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Urinary Microbiome Associated with Chronic Allograft Dysfunction in Kidney Transplant Recipients

Jennifer F. Wu, DO¹, Amutha Muthusamy, MS², Gabriel A. Al-Ghalith, BS³, Dan Knights, PhD⁴, Bin Guo, MS⁵, Baolin Wu, PhD⁵, Rory P. Remmel, PhD⁶, David P. Schladt, MS², Maria-Luisa Alegre, MD, PhD⁷, William S. Oetting, PhD⁸, DR. Pamala A. Jacobson, PharmD⁸, DR. Ajay K. Israni, MD, MS^{1,2}

¹Department of Medicine, Nephrology Division, Hennepin Healthcare, Minneapolis, MN

²Hennepin Healthcare Research Institute, Minneapolis, MN

³Bioinformatics and Computational Biology, University of Minnesota, Minneapolis MN

⁴Department of Computer Science and Biotechnology Institute, University of Minnesota, Minneapolis, MN

⁵Division of Biostatistics, University of Minnesota, Minneapolis, MN

⁶Department of Medicinal Chemistry, University of Minnesota, Minneapolis, MN

⁷Department of Medicine, Section of Rheumatology, University of Chicago, Chicago, IL

⁸Department of Experimental and Clinical Pharmacology, University of Minnesota, Minneapolis, MN

Abstract

Background: We performed a study to identify differences in the urinary microbiome associated with chronic allograft dysfunction (CAD) and compared the urinary microbiome of male and female transplant recipients with CAD.

Methods: This case-control study enrolled 67 patients within the Deterioration of Kidney Allograft Function (DeKAF) Genomics cohort at two transplant centers. CAD was defined as a greater than 25% rise in serum creatinine relative to a 3 month post-transplant baseline. Urine

Corresponding Author: Ajay K. Israni, MD, MS, Department of Nephrology, Hennepin County Medical Center, 701 Park Avenue, Minneapolis, MN 55415, isran001@umn.edu.

Authorship Statement

Jennifer F. Wu, DO – writing of the manuscript, research design

Amutha Muthusamy MS – research performance, data analysis, writing of the manuscript

Rory Remmel, PhD - writing of the manuscript, research design

David P. Schladt, MS – participated in data analysis, writing of the manuscript

Pamala Jacobson, PharmD – co-principal investigator, writing of the manuscript, research design

Gabe A. Al-Ghalith BS - writing of the manuscript, analysis

Dan K. Knights, PhD - writing of the manuscript, research design, data analysis

Bin Guo, MS - writing of the manuscript, analysis

Baolin Wu, PhD – data analysis, analytic tool development, writing of the manuscript

Maria-Luisa Alegre, MD, PhD - participated in the writing of the manuscript, research design

William Oetting, PhD - writing of the manuscript, research design

Ajay K. Israni, MD, MS – principal investigator, research design, writing of the manuscript

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samples from patients with and without CAD were analyzed using 16S V4 bacterial ribosomal DNA sequences.

Results: *Corynebacterium* was more prevalent in female and male patients with CAD compared to non-CAD female patients ($p=0.0005$). A total 21 distinct Operational Taxonomic Unit (OTUs) were identified as significantly different when comparing CAD and non-CAD patients using (Kruskal-Wallis, $p<0.01$). A subset analysis of female patients with CAD compared to non-CAD females identified similar differentially abundant OTUs, including the genera *Corynebacterium* and *Staphylococcus* (Kruskal-Wallis; $p=0.01$; $p=0.004$ respectively). Male CAD vs. female CAD analysis showed greater abundance of phylum *Proteobacteria* in males.

Conclusion: There were differences in the urinary microbiome when comparing female and male CAD patients with their female non-CAD counterparts and these differences persisted in the subset analysis limited to female patients only.

Introduction

Chronic allograft dysfunction (CAD) is a potential complication in kidney transplant recipients and can herald allograft failure. Modern immunosuppression and antimicrobial prophylaxis has reduced acute allograft losses, but long-term fibrotic changes over the lifespan of the allograft have persisted¹. Much research has been conducted to elucidate causative factors in the development of CAD. The microbiome of transplant recipients is increasingly studied as a factor associated with CAD. The gut microbiome in allograft recipients has been studied, both to quantify alterations in normal gut flora following immunosuppressive therapies²⁻⁴ as well as the effects of microbiome alterations on immune function, though these studies have looked at mouse models⁵ or non-transplant human cohorts⁶. Alterations in the normal gut microbiome have been shown to stimulate intestinal innate and adaptive immune mechanisms, pro-inflammatory cytokines, and inflammation⁷. Conversely, absence of regulatory T cells in mice was associated with increased Th2-type inflammation as well as alterations in microbiota, suggesting that chronic inflammation can result in dysbiosis⁸. Thus, the relationship between the microbiome and the transplanted host appears to be bidirectional^{8,9}.

