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Fibroblast Growth Factor-23 and Chronic Allograft Injury in Pediatric Renal Transplant Recipients: a Midwest Pediatric Nephrology Consortium Study

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Abstract

Background—The chronic kidney disease-mineral bone disorder (CKD-MBD) produces fibroblast growth factor-23 (FGF23) and related circulating pathogenic factors that are strongly associated with vascular injury and declining kidney function in native CKD. Similarly, chronic renal allograft injury (CRAI) is characterized by vascular injury and declining allograft function in transplant CKD. We hypothesized that circulating CKD-MBD factors could serve as non-invasive biomarkers of CRAI.

Methods—We conducted a cross-sectional, multicenter, case-control study. Cases (n=31) had transplant function > 20 mL/min/1.73 m² and biopsy-proven CRAI. Controls (n=31) had

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AUTHOR CONTRIBUTIONS

M.E.S.: study design; enroll subjects; draft/edit manuscript; assay performance; data analysis.

I.F.A.: enroll subjects, edit/revise manuscript.

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transplant function $> 90 \text{ mL/min/1.73 m}^2$ and/or a biopsy with no detectable abnormality in the previous 6 months. We measured plasma CKD-MBD factors at a single time point using ELISA.

Results—Median (range) FGF23 levels were over 2-fold higher in CRAI versus controls [106 (10-475) pg/mL versus 45 (8-91) pg/mL; $P<0.001$]. FGF23 levels were inversely correlated with transplant function ($r^2 = -0.617$, $P<0.001$). Higher FGF23 levels were associated with increased odds of biopsy-proven CRAI after adjusting for transplant function, clinical, and demographic factors [OR (95% CI) 1.43 (1.23, 1.67)]. Relationships between additional CKD-MBD factors and CRAI were attenuated in multivariable models.

Conclusions—Higher FGF23 levels were independently associated with biopsy-proven CRAI in children.

Keywords

chronic allograft nephropathy; chronic rejection; pediatric kidney transplantation; biomarkers

INTRODUCTION

Kidney transplantation is the optimal treatment for end-stage renal disease (ESRD) in children. Despite advances in short-term patient and allograft survival over the last few decades, improvements in long-term patient and allograft survival have been less dramatic(1, 2). Chronic renal allograft injury (CRAI) and cardiovascular disease are now among the leading causes of allograft failure and death in kidney transplant recipients, respectively(3, 4). There are no effective therapies for CRAI and current diagnostic strategies are inadequate(5). Kidney biopsies are expensive and invasive, and while creatinine-based estimates of allograft function can be associated with CRAI they provide no therapeutic target or insight into mechanisms of disease. This is particularly relevant in pediatric recipients of adult kidneys, where creatinine-based estimates are insensitive for detecting modest changes in allograft function, which often delays the diagnosis of CRAI(6). Novel biomarkers are needed to allow non-invasive detection of CRAI and identify new therapeutic targets and potential mechanisms of disease.

CRAI is characterized by cumulative vascular injury that leads to progressive renal fibrosis and transplant vasculopathy(7, 8). The transplant vasculature (particularly the endothelium) is the final common target of many immune and non-immune sources of injury(9). CRAI leads to a progressive decline in kidney function that is also associated with an immense burden of cardiovascular risk, just as in patients with native kidney disease(4, 10, 11). One potential source of non-immune renal and extrarenal vascular injury in kidney transplant recipients is the chronic kidney disease-mineral bone disorder (CKD-MBD).

The CKD-MBD represents a disruption in systems biology between the kidney, skeleton, and cardiovascular system(12). In native CKD, the earliest sign of the CKD-MBD is increased circulating levels of fibroblast growth factor-23 (FGF23), but we and others have demonstrated that additional circulating CKD-MBD factors may be pathogenic for the disease, including soluble klotho, Dickkopf-related protein-1 (DKK1), sclerostin, and activin-A(13-16). In adults, FGF23 levels are associated with increased risk for progression

of native CKD, and are associated with increased risk for allograft failure and all-cause mortality in kidney transplantation(17-20). Soluble klotho, DKK1, sclerostin, and activin-A have been associated with declining eGFR and vascular disease in native CKD(14, 16, 21-24). While the role of these CKD-MBD factors in adults with native and transplant CKD has become clearer, the significance of these factors in children with native and transplant kidney disease, including CRAI, is largely unknown.

Accordingly, in this study we hypothesized that in children with CRAI, cumulative transplant injury produces differential expression of circulating CKD-MBD factors that could serve as non-invasive biomarkers of disease. To test this hypothesis, we conducted a multicenter case-control study of pediatric kidney transplant recipients to determine whether circulating CKD-MBD factors are associated with biopsy-proven CRAI.

