evaluated all allograft recipients in a cross-sectional study design of all-comers in a transplant clinic irrespective of allograft function. This *heuristic* approach offers a non-invasive approach to test the role of podocyte depletion in relation to long-term allograft outcomes in the general KT population, which would otherwise require a 15-20-year study period. We tested the hypothesis that glomerular stresses resulting in accelerated podocyte loss into the urine that are initiated immediately following transplantation continue throughout allograft lifespan reflecting a putative glomerular injury process that could drive long term allograft failure. We observed evidence of accelerated podocyte loss throughout 17-19-years post-transplantation consistent with the previously reported high prevalence of glomerulosclerosis in long-term (as opposed to short term) protocol allografts biopsies.[4,5] Furthermore, hypertrophic stress (as indicated by donor and recipient body size mismatch) at the time of transplantation was strongly associated with long-term

podocyte loss and podocyte stress. Finally, podocyte loss was related to increased urine TGFbeta1, lower eGFR, and proteinuria, thereby linking long-term podocyte loss with allograft survival.

The most obvious mechanism that could cause the observed changes in podocyte stress and loss starting immediately after transplantation and persisting long-term is the hyperfiltration and rapid compensatory kidney growth processes inherent in KT where a single kidney has to take up the functions that would normally be performed by two kidneys. This concept is also supported by finding that both donor and recipient BMI were significantly associated with podocyte loss but not with the tubular marker. Furthermore, the interaction between smaller donor and larger recipient BMI magnified the impact of either alone. Donor BMI and recipient BMI and donor and recipient kidney size mismatch (using BMI and BSA as surrogates) have long been recognized as having the potential to drive long term transplant outcomes as originally suggested by Brenner and colleagues.[27,28] Consistent with this concept, recipient to donor body size mismatch has also been associated with more proteinuria and glomerulosclerosis.[11]

Immediately post-transplantation, both glomerular and tubular compartments of the allograft undergo various stresses related to well-accepted causes of early graft dysfunction (ischemia-reperfusion, drug toxicities, and immune activation). However, while tubular loss markers return to normal, those associated with podocyte injury and stress continue to increase. Furthermore, the ratio of podocyte marker to tubular marker, increases over time compatible with preferential glomerular injury over tubular injury with an increasing amount of time post-transplantation.

It is likely that hypertrophic stress would also promote other pathologic processes that could shorten allograft lifespan. For example, hypertrophic stresses in the allograft could promote HLA system proteins' expression in the graft that triggers host immune responses to cause rejection. Indeed, in animal models of transplantation, reduced nephron mass is reported to be associated with increased expression of ICAM-1 and MHC class II antigens, cellular infiltration, and expression of all T cell and associated macrophage products, thereby offering evidence that reduced nephron mass might amplify immune-mediated injury.[29] In the current study, we also observed a strong association of donor BMI, recipient BMI, and their interaction with increased urinary TGFbeta1, thereby supporting a potential role of hypertrophic stress in driving immune-mediated injury. Thus, mitigating the impact of hypertrophic stress might also impact immune-mediated allograft injury that together with glomerular disease is thought to be a leading cause of late graft failure.[3,30–32]

While our approach provides an approximation of patterns of nephron segment-specific injury in kidney allografts over time, it has inherent weaknesses. We used aquaporin2 as a marker representing the distal nephron farthest downstream for comparison to the upstream glomerulus. In the allograft, proximal tubular cells are a major target of ischemia-reperfusion injury, drug toxicities, and immune injury. Thus, the aquaporin2 distal tubular marker does not directly report proximal tubular cell injuries, although nephron death from any cause will also result in detachment of distal tubular cells. We, therefore, cannot conclude from these data that persistent proximal tubular injury is not occurring at some level sufficient to impact survival in long-term allografts. RNA quality was not measured in the UP samples because it has not proven to be a useful parameter[22]; however, by comparing the ratio of two transcripts measured in the same UP sample by the same method at the same time the possibility that variation in RNA quality or quantity over time after transplantation could be responsible for the observed result is minimized. It is also important to note that there remains an inherent survivor bias in our study, and on average allografts evaluated had mean eGFR of 58 ml/min. However, despite this selection bias towards surviving allografts with better function, we still observed evidence of podocyte stress and detachment thereby supporting the hypothesis that there is ongoing subclinical glomerular injury even with relatively normal kidney function, although worse function was associated with higher podocyte loss rate as previously reported.[17] Confirmation of findings from this cross-sectional study will require a longitudinal study of individual patients over a 15-20-year period. It is also important to note that association does not prove causality, although, most of our findings related to urinary pellet mRNA markers are in line with what we have observed in model systems of nephrectomy induced glomerular and renal disease progression where causation has been established.[13,14,33,34] Lastly, although podocyte loss was associated with TGFbeta1, the directionality of this association remains unclear.