One promising area of study is the human urinary microbiome and its relation to kidney allograft function. Once felt to be a sterile body site, there is increasing knowledge of the composition of the urinary microbiome in healthy hosts¹⁰⁻¹³. Alterations in the urinary microbiome have been explored in a variety of lower urinary tract disorders such as urinary incontinence, interstitial cystitis, urologic cancers, and chronic prostatitis¹⁰, but little is yet known about urinary microbiome alterations in renal allograft dysfunction. Quantification of the urinary microbiome in renal allograft recipients has been performed in small cohorts^{14,15} and dysregulation of the microbiome has been associated with interstitial fibrosis and tubular atrophy (IFTA)¹⁶. However, to our knowledge, the urinary microbiome has not been studied in CAD prior to IFTA development. We hypothesized that CAD is associated with alterations in the urinary microbiome in renal transplant recipients. To test this hypothesis, we performed a case-control analysis of urine microbiome from kidney allograft recipients with and without CAD using 16S ribosomal RNA (rRNA) V4 microbial sequences for

bacteria as well as Internal Transcribed Spacer rRNA (ITS1 and ITS2 rRNA) region for fungi.

The objective was to compare the urinary microbiome profile in female and male kidney transplant recipients with CAD compared to female controls without CAD. Another objective was to compare the urinary microbiome profile of male kidney transplant recipients with CAD to those of female transplant recipients with CAD. The last objective was to compare urinary microbiome profile in females with CAD and those without CAD.

Materials and Methods

Study Design

This is a case-control study nested within existing cohorts. Adult participants were previously enrolled in the two prospective cohorts of the Deterioration of Kidney Allograft Function (DeKAF) Genomics study (NCT00270712), which enrolled from 2005–2010 or into the Genomics of Transplantation (GEN03) study (NCT01714440) enrolling from 2012–2016. These subjects from the transplant clinics at Hennepin County Medical Center and the University of Minnesota were enrolled as cases or controls during follow-up visits.

Cases were defined as adult male and female subjects at least one year post-transplantation returning to clinic with serum creatinine (SCr) persistently 25% above their 3 month post-transplantation baseline. If both the most recent SCr value and the closest previous value at least one week apart were 25% above the baseline value, this was considered a persistent rise. Controls were defined as female subjects at least one year post-transplantation returning to clinic with SCr persistently the same or better than their 3 month post-transplantation baseline. Similarly, if the most recent SCr and the closest prior value at least one week before were the same or better than baseline, it was considered persistently similar to baseline SCr.

The 3 month post-transplantation SCr had already been obtained in our database by using three SCr measurements at least one week apart. Exclusion criteria comprised fever $\geq 100^{\circ}\text{F}$ in the last 72 hours, recent acute infection with use of antibiotics, or probiotic use at the time of visit for microbiome sample collection. This study was approved by institutional IRB at University of Minnesota and all subjects gave written informed consent.

Sample Collection

Patients collected clean-catch urine samples in a sterile container during the study visit. These samples were placed on ice and promptly transported to the laboratory, where they were stored in an -80°C freezer until the time of DNA extraction.

Microbial and Fungal DNA Extraction

For the isolation of DNA, urine samples were gradually thawed in a laminar airflow hood to avoid cross-contamination. A modified MO BIO protocol including enzymatic lysis^{17–19} and addition of 0.5mm beads²⁰ was followed to aid in isolation of fungal DNA. After enzymatic lysis, the samples were transferred to a bead tube containing a mixture of 0.5 mm beads (MO BIO Laboratories) and 0.1mm beads from the Mo Bio Powerlyzer power soil kit (MO

BIO Laboratories). The standard manufacturer's protocol was followed for all the subsequent steps. DNA was quantitated using a NanoDrop spectrophotometer (ThermoFisher). Negative extraction controls and mock community cultures and DNA extracts (Zymoresearch) were processed in the same fashion.

16S and ITS1/ITS2 Sequencing and Amplification

Those samples that met a qPCR threshold concentration of at least 1000 molecules/ μ L for 16S V4, ITS1 and ITS2 were processed for amplicon library creation and sequencing at the University of Minnesota Genomics Center. Ribosomal 16S V4 and fungal ITS1 and ITS2 regions were amplified using a dual-index DNA barcoding approach with KAPA Hi-Fidelity HotStart DNA polymerase PCR kit (Kapa Biosystems) and libraries were created using a Nextera XT system (Illumina)^{21,22}. Samples were spiked with 15% phiX and sequenced with MiSeq 600 cycle V3 kit (Illumina) to generate 50,000–100,000 reads per sample.

