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# Podocyte stress and detachment measured in urine are related to mean arterial pressure in healthy humans



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Hypertension-associated progressive glomerulosclerosis is a significant driver of both *de novo* and all-cause chronic kidney disease leading to end-stage kidney failure. The progression of glomerular disease proceeds via continuing depletion of podocytes from the glomeruli into the ultrafiltrate. To non-invasively assess injury patterns associated with mean arterial pressure (MAP), we conducted an observational study of 87 healthy normotensive individuals who were cleared for living kidney donation. Urine pellet podocin and aquaporin2 mRNAs normalized to the urine creatinine concentration (UPod:Creat ratio and UAqp2:Creat ratio) were used as markers of podocyte detachment and tubular injury, respectively. The ratio of two podocyte mRNA markers, podocin to nephrin (UPod:Neph) as well as the ratio of podocin to the tubular marker aquaporin2 (UPod:Aqp2) estimated the relative rates of podocyte stress and glomerular vs. tubular injury. The MAP was positively correlated with the UPod:Neph and UPod:Aqp2, thereby confirming the relationship of MAP with podocyte stress and the preferential targeting of the glomerulus by higher MAP. In multivariable linear regression analysis, both UPod:Neph and UPod:Creat, but not UAqp2:Creat or proteinuria, were both significantly related to a range of normal MAP (70 to 110 mm Hg). Systolic, as opposed to diastolic or pulse pressure was associated with UPod:Creat. Thus, higher podocyte stress and detachment into the urine are associated with MAP even in a relatively “normal” range of MAP. Hence, urine pellet mRNA monitoring can potentially identify progression risk before the onset of overt hypertension, proteinuria or chronic kidney disease.

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KEYWORDS: glomerulosclerosis; hypertension; podocyte

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With the rising global prevalence of hypertension (HTN), the burden of chronic kidney disease (CKD) is expected to continue to increase.<sup>1</sup> Superimposed HTN is well known to accelerate all-cause CKD as proven by the efficacy of blood pressure control in delaying the onset of end-stage kidney disease (ESKD). Accumulating evidence suggests that the degree to which primary HTN drives CKD and ESKD risk is substantial. For example, in 2016, among 121,198 patients with incident ESRD, 29% carried a diagnosis of primary HTN.<sup>2</sup> Epidemiological data demonstrate a strong relationship between systemic blood pressure in the high-normal range and long-term CKD/ESRD risk although, in these reports, baseline renal function was not prospectively evaluated and thus the presence of underlying kidney disease could not be excluded.<sup>3,4</sup> Furthermore, in a metanalysis that included individuals with baseline estimated glomerular filtration rate (eGFR) >60 ml/min per 1.73 m<sup>2</sup> and without proteinuria or hematuria by dipstick analysis, even modest elevation in systemic blood pressure was independently associated with an increased ESRD incidence.<sup>5</sup>

Despite the high prevalence of HTN and its strong association with increased CKD/ESRD risk, mechanisms that initiate, drive, and amplify HTN-mediated kidney injury are not well defined. In an autopsy study of individuals without CKD who died in motor vehicle accidents, those with a history of HTN had fewer larger glomeruli and more glomerulosclerosis than those who were normotensive.<sup>6</sup> In studies of Aboriginal Australians as well as African Americans in Mississippi, those with a history of HTN were also found to have lower nephron numbers and larger glomeruli.<sup>7,8</sup> Furthermore, biopsy data from the African American Study of Kidney Disease revealed that among nondiabetic, hypertensive individuals with established mild-to-moderate CKD, global glomerulosclerosis involved 43% ± 26% of glomeruli, and the extent of glomerulosclerosis correlated strongly with systolic blood pressure.<sup>9</sup> These data point to a cause-effect relationship between increased blood pressure and glomerular disease progression.

Podocytes are cells of the glomerular filter with limited capacity for replacement. The podocyte depletion hypothesis for progression of glomerular diseases is based on the concept that diverse stresses induce an accelerated loss of podocytes

**Table 1 | Demographic characteristics of the cohort of prospective kidney donors**

| Demographic and clinical characteristics                                    | Mean $\pm$ 1 SD           |
|---|---------------------------|
| Age, yr   | 43.5 $\pm$ 12.5           |
| Male gender, n (%)  | 35 (40.2)                 |
| White race, n (%)   | 68 (78)                   |
| Living related, n (%)   | 40 (46)                   |
| BMI, kg/m <sup>2</sup>  | 27 $\pm$ 4.7              |
| Serum creatinine, mg/dl   | 0.83 $\pm$ 0.16           |
| eGFR (CKD-EPI), ml/min per 1.73 m <sup>2</sup>                              | 97.3 $\pm$ 15.6           |
| Measured GFR  |                           |
| Urine based, ml/min, n = 50   | 127.1 $\pm$ 29.3          |
| Urine based indexed to BSA, ml/min per 1.73 m <sup>2</sup> , n = 50         | 120.3 $\pm$ 31.7          |
| Iothalamate indexed to BSA, ml/min per 1.73 m <sup>2</sup> , n = 37 (range) | 108.8 $\pm$ 18.9 (83–163) |
| SBP, mm Hg  | 127.5 $\pm$ 13.7          |
| DBP, mm Hg  | 73 $\pm$ 9.9              |
| MAP, mm Hg  | 91.2 $\pm$ 9.3            |
| Urine markers   |                           |
| UProtein:creatinine ratio, n = 87   | 0.05 $\pm$ 0.02           |
| UPodocin mRNA:creatinine ratio, log <sub>2</sub> -transformed, n = 72       | -16.5 $\pm$ 2.35          |
| UNephrin:creatinine ratio, log <sub>2</sub> -transformed, n = 83            | -12.85 $\pm$ 2.16         |
| UAquaporin2:creatinine ratio, log <sub>2</sub> -transformed, n = 82         | -13.13 $\pm$ 2.87         |

BMI, body mass index; BSA, bovine serum albumin; CKD-EPI, chronic kidney disease epidemiology collaboration; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; MAP, mean arterial pressure; SBP, systolic blood pressure. Living related is the proportion of potential donors who were donating a kidney to a recipient who was related (sibling, parent, or child) to the donor and thereby might have a genetic susceptibility to developing kidney disease.

