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Design and Methods of the Validating Injury to the Renal Transplant Using Urinary Signatures (VIRTUUS) Study in Children

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Background. Lack of noninvasive diagnostic and prognostic biomarkers to reliably detect early allograft injury poses a major hindrance to long-term allograft survival in pediatric kidney transplant recipients. **Methods.** Validating Injury to the Renal Transplant Using Urinary Signatures Children's Study, a North American multicenter prospective cohort study of pediatric kidney transplant recipients, aims to validate urinary cell mRNA and metabolite profiles that were diagnostic and prognostic of acute cellular rejection (ACR) and BK virus nephropathy (BKVN) in adult kidney transplant recipients in Clinical Trials in Organ Transplantation-4. Specifically, we are investigating: (1) whether a urinary cell mRNA 3-gene signature (*18S*-normalized *CD3ε, CXCL10* mRNA, and *18S* ribosomal RNA) discriminates biopsies with versus without ACR, (2) whether a combined metabolite profile with the 3-gene signature increases sensitivity and specificity of diagnosis and prognostication of ACR, and (3) whether *BKV-VP1* mRNA levels in urinary cells are diagnostic of BKVN and prognostic for allograft failure. **Results** . To date, 204 subjects are enrolled, with 1405 urine samples, including 144 biopsy-associated samples. Among 424 urine samples processed for mRNA, the median A260:280 ratio (RNA purity) was 1.91, comparable with Clinical Trials in Organ Transplantation-4 (median 1.82). The quality control failure rate was 10%. Preliminary results from urine supernatant showed that our metabolomics platform successfully captured a broad array of metabolites. Clustering of pool samples and overlay of samples from various batches demonstrated platform robustness. No study site effect was noted. **Conclusions.** Multicenter efforts to ascertain urinary biomarkers in pediatric kidney transplant recipients are feasible with high-quality control. Further study will inform whether these signatures are discriminatory and predictive for rejection and infection.

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INTRODUCTION

Advances in immunosuppressive regimens have significantly improved short-term allograft survival for kidney transplant recipients. Yet, long-term allograft survival remains static. As per the 2018 Scientific Registry of Transplant Recipients annual report, in the 2016–2017 cohort, the overall incidence of acute rejection within the first year was 11.4%, with some variation by age: highest for ages <6 y (12.5%) and lowest for ages 6–10 y (7.9%).[1] Adolescents with kidney transplants have a lower 5-y allograft survival than any other age group besides patients aged >65 y. Once diagnosed with rejection, they do not respond as well to treatment, exhibiting fewer complete rejection reversals and greater residual allograft dysfunction.[2] Over a lifetime, pediatric chronic kidney disease patients will require several transplants, with each transplant contributing cumulative immunological and infectious risk.

The reasons for poor pediatric allograft outcomes are likely multifactorial but are chiefly the consequences of over- or underimmunosuppression. Allograft injury occurs primarily because of acute cellular rejection (ACR) or antibody-mediated rejection (AMR) and viral infections, such as BK virus–associated nephropathy (BKVN). These allograft insults are generally detected when the serum creatinine rises; however, serum creatinine has low sensitivity and specificity for early kidney allograft damage, hindering early diagnosis and intervention. The current gold standard for diagnosing allograft injury is core needle biopsy; however, biopsies are highly invasive, incur risk of bleeding and graft loss, are subject to sampling error, and lack sensitivity and specificity for early injury.

There is a critical need to identify and characterize noninvasive markers of early histologic injury in the pediatric setting. A few studies in pediatric kidney transplant recipients have shown promise for the ability of urinary metabolomics to detect ACR, AMR, and BKVN; however, they have been limited by small sample size and lack of a diverse cohort.[3-5] The Clinical Trials in Organ Transplantation (CTOT)-04 study, a National Institutes of Health–sponsored, multicenter, prospective study of adult kidney allograft recipients in the United States, was able to diagnose and predict ACR using urinary cell mRNA and metabolite profiles with high sensitivity and specificity.[6,7] In addition, the CTOT-04 investigators validated a urinary cell mRNA signature that distinguishes acute rejection from acute tubular injury and ACR from AMR as well as a urinary cell mRNA signature diagnostic and prognostic of BKVN.[6-9] With the Validating Injury to the Renal Transplant Using Urinary Signatures Children's Study (VIRTUUS) we seek to validate these existing adult noninvasive diagnostic and prognostic biomarkers to characterize allograft injury in a large, diverse cohort of pediatric kidney allograft recipients.