PATIENTS AND METHODS

Study Design and Population

This was a cross-sectional, case-control study that enrolled pediatric kidney transplant recipients from 6 centers in the Midwest Pediatric Nephrology Consortium. The Institutional Review Board (IRB) at each center approved the study protocol. Subjects were enrolled between July 2011 and June 2014. All subjects aged 18-21 years provided written informed consent, whereas subjects aged 3-17.9 years had a parent or guardian provide written informed consent. Informed assent was obtained where appropriate and in accordance with local IRB guidance. All recruitment and consent procedures involving human subjects adhered to guidelines set forth in the Declaration of Helsinki. All clinical and research activities in transplant recipients reported here adhered to the principles of the Declaration of Istanbul.

Inclusion criteria were prevalent kidney transplant recipients aged 3-21 years who received a kidney transplant at least 1 year prior to enrollment. The CRAI group (cases) included subjects with eGFR (traditional Schwartz calculation in subjects < 18 years, CKD-EPI 2009 calculation in subjects ≥ 18 years)(25, 26) at least 20 mL/min/1.73 m² and biopsy-proven CRAI prior to enrollment. Biopsy-proven CRAI was defined from pathology reports as any degree of interstitial fibrosis/tubular atrophy (IF/TA), transplant glomerulopathy (TG), and/or allograft vasculopathy (AV) according to Banff 2005 and 2009 criteria, regardless of severity(8, 27). Subjects were not included if the biopsy with CRAI had concomitant evidence of acute cellular or antibody-mediated rejection. The No CRAI group (controls) included subjects with traditional Schwartz or CKD-EPI eGFR > 90 mL/min/1.73 m² and/or a biopsy within the previous 6 months demonstrating no evidence of CRAI. We did not use the modified bedside Schwartz equation since at the time of study design it had only been validated in children with native CKD and a measured GFR between 15-75 mL/min/1.73 m²(28). Additionally, a subset (n=7) of the No CRAI group had measured GFR within 6 months of enrollment as standard of care; all had measured GFR > 90 mL/min/1.73 m² but 5/7 subjects had modified bedside Schwartz eGFR < 90 mL/min/1.73 m² and would have been excluded from the study based on eGFR alone. Therefore, we elected to use the traditional bedside Schwartz eGFR for enrollment, classification of stage of CKD-T, and

primary data analysis, but also reported the modified Schwartz eGFR to allow comparison with future studies.

Exclusion criteria for cases and controls were repeat kidney transplants, recipients of another solid organ transplant, acute cellular or antibody-mediated rejection within 6 months prior to enrollment (since acute inflammation could affect expression of CKD-MBD factors), and eGFR < 20 mL/min/1.73 m². Each subject completed a single study visit. We did not specifically instruct subjects to fast overnight, but most blood samples were drawn in the early morning at the time of standard-of-care tests. We used unique identifiers that maintained privacy of the participants and blinded those performing the assays to case-control status.

Exposures and Outcomes

The primary outcome was biopsy-proven CRAI. The primary exposure was plasma FGF23 levels modeled as a continuous variable. The secondary outcome was stage of CKD-T based on a traditional Schwartz calculation or CKD-EPI calculation: stage 1T (> 90 mL/min/1.73 m²), stage 2T (60-89 mL/min/1.73 m²), and stage 3T/4T (15-59 mL/min/1.73 m²). Stage 3T and 4T were combined into a single subgroup because of limited subjects with stage 4T. We also assessed the subtype of CRAI as a secondary outcome, although most patients had multiple concurrent subtypes present and the study was not adequately powered to detect differences in biomarkers according to CRAI subtype. Secondary exposures included plasma FGF23 levels modeled as a categorical variable, plasma DKK1, sclerostin, soluble klotho, and activin-A levels. Additional secondary exposures included a history of biopsy-proven acute cellular or antibody-mediated rejection (> 6 months prior to enrollment), donor type [living related/unrelated donor (LD) or deceased donor (DD)], eGFR (CKD-EPI calculation, traditional and CKD-Schwartz calculations)(6), transplant vintage (years from transplantation to enrollment), current prednisone use (at enrollment), current calcitriol use (at enrollment), systolic/diastolic blood pressure, hypertension (blood pressure > 95th percentile for age/sex/height(29) or taking antihypertensive medication), plasma calcium, and plasma phosphorus.

We used commercial ELISA kits to measure each CKD-MBD factor in duplicate [FGF23 (intact assay), Kainos Laboratories, Tokyo, Japan; DKK1, R&D Systems, Minneapolis, MN; sclerostin, R&D Systems, Minneapolis, MN; soluble klotho, IBL-America, Minneapolis, MN; and activin-A, R&D Systems, Minneapolis, MN]. The intra-assay and inter-assay coefficients of variation from the manufacturer's protocols were as follows: FGF23: 2.6% and 2.8%; soluble klotho: 3.1% and 6.9%; DKK1: 3.9% and 6.1%; sclerostin: 2.0% and 9.5%; activin-A: 4.3% and 5.9%. All other standard-of-care biochemical measures were generated by the core laboratory at each center.