(measurable noninvasively in the urine pellet) proven to cause podocyte depletion, leakiness of the glomerular filter (proteinuria), glomerulosclerosis, and nephron loss culminating in CKD and ESKD.<sup>10–25</sup> Urine pellet podocin mRNA loss is therefore a sensitive and specific marker of glomerular damage that precedes the onset of both proteinuria and evidence of glomerular disease in kidney biopsies.<sup>13,25</sup> Normal aging-associated podocyte loss is also accelerated in association with diverse glomerular diseases to drive progression.<sup>26</sup> HTN-associated glomerulosclerosis, like all other forms of glomerulosclerosis evaluated so far, is also associated with reduced podocyte number and density, implying that similar mechanisms are operating in HTN.<sup>27</sup>

To examine the relationship of systemic blood pressure with podocyte stress and loss in healthy individuals, we evaluated a cohort of rigorously phenotyped individuals assessed to have no identifiable medical or other contraindication to their serving as kidney donors and who subsequently donated a kidney for transplantation. Protocol kidney biopsies performed at the time of implantation into the recipient provided parallel histologic information confirming normal kidney structure. The relationship of nephron segment-specific glomerular and tubular urine pellet mRNA markers of injury with systemic blood pressure was tested.

## RESULTS

Table 1 shows demographic data for 87 healthy donors who participated in this analysis before undergoing nephrectomy.

## Relationship of mean arterial pressure (MAP) with podocyte and tubular mRNA markers and proteinuria

Figure 1 shows unadjusted data, and Figure 2 shows the same data adjusted for clinical variables including age, race, gender, baseline kidney function, relatedness to recipient, and body mass index.

## Glomerular versus tubular cell injury patterns

Figures 1a and 2a show that the ratio of a glomerular podocyte mRNA marker (Podocin) to a tubular mRNA marker (Aquaporin2) (UPod:Aqp2 ratio) measured in the same sample at the same time by the same method demonstrates a strong correlation ( $r = 0.35$ ,  $P = 0.003$ ) with MAP in unadjusted data. This relationship was further strengthened when data were adjusted for the clinical variables ( $P < 0.0001$ ) (Table 2). This result is compatible with higher MAP being related to preferential glomerular versus tubular injury.

## Podocyte stress

The ratio of 2 podocyte-specific mRNAs (podocin and nephrin) (UPod:Neph) measured in the same sample at the same time is a measure of podocyte stress that reflects relative downregulation of nephrin mRNA compared with podocin mRNA in stressed podocytes.<sup>18</sup> Figure 1b shows that the UPod:Neph ratio correlated significantly in relation to MAP in unadjusted data ( $r = 0.24$ ,  $P = 0.04$ ). When the data were adjusted for clinical variables, this association is strengthened ( $P = 0.002$ ) (Figure 2b, Table 2).

## Podocyte detachment

Figures 1c and 2c show unadjusted and adjusted urine pellet podocin mRNA values expressed in relation to urine creatinine concentration (UPod:Creat ratio) as a measure of podocyte detachment in the urine. The relationship of MAP with the unadjusted UPod:Creat ratio was not significant ( $P = 0.10$ ); however, when the values were adjusted, a significant relationship was observed ( $P = 0.04$ ) with a slope indicating a 6-fold increased UPod:Creat ratio over a MAP range of 70–110 mm Hg (Table 3).

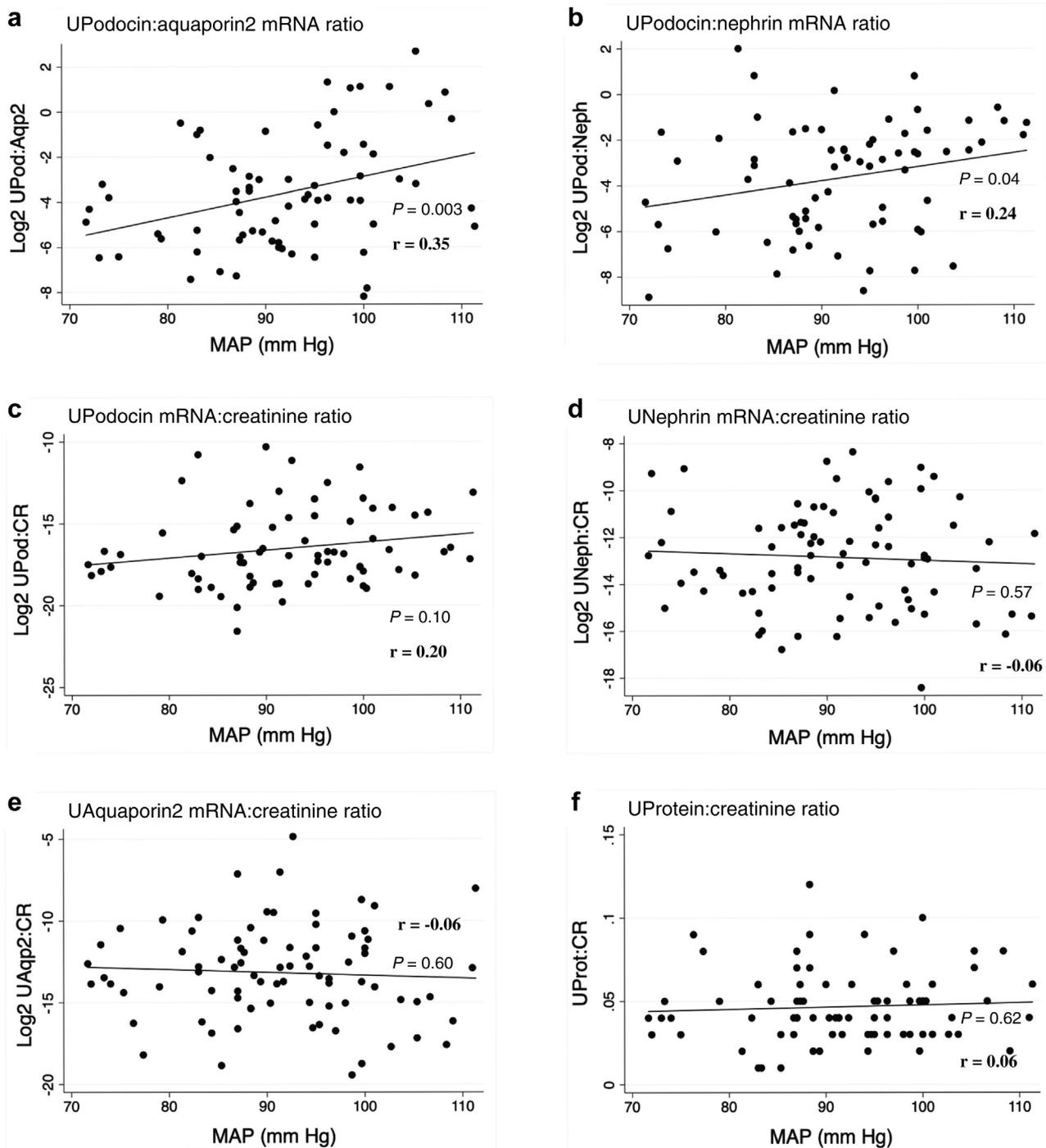
Figures 1d and 2d show similar analysis for urine nephrin mRNA that was not significantly correlated with MAP even when adjusted for clinical variables. The decreasing slope of nephrin mRNA with higher MAP reflects relative downregulation of nephrin mRNA versus podocin mRNA associated with podocyte stress as outlined above.