MATERIALS AND METHODS

Study Design and Population

The VIRTUUS study is an observational prospective cohort study. Kidney transplant recipients aged between 2 and 18 y are eligible for inclusion. Exclusion criterion include multiorgan transplant recipients. There are 12 study sites across the United States and Canada, with 2 core laboratories for performing the study assays (Figure 1). We are recruiting 450 incident kidney allograft recipients from the participating pediatric kidney transplant centers over a period of 4 y. The study sites were chosen to represent geographically diverse regions with large pediatric transplant volumes, diverse patient populations, and

FIGURE 1. VIRTUUS Clinical Sites and Core Labs across the United States and Canada. VIRTUUS, Validating Injury to the Renal Transplant Using Urinary Signatures.

robust research infrastructure to promote the feasibility of study recruitment, retention, and reliable sample collection and provide rich diversity in urine proteomic and metabolomic samples. The study was approved by the Children's Hospital of Philadelphia Internal Review Board (#IRB17-013841).

Study Aims

The study will investigate whether the adult urinary cell 3-gene mRNA signature consisting of *18S*-normalized *CD3E* and *CXCL10* mRNAs and *18S* ribosomal RNA (*18S rRNA*) will distinguish biopsies with ACR from the biopsies without rejection. The longitudinal trajectory of the signature will also be evaluated for its ability to prognosticate ACR. Chemokines such as *CXCL10* play an important role in leukocyte trafficking and recruitment during the inflammatory response in allograft rejection. CD3-epsilon polypeptide forms the T-cell receptor–CD3 complex. *CD3E* plays an important role in signal transduction during T-cell activation in response to antigen-presenting cells. *18S rRNA* was measured in the CTOT-04 study to ensure that cells with measurable transcripts were present in the urinary cell pellet and as quality control (QC) parameters for the RNA isolated from the urine cell pellet.

We will also measure levels of *BK virus VP1 mRNA* and *plasminogen activator inhibitor-1* (*PAI-1*) mRNA and evaluate their ability to predict BKVN and BKVN-associated allograft failure. Increased levels of *PAI-1* mRNA have been associated with inflammation. *PAI-1* is thought to play an important role in the increase in extracellular matrix deposition in allografts by inhibiting plasmin that promotes fibrin degradation.

Urine metabolites will be measured in the urine supernatant and their ability to predict allograft injury will be evaluated. In addition, we will also evaluate if a combination of pediatric-specific urine metabolites and urine mRNA signatures is better able to predict allograft injury.

Study Procedures

Urine samples are collected on post-transplant days 3, 7, 15, and 30, and at months 2, 3, 4, 5, 6, 9, and 12 (longitudinal urine samples) and at the time of clinically indicated or surveillance kidney allograft biopsy as well as 2–6 wk thereafter (biopsy-matched urine samples). Urine collection will be coordinated by the study coordinator at each institution and will occur at scheduled standard of care or biopsy visits. Our protocol for urine cell pellet preparation for mRNA profiling is consistent with the protocol implemented in the CTOT-04 study.[6] Urine samples will be processed for generation of urine pellets and supernatant and will be stored locally at −80 °C. Samples will be shipped in batches at 6-mo intervals to the core mRNA and metabolomics processing laboratories.

