Renal Transplant Injury and the Renin-Angiotensin System in Kids (RETASK)
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Abstract
Kidney disease is a major source of childhood morbidity and mortality. Kidney transplants improve quality of life and life expectancy. Renal graft injury is a significant problem and early identification is critical. No biomarkers exist to identify subclinical injury. Biopsies are the gold standard but are invasive and can miss early injury. The renin-angiotensin system (RAS) is activated in renal injury. The alternative RAS pathway ACE2/Ang-(1-7)/mas is important in counteracting these harmful effects. The components of these pathways are excreted in the urine and are altered in renal injury. The aims of this proposal are to develop noninvasive biomarkers of 1) early and 2) chronic subclinical graft injury. The project was an observational study with a convenience sample of children undergoing renal transplantation. The profile of these pathways in the urine will be compared to outcomes of 1) renal biopsy-confirmed early graft injury and 2) chronic graft injury using Cortical Fractional Interstitial Fibrosis Volume (V_{IntFib}) on biopsy specimens. The components of this pathway are novel potential biomarkers and have not been previously evaluated in this population. The findings of this proposal may enable more prompt diagnosis and more effective treatment of renal graft injury in children.

Specific Aims
In pediatric kidney transplant patients, rejection, medication toxicity and ischemia cause early and chronic renal allograft injury, which reduces graft lifespan and patient survival\(^1\). Early identification of injury would facilitate prevention and treatment. The gold standard surveillance biopsy has limitations including delayed discovery of injury\(^2,3\). No noninvasive test identifies graft injury before it is clinically apparent\(^4,5,6\). This project’s goal is to develop a novel early marker of subclinical graft injury to facilitate prompt recognition and treatment. Kidney damage activates the renin-angiotensin system (RAS), and the ACE2/Ang-(1-7)/mas pathway counteracts this damage\(^7\). The balance, or ratio, between levels of RAS and the ACE2 pathway may be clinically important because the ACE2 pathway counteracts RAS-mediated injury. An increase in ACE and Ang II expression and a decrease in ACE2 and Ang-(1-7) expression on tubular cells may promote renal injury. Tubular damage may increase urinary loss of protective ACE2 and Ang-(1-7), propagating renal damage by allowing ACE and Ang II to stimulate inflammation and fibrosis unopposed.

**Aim 1:** Diagnose early subclinical graft injury in renal transplant patients with the ratios from ACE2 to ACE and from Ang-(1-7) to Ang II in urine.
**Hypothesis 1:** A shift in the urinary ACE2 to ACE and Ang-(1-7) to Ang II ratios towards ACE2 and Ang-(1-7) predicts early subclinical graft injury diagnosed on renal biopsy.
Aim 2: Diagnose chronic subclinical graft injury using the ratios of ACE2 to ACE and Ang-(1-7) to Ang II in the urine.

Hypothesis 2: A shift in the ACE2 to ACE and Ang-(1-7) to Ang II ratios in the urine towards ACE2 and Ang-(1-7) predicts chronic subclinical graft injury on renal biopsy.

Background/Significance
Angiotensin-converting enzyme (ACE) cleaves angiotensin (Ang) I into Ang II, which operates through the Ang II type 1 receptor (AT$_1$). The ACE2/Ang-(1-7)/mas pathway is an alternative, less characterized pathway that operates through the mas receptor$^8$. ACE2 cleaves Ang II into Ang-(1-7) (figure 1). The exact mechanism of action of Ang-(1-7) is unknown but most data support its anti-inflammatory, anti-fibrotic and immune modulatory effects$^9$-$^{17}$. ACE2, Ang-(1-7) and mas expression is reciprocal to ACE, Ang II and AT$_1$ expression$^{10,12}$. In the kidney, expression of ACE2 and Ang-(1-7) is greatest in the proximal tubules, especially the brush border membrane$^7,10,12,18,19$. The balance between the ACE and ACE2 pathways is critical in preventing renal fibrosis$^7,10,12$. Ang-(1-7) directly blocks Ang II and Ang II’s downstream effects through the AT$_1$ receptor$^{10,14,16}$-$^{27}$. ACE inhibitors (ACEi) and AT$_1$ blockers (ARB) reduce proteinuria and graft injury and improve graft function, independent of blood pressure$^{32,37,38}$. The ACE2 pathway may be responsible for these effects because it is enhanced at the expense of the RAS$^{32,37-42}$.

Tubulointerstitial damage decreases ACE2 expression, enhancing ACE and propagating injury$^{43,44}$. Urine ACE2 protein is elevated in renal transplant patients, independent of serum levels$^{45,46}$. These findings suggest that tubular damage causes shedding of ACE2 and Ang-(1-7) from the apical membrane of tubular epithelial cells into the tubular lumen and urine. Urinary excretion of ACE2 and Ang-(1-7) is altered in relation to ACE and Ang II. The ratios of ACE2/ACE and Ang-(1-7)/Ang II in urine could be noninvasive markers of subclinical graft injury. It is important to evaluate the balance between the ACE and ACE2 pathways because increased ACE2 could be due to increased expression of ACE2 or decreased ACE. ACE2/Ang-(1-7)/mas directly alters renal inflammation, making it an intriguing biomarker and therapeutic target.