Sequence Analysis

The resulting 16SrRNA raw sequence information was converted to fastq files (Illumina) and processed for quality. Adaptors were removed and remaining sequences were filtered using a threshold of Q37, and sequences below 33 base pairs were removed and paired ends were merged using the computational tool FLASH^{23,24}. Data were quality controlled and stitched using the NINJA-SHI7 fastq-to-fasta pipeline²⁵. Operational taxonomic units (OTUs) were selected using the NINJA-OPS²⁶ pipeline and the Greengenes for bacterial reference database, and RTL²⁷ and UNITE²⁸ for fungal reference databases (using a threshold of 97% similarity). Further taxonomic compositional and diversity analyses were performed using QIIME 1.9.1²⁹.

Sequences of Microbiota

77 urine DNA samples were amplified for 16S V4, ITS1 and ITS2 regions separately and were sequenced on MiSeq. A total of 3,390,054 reads were obtained with 16S V4 sequencing from these samples, averaging 36,208 reads per sample. After sequencing, ten samples were removed from the analysis (one withdrawal from the study, four samples with low microbiome sequence coverage, and five did not meet case or control eligibility requirements), resulting in 67 total samples (35 cases and 32 controls). The sequences from the mock community cultures and DNA were also examined for the presence and identification of the standard microorganisms. The sequences from the negative extraction controls looked clean and helped to rule out environmental contamination. The ITS1, ITS2 sequencing of these samples resulted in an average of 19,070 reads per sample with a median of 5,439 reads and 13,865 reads per sample with a median of 3,681 reads, respectively.

Statistical Analysis

For each individual OTU, we applied the Wilcoxon rank-sum test to compare differences between case and control groups. In addition, we also evaluated the aggregated OTU distribution difference at the genus level between the two groups using the kernel RV coefficient (KRV) test^{30,31}. To correct for multiple testing, we controlled the false discovery

rate (FDR) at the 0.05 level identify significant tests. Kruskal-Wallis nonparametric ANOVA using QIIME was also performed on taxonomic counts data to identify unique statistically significant OTUs, which corroborated with kernel RV tests. Furthermore, linear discriminant analysis effect size (LEfSe) was also employed to identify differentially abundant OTUs specific to the cohorts (case/control) for determining the biomarkers. In this analysis, LEfSe software determines biomarkers by relative abundance, emphasizing both statistical significance as well as biological consistency and effect relevance³².

Results

The characteristics of the study participants are outlined in Table 1. Several subjects in the case and control cohorts underwent allograft biopsies for elevations in SCr above 3-month baseline or for suspected acute rejection (AR) episodes prior to urinary microbiome sample collection. For some subjects, this was the first allograft biopsy obtained for persistent elevation in SCr $\geq 25\%$ above 3-month post-transplantation baseline, and is hereafter referred to as the index biopsy. These results are summarized in Table 2A. Among the 35 cases, 19 subjects had at least one biopsy performed, of which 11 were index biopsies. Among the 32 controls, 11 had at least one biopsy, of which one was an index biopsy. A total six AR events occurred: 4 among cases and 2 among controls. As expected, there were more index biopsies and AR events among cases than among controls. The pathologic findings for biopsies closest to the microbiome sample collection and the presence of donor-specific antibodies near time of biopsy are summarized in Table 2B and 2C. Among cases, the mean time from biopsy to microbiome sample collection was $4.98 (\pm 2.77)$ years, interquartile range (IQR) was 6.43 years. Among controls, the mean time from biopsy to microbiome sample collection was $7.15 (\pm 3.86)$ years, IQR was 8.89 years.

Urinary Microbial Diversity in Cases and Controls

Each urine sample contained a median of $23,566 \pm 9,320$ bacterial OTUs. Samples ranged from 5423 (min) to 38,373 (max) OTUs/sample. In contrast, there were only a median of $690 \pm 21,399$ fungal OTUs for ITS1 and median of 144 ± 3028 fungal OTUs for ITS2 using the most comprehensive RTL fungal database²⁷ among all samples. When UNITE database was used as reference, the number of uniquely identified OTUs were less than that identified by RTL database. Thus, the total count of uniquely identified fungal OTUs ranged from 55–95, in contrast to 5,133 bacterial taxa (Supplemental Figures 2 and 3). Therefore, further analysis was restricted to bacterial microbiome due to this low yield. The overall bacterial taxonomic compositions at phyla level for these samples are shown in Supplemental Figure 1.