Training of Study Coordinators and Laboratory Technicians

Study coordinators and laboratory technicians from each site were trained for the collection and processing of urine samples to generate urine cell pellets and supernatant as per the validated protocol used in the CTOT-04 study.[6] Coordinator/technician conference calls are held at regular intervals to review mRNA samples QC results and to review the site storage and processing practices to improve QC pass rates for the urine cell pellets. Training webinars, video guides, and a detailed standard operating procedures manual were developed for coordinators to ensure accurate data entry into a Research Electronic Data Capture database. Additional training of site coordinators was also provided by individual site principal investigators to ensure the accuracy of data and interpretations of clinical reports. Ongoing training and education of data entry are provided during coordinator meetings.

Clinical Data

Data on key covariates and demographics of interest are collected for each participant and will be entered by the research coordinator at each site in a secure Research Electronic Data Capture database at regular intervals. Table 1 reflects the pertinent clinical and demographics data collection time points over the study period across all sites.

TABLE 1.

Clinical and demographic data collection for VIRTUUS study

	Days posttransplant				Months posttransplant							If biopsy done	
	3	7	15	30	2	3	4	5	6	9	12	At time of biopsy	2 wk post biopsy
Urine collection	•	•	•	•	•	•	•	•	•	•	•	•	•
Demographics: age at transplant, sex, race, ethnicity	•												
ESKD data: cause of ESKD	•												
Transplant data													
Transplant#, donor type	•												
HLA match, PRA, EBV, and CMV donor/recipient status	•												
Induction + baseline immunosuppression	•												
Clinical data													
Serum creatinine and height	•	•	•	•	•	•	•	•	•	•	•	•	•
Maintenance immunosuppression	•	•	•	•	•	•	•	•	•	•	•	•	•
Tacrolimus or sirolimus level	•	•	•	•	•	•	•	•	•	•	•	•	•
Viral surveillance													
EBV, CMV, and BKV PCR (per each site's protocol)				•		•			•	•	•		
Rejection data													
Rejection yes/no; (biopsy data form if yes)												•	
Posttransplant donor-specific antibodies				•		•			•		•	•	

BKV, BK virus; CMV, cytomegalovirus; EBV, Epstein-Barr virus; ESKD, end-stage kidney disease; PRA, panel reactive antibodies; PCR, polymerase chain reaction; VIRTUUS, Validating Injury to the Renal Transplant Using Urinary Signatures.

Renal Allograft Biopsies

Biopsies will only be performed where clinically indicated ("for cause") or where they are part of the standard of care ("surveillance biopsy") for a given site. Allograft biopsy specimens are reviewed by each center's pathologist using the latest Banff classification in existence. All urine-matched biopsy samples will be collected on the day of biopsy and no later than 72 h after biopsy (but will occur before any treatment is given). Using a standardized biopsy data collection form, information will be entered by the research coordinator at each center. Data will be collected on the reason for biopsy, adequacy of the biopsy specimen, and recording of the biopsy results (for adequate biopsies) using the Banff classification. In case of ambiguities, clarification will be sought from the pathology team by the site principal investigator. The pathologist and research coordinator at each site will be blinded to the results of the mRNA and metabolome profiles.

In the adult CTOT-4 study, rejection was defined using the Banff classification prevalent at that time. All grades of ACR, starting from1A and higher were included in the rejection cohort. Additional analyses were done in the CTOT-4 study to evaluate the specificity of the 3-gene signature and its ability to discriminate between different grades of ACR. Given the lag time of research and analysis and changes in Banff over time, we also propose to use whatever Banff classification is in existence at the time of final analysis and biopsies with a definitive diagnosis of acute cellular or AMR will be used for ascertaining associations with mRNA and metabolomic profiles. If sample size allows, the urinary levels of mRNA and diagnostic signature score will be compared between those with borderline changes on the biopsy and confirmed ACR and between different grades of the Banff classification of ACR.