## Tubular cell injury

Figures 1e and 2e show similar data for aquaporin2 mRNA, a connecting tubule, and a collecting duct marker. No significant relationship to MAP was observed.

## Proteinuria

Figures 1f and 2f show that the spot urine protein:creatinine ratio (UProt:Creat ratio) was not significantly related to MAP

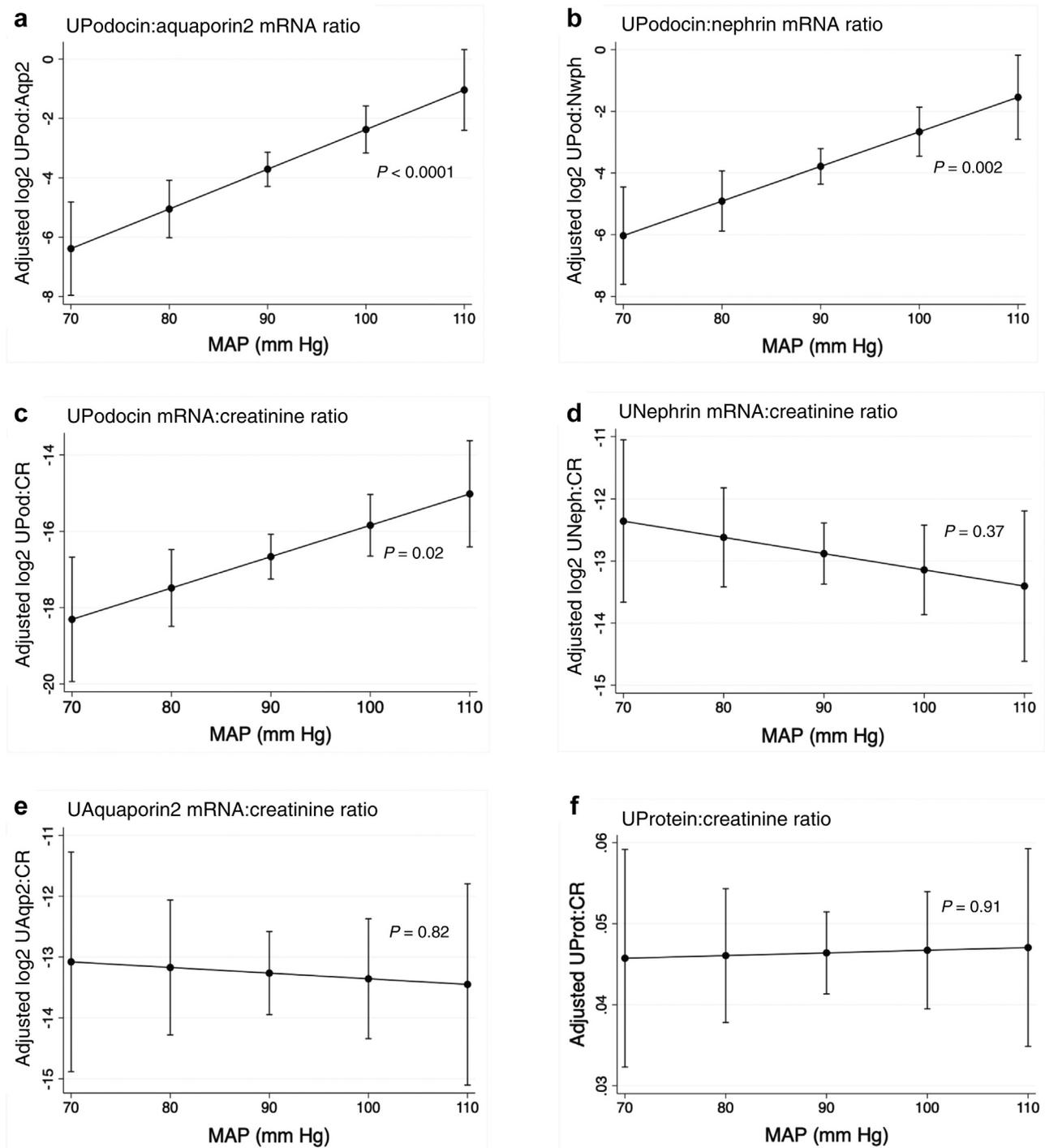


**Figure 1 | Unadjusted urine pellet marker data plotted against mean arterial pressure (MAP).** (a) The ratio of a glomerular podocyte mRNA marker (podocin) to a tubular mRNA marker (aquaporin2) provides a readout of relative glomerular versus tubular injury. Even in unadjusted data, the relationship was highly significant ( $P = 0.003$ ), demonstrating preferential glomerular versus tubular injury in relation to MAP. (b) The ratio of 2 podocyte-specific mRNA markers (podocin and nephrin shown as the podocin:nephrin mRNA ratio) in which relative downregulation of the nephrin mRNA versus podocin mRNA expression provides a readout for podocyte stress. Even in unadjusted data, the relationship with MAP reached statistical significance ( $P = 0.04$ ). (c–e) Urine pellet mRNAs (podocin, nephrin, and aquaporin2) shown in relation to urine creatinine concentration analogous to the urine protein:creatinine ratio shown in (f). None of these relationships reached statistical significance.