Statistical Considerations

Based on our previous studies of FGF23 in native stage 1-3 CKD(30, 31), we determined a sample size of 28 cases and 28 controls could detect an effect size of 0.75 with 80% power and alpha of 0.05. Each exposure variable was tested for normality using a 1-sample Kolmogorov-Smirnov test. Non-normally distributed continuous variables were compared

using the Mann-Whitney-U test and are presented as median (minimum, maximum). Normally distributed continuous variables were compared using student's t-test and are presented as mean $\pm$ standard deviation. Variables were compared between stages of CKD using the Kruskal-Wallis test (non-normally distributed) or one-way ANOVA (normally distributed). Categorical variables were compared using a chi-square test or Fisher's exact test. Bivariate correlations of continuous variables were determined using Spearman's rho test. We used receiver operating characteristic (ROC) analysis to examine the diagnostic performance of FGF23 for biopsy-proven CRAI. We used the Youden index to determine the optimal cutoff point with maximal sensitivity and specificity to model FGF23 levels as a categorical variable(32).

A univariable logistic regression model was built to measure associations between each primary and secondary exposure and CRAI. Exposures that were significantly associated with the primary outcome in univariable analysis ($P < 0.10$) were entered into a forward stepwise multivariable model. We used stringent entry criteria in an attempt to reduce Type II errors and prevent entry of excessive numbers of covariates in the multivariable model relative to our modest cohort size. For similar reasons, we built a separate prespecified multivariable logistic regression model to adjust the effect of the primary exposure for the following potential confounders regardless of their performance in univariable analysis: age, sex, donor type, and transplant vintage. All statistical tests were two-tailed with statistical significance defined as $P < 0.05$. All analyses were performed with SPSS Statistics version 22 (IBM, Armonk, NY).

Sensitivity analyses were performed to examine the effect of misclassification into the No CRAI group. The clinical correlate would be a subject with focal CRAI that was unsampled by the renal biopsy, or a subject with undiagnosed CRAI because no for-cause biopsy was performed in the setting of normal eGFR. We randomly selected 5 subjects from the No CRAI group and reclassified them into the CRAI group to simulate a 15% misclassification error. Random selection was performed using an SPSS Statistics module. Associations between primary and secondary exposures and CRAI were explored with univariable and multivariable models as in the original dataset.

RESULTS

We enrolled 31 subjects in the No CRAI group and 31 subjects in the CRAI group, exceeding our prespecified enrollment targets to power differences in the primary exposure between groups. In the CRAI group, biopsies were performed between 1 and 12 years post-transplant; indications were increased creatinine above baseline in 25 subjects and proteinuria in 6 subjects. Multiple subtypes of CRAI were coexistent in many subjects: 18/31 (58%) had interstitial fibrosis/tubular atrophy, 17/31 (55%) had transplant glomerulopathy and 9/31 (29%) had allograft vasculopathy. In the No CRAI group, 13 subjects were enrolled based on a normal transplant biopsy, whereas 18 subjects were enrolled based on eGFR > 90 mL/min/1.73 m² alone. Biopsies in the No CRAI group were performed between 1 and 7 years post-transplant; indications were surveillance in 9 subjects and increased creatinine above baseline in 4 subjects. No biopsies in either group were read as recurrence of primary disease or calcineurin inhibitor toxicity. All remote rejection

episodes > 6 months prior to enrollment were due to acute cellular rejection; there were no reports of remote antibody-mediated rejection.

We did not collect data regarding donor specific antibody (DSA) positivity prior to enrollment, only at the time of biopsy if performed. Only 1 subject in the CRAI group (biopsy with both IF/TA and AV subtypes) had a detectable donor-specific antibody (DSA) at the time of the biopsy but no concurrent evidence of acute or chronic cellular or antibody-mediated rejection, respectively. Notably, the presence of DSA was assessed at the time of biopsy in 17/31 biopsied subjects from the CRAI group and 11/13 biopsied subjects from the No CRAI group. The presence of peritubular capillary C4d was assessed in all biopsies and the proportion of C4d-positive biopsies was similar between groups. Comparisons of demographic data between CRAI and No CRAI are displayed in Table 1.

We found significantly higher FGF23 levels and lower soluble klotho levels in the CRAI group versus No CRAI group. We found no significant differences between groups with respect to the other CKD-MBD factors DKK1, sclerostin, and activin-A (see Table 2a). In ROC analysis, FGF23 performed very well in distinguishing subjects in the CRAI group from the No CRAI group, with an area under the curve of 0.852 ($P < 0.001$, see Figure 1). Using the Youden index, we determined the optimal cutoff for FGF23 at 70 pg/mL and modeled FGF23 as a dichotomous categorical variable. Subjects with FGF23 levels ≥ 70 pg/mL had significantly higher odds of CRAI [crude OR (95% CI) of 35.4 (6.9, 180.8)]. This relationship remained significant when adjusted for eGFR as a continuous variable [adjusted OR (95% CI) of 7.4 (1.2, 46.9)]. We also adjusted for eGFR modeled as a dichotomous categorical variable (overall median of 76 mL/min/1.73 m²). Subjects with FGF23 levels ≥ 70 pg/mL had significantly higher odds of CRAI after adjusting for eGFR as a dichotomous variable [adjusted OR (95% CI) of 16.1 (2.7, 97.4)]. This relationship was maintained when adjusting for eGFR dichotomized at a lower threshold of 60 mL/min/1.73 m² [adjusted OR (95% CI) of 22.0 (4.1, 118.7)]. Overall, using a cutoff for FGF23 of 70 pg/mL diagnosed biopsy-proven CRAI with comparable or superior sensitivity and specificity versus eGFR cutoffs of 76 or 60 mL/min/1.73 m² (see Figure 2).