Whole-Urinary Urine Metabolomics Methodology and Processing

Sample Preparation and Data Acquisition

Urine samples will be thawed, prepared, and analyzed randomly as previously described.[10] Briefly, hydrophobic and hydrophilic metabolites are prepared by adding 3-fold water and acetonitrile spiked-in with 16 internal standards, respectively. Samples will be centrifuged at 20 000*g* for 10 min at 4 °C and analyzed using hydrophilic interaction liquid chromatography (HILIC) and reversed-phase liquid chromatography (RPLC) separation in both positive and negative ionization modes. Data are acquired on a Thermo Q Exactive HF mass spectrometer for HILIC and a Thermo Q Exactive mass spectrometer for RPLC. Both instruments are equipped with a heated electrospray ionization-II probe and operated in full mass spectrophotometry (MS) scan mode. MS/MS data are acquired on a pool sample (QC) consisting of an equimolar mixture of all samples in the study. HILIC experiments are performed using a ZIC-HILIC column 2.1 × 100 mm, 3.5 µm, 200 Å (Merck Millipore) and mobile-phase solvents consisting of 10 mmol/L ammonium acetate in 50/50 acetonitrile/water (a) and 10 mmol/L ammonium acetate in 95/5 acetonitrile/water (b). RPLC experiments are performed using an Hypersil GOLD column 2.1 × 150 mm, 1.9 µm, 175 Å (Thermo) and mobile-phase solvents consisting of 0.06% acetic acid in water (a) and 0.06% acetic acid in methanol (b). Data quality are ensured by (1) injecting 6 and 12 pool samples to equilibrate the liquid chromatography–mass spectrometry system before running the sequence for RPLC and HILIC, respectively; (2) injecting a pool sample every 10 injections to control for signal deviation with time; and (3) checking mass accuracy, retention time, and peak shape of internal standards in each sample.

Data Processing

Data from each mode are independently analyzed using Progenesis QI software (v2.3) (Nonlinear Dynamics). Metabolic features from blanks and those with insufficient linearity upon dilution in QC samples ($r < 0.6$) are discarded. Only metabolic features present in >2/3 of the samples are kept for further analysis. Inter- and intrabatch variation are corrected using systematic error removal using random forest normalization.[5] Differential dilution across samples are corrected using probabilistic quotient normalization.[11] Data are acquired in 8 batches. Missing values are imputed by drawing from a random distribution of low values in the corresponding sample. Data from each mode are merged and metabolites are putatively identified by matching fragmentation spectra and retention time to analytical-grade standards when possible or matching experimental MS/MS to fragmentation spectra in publicly available databases. Structural characterization of metabolites is obtained using ClassyFire classification system.

Urine mRNA Processing

Urine cell pellets will be used for mRNA profiling. Urinary cell mRNA transcripts for *CD3ε*, *CXCL10*, *TGFβ1*, *BKV VP1*, *PAI-1*, and *18S rRNA* will be quantified with the validated preamplification-enhanced real-time quantitative polymerase chain reaction assays as described previously.[6] The results will be reported as mRNA copies per microgram of total RNA and *18S rRNA* normalized copies per microgram of total RNA. Transforming growth factor-beta1 (*TGFβ*1) mRNA and *18S rRNA* were measured in the adult CTOT-04 study to ensure that cells with measurable transcripts were present in the urinary cell pellet and as QC parameters for the RNA isolated from the urine cell pellet. A urinary cell pellet sample will be classified as QC passed if the *18S rRNA* copy number is $\geq 5 \times 10^7$/µg of total RNA and its *TGFβ1* mRNA copy number is ≥100 copies/µg of RNA. The staff members at the laboratory will be unaware of the clinical information, including the results of kidney allograft biopsies.

Statistical Analyses

Our first hypothesis is that the 3-gene diagnostic signature, composed of 18S-normalized *CD3ε* and *CXCL10* mRNAs and *18S rRNA* in urinary cells will be (1) diagnostic and prognostic of ACR in pediatric recipients of kidney allografts and (2) able to distinguish biopsy-confirmed ACR from AMR and biopsy-confirmed ACR from acute tubular injury in pediatric recipients. Kruskal-Wallis and Mann-Whitney tests will be used to compare levels across the groups with biopsy-proven rejection, those with biopsy showing no rejection, and in those with stable allograft function. Logistic regression analysis will be used to identify whether the urinary cell mRNA levels will discriminate between biopsy specimens showing ACR versus those not showing any rejection. The ability of the diagnostic signature to discriminate ACR biopsies from no rejection biopsies will be evaluated by the area under the receiver operating characteristic curve of the fitted model, as well as sensitivity and specificity for diagnosing ACR. The ability of the model to accurately predict the probability