over the range 70–110 mm Hg even when adjusted for clinical variables. This result might in part reflect the stringent selection criteria used for living donors wherein proteinuria would have led to exclusion.

#### The relationship of podocyte stress and detachment to MAP was higher in females

There were no significant differences in the mean UPod:Creat, UNeph:Creat, and UAqp2:CR ratios between males and



**Figure 2 | Urine pellet marker data adjusted for clinical variables shown in Tables 2 and 3 plotted against mean arterial pressure (MAP).** (a) The ratio of a glomerular podocyte mRNA marker (podocin) to a tubular mRNA marker (aquaporin2) provides a readout of relative glomerular versus tubular injury. A highly significant relationship with MAP was observed ( $P < 0.0001$ ), indicating preferential glomerular versus tubular injury in relation to MAP. (b) The ratio of 2 podocyte-specific mRNA markers (podocin and nephrin shown as the podocin:nephrin mRNA ratio) in which relative downregulation of the nephrin mRNA versus podocin mRNA expression provides a readout for podocyte stress. A highly significant relationship between podocyte stress and MAP was observed ( $P = 0.002$ ). (c–e) Urine pellet mRNAs (podocin, nephrin, and aquaporin2) shown in relation to urine creatinine concentration analogous to the urine protein:creatinine ratio shown in (f). Only the urine podocin mRNA:creatinine ratio was significantly related to MAP ( $P = 0.04$ ). The urine nephrin mRNA:creatinine ratio showed a downward trend with increasing MAP that did not reach statistical significance. No relationship of MAP to either the tubular marker (aquaporin2) or proteinuria was observed. Note: predictive margins were calculated after the linear regression command to test the effect of increments in MAP on the urine marker of interest keeping all other covariates constant.

**Table 2 | Multivariable linear regression: relationship of clinical variables listed top to bottom to the urine mRNA markers expressed in relation to urine creatinine concentration analogous to the urine protein:creatinine ratio**

| Variables              | UPodocin:creatinine ratio<br>(n = 72) |         | UNephrin:creatinine ratio<br>(n = 83) |         | Aquaporin2:creatinine ratio<br>(n = 82) |         |
|------------------------|---------------------------------------|---------|---------------------------------------|---------|---|---------|
|                        |                                       | P value |                                       | P value |   | P value |
| MAP, mm Hg             | 0.08 ± 0.03                           | 0.02    | -0.03 ± 0.03                          | 0.37    | -0.009 ± 0.04                           | 0.82    |
| Age, yr                | 0.01 ± 0.03                           | 0.64    | 0.005 ± 0.03                          | 0.86    | 0.02 ± 0.03                             | 0.60    |
| Male                   | -0.83 ± 0.61                          | 0.18    | 0.84 ± 0.52                           | 0.11    | 0.30 ± 0.73                             | 0.69    |
| White race             | -0.88 ± 0.70                          | 0.22    | -1.18 ± 0.59                          | 0.06    | -0.84 ± 0.82                            | 0.31    |
| BMI, kg/m <sup>2</sup> | -0.10 ± 0.06                          | 0.10    | -0.06 ± 0.06                          | 0.24    | -0.08 ± 0.07                            | 0.29    |
| eGFR, ml/min           | 0.02 ± 0.02                           | 0.32    | 0.02 ± 0.02                           | 0.44    | 0.03 ± 0.03                             | 0.25    |
| Related                | -0.81 ± 0.62                          | 0.19    | -0.53 ± 0.50                          | 0.30    | -0.67 ± 0.70                            | 0.34    |

BMI, body mass index; eGFR, estimated glomerular filtration rate; MAP, mean arterial pressure; Related, whether or not the prospective kidney donor was related to the recipient (sibling, parent, or child) as a potential indication of underlying genetic susceptibility to progression. Data are mean ± SD.

females. However, as shown in Table 3, for any given level of MAP, females had higher podocyte stress (UPod:Neph mRNA ratio) and detachment (UPod:Creat ratio) compared with males. This sex difference persisted in a subgroup analysis that accounted for donor body surface area to reflect differences in body size (data not shown).

#### Systolic blood pressure, diastolic blood pressure, and pulse pressure in relation to urine markers

The independent addition of systolic blood pressure and diastolic blood pressure to the multivariable linear regression showed that the addition of systolic blood pressure, but not diastolic blood pressure, leads to loss of a significant relationship between urine mRNA markers and MAP (data not shown). This demonstrates that the relationship of MAP and markers is driven predominantly by systolic blood pressure. Pulse pressure did not have an independent relationship with any of the urine markers.

#### Kidney structural changes

Amongst 39 implant biopsies available for analysis, no statistically significant relationship of % globally sclerotic glomeruli with UPod:Creat ratio, UPod:Neph ratio, or

UPod:Aqp2 ratio was observed. In addition, MAPs did not correlate with degree of arteriosclerosis ( $r = 0.03$ ,  $P = 0.82$ ) or percent of globally sclerotic glomeruli ( $r = 0.05$ ,  $P = 0.77$ ) in this cohort. No significant relationship was found between degree of arteriosclerosis and % globally sclerotic glomeruli ( $r = 0.02$ ,  $P = 0.89$ ).