In subgroup analyses, we found higher FGF23 and lower soluble klotho levels for subjects with biopsies containing each subtype of CRAI compared to the No CRAI group (see Table 2b). Since multiple subtypes of CRAI were often present concurrently in a given biopsy, we could not make direct comparisons between subgroups. Alternatively, we compared FGF23 levels between subjects with biopsies containing each CRAI subtype, biopsies containing the other CRAI subtypes, and subjects in the No CRAI group, conducting separate analyses for IF/TA, TG, and AV. As in the combined CRAI group, we found significantly higher FGF23 levels for each CRAI subtype compared to the No CRAI group, with the TG subtype showing the highest FGF23 levels (see Figure 3).

We found significantly higher levels of FGF23 across worsening stages of CKD-T, consistent with previous reports of native CKD that showed an inverse correlation between FGF23 and kidney function. Soluble klotho levels declined across worsening stages of CKD-T as in previous reports of native CKD (see Table 3). As expected, FGF23 and soluble

klotho levels each correlated strongly with eGFR ($r^2 = -0.617$ and 0.380 , respectively; both $P < 0.01$).

Univariate logistic regression models demonstrated that each 5 pg/mL increase in plasma FGF23 was associated with a 26% increase in odds of having CRAI. Lower soluble klotho levels, lower traditional eGFR (continuous variable), current prednisone use, history of acute rejection, and living donor type were also associated with increased odds of CRAI (see Table 4a). We used forward stepwise entry (likelihood ratio method) to fit the best multivariable regression model using these 6 covariates. The final model included FGF23, eGFR, history of acute rejection, and donor type and explained 87% of the variance in the primary outcome (df 4, chi-square 65.316, $P < 0.001$, r^2 0.868). Plasma FGF23 levels, history of acute rejection, and living donor type remained significantly associated with CRAI in this adjusted model, but the relationship between eGFR and CRAI was attenuated (see Table 4a). We repeated this first multivariable analysis with forced entry of interaction terms FGF23 $\times$ history of acute rejection and FGF23 $\times$ eGFR, but neither were significantly associated with CRAI (data not shown). The second multivariable logistic regression model used forced entry to adjust the effect of FGF23 for prespecified demographic factors of interest. FGF23 levels remained associated with CRAI after adjusting for demographic factors and explained 58% of the variance in the primary outcome of biopsy-proven CRAI (df 5, chi-square 25.504, $P < 0.001$, r^2 0.577, see Table 4a).

Sensitivity analyses also demonstrated significant associations between higher FGF23 levels, lower soluble klotho levels, lower eGFR (continuous variable), history of acute rejection, current prednisone use and CRAI in univariable logistic regression, but these relationships were all attenuated in multivariable logistic regression. Only a history of acute rejection remained significantly associated with CRAI in multivariable sensitivity analysis (see Table 4b). FGF23 remained significantly associated with CRAI in a second multivariable sensitivity analysis that adjusted for prespecified demographic factors using the same 15% misclassification scheme (see Table 4b).

DISCUSSION

This cross-sectional, multicenter case-control study tested the hypothesis that cumulative transplant injury produces differential expression of circulating CKD-MBD factors that could serve as non-invasive biomarkers of CRAI in children. Higher FGF23 and lower soluble klotho levels were significantly associated with biopsy-proven CRAI in univariable models, but only FGF23 remained significantly associated with CRAI in separate models adjusted for eGFR and prespecified demographic factors. Furthermore, a cutoff value for FGF23 of 70 pg/mL diagnosed CRAI with excellent sensitivity and specificity. Elevated FGF23 levels were associated with all 3 subtypes of CRAI (especially TG) versus the No CRAI group, but the study was not adequately powered to detect subgroup differences in FGF23 levels.

To our knowledge, this is the first study to examine relationships between circulating CKD-MBD factors and biopsy-proven CRAI in children. Wesseling-Perry et al conducted a prospective cohort study of FGF23 in 68 pediatric kidney transplant recipients,

demonstrating that higher levels were associated with a 50% decline in eGFR and higher acute rejection rates over 2 years of clinical follow-up, but did not examine relationships between FGF23 and CRAI. Similar to our study, subjects with CKD-3T/4T had 2-fold higher FGF23 levels than subjects with CKD-1T/2T(33). Recently, van Husen et al reported serum FGF23 levels in 57 pediatric renal transplant recipients analyzed by stage of CKD-T. They found similar FGF23 levels and trends across worsening stages of CKD-T as in our study, but included no assessment of biopsy-proven CRAI or other CKD-MBD factors(34). Wan et al examined both FGF23 and soluble klotho in a pediatric CKD cohort that included 44 transplant recipients with stage CKD-2T but no specific assessment of CRAI(35). Overall, our study is among the largest to examine CKD-MBD factors in pediatric kidney transplantation, which substantially adds to the literature by evaluating a broad panel of CKD-MBD factors in subjects with biopsy-proven CRAI and including a control group with normal estimated allograft function.

