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, 18-27]. 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 ≥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.

<u>Primary outcome for Aim 1: Early subclinical graft injury.</u>
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.

<u>Primary outcome for Aim 2: Chronic subclinical graft injury.</u>

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

1. Renders L, Heemann U. Chronic renal allograft damage after transplantation: what are the reasons, what can we do? Current Opinion in Organ Transplantation. 2012; **17**(6): 634-9.
2. Furness PN, Taub N. International variation in the interpretation of renal transplant biopsies: Report of the CERTPAP Project. Kidney International. 2001; **60**: 1998-2012.
3. Solez K, Colvin RB, Racusen LC, Haas M, Sis B, Mengel M, et al. Banff 07 Classification of Renal Allograft Pathology: Updates and Future Directions. American Journal of Transplantation. 2008; **8**(4): 753-60.
4. Halawa A. The early diagnosis of acute renal graft dysfunction: A challenge we face. The role of novel biomarkers. Ann Transplant. 2011; **16**(1): 90-8.
5. Hall IE, Yarlagadda SG, Coca SG, et al. IL-18 and urinary NGAL predict dialysis and graft recovery after kidney transplantation. J Am Soc Nephrol. 2010; **21**: 189-97.
6. Schaub S, Mayr M, Honger G, et al. Detection of subclinical tubular injury after renal transplantation: comparison of urine protein analysis with allograft histopathology. Transplantation. 2007; **84**(1): 104-12.
7. Rüster C, Wolf G. Renin-angiotensin-aldosterone system and progression of renal disease. J Am Soc Nephrol. 2006; (17): 2985-91.
8. Iwai M, Horiuchi M. Devil and angel in the renin-angiotensin system: ACE-angiotensin II-AT1 receptor axis vs. ACE2-angiotensin-(1-7)-Mas receptor axis. Hypertens Res. 2009; (32): 533-6.
9. Ferrario CM. ACE2: More Ang-(1-7) or less Ang II? Curr Opin Nephrol Hypertens. 2011; (20): 1-6.
10. Ferrario CM, Varagic J. The Ang-(1-7)/ACE2/mas axis in the regulation of nephron function. Am J Physiol Renal Physiol. 2010; (298): F1297-305.
11. Santos RAS, Ferreira AJ, Pinheiro SVB, et al. Angiotensin-(1-7) and its receptor as potential targets for new cardiovascular drugs. Expert Opin Investig Drugs. 2005; (14): 1019-31.
12. Santos RAS, Ferreira AJ, Simões e Silva AC. Recent advances in the angiotensin-converting enzyme2-angiotensin(1-7)-Mas axis. Exp Physiol. 2008; (93.5): 519-27.

13. Dilauro M, Burns KD. Angiotensin-(1-7) and its effects in the kidney. Scien World J. 2009; (9): 522-35.
14. Heringer-Walther S, Eckert K, Schumacher S-M, Uharek L, Wulf-Goldenberg A, Gembardt F, et al. Angiotensin-(1–7) stimulates hematopoietic progenitor cells in vitro and in vivo. Haematologica. 2009; **94**(6): 857-60.
15. Geara AS, Azzi J, Jurewicz M, Abdi R. The renin-angiotensin system: an old, newly discovered player in immunoregulation. Transplantation Reviews. 2009; **23**(3): 151-8.
16. Miyake-Ogawa C, Miyazaki M, Abe K, et al. Tissue-specific expression of renin-angiotensin system components in IgA nephropathy. Am J Nephrol. 2005; (25): 1-12.
17. Jurewicz M, McDermott DH, Sechler JM, Tinckam K, Takakura A, Carpenter CB, et al. Human T and Natural Killer Cells Possess a Functional Renin-Angiotensin System: Further Mechanisms of Angiotensin II–Induced Inflammation. Journal of the American Society of Nephrology. 2007; **18**(4): 1093-102.
18. Su Z, Zimpelmann J, Burns KD. Angiotensin-(1-7) inhibits angiotensin II-stimulated phosphorylation of MAP kinases in proximal tubular cells. Kidney Int. 2006; (69): 2212-8.
19. Moon JY. ACE2 and angiotensin-(1-7) in hypertensive renal disease. Electrolyte Blood Press. 2011; (9): 41-4.
20. Ahmed AK, El Nahas AM, Johnson TS. Changes in matrix metalloproteinases and their inhibitors in kidney transplant recipients. Experimental and Clinical Transplantation. 2012; **10**(4): 332-43.
21. Castoldi G, Di Gioia CRT, Travaglini C, Busca G, Redaelli S, Bombardi C, et al. ANGIOTENSIN II INCREASES TISSUE-SPECIFIC INHIBITOR OF METALLOPROTEINASE-2 EXPRESSION IN RAT AORTIC SMOOTH MUSCLE CELLS IN VIVO: EVIDENCE OF A PRESSURE-INDEPENDENT EFFECT. Clinical and Experimental Pharmacology and Physiology. 2007; **34**(3): 205-9.
22. Mazanowska O, Kamińska D, Krajewska M, Żabińska M, Kopeć W, Boratyńska M, et al. Imbalance of Metallaproteinase/Tissue Inhibitors of Metalloproteinase System in Renal Transplant Recipients With Chronic Allograft Injury. Transplantation Proceedings. 2011; **43**(8): 3000-3.
23. Ling XB, Sigdel TK, Lau K, Ying L, Lau I, Schilling J, et al. Integrative Urinary Peptidomics in Renal Transplantation Identifies Biomarkers for Acute Rejection. Journal of the American Society of Nephrology. 2010; **21**(4): 646-53.
24. Lutz J, Yao Y, Song E, Antus B, Hamar P, Liu S, et al. Inhibition of Matrix Metalloproteinases During Chronic Allograft Nephropathy in Rats. Transplantation. 2005; **79**(6): 655-61.
25. Pan C-H, Wen C-H, Lin C-S. Interplay of angiotensin II and angiotensin(1–7) in the regulation of matrix metalloproteinases of human cardiocytes. Experimental Physiology. 2008; **93**(5): 599-612.
26. Clark MA, Tallant EA, Diz DI. Downregulation of the At1A receptor by pharmacologic concentrations of angiotensin-(1-7). J Cardiovasc Pharmacol. 2001; (37): 437-48.
27. Brosnihan KB, Neves LAA, Joyner J, et al. Enhanced renal immunocytochemical expression of Ang-(1-7) and ACE2 during pregnancy. Hypertens. 2003; (42(2)): 749-53.
28. Koka V, Huang XR, Chung AC, et al. Angiotensin II up-regulates angiotensin I-converting enzyme (ACE), but down-regulates ACE2 via the AT1-ERK/p38 MAP kinase pathway. Am J Pathol. 2008; (172): 1174-83.
29. Dong J, Wong SL, Lau CW, Lee HK, Ng CF, Zhang L, et al. Calcitriol protects renovascular function in hypertension by down-regulating angiotensin II type 1 receptors and reducing oxidative stress. European Heart Journal. 2012; **33**(23): 2980-90.
30. Dai B, David V, Martin A, Huang J, Li H, Jiao Y, et al. A Comparative Transcriptome Analysis Identifying FGF23 Regulated Genes in the Kidney of a Mouse CKD Model. PLoS ONE. 2012; **7**(9): e44161.
31. Ejaz AA, Kambhampati G, Ejaz NI, et al. Post-operative serum uric acid and acute kidney injury. J Nephrol. 2012; **25**(4): 497-505.
32. El-Agroudy AE, Hassan NA, Foda MA, Ismail AM, El-Sawy EA, Mousa O, et al. Effect of Angiotensin II Receptor Blocker on Plasma Levels of TGF-β1 and Interstitial Fibrosis in Hypertensive Kidney Transplant Patients. American Journal of Nephrology. 2003; **23**(5): 300-6.