#### Missing data

Table 1 highlights outcome variables (urine markers) with missing data. The missing data were due to nondetectable mRNA levels in the urine marker of interest. None of the predictor variables had missing data. There was no difference in predictor variables between those with missing and non-missing urine markers; thus missingness appears to be random.

#### DISCUSSION

We demonstrate in a well-phenotyped healthy nonhypertensive human cohort that a higher MAP was significantly associated with preferential glomerular versus tubular injury, podocyte stress, and an increased amount of podocyte mRNA in urine (UPod:Creat ratio). In contrast, MAP was not associated with either proteinuria (UProt:Creat ratio) or a marker of tubular cell loss (UAqp2:Creat) ratio. These findings offer the first evidence for how systemic blood pressure, even in the “normal” range, might initiate, drive, and amplify podocyte stress and depletion from glomeruli over time, even in individuals perceived by conventional phenotyping to be very healthy.

Interpretation of these data requires understanding what the mRNA marker assays report and caveats related to this approach. First, mRNA markers are measured in the low-speed urine pellet that contains whole cells and is not designed to enrich subcellular particles such as exosomes. This low-speed pellet is equivalent to that used routinely by nephrologists to look for casts, cells, and crystals in clinical urine samples. Podocyte damage or death such that soluble mRNA is released into the filtrate would not be measured by the assay because the centrifugation step would not pellet soluble mRNAs, because the pellet is washed to remove soluble proteins before processing, and because urine contains high quantities of RNase that would destroy soluble mRNAs. Thus any circumstance that accelerates detachment of whole cells (e.g., podocytes tubular cells or inflammatory cells from

**Table 3 | Multivariable linear regression: relationship of clinical variables listed top to bottom at left to the ratio of 2 urine mRNA markers measured in the same assay by the same method at the same time**

| Variables              | UPod:Aqp2 mRNA ratio (n = 71) |         | UPod:Neph mRNA ratio (n = 71) |         |
|------------------------|-------------------------------|---------|-------------------------------|---------|
|                        |                               | P value |                               | P value |
| MAP, mm Hg             | 0.13 ± 0.03                   | 0.001   | 0.11 ± 0.03                   | 0.002   |
| Age, yr                | -0.01 ± 0.03                  | 0.61    | 0.01 ± 0.003                  | 0.66    |
| Male                   | -1.70 ± 0.60                  | 0.006   | -1.72 ± 0.60                  | 0.006   |
| White race             | 0.61 ± 0.68                   | 0.37    | 0.24 ± 0.68                   | 0.73    |
| BMI, kg/m <sup>2</sup> | -0.05 ± 0.06                  | 0.44    | -0.08 ± 0.06                  | 0.19    |
| eGFR, ml/min           | -0.01 ± 0.02                  | 0.61    | 0.009 ± 0.02                  | 0.69    |
| Related                | -0.33 ± 0.60                  | 0.58    | -0.29 ± 0.60                  | 0.63    |

BMI, body mass index; eGFR, estimated glomerular filtration rate; MAP, mean arterial pressure; Related, whether or not the prospective kidney donor was related to the recipient (sibling, parent, or child) as a potential indication of underlying genetic susceptibility to progression.

Urine podocin:aquaporin2 mRNA ratio is a measure of relative glomerular vs. tubular injury. The ratio of 2 podocyte-specific mRNAs (podocin and nephrin) is a measure of podocyte stress.

Data are mean ± SD.

the nephron itself or cells from the downstream urinary tract) that remain sufficiently intact to preserve their mRNAs during passage down the nephron and urinary tract, storage in the bladder, voiding, and pelleting by centrifugation will be captured in the output from these mRNA assays. Transcripts used are kidney cell-specific to allow assessment of kidney cell-specific effects as opposed to “housekeeping mRNAs” such as glyceraldehyde-3-phosphate dehydrogenase or beta-actin that are expressed by all cells present in the urine sample and thereby are not useful for this analysis. Quantitation of mRNA amount by quantitative polymerase chain reaction is not necessarily a measure of cell number because changes in cell size and synthetic activity will also result in changes in cellular mRNA content. On the other hand, a podocyte’s job is to completely cover the filtration surface with foot processes so that loss of a single larger cell might be equivalent to the loss of 2 smaller cells with respect to functional compromise. Thus, transcript quantity (“amount”) may be a preferable averaging mechanism than would cell number. Furthermore, the data are adjusted for age, body mass index, and baseline eGFR each of which might impact podocyte volume. A potential concern is that because the amount of podocyte detachment could not be directly measured in the kidneys of the living related kidney donors before donation, the interpretation of the UPod:Creat ratio as representing podocyte detachment might not be justified. However, because the assay measures transcript that is absolutely podocyte specific in pelleted cells recovered from urine, these cells must have “detached” from the glomerular basement membrane. Furthermore, we have previously proven the relationship between the UPod:Creat ratio and rate of loss of podocytes from glomeruli in both animal models and human diseases.<sup>16,17,24,28</sup> We therefore believe that it is reasonable to extend this interpretation to normal healthy subjects with the caveat that it has not yet been directly proven in this special case. Podocin mRNA is a robust podocyte marker in contrast to nephrin mRNA that is more readily downregulated in response to stress conditions. Therefore, the ratio of 2 podocyte-specific markers whose functions are differentially regulated but can be measured by the same method in the same sample at the same time provides a useful internally controlled marker for podocyte stress.<sup>18</sup> Finally, we note that this approach assumes that cells that appear in the urine pellet have undergone stresses resulting in their detachment and therefore are a representative of a subset of the *in situ* cell population that reflects the glomerular biology at a point in time.