of an ACR will be assessed with the Hosmer-Lemeshow test. In the CTOT-04 study, a 3-gene model of 18S-normalized *CD3ε* mRNA, 18S-normalized *CXCL10* mRNA and *18S rRNA* (all logged) was the best-fitting parsimonious model yielding the following diagnostic signature: = −6.1487 + 0.8534 log10(*CD3ε/18S*) + 0.6376 log10 (*CXCL10*/18S) + 1.6464 log10(18S).[6] Our validation study will use this equation, and the diagnostic signatures Youdens cut point −1.213 that maximized the sensitivity and specificity to determine whether the 3-gene signature is diagnostic and prognostic of ACR in pediatric recipients of kidney allografts.

Our second hypothesis is that the combined metabolomics profiles (ratios of 3-sialyllactose to xanthosine) and 3-gene signature are diagnostic and prognostic of ACR in pediatric recipients. In the CTOT-04 study, the linear combination of log (3-sialyllactose/xanthosine) and the 3-gene signature that maximized the area under the receiver operating characteristic curve was: 3-gene-mRNA signature + 1.1164 × log (3SL/X).[7] In keeping with good practice for biomarker validation studies, we will determine the diagnostic and the prognostic performance of the previously developed model for the composite metabolite and the 3-gene mRNA signature. Although we will attempt to replicate this previous finding, we will avail of the whole-urinary metabolite data sets to maximize the discovery power for ACR/AMR, BKVN, and other posttransplant complications.

Finally, our third hypothesis is that *BKV VP1* mRNA levels will be diagnostic of BKVN and a 2 variable prediction model composed of urinary cell level of *PAI-1* mRNA and serum creatinine level, both measured at the time of BKVN biopsy diagnosis, will predict allograft failure.[8] A receiver operating characteristic curve analysis will be used to determine if the cutoff value of 6.5×10^5 *BKV VP1* copy number has the sensitivity and specificity to distinguish between patients with and without BKVN. Fisher exact test will be used to test the association between the BKVN status and a dichotomized *BKV VP1* mRNA level based on the above cutoff. The previously used composite score equation: −10.61 + 1.14 ln (*PAI-1/18S* rRNA) + 0.72 (serum creatinine mg/dL) will be used to determine whether the prediction model predicts graft failure during the 36 mo following BKVN biopsy diagnosis.[8] Additional logistic regression models will be created using serum creatinine, BKVN biopsy stage, urine mRNA for *BKV VP1* and *PAI-1*, and serum BKV DNA copy number to identify independent predictors of graft failure.[9]

Sample Size and Power Estimation

For testing the predictive models built using adult kidney allograft samples in pediatric subjects, with the sensitivity and specificity of the diagnostic signatures both at 80%, a sample size of 14 in the ACR and AMR group and 28 in the no ACR group would provide 98% power to detect a significant 2 × 2 association between the diagnostic signature (dichotomized at the prespecified cutoff point) and biopsy status using a 2-tailed alpha = 0.05 test. With projected samples sizes of 65 ACR and 35 AMR, this proposed study has a sample size large enough to have adequate power to achieve our goal for validating the adult signature in pediatric kidney allograft recipients.

For testing the predictive models for BKVN, built using adult kidney allograft samples in pediatric kidney allograft subjects, with the sensitivity and specificity of the diagnostic signatures both at 80%, a sample size of 14 in the BKVN group and 28 in the control group would provide 98% power to detect a significant 2 × 2 association between the diagnostic signature (dichotomized at the prespecified cutoff point) and biopsy status using a 2-tailed alpha = 0.05 test. We anticipate a sample size of 35 BKVN cases, suggesting adequate power to achieve our goal for validating the adult signature in pediatric kidney allograft recipients.