33. Haririan A, Metireddy M, Cangro C, Nogueira JM, Rasetto F, Cooper M, et al. Association of Serum Uric Acid With Graft Survival After Kidney Transplantation: A Time-Varying Analysis. American Journal of Transplantation. 2011; **11**(9): 1943-50.
34. Kanellis J, Watanabe S, Li JH, Kang DH, Li P, Nakagawa T, et al. Uric Acid Stimulates Monocyte Chemoattractant Protein-1 Production in Vascular Smooth Muscle Cells Via Mitogen-Activated Protein Kinase and Cyclooxygenase-2. Hypertension. 2003; **41**(6): 1287-93.
35. Numakura K, Satoh S, Tsuchiya N, Saito M, Maita S, Obara T, et al. Hyperuricemia at 1 Year After Renal Transplantation, Its Prevalence, Associated Factors, and Graft Survival. Transplantation. 2012; **94**(2): 145-51.
36. Feig DI. Hyperuricemia and Hypertension. Advances in Chronic Kidney Disease. 2012; **19**(6): 377-85.
37. Szabo A, Lutz J, Schleimer K, et al. Effect of angiotensin-converting enzyme inhibition on growth factor mRNA in chronic renal allograft rejection in the rat. Kidney International. 2000; **57**: 982-91.
38. Tylicki L, Biedunkiewicz B, Chamienia A, Wojnarowski K, Zdrojewski Z, Aleksandrowicz E, et al. Renal Allograft Protection with Angiotensin II Type 1 Receptor Antagonists. American Journal of Transplantation. 2007; **7**(1): 243-8.
39. Luque M, Martin P, Martell N, et al. Effects of captopril related to increased levels of prostacyclin and angiotensin-(1-7) in essential hypertension. Hypertens. 1996; (14): 799-805.
40. Shah P, Kute V, Saboo D, Vanikar A, Patel H, Trivedi H, et al. Safety and efficacy of angiotensin converting enzyme inhibitors and angiotensin receptor blockers in chronic allograft injury; 2011.
41. Arbeiter K, Pichler A, Stemberger R, et al. ACE inhibition in the treatment of children after renal transplantation. Pediatric Nephrology. 2004; **19**(2): 222-6.
42. Oudit GY, Herzenberg AM, Kassiri Z, et al. Loss of angiotensin-converting enzyme-2 leads to the late development of angiotensin II-dependent glomerulosclerosis. Am J Pathol. 2006; (168): 1808-20.
43. Mizuiri S, Hemmi H, Arita M, et al. Expression of ACE and ACE2 in individuals with diabetic kidney disease and healthy controls. Am J Kidney Dis. 2008; (51): 613-23.
44. Mizuiri S, Hemmi H, Arita M, et al. Increased ACE and decreased ACE2 expression in kidneys from patients with IgA nephropathy. Nephron Clin Pract. 2011; (117): c57-66.
45. Mizuiri S, Aoki T, Hemmi H, et al. Urinary angiotensin-converting enzyme 2 in patients with CKD. Nephrol. 2011; (16): 567-72.
46. Xiao F, Hiremath S, Knoll G, et al. Increased Urinary Angiotensin-Converting Enzyme 2 in Renal Transplant Patients with Diabetes. PLoS One. 2012; **7**(5): e37649.
47. Grimm PC, Nickerson P, Gough J, et al. Computerized image analysis of sirius red-stained renal allograft biopsies as a surrogate marker to predict long-term allograft function. J Am Soc Nephrol. 2003; **14**: 1662-8.