In addition to CKD-MBD factors, we examined relationships between other secondary exposures and CRAI in children. We were surprised to find a higher proportion of living donor recipients in the CRAI group since they traditionally have superior allograft survival(36). The difference could be attributable to ascertainment bias since there was a non-significant trend toward longer transplant vintage in the CRAI group, allowing more time for progression of mild disease. This could have diminished the strength of association between FGF23 and CRAI, if FGF23 levels are directly correlated to the severity of disease as well as presence of disease. This relationship bears watching in future studies of pediatric CRAI. We also demonstrated a significant association between a history of biopsy-proven acute rejection and CRAI, which has been established by numerous previous studies and lends validity to our findings. Importantly, while the parameter estimate for a history of acute rejection was large and imprecise in our final multivariable model (likely attributable to only 1 subject with a history of acute rejection in the No CRAI group), its inclusion did not attenuate the relationship between FGF23 levels and CRAI.

Our study has several strengths compared to previous pediatric transplant studies of CKD-MBD factors. These include our multicenter design that included biopsy data and a control group with normal estimated transplant function. We examined a panel of established and novel CKD-MBD factors, providing important knowledge on these biomarkers in pediatric kidney transplantation. Ideally, using a pediatric cohort should have allowed us to examine associations of these factors with CRAI with less confounding by hypertension, obesity, and advanced age, factors common in adult transplant studies. However, it is important to note that 34% of our pediatric cohort had hypertension and the CRAI group had a higher mean diastolic blood pressure, although still within the normal range for age/height for most subjects. Also, while median BMI was normal, obese BMIs were seen within each study group (highest BMI was 33 kg/m² in the CRAI group and 42 kg/m² in the No CRAI group). This highlights the prevalence of hypertension and abnormal BMI in pediatric kidney transplant recipients, which have both been associated with inferior patient and allograft outcomes. Despite significant differences in eGFR between study groups, we had adequate enrollment across a range of eGFR for adjustment in multivariable analyses.

Limitations of our study include our modest cohort size that limited our ability to detect smaller differences in the exposures between groups. The lack of complete biopsy data in the No CRAI group could have introduced misclassification bias, which we attempted to address with strict eGFR enrollment criteria and sensitivity analyses. Future studies should include more subjects in the No CRAI group with surveillance biopsies and a broader range of eGFR to further address this issue. The reasoning to use traditional Schwartz eGFR was discussed in the methods, but we still may have overestimated the true GFR and incorrectly assigned some subjects without biopsy data into the No CRAI group. Sensitivity analyses demonstrated that a 15% misclassification error would have attenuated the relationship between FGF23 and CRAI in multivariable analysis. Therefore, our findings must be interpreted with caution until confirmed in larger validation studies. Many centers did not include Banff scoring for CRAI components on their pathology reports, so we were unable to report severity of the 3 subtypes of CRAI or correlate FGF23 levels directly to Banff scores. Future multicenter studies of CRAI should include Banff severity scoring of individual chronic injury lesions, performed by a central blinded pathologist if feasible (to enhance consistency and limit bias), in order to provide necessary data regarding associations of CKD-MBD factors with subtypes and severity of CRAI.

While no subjects had a remote history of acute antibody-mediated rejection > 6 months prior to enrollment, we did not specifically collect historical DSA data. While there were similar proportions of DSA-positive subjects in each group at the time of biopsy, it is conceivable that some subjects had a history of DSA without antibody-mediated rejection that could have influenced the presence of CRAI or FGF23 levels, and future studies should adjust for potential confounding from the presence of DSA. While there were no significant differences between groups with respect to maintenance immunosuppressive agents, we did not collect data on induction regimens that could have modified the association of FGF23 with CRAI. Additionally, we did not collect iron levels or a history of iron deficiency, which can influence expression of FGF23. While the proportions of subjects taking iron supplements was similar between groups, this may not be a good surrogate for iron deficiency. Finally, we did not collect information on recurrent pyelonephritis, which is a major contributor to interstitial fibrosis in CRAI and may have influenced FGF23 levels. Future studies of CKD-MBD factors and CRAI should take into account and adjust for these and other various confounding factors whenever possible.

Given the cross-sectional design of this study, we can only speculate on the potential mechanistic relevance of circulating CKD-MBD factors in CRAI. One possible mechanism is FGF23-mediated transplant vascular injury through direct activation of endothelial FGF-receptors, similar to animal models of FGF23-mediated cardiovascular toxicity and endothelial injury(20, 37). Kidney transplants affected by CRAI may release additional unidentified pathogenic factors that stimulate FGF23 secretion from osteocytes, making FGF23 a biomarker of proximal events that are important in the pathogenesis of CRAI.