An additional goal of our data analysis would be to see if differences exist in the above associations based on the sex of participants, as studies have shown sex to be an important determinant of the metabolome as well as transplant outcomes. We do not have data on Tanner staging and pubertal status, which is a limitation, but we will include age as a covariate in the analysis to ascertain if there are age-related differences in metabolomic and mRNA profiles and outcomes.

Preliminary Results

Clinical Data

VIRTUUS study enrollment commenced in 2018. Since study onset, 204 subjects have been enrolled. One thousand four hundred five urine samples have been collected so far. Only 7 participants (3%) have withdrawn from the study. Among 204 study subjects to date, median age was 13 y (interquartile range [IQR], 8–16 y) and 54% were male. Racial distribution was 63% White, 11% Black, and 8% Asian. Hispanic ethnicity was reported by 28%. Cause of end-stage kidney disease was congenital anomalies of the kidney and urinary tract in 61%. Eight percent of the cohort had >1 transplant. Thirty-eight percent of the transplants were living donor and 62% were deceased donor transplants. An allograft biopsy was conducted for 144 patients. Of these, 39% were protocol biopsies, 43% were for elevated creatinine and concern for rejection, 6% for recurrent disease, and 7% were for follow-up after treatment for rejection. Thirty-three percent of the biopsy results were reported as abnormal, 23% as unknown, and 44% as normal.

Quality Control of Urinary Cell Pellet Samples

The centralized laboratory at Weill Cornell Medicine has processed 569 urine cell pellet samples. Total RNA was isolated and RNA purity was measured (ascertained using the ratio of absorbance at 260 and 280 nm). The median urine volume collected was 53 mL (IQR, 33–68 mL). The median A260/280 ratio (RNA purity) was 1.91 (IQR, 1.82–1.97), comparable with the CTOT-04 study being 1.82 (IQR, 1.64–1.89). A urinary cell pellet sample was classified as QC passed if the *18S rRNA* copy number was ≥5 × 107/μg of total RNA and its *TGFβ1* mRNA copy number was ≥100 copies/μg of RNA. The QC failure rate to date is 17.6%.

Metabolomics Preliminary Quality Control Data

Untargeted metabolomics analysis of urines samples resulted in the robust monitoring of 9000+ metabolic features. Two hundred sixty urine samples collected from 55 pediatric individuals have been analyzed to date. The distribution of MS signal was variable between samples because of differential dilutions. Application of probabilistic quotient normalization resulted in the correction of the dilution effect. Principal component analysis shows that pool samples (QC) cluster together and samples acquired in multiple batches overlay, demonstrating a good technical reproducibility and no batch effect, respectively. The whole-urinary metabolite preliminary QC

data demonstrate that our metabolomics pipeline successfully captured a broad array of metabolites in urine from a pediatric population. Clustering of pool samples and overlay of samples from various batches demonstrate the robustness of the platform. Urine samples collected from different sites did not show any clear site-specific batch effects, and thus, they can be analyzed together downstream for broader meta-analyses.

Hierarchical clustering shows that samples collected from the same individual tend to cluster together. This is consistent with previous studies showing high individuality of metabolic profiles.[12] In addition, urine metabolic profiles were not strongly impacted by participant age. Our optimized liquid chromatography–mass spectrometry method enabled the coverage of a wide variety of metabolites. Altogether, our preliminary analysis validates sample collection, processing, and analysis protocols for metabolomics investigation and suggests the feasibility of urine untargeted metabolomics for discovering biomarkers of kidney rejection in a pediatric cohort.