In summary, the data presented are consistent with the hypothesis that injury to the glomerulus is initiated immediately post-transplant driven in part by hypertrophic stresses caused by transitioning to a single kidney state that is inherent in the transplantation process itself, and that these stresses persist throughout the lifespan of the allograft. The impact of hypertrophy, as opposed to other potential mechanisms, is suggested by the finding that the signal was amplified by a larger size mismatch between recipient and donor, and vice versa. If this concept is correct, then extending the average allograft lifespan beyond the current average 15-years will require mitigation of hypertrophic stress-induced glomerular injury in addition to ongoing efforts to reduce immune mediate microvascular injury.

ACKNOWLEDGMENTS

We gratefully acknowledge the support of the University of Michigan transplant study coordinators and the transplant patients who consented for their urine samples to be used for the study. ASN acknowledges support from University of Michigan O'Brien Kidney Translational Core Center P30 DK08194, the Michigan Institute for Clinical and Health Research (MICHR) First Pathway Award (grant UL1TR002240), and the National Institutes of Health (grant K23 DK 125529). RW acknowledges the support of the National Institutes of Health (grants R01 DK 46073 and R01 DK 102643 and the University of Michigan O'Brien Kidney Translational Core Center P30 DK081943.

CONFLICT OF INTEREST

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

Abhijit S. Naik and Roger C. Wiggins designed the study. Jawad Aqeel, Su Q Wang, and Mahboob Chowdhury carried out the experiments.

Abhijit S. Naik analyzed the data. Kevin He provided statistical input for analysis. Abhijit S. Naik and Roger C. Wiggins wrote the first and final draft of the manuscript with input from Kevin He.

DATA AVAILABILITY STATEMENT

The data is available from the authors at request.