Wang *et al.*<sup>27</sup> compared intrarenal mRNA expression, podocyte number, and density between controls and those with a diagnosis of biopsy-proven hypertensive nephrosclerosis. They reported that kidneys from hypertensive subjects had a lower intrarenal expression of podocin, nephrin, and synaptopodin mRNAs corresponding with higher urinary mRNA expression suggesting net depletion of podocytes. Furthermore, podocyte density and intrarenal gene expression of podocyte-associated molecules have been reported to

be significantly inversely correlated with systemic blood pressure.<sup>27</sup> We also observed that HTN-associated nephropathy is associated with increased urine pellet podocin mRNA.<sup>19</sup> Similarly, in model systems, Kretzler *et al.*<sup>29</sup> reported accelerated podocyte injury and loss in uninephrectomized desoxycorticosterone hypertensive male Munich-Wistar rats.

In contrast to overt HTN, where net podocyte depletion may have been either the cause or a consequence of HTN, in this report, we show that both podocyte stress and detachment are related to levels of blood pressure generally considered to be in normal ranges (MAP 70–110 mm Hg). Furthermore, this occurred in the absence of underlying renal disease as assessed by careful clinical phenotyping, including renal biopsy in a subset of cases. We have previously shown that podocyte mRNA markers are present in the urine pellet of healthy individuals that we interpreted as a “normal” amount of podocyte loss quantitatively related to a “normal” rate of loss of podocytes from glomeruli with age (approximately 2 podocytes per glomerulus per year out of an average total of approximately 580 podocytes per glomerulus present at birth).<sup>24,30</sup> In addition, we have reported that during normal aging, glomeruli become progressively depleted of podocytes and at the same time, glomerular volume tends to increase with age in the general population in part due to compensatory nephron hypertrophy as nephrons are lost over time, weight gain, diabetes, and other factors.<sup>26,31</sup> The combination of increasing glomerular volume and podocyte loss results in decreased podocyte density that can quantitatively account for the increasing degree of global glomerulosclerosis associated with aging as well as serve as a mechanism explaining the powerful relationship between increasing age and ESKD.<sup>23</sup>

Higher levels of blood pressure in the normal ranges are reported to be associated with increased risk for CKD and progression to ESKD.<sup>3–5</sup> Figure 2c shows that there was a 6-fold increase in podocyte detachment observed over the range of MAP evaluated (70–110 mm Hg). Could this degree of increased podocyte loss cause the reported increased incidence of long-term progression associated with high-normal MAP? Let us assume that the 3-fold increased urine pellet podocin mRNA signal was linearly related to a 3-fold increased rate of podocyte loss from the glomeruli. On the basis of the average rate of podocyte loss from glomeruli of healthy nonhypertensive subjects of 2 podocytes/glomerulus/yr, we can thus estimate the impact of a 3-fold increased podocyte detachment if the MAP was increased from 90 to 110 mm Hg.<sup>24,26,30</sup> In this case, the rate of podocyte loss from glomeruli would be increased from 2 to 6 podocytes/glomerulus/yr. At this loss rate over a lifespan of 80 years, glomeruli would lose  $80 \times 6 = 480$  podocytes/glomerulus of a total of approximately 580 podocytes/glomerulus present at birth to leave 100 podocytes/glomerulus remaining by 80 years of age. If glomerular volume did not increase and remained at approximately  $2.0 \times 10^6 \mu\text{m}^3$ , this would give a podocyte density by 80 years of age of  $100/2 = 50$  per  $10^6 \mu\text{m}^3$ . We know that

proteinuria and progressive glomerulosclerosis occur when podocyte densities reach  $<100$  per  $10^6 \mu\text{m}^3$  and ESKD supervenes at densities below 35–50 per  $10^6 \mu\text{m}^3$ .<sup>21</sup> Furthermore, if the glomerular volume were to increase as commonly occurs with increasing age in association with weight gain, type 2 diabetes, nephron loss for any cause, or when compensatory kidney hypertrophy occurs after donating one kidney, then the podocyte density would be lower. For example, if the glomerular volume were to double to  $4.0 \times 10^6 \mu\text{m}^3$ , then the estimated podocyte density at 80 years would be  $100/4 = 25$  per  $10^6 \mu\text{m}^3$ , a level at which ESKD would already have supervened. These estimates demonstrate that the observed increased rate of podocyte detachment associated with MAP in the higher-normal ranges (e.g., at 110 mm Hg) would be predicted to cause significant podocyte depletion as aging proceeds and approximate the observed increased prevalence of CKD and ESKD with HTN.<sup>3–5</sup>

Data in this report imply that UPod:CR is a continuous variable related to ambient blood pressure (hemodynamic transcapillary glomerular pressure) as opposed to some hypothetical critical level of MAP above which podocyte loss becomes accelerated and increases progression risk. If higher glomerular filtration pressure *per se* is a driver of podocyte loss, then blood pressure reduction would be expected to reduce this loss thereby delay the critical reduction in podocyte density resulting in glomerulosclerosis, nephron loss, and CKD as is well documented by numerous clinical trials. The relationship between MAP and podocyte loss will also be impacted by genetic and acquired factors that determine the capacity of podocytes to remain attached to the glomerular basement membrane. For example, in Alport syndrome, abnormal glomerular basement membrane type IV collagens predispose to accelerated podocyte loss at an average 11-fold increased rate from birth that can quantitatively account for the average age of onset of ESKD in children with Alport syndrome at approximately 21 years.<sup>24</sup> In another example, the average 6-fold increased urine pellet podocin mRNA observed in allograft recipients can quantitatively account for the shorter-than expected life of transplanted kidneys at approximately 15 years.<sup>23,28</sup> In more rapidly progressing forms of primary glomerular disease (e.g., childhood focal segmental glomerulosclerosis), urine pellet podocin mRNA is increased to 30- to 100-fold above control, accounting for a rapid eGFR decline and ESKD onset unless the underlying disease process (e.g., postinfectious glomerulonephritis) resolves when urine pellet podocin mRNA returns to control levels.<sup>19</sup> The availability of a noninvasive podocyte loss rate estimate, therefore, provides an approach by which both individualized early detection of progression risk at a point in time before the onset of proteinuria or CKD can be detected and quantified, and monitoring of the efficacy of prevention strategies can be implemented.<sup>21</sup> In the particular case of kidney donation, this approach could potentially serve to identify higher-risk individuals for whom donating a kidney would be projected to lead to CKD/ESKD during their

expected lifespan unless prospective prevention measures were to be implemented at the time of kidney donation and maintained throughout postdonation life.