In conclusion, higher FGF23 levels were associated with biopsy-proven CRAI in children independently of traditional Schwartz eGFR and multiple demographic factors, and if confirmed in validation cohorts may serve as a non-invasive biomarker of disease. Additional studies are essential to determine whether FGF23 and related CKD-MBD factors

are also pathogenic for disease, including whether they could serve as both diagnostic and therapeutic targets to reduce non-immune kidney transplant injury and improve long-term patient and graft outcomes. Longitudinal studies in children are needed to provide important data regarding the value of FGF23 as a predictive biomarker that may allow for early diagnosis and treatment of CRAI. Given the strong association between increased FGF23 levels, CKD progression, and cardiovascular risk across the continuum of native and transplant kidney disease in adults, more studies are needed to further explore similar associations in children.

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Figure 1. Receiver operating characteristic (ROC) analysis demonstrating the performance of FGF23 as a biomarker for biopsy-proven chronic renal allograft injury (CRAI)
Area under the curve is significant at $P < 0.001$.

2b

Cutoff Value	Sensitivity	Specificity	PPV	NPV
FGF23 $\geq$ 70 pg/mL	71%	94%	92%	76%
eGFR $\leq$ 75 mL/min/1.73 m2	81%	87%	86%	82%
eGFR $\leq$ 60 mL/min/1.73 m2	52%	94%	89%	66%

Figure 2. Categorical FGF23 levels discriminate between subjects with and without chronic renal allograft injury (CRAI) with excellent sensitivity and specificity compared to eGFR

(a) FGF23 levels were dichotomized at the optimal cutoff value of 70 pg/mL, as determined by the Youden Index (dotted horizontal line). Closed circles are subjects from the CRAI group, whereas open circles are subjects from the No CRAI group. Univariable crude odds ratio (OR) plus 95% confidence interval (CI) for CRAI with FGF23 ≥ 70 pg/mL was 35.4 (6.9, 180.8), with an adjusted OR (95% CI) of 7.4 (1.2, 46.9) after adjusting for eGFR as a continuous variable. (b) Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for diagnosis of biopsy-proven CRAI with categorized FGF23, categorized eGFR overall median 76 mL/min/1.73 m², and categorized eGFR ≥ 60 mL/min/1.73 m².

3a

Figure 3. FGF23 levels are elevated in all 3 subtypes of chronic renal allograft injury (CRAI) FGF23 levels were compared for interstitial fibrosis/tubular atrophy (IF/TA), transplant glomerulopathy (TG) and allograft vasculopathy (AV) to the No CRAI group. Since many subjects had overlapping subtypes of CRAI that were not mutually exclusive, we could not perform direct comparisons between subtypes. **(a)** FGF23 levels are elevated in subjects with the IF/TA subtype versus the No CRAI group, but less so than in subjects with non-IF/TA subtypes of CRAI (TG and/or AV). **(b)** FGF23 levels are elevated in subjects with the TG subtype versus the No CRAI group, and more so than in subjects with non-TG subtypes of CRAI (IF/TA and/or AV). **(c)** FGF23 levels are elevated in subjects with the AV subtype versus the No CRAI group, but less so than in subjects with non-AV subtypes of CRAI (IF/TA and/or TG). In each figure, $P < 0.001$ for the trend between groups by the Kruskal-Wallis test.

Table 1**Demographic data**

Comparison of demographic variables between chronic renal allograft injury (CRAI) and No CRAI groups. Normally distributed continuous variables were compared using student's t-test and are presented as mean $\pm$ standard deviation. Non-normally distributed continuous variables were compared using the Mann-Whitney-U test and are presented as median (minimum, maximum). Categorical variables were compared using a chi-square test or Fisher's exact test.

Characteristic	CRAI (n=31)	No CRAI (n=31)	P-value
Age (yr)	13.5 $\pm$ 4.1	12.9 $\pm$ 4.4	0.64
Sex (Male/Female)	21/10	23/8	0.58
Donor type			
Living (Related or Unrelated)	18 (58.1)	7 (22.6)	0.004
Deceased	13 (41.9)	24 (77.4)	
Race (no., %)			
Caucasian	23 (74.2)	25 (80.6)	
African-American	6 (19.4)	3 (9.7)	0.53
Other	2 (6.4)	3 (9.7)	
Height (cm)	149 $\pm$ 19	150 $\pm$ 21	0.87
Body mass index (kg/m ²)	21 (15, 33)	21 (15, 42)	0.76
Plasma creatinine (mg/dL)	1.6 $\pm$ 0.8	0.84 $\pm$ 0.3	<0.001
Estimated GFR (mL/min/1.73m²)			
Traditional Schwartz or CKD-EPI	55 $\pm$ 24	110 $\pm$ 35	<0.001
CKD-Schwartz	43 $\pm$ 18	77 $\pm$ 19	<0.001
Stage of Transplant CKD^a (no., %)			
Stage 1T (>90 mL/min/1.73m ²)	1 (3.2)	26 (83.9)	
Stage 2T (60-90 mL/min/1.73m ²)	14 (45.2)	3 (9.7)	<0.001
Stage 3T/4T ^b (15-30 mL/min/1.73m ²)	16 (51.6)	2 (6.4)	
Transplant vintage (yr)	6.0 (2.0, 14.8)	3.5 (1.0, 12.0)	0.07
History of acute rejection ^c (no., %)	10 (32%)	1 (3%)	0.006
DSA at time of biopsy (no./assessed, %)	1/17 (6%)	0/11 (0%)	1.00
Peritubular capillary C4d+ (no., %)	2 (6%)	0 (0%)	0.23
Hypertension (no., %)	9 (29%)	12 (38%)	0.51
Systolic blood pressure (mmHg)	117 $\pm$ 16	111 $\pm$ 15	0.27
Diastolic blood pressure (mmHg)	75 $\pm$ 9	67 $\pm$ 9	0.01
Calcineurin inhibitor use (Tac/CsA)	30/0	29/2	0.18
Antimetabolite use (MMF/Aza)	29/2	29/2	1.00
Sirolimus use (no., %)	1 (3%)	0 (0%)	1.00
Prednisone use (no., %)	22 (71%)	12 (38%)	0.01
Iron supplementation (no., %)	2 (6%)	2 (6%)	1.00
Calcitriol use (no., %)	2 (6%)	0 (0%)	0.24