DISCUSSION

Urine biomarkers are likely more reflective of the ongoing immunological and inflammatory processes in the allograft during the process of ACR.[4,9,13-16] Studies by investigators who pioneered urine mRNA profiling have shown that accurate and noninvasive assessment of renal allograft status in adults is feasible by evaluation of urinary mRNA and metabolite signatures.[6,7] These findings that noninvasive diagnoses of ACR in adults are practical and feasible and that they can reduce biopsy-associated complications and biopsy reading-associated ambiguities have enormous implications if they can be translated into the pediatric setting. We believe that these adult urinary cell mRNA and urine supernatant metabolite biomarkers, identified in the most comprehensive well-powered prospective adult studies performed to date, once validated by the aims proposed in this study, can be translated into clinically applicable assays for the pediatric population. Because children require anesthesia and often hospitalization for biopsies, noninvasive markers have the potential for tremendously reducing patient and caregiver burden and anxiety, caregiver time off work, and healthcare utilization and provide a means to select patients most in need of invasive biopsies. Even more importantly, if noninvasive markers are able to detect early, subclinical allograft injury, there is immeasurable potential for improving long-term transplant outcomes and allowing early intervention before chronic injury.

The VIRTUUS study will rigorously evaluate and validate urinary cell mRNA and metabolomic signatures across a large, unique, diverse cohort of children of different ages and racial/ethnic backgrounds. Our investigative team has assembled an outstanding collaboration between international experts in pediatric kidney transplantation and translational research, including collaborators who conducted the successful CTOT-04 study upon which the VIRTUUS research strategy is based. As such, the VIRTUUS study team is distinctly poised to achieve its aims and gain novel insights into the relationship between immunosuppressive treatment and efficacy for preventing rejection and the sequelae of BK viremia among pediatric kidney allograft recipients.

In the process of validation, we may also identify novel signatures that are unique to pediatric kidney allograft recipients. In addition, the effects of age and sex on the immune response and metabolomic profiles can also be evaluated. Such discoveries will contribute greatly to the overall understanding of the cause and pathophysiology of kidney allograft injury and the relationship between injury and immunosuppressive treatments in children.

There are a number of unique challenges in conducting a multicenter cohort study of children. We acknowledge that urinary metabolites patterns may differ among adults and pediatric renal allograft recipient populations as up to 50% of the urinary metabolites are derived from the human microbiota that are heavily influenced by diet and other environmental perturbations. mRNA profiles predictive of allograft dysfunction might be different in children. However, we have previously studied the urine mRNA for *CD3Ɛ, CXCL 10, and 18S rRNA* cross-sectionally in a small cohort of prevalent pediatric kidney transplant recipients and found them to be elevated in patients with donor-specific antibodies and past rejection episodes, indicating similar mechanisms and pathways of immune activation in pediatric patients.[17] For these reasons, it is imperative to validate the adult findings in a large, diverse pediatric cohort.

There are also unique sampling challenges. For example, we may face difficulties obtaining urine from children who are not yet able to void independently and it may be difficult to interpret urine samples from children who have altered genitourinary tracts associated with congenital urological diseases, such as patients with vesicostomies, augmented bladders, or ileal conduits. We hope to learn more about the feasibility and interpretation of our approach by including such patients in our cohort.

Not unique to pediatrics, it is well understood that sampling issues are prevalent with metabolomics wet-lab pipelines including batch effects from collection and storage, for example, oxidation of samples (even in −80°C conditions). We have attempted to mitigate these issues by training all laboratory personnel at the sites in the urine collection, processing, and storage protocols to promote best practices for consistent sample collection and processing. The metabolomics core group has pioneered many metabolomics wet-lab analytical pipelines to control for batch effects.[18-21] Nevertheless, monthly conference calls will also specifically address any problems with sampling or processing across study sites.

Despite several challenges, the creation of this broad consortium will support future collaborative studies of large pediatric kidney transplant recipient cohorts, allowing ample sample size to evaluate other unanswered questions such as transplant outcomes based on immunosuppression protocols, comparing practice patterns of monitoring for viral infections and donor-specific antibodies across a heterogeneous pediatric kidney transplant population, and opening up opportunities for clinical trials that are notoriously challenging in pediatrics because of smaller sample sizes. Further, the VIRTUUS study will enable the initiation of a biobank for future studies of exome sequencing, cell-free DNA, RNA sequencing, small RNA sequencing proteomics, and untargeted metabolomics to provide further insight into the ongoing immunological activity and its effects at different time points in a kidney allograft.

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