Approach/Methods
This was an observational study with a convenience sample of patients recruited from Lucile Packard Children’s Hospital’s kidney transplant evaluation clinic from 2013 to 2015; 29 subjects completed the study. The recipient ages ranged 1 to 20 years and 34% were living donors. Stanford University School of Medicine IRB approval was obtained (IRB 5136) and informed consent and assent were obtained. Data was collected from information gathered per standard of care. Baseline and post-transplant data included age, sex, self-reported race (Black, White, Hispanic, Asian, or Other), presence of obesity (body mass index $\geq$95th percentile for age and sex), primary cause of end stage renal disease, dialysis modality, time on dialysis, presence of hypertension, use of ACEi/ARB, presence
of left ventricular hypertrophy, sensitization at transplant, creatinine, estimated glomerular filtration rate (eGFR) by modified Schwartz equation and urine creatinine and protein. Post-transplant data included deceased versus living donor transplant, antigen match, cold ischemia time, immunosuppression including drug levels, and protocol, and indication biopsy results. Specific to the study, plasma and urine protein ACE, ACE2, Ang II, and Ang-(1-7) were collected and analyzed. Data was collected at baseline and post-operatively at 12 hours, 24 hours, daily for 1 week, weekly for 1 month and monthly until 6 months post-transplant. Samples were collected at any clinically significant event concerning for graft injury, including a rise in creatinine, rejection, medication toxicity, and infection.

Subjects underwent a six-month surveillance protocol biopsy and some received three-month surveillance protocol or indication biopsies per standard of care. For the study, existing biopsy tissue was stained with Sirius Red, providing a measure of fibrosis called \( V_{IntFib} \) that was quantified by computerized image analysis\(^{47} \). Fibrosis is an established measurement of chronic graft injury. ACE, ACE2, Ang II, and Ang-(1-7) protein were measured by radioimmunoassays at the Hypertension and Vascular Research Biomarker Analytic Core at Wake Forest School of Medicine. All data, including protected health information, was stored securely on a HIPAA-compliant REDCap Database hosted by Stanford University School of Medicine. Summary statistics, including frequencies for categorical variables and mean with standard deviation (SD) or median with interquartile range for continuous variables will describe clinical and demographic characteristics. Graphical exploration will assess the data and quantile plots will evaluate the need for log transformation. Measurements below the lower limit of detection of the laboratory will be assigned a value of the lower limit of detection divided by the square root of two. Spearman rank correlation coefficients will be used to assess the correlation between ACE2, ACE, Ang-(1-7), Ang II and the continuous covariates and outcomes. We will estimate sensitivity, specificity, and the area under the receiver operator characteristics curve to assess the clinical predictive value of the biomarkers.

Primary outcome for Aim 1: Early subclinical graft injury.
Cox regression will be used to estimate hazard ratios for the association of biomarker levels with the outcome biopsy-proven graft injury, adjusting for potential confounders. Robust standard errors and clustering by subject ID for patients diagnosed with more than one episode of graft injury during the study period will be used. The time axis will be six months, starting at the time of transplant or time of recovery from the preceding acute injury event, and ending at six months post-transplant. Observations will be right-censored at the time of graft loss, loss to follow up, or withdrawal from the study. The model will be compared to other models that include the time dependent-covariates serum creatinine and eGFR.

Primary outcome for Aim 2: Chronic subclinical graft injury.
Cox regression will be utilized to estimate hazard ratios for the association of biomarker levels with the outcome biopsy-proven graft fibrosis, adjusting for potential confounders. The time axis will begin at the time of transplant and end at six months post-transplant. Observations will be right-censored at the time of graft loss, loss to follow up, or withdrawal from the study. The model will be compared to other models that include the time dependent-covariates serum creatinine and eGFR.

**Missing Data**
Subjects may miss lab collections or may be lost to follow up. However the reasons for missing data are presumed to be independent of the values of the predictors ACE, ACE2, Ang II, and Ang-(1-7). Clinical data including covariates and outcomes will be recorded even if a subject is lost to follow up. Therefore missing data will be presumed to be missing completely at random. We chose to handle missing covariate values by multiple imputations due to the longitudinal data and high rate of missing data (23%).

**Power**
The study was estimated to enroll 30 subjects (final enrollment 29), based on anticipated subjects and estimated incidence of kidney injury (10% in first six months post-transplant). The study was powered to detect a Pearson correlation of 0.56 with a two-sided \( p \) value of 0.025.

**References**