Characteristic	CRAI (n=31)	No CRAI (n=31)	P-value
Total plasma calcium (mg/dL)	9.5 ± 0.4	9.6 ± 0.4	0.33
Plasma phosphorus (mg/dL)	4.0 ± 0.8	4.1 ± 0.6	0.55
Calcium × phosphorus product (mg ² /dL ²)	38 ± 8	39 ± 7	0.43

^aStage of CKD-T is based on estimated GFR (traditional Schwartz calculation) for subjects aged < 18 years and CKD-EPI calculation for subjects 18 years.

^bStage 3T/4T were combined into a single subgroup because of relatively few subjects with CKD 4T.

^cHistory of acute rejection includes both acute cellular and antibody-mediated rejection.

Table 2
Comparison of plasma chronic kidney disease-mineral bone disorder (CKD-MBD) factors between chronic renal allograft injury (CRAI) and No CRAI groups

Non-normally distributed continuous variables are presented as median (minimum, maximum). **(a)** Comparison of CRAI versus No CRAI groups by Mann-Whitney U test. **(b)** Comparison of biopsies with each subtype of CRAI [interstitial fibrosis/tubular atrophy (IF/TA), transplant glomerulopathy (TG), and allograft vasculopathy (AV)] versus the No CRAI group by Mann-Whitney U test. Many subjects had biopsies with more than one subtype of CRAI, so comparisons between subtypes of CRAI were not performed.

2a)				
Outcome Variable	Total cohort (n=62)	CRAI (n=31)	No CRAI (n=31)	P-value^a
Plasma FGF23 (pg/mL)	58 (8, 475)	106 (10, 475)	45 (8, 91)	<0.001
Plasma soluble klotho (pg/mL)	1728 (546, 5947)	1715 (546, 2618)	1767 (577, 5947)	0.03
Plasma DKK1 (pg/mL)	744 (31, 3346)	688 (31, 3346)	780 (345, 3152)	0.33
Plasma sclerostin (pg/mL)	353 (132, 967)	387 (166, 967)	353 (132, 798)	0.73
Plasma activin-A (pg/mL)	424 (182, 1115)	435 (207, 653)	405 (182, 1115)	0.54

2b)				
Outcome Variable	No CRAI (n=31)	Biopsies with IF/TA (n=18)	Biopsies with TG (n=17)	Biopsies with AV (n=9)
Plasma FGF23 (pg/mL)	45 (8, 91)	68 (10, 355) **	147 (40, 475) ***	91 (46, 119) ***
Plasma soluble klotho (pg/mL)	1767 (577, 5947)	1265 (546, 2618) *	1719 (897, 1783)	1153 (546, 2254) *
Plasma DKK1 (pg/mL)	780 (345, 3152)	698 (279, 3346)	630 (31, 1125)	944 (341, 3346)
Plasma sclerostin (pg/mL)	353 (132, 798)	391 (200, 967)	397 (166, 624)	500 (213, 967)
Plasma activin-A (pg/mL)	405 (182, 1115)	435 (207, 653)	407 (207, 449)	490 (412, 653)

or 0.05 and 2009 f CRAI levels and OGFR for each subject did not alter the beta coefficients of our regression analyses, so those at

^a Comparison of CRAI and No CRAI groups by Mann-Whitney U test.

* P < 0.05

** P < 0.01

*** P < 0.001

Table 3
Comparison of plasma chronic kidney disease-mineral bone disorder (CKD-MBD) factors
between stages of CKD-T

Non-normally distributed continuous variables are presented as median (minimum, maximum).