## METHODS

The study was an observational cohort study conducted at the University of Michigan under institutional review board approval numbers HUM00025707 and HUM00055525 from January 1, 2015, to June 30, 2016. Urine samples were collected from 100 potential living donors of whom 87 eventually donated a kidney and were included in the study. Clinical and demographic information were collected using the electronic medical record as well as EMERSE system, as shown in Table 1.<sup>32</sup> The modified STROBE checklist for observational studies was used to ensure good reporting guidelines.

### Blood pressure measurement

Systemic blood pressure was measured using an automated oscillometer (GE Dynamap Procare 300, Milwaukee, WI). The second blood pressure value was used to calculate MAP.

### GFR estimation

GFRs were estimated for all patients using 2 methods due to a change in protocol. In the earlier part of the study ( $n = 50$ ), GFR was quantitated using 24-hour urine creatinine clearances. In the later part of the study ( $n = 37$ ), iothalamate clearance was used. eGFR for all patients was calculated using the creatinine-based CKD-epidemiology formula.<sup>33</sup> In this cohort, creatinine clearance showed no significant correlation with eGFR calculated by the CKD-epidemiology formula ( $r = 0.03$ ,  $P = 0.80$ ). Indexing of 24-hour urine creatinine clearance with body surface area did not change this relationship. Iothalamate GFR was weakly correlated with CKD-epidemiology ( $r = 0.35$ ,  $P = 0.04$ ), consistent with previous reports.<sup>34</sup>

### Biopsies

Although postperfusion biopsies are part of the University of Michigan transplant protocol, only 44% of samples in this cohort were accompanied by postperfusion biopsies. The major cause of absent biopsy was that the donor kidney was shipped to another center as part of a paired exchange program or when the recipient was considered to be at an increased risk of bleeding.

### Demographic data

Donor age at the time of urine collection, self-identified race, gender, relationship to the recipient (related and unrelated), and body mass index were collected from the medical record.

### Urine sample processing and mRNA assay

Urine pellet mRNA was assayed as previously described.<sup>19</sup> The surplus urine sample produced by prospective donors attending clinic was stored at 4 °C in a plastic container for up to 8 hours. Urine was gently shaken to resuspend all contents and then transferred to a 50 ml plastic centrifuge tube for centrifugation at 4000 rpm ( $3200 \times g$ ) at 4 °C for 15 minutes in a table-top centrifuge. Supernatant (1.8 ml) 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  $\mu\text{l}$  diethylpyrocarbonate-treated phosphate-buffered saline at 4 °C and then transferred to a 1.7 ml plastic centrifuge tube. A second 750  $\mu\text{l}$  aliquot of diethylpyrocarbonate-treated phosphate-buffered saline 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 in 1.5 ml diethylpyrocarbonate-treated phosphate-buffered saline was centrifuged at 12,000 rpm for 5 minutes at 4 °C. The supernatant was discarded. To the remaining washed pelleted material was added 350 µl of RLT buffer containing β-mercaptoethanol at 10 µl/ml according to the RNeasy Qiagen protocol (Germantown, MD). The pellet was suspended in the RLT/β-mercaptoethanol buffer and then frozen at −80 °C for assay. The total urine pellet RNA was isolated using the RNeasy Mini Kit protocol (catalog no. 74106; Qiagen). Quantitation of the absolute nephrin, podocin, TGF-β1, and aquaporin-2 mRNA 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 were as follows: human NPHS1 (nephrin; catalog no. s00190446\_m1), human NPHS2 (podocin; catalog no. Hs00922492\_m1), human aquaporin2 (cat. no. Hs00166640\_m1). All data were from 2 µl samples measured in duplicate. Assays were accepted only if  $R^2$  was 0.97 for standard curves using SDS 2.2.2 software (Applied Biosystems). cDNAs of known sequence and concentration were used as standards for each assay. We previously reported analysis urine RNA quality, recovery, and stability. The coefficient of assay variation is 35%.

### Outcome variables

We used the 2 quantitated podocyte markers (Podocin and Nephlin mRNA) and a tubular marker (Aquaporin2 mRNA) normalized to urine creatinine (all as continuous variables) to assess nephron segment-specific patterns of injury. Podocyte injury leads to relative downregulation of nephrin versus podocin mRNA expression and thus an increasing UPod:Neph ratio is suggestive of podocyte stress.<sup>18</sup> The urine podocin mRNA to aquaporin mRNA ratio (UPod:Aqp2) was used to assess evidence of preferential glomerular versus tubular injury. Spot urine protein to creatinine ratio was used (UProt:CR) as a measure of urinary protein loss rate.

### Model building and predictor variables

To assess potential predictors of relevant urine markers, a literature-driven *a priori* approach was used including age, race, gender, baseline kidney function, and body mass index, which have all been associated with progressive kidney disease. In addition, because donors who are related to their recipients have a higher risk of kidney disease, we used relatedness as another independent variable in the model. Relatedness was defined as a sibling, child, or parent. To assess for the possibility of multicollinearity in these models, we independently tested for collinearity between dependent variables and further assessed collinearity using assessment of the variance inflation factor in the multivariable regression model.<sup>35</sup>

Correlational analysis was performed using Spearman's "r." Linear regression was used to assess the relationship with each outcome's variable with the predefined clinical predictors in the model. Predictive margins were calculated after the regression command to test the effect of increments in MAP on the urine marker of interest keeping all other covariates constant. The "margins" and "marginplot" commands were used to generate margins and plots of those margins.<sup>36,37</sup> All analysis was performed using Stata 15 I/C (College Station, TX).

### DISCLOSURE

All the authors declared no competing interests.