ORCID

Abhijit S. Naik https://orcid.org/0000-0003-1602-9425

REFERENCES

1. Lamb KE, Lodhi S, Meier-Kriesche HU. Long-term renal allograft survival in the United States: a critical reappraisal. *Am J Transplant*. 2011;11(3):450-462.
2. Loupy A, Hill GS, Nochy D, et al. Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure. *Am J Transplant*. 2010;10(4):952; author reply 953.
3. Einecke G, Sis B, Reeve J, et al. Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure. *Am J Transplant*. 2009;9(11):2520-2531.
4. Stegall MD, Cornell LD, Park WD, et al. Renal allograft histology at 10 years after transplantation in the tacrolimus era: evidence of pervasive chronic injury. *Am J Transplant*. 2018;18(1):180-188.
5. Nankivell BJ, Borrows RJ, Fung CL, et al. The natural history of chronic allograft nephropathy. *N Engl J Med*. 2003;349(24):2326-2333.
6. Bosma RJ, Kwakernaak AJ, van der Heide JJ, et al. Body mass index and glomerular hyperfiltration in renal transplant recipients: cross-sectional analysis and long-term impact. *Am J Transplant*. 2007;7(3):645-652.
7. Brenner BM, Lawler EV, Mackenzie HS. The hyperfiltration theory: a paradigm shift in nephrology. *Kidney Int*. 1996;49(6):1774-1777.
8. Brenner BM, Milford EL. Nephron underdosing: a programmed cause of chronic renal allograft failure. *Am J Kidney Dis*. 1993;21(5 Suppl 2):66-72.
9. el-Agroudy AE, Hassan NA, Bakr MA, et al. Effect of donor/recipient body weight mismatch on patient and graft outcome in living-donor kidney transplantation. *Am J Nephrol*. 2003;23(5):294-299.
10. Kasiske BL, Snyder JJ, Gilbertson D. Inadequate donor size in cadaver kidney transplantation. *J Am Soc Nephrol*. 2002;13(8):2152-2159.
11. Giral M, Foucher Y, Karam G, et al. Kidney and recipient weight incompatibility reduces long-term graft survival. *J Am Soc Nephrol*. 2010;21(6):1022-1029.
12. Wiggins JE, Goyal M, Sanden SK, et al. Podocyte hypertrophy, "adaptation," and "decompensation" associated with glomerular enlargement and glomerulosclerosis in the aging rat: prevention by calorie restriction. *J Am Soc Nephrol*. 2005;16(10):2953-2966.
13. Fukuda A, Chowdhury MA, Venkatareddy MP, et al. Growth-dependent podocyte failure causes glomerulosclerosis. *J Am Soc Nephrol*. 2012;23(8):1351-1363.
14. Nishizono R, Kikuchi M, Wang SQ, et al. FSGS as an adaptive response to growth-induced podocyte stress. *J Am Soc Nephrol*. 2017;28(10):2931-2945.
15. Yang Y, Hodgin JB, Afshinnia F, et al. The two kidney to one kidney transition and transplant glomerulopathy: a podocyte perspective. *J Am Soc Nephrol*. 2015;26(6):1450-1465.
16. Naik AS, Afshinnia F, Cibrik D, et al. Quantitative podocyte parameters predict human native kidney and allograft half-lives. *JCI Insight*. 2016;1(7):e86943.
17. Naik AS, Afshinnia F, Aqeel J, et al. Accelerated podocyte detachment early after kidney transplantation is related to long-term allograft loss of function. *Nephrol Dial Transplant*. 2019;34:1232-1239.
18. Wickman L, Afshinnia F, Wang SQ, et al. Urine podocyte mRNAs, proteinuria, and progression in human glomerular diseases. *J Am Soc Nephrol*. 2013;24(12):2081-2095.
19. Fukuda A, Sato Y, Iwakiri T, et al. Urine podocyte mRNAs mark disease activity in IgA nephropathy. *Nephrol Dial Transpl*. 2015;30(7):1140-1150.
20. Naik AS, Le D, Aqeel J, et al. Podocyte stress and detachment measured in urine are related to mean arterial pressure in healthy humans. *Kidney Int*. 2020;98:699-707.
21. Levey AS, Coresh J, Greene T, et al. Expressing the modification of diet in renal disease study equation for estimating glomerular filtration rate with standardized serum creatinine values. *Clin Chem*. 2007;53(4):766-772.
22. Sato Y, Wharram BL, Lee SK, et al. Urine podocyte mRNAs mark progression of renal disease. *J Am Soc Nephrol*. 2009;20(5):1041-1052.
23. Fukuda A, Wickman LT, Venkatareddy MP, et al. Urine podocin:nephrin mRNA ratio (PNR) as a podocyte stress biomarker. *Nephrol Dial Transplant*. 2012;27(11):4079-4087.
24. Hardin JW, Hilbe JM. *Generalized Estimating Equations*. Chapman and Hall/CRC; 2002.
25. Liang K-Y, Zeger SL. Longitudinal data analysis using generalized linear models. *Biometrika*. 1986;73(1):13-22.
26. Amer H, Fidler ME, Myslak M, et al. Proteinuria after kidney transplantation, relationship to allograft histology and survival. *Am J Transplant*. 2007;7(12):2748-2756.
27. Naik AS, Sakhuja A, Cibrik DM, et al. The impact of obesity on allograft failure after kidney transplantation: a competing risks analysis. *Transplantation*. 2016;100(9):1963-1969.
28. Naik AS, Zhong Y, Parasuraman R, et al. The temporal and long-term impact of donor body mass index on recipient outcomes after kidney transplantation - a retrospective study. *Transpl Int*. 2020;33(1):59-67.
29. Azuma H, Nadeau K, Mackenzie HS, et al. Nephron mass modulates the hemodynamic, cellular, and molecular response of the rat renal allograft. *Transplantation*. 1997;63(4):519-528.
30. Sellares J, de Freitas DG, Mengel M, et al. Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. *Am J Transplant*. 2012;12(2):388-399.
31. Gaston RS, Cecka JM, Kasiske BL, et al. Evidence for antibody-mediated injury as a major determinant of late kidney allograft failure. *Transplantation*. 2010;90(1):68-74.
32. El-Zoghby ZM, Stegall MD, Lager DJ, et al. Identifying specific causes of kidney allograft loss. *Am J Transplant*. 2009;9(3):527-535.
33. Wharram BL, Goyal M, Wiggins JE, et al. Podocyte depletion causes glomerulosclerosis: diphtheria toxin-induced podocyte depletion in rats expressing human diphtheria toxin receptor transgene. *J Am Soc Nephrol*. 2005;16(10):2941-2952.
34. Fukuda A, Wickman LT, Venkatareddy MP, et al. Angiotensin II-dependent persistent podocyte loss from destabilized glomeruli causes progression of end stage kidney disease. *Kidney Int*. 2012;81(1):40-55.

SUPPORTING INFORMATION

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

How to cite this article: Naik AS, Aqeel J, Wang SuQ, Chowdhury M, He K, Wiggins RC Urine marker analysis identifies evidence for persistent glomerular podocyte injury across allograft lifespan. *Clin Transplant*. 2021;35:e14457. https://doi.org/10.1111/ctr.14457