Outcome Variable	CKD 1T (n=27)	CKD 2T (n=17)	CKD 3T/4T (n=18)	P-value ^a
Plasma FGF23 (pg/mL)	41 (8, 82)	64 (20, 137)	141 (10, 475)	<0.001
Plasma soluble klotho (pg/mL)	2073 (577, 5947)	1678 (799, 2093)	1710 (546, 2254)	0.04
Plasma DKK1 (pg/mL)	793 (345, 3152)	650 (31, 3346)	665 (31, 2400)	0.28
Plasma sclerostin (pg/mL)	390 (132, 798)	423 (166, 967)	358 (200, 525)	0.23
Plasma activin-A (pg/mL)	413 (182, 1115)	433 (207, 675)	452 (425, 653)	0.57

^a Comparison across stages of CKD using Kruskal-Wallis test.

Table 4
Multivariable modeling of exposures associated with chronic renal allograft injury (CRAI)

(a) Univariable and multivariable logistic regression model of exposures associated with CRAI. **(b)** Sensitivity analyses of univariable and multivariable logistic regression modeling of exposures associated with CRAI.

4a)						
Parameter	Univariable OR (95% CI)	P-value	Multivariable OR (95% CI)^a	P-value	Multivariable OR (95% CI)^b	P-value
Each 5 pg/mL increase in plasma FGF23	1.26 (1.19, 1.35)	<0.001	1.43 (1.23, 1.67)	0.02	1.32 (1.19, 1.46)	0.01
Each 1 mg/dL increase in traditional Schwartz eGFR	0.93 (0.90, 0.96)	<0.001	0.96 (0.92, 1.002)	0.06		
Prednisone use (current)	3.87 (1.34, 11.17)	0.01				
History of acute rejection	14.29 (1.70, 120.20)	0.01	1084.11 (8.41, 139717.90)	0.005		
Plasma phosphorus (mg/dL)	0.77 (0.33, 1.80)	0.54				
Plasma soluble klotho (pg/mL)	0.999 (0.998, 1.000)	0.03				
Plasma DKK1 (pg/mL)	1.00 (0.99, 1.00)	0.32				
Plasma sclerostin (pg/mL)	0.99 (0.99, 1.00)	0.50				
Plasma activin-A (pg/mL)	1.00 (0.99, 1.01)	0.32				
Age (yrs)	1.04 (0.90, 1.20)	0.63			0.95 (0.77, 1.17)	0.65
Female sex (male as reference category)	1.37 (0.46, 4.12)	0.58			3.07 (0.55, 17.17)	0.20
DD donor type (LD as reference category)	0.21 (0.07, 0.64)	0.006	0.02 (0.001, 0.75)	0.03	0.09 (0.01, 0.65)	0.02
Transplant vintage (yrs)	1.12 (0.96, 1.30)	0.14			1.06 (0.79, 1.40)	0.71
4b)						
Parameter	Univariable OR (95% CI)	P-value	Multivariable OR (95% CI)^c	P-value	Multivariable OR (95% CI)^d	P-value
Each 5 pg/mL increase in plasma FGF23	1.23 (1.15, 1.31)	0.001	1.14 (1.05, 1.24) ^e	0.12	1.23 (1.12, 1.34)	0.02
Each 1 mg/dL increase in traditional Schwartz eGFR	0.94 (0.92, 0.97)	<0.001	0.96 (0.92, 1.001)	0.06		
Prednisone use (current)	3.20 (1.12, 9.15)	0.03	0.61 (0.10, 3.92)	0.60		
History of acute rejection	9.62 (1.15, 80.72)	0.04	40.45 (1.24, 1322.11)	0.04		
Plasma soluble klotho (pg/mL)	0.999 (0.998, 1.000)	0.02	0.999 (0.998, 1.001)	0.34		
Age (yrs)	1.01 (0.88, 1.16)	0.89			0.97 (0.82, 1.14)	0.71
Female sex (male as reference category)	0.87 (0.29, 2.62)	0.80			1.10 (0.24, 4.98)	0.90
DD donor type (LD as reference category)	0.37 (0.12, 1.09)	0.07	0.80 (0.12, 5.31)	0.82	0.41 (0.09, 1.91)	0.25
Transplant vintage (yrs)	1.14 (0.97, 1.35)	0.10			1.16 (0.92, 1.46)	0.22

^aMultivariable model with forward stepwise entry of significant parameters (P<0.10) from univariable model (FGF23, eGFR, current prednisone use, history of acute rejection, soluble klotho, and donor type). Soluble klotho and current prednisone use were excluded from the final model that was the best fit for the data.

^b Multivariable model with forced entry of FGF23, age, sex, donor type and transplant vintage.

* eGFR refers to traditional Schwartz calculation (subjects aged < 18 years) or CKD-EPI calculation (≥ 18 years).

^c Multivariable model with forced entry of significant parameters ($P < 0.10$) from univariable model.

^d Multivariable model with forced entry of age, sex, donor type and transplant vintage.

^e Not statistically significant at the $P < 0.05$ level as the odds ratio (OR) and 95% confidence interval (CI) for each 1 pg/mL increase in FGF23 includes 1.00, but modifying the OR to reflect each 5 pg/mL increase in FGF23 excludes 1.00 from the 95% CI as an arithmetic artifact.