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### REFERENCES

1. Fisher ND, Curfman G. Hypertension—a public health challenge of global proportions. *JAMA*. 2018;320:1757–1759.
2. United States Renal Data System. 2016 USRDS annual data report: Epidemiology of kidney disease in the United States. National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, 2016.
3. Klag MJ, Whelton PK, Randall BL, et al. End-stage renal disease in African-American and white men: 16-year MRFIT findings. *JAMA*. 1997;277:1293–1298.
4. Tozawa M, Iseki K, Iseki C, et al. Blood pressure predicts risk of developing end-stage renal disease in men and women. *Hypertension*. 2003;41:1341–1345.
5. Hsu C. Does treatment of non-malignant hypertension reduce the incidence of renal dysfunction? A meta-analysis of 10 randomised, controlled trials. *J Hum Hypertens*. 2001;15:99.
6. Keller G, Zimmer G, Mall G, et al. Nephron number in patients with primary hypertension. *N Engl J Med*. 2003;348:101–108.
7. Hoy WE, Hughson MD, Bertram JF, et al. Nephron number, hypertension, renal disease, and renal failure. *J Am Soc Nephrol*. 2005;16:2557–2564.
8. Samuel T, Hoy WE, Douglas-Denton R, et al. Determinants of glomerular volume in different cortical zones of the human kidney. *J Am Soc Nephrol*. 2005;16:3102–3109.
9. Fogo A, Breyer JA, Smith MC, et al. Accuracy of the diagnosis of hypertensive nephrosclerosis in African Americans: a report from the African American Study of Kidney Disease (AASK) Trial. *Kidney Int*. 1997;51:244–252.
10. Kriz W, Gretz N, Lemley KV. Progression of glomerular diseases: is the podocyte the culprit? *Kidney Int*. 1998;54:687–697.
11. Kim YH, Goyal M, Kurnit D, et al. Podocyte depletion and glomerulosclerosis have a direct relationship in the PAN-treated rat. *Kidney Int*. 2001;60:957–968.
12. 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;16:2941–2952.
13. Yu D, Petermann A, Kunter U, et al. Urinary podocyte loss is a more specific marker of ongoing glomerular damage than proteinuria. *J Am Soc Nephrol*. 2005;16:1733–1741.
14. Wiggins RC. The spectrum of podocytopathies: a unifying view of glomerular diseases. *Kidney Int*. 2007;71:1205–1214.
15. Sato Y, Wharram BL, Lee SK, et al. Urine podocyte mRNAs mark progression of renal disease. *J Am Soc Nephrol*. 2009;20:1041–1052.
16. 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;81:40–55.
17. Fukuda A, Chowdhury MA, Venkatareddy MP, et al. Growth-dependent podocyte failure causes glomerulosclerosis. *J Am Soc Nephrol*. 2012;23:1351–1363.

18. Fukuda A, Wickman LT, Venkatarreddy MP, et al. Urine podocin:nephrin mRNA ratio (PNR) as a podocyte stress biomarker. *Nephrol Dial Transplant*. 2012;27:4079–4087.
19. Wickman L, Afshinnia F, Wang SQ, et al. Urine podocyte mRNAs, proteinuria, and progression in human glomerular diseases. *J Am Soc Nephrol*. 2013;24:2081–2095.
20. Kriz W, Lemley KV. A potential role for mechanical forces in the detachment of podocytes and the progression of CKD. *J Am Soc Nephrol*. 2015;26:258–269.
21. Kikuchi M, Wickman L, Hodgkin JB, et al. Podometrics as a potential clinical tool for glomerular disease management. *Semin Nephrol*. 2015;35:245–255.
22. Fukuda A, Sato Y, Iwakiri T, et al. Urine podocyte mRNAs mark disease activity in IgA nephropathy. *Nephrol Dial Transplant*. 2015;30:1140–1150.
23. Naik AS, Afshinnia F, Cibrik D, JB, et al. Quantitative podocyte parameters predict human native kidney and allograft half-lives. *JCI Insight*. 2016;1:86943.
24. Ding F, Wickman L, Wang SQ, et al. Accelerated podocyte detachment and progressive podocyte loss from glomeruli with age in Alport Syndrome. *Kidney Int*. 2017;92:1515–1525.
25. 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.
26. Hodgkin JB, Bitzer M, Wickman L, et al. Glomerular aging and focal global glomerulosclerosis: a podometric perspective. *J Am Soc Nephrol*. 2015;26:3162–3178.
27. Wang G, Lai FM, Kwan BC, et al. Podocyte loss in human hypertensive nephrosclerosis. *Am J Hypertens*. 2009;22:300–306.
28. Yang Y, Hodgkin JB, Afshinnia F, et al. The two kidney to one kidney transition and transplant glomerulopathy: a podocyte perspective. *J Am Soc Nephrol*. 2015;26:1450–1465.
29. Kretzler M, Koepfen-Hagemann I, Kriz W. Podocyte damage is a critical step in the development of glomerulosclerosis in the uninephrectomised-desoxycorticosterone hypertensive rat. *Virchows Archiv*. 1994;425:181–193.
30. Kikuchi M, Wickman L, Rabah R, et al. Podocyte number and density changes during early human life. *Pediatric Nephrology*. 2017;32:823–834.
31. 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;16:2953–2966.
32. Hanauer DA, Mei Q, Law J, et al. Supporting information retrieval from electronic health records: a report of University of Michigan's nine-year experience in developing and using the Electronic Medical Record Search Engine (EMERSE). *J Biomed Inform*. 2015;55:290–300.
33. Levey AS, Stevens LA, Schmid CH, et al. A new equation to estimate glomerular filtration rate. *Ann Intern Med*. 2009;150:604–612.
34. Rule AD, Bailey KR, Lieske JC, et al. Estimating the glomerular filtration rate from serum creatinine is better than from cystatin C for evaluating risk factors associated with chronic kidney disease. *Kidney Int*. 2013;83:1169–1176.
35. Acock AC. *A Gentle Introduction to Stata*. Stata Press; 2008.
36. Williams R. Using the margins command to estimate and interpret adjusted predictions and marginal effects. *Stata J*. 2012;12:308–331.
37. Mitchell MN. *Interpreting and Visualizing Regression Models Using Stata*. Vol 5. College Station, TX: Stata Press; 2012.