Bacterial sample diversity was measured in two ways. All the samples were rarefied to 1000 sequences per sample before performing the diversity analyses to avoid bias. Shannon diversity index measures microbial diversity within a grouping, reflecting both richness (the number of taxa within a community) and evenness (the relative abundance of taxa in each sample). The mean Shannon diversity index was 4.66 ± 0.85 in the controls and 4.428 ± 0.77 in cases (Monte Carlo permuted two sample t-test; $p=0.337$) (Supplemental Figure 4a). The mean Chao1 index was 150.82 ± 61.5 in the controls and 138.76 ± 32.63 in cases (Monte

Carlo permuted two sample t-test; $p=0.335$) (Supplemental Figure 4b). We also measured beta diversity, or differences between differing communities, by weighted and unweighted UniFrac ANalysis Of SIMilarity (ANOSIM) calculations in QIIME (Supplemental Figure 5). There was no statistically significant difference in beta diversity by weighted and unweighted UniFrac (ANOSIM; $p=0.09$ and $p=0.07$ respectively).

Analysis of Cases vs Controls

No individual OTU showed any significant association with CAD when comparing cases and controls. However, when all OTUs were aggregated and analyzed with KRV testing^{30,31}, there was a statistically significant difference in OTUs between cases and controls ($p=0.016$). At the genus level, 378 distinct genera were identified but only 79 existed in greater than 10% of samples. Using KRV, when controlling for false discovery rate (FDR) at 0.05, we identified one significant genus, *Corynebacterium*, preferentially associated with cases of CAD versus controls ($p=0.0005$) (Supplemental Figure 6). Of the selected OTUs, 21 were found to be in statistically significant higher abundance in cases vs. controls ($p<0.01$) using Kruskal-Wallis one-way ANOVA testing (Table 3). Among them is the genus *Corynebacterium* which was found to be in corroboration with the KRV testing ($p=0.0008$). This is also evident from the heat map constructed with the relative abundance of the most significant taxa across all the samples (Supplemental Figure 7).

LEfSe analysis identified 22 discriminant features or distinct OTUs within the cohorts. Using a linear discriminant analysis (LDA) score threshold of > 2.0 , ten OTUs within the control set and twelve OTUs within the cases were identified (Figures 1 and 2). With this analytic framework, significant taxonomic groupings that emerged were the class *Actinobacteria* (includes the genus *Corynebacterium*), order *Bacillales* (includes the genus *Staphylococcus*), and genus *Corynebacterium* within cases, as well as the class *Cytophagia* which were more prevalent in controls.

Subset Analysis of Female Cases vs Female Controls

An analysis using KRV, comparing female cases versus female controls found a similar trend towards significance ($p=0.13$) but was underpowered. The Kruskal-Wallis testing between female cases versus female controls also identified the genus *Corynebacterium* with p-value 0.01 similar to all cases vs. controls analysis (Table 4).

LEfSe analysis performed between female cases versus female controls showed that the same taxonomic groupings as all cases vs. controls analysis emerged, such as class *Actinobacteria*, genus *Staphylococcus* for cases and class *Cytophagia* for controls which were significantly different (Figure 3).

Analysis of Cases Based on Sex

Because the initial study was designed as a sex supplement to the existing cohorts to analyze sex differences in post-transplantation outcomes, the control cohort was restricted to female participants. Once it was determined that there were significant differences between the cases and controls, we analyzed male versus female cases of CAD. Mean Shannon diversity index for male cases was 4.9 ± 0.6 and for female cases was 4.1 ± 0.7 (Monte Carlo permuted

two sample t-test; $p=0.002$) Mean Chao1 index for male cases was 243.93 ± 42.72 and for female cases was 217.55 ± 62.06 (Monte Carlo permuted two sample t-test; $p=0.183$) (Supplemental Figure 8). Despite a higher mean Shannon index for male cases, the calculated equitability (a measure of homogeneity within the sample) was higher in males (82.5%) compared with females (95%), reflecting a more homogenous male urinary microbiome compared to female cases. Weighted and unweighted UniFrac revealed significant grouping comparing male vs female cases (ANOSIM; $p=0.007$) (Supplemental Figure 5).

When KRV testing was done between female and male cases, there was a significant difference between female cases and male cases ($p\text{-value} < 0.01$). When FDR was controlled at 0.05 level, the microbiome class *Bacilli* ($p=8.00 \times 10^{-4}$) and the order *Lactobacillales* ($p=0.0027$) were found to be significantly associated with female cases.

LEfSe analysis of the male versus female cases of CAD was also performed using LDA threshold > 3.0 (Figure 4). Higher LDA scores correlate with more “separate” or distinct groupings or unique microbial signatures. Among male cases, the predominant phylum found was the Gram-negative *Proteobacteria*, which includes *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, *Yersinia*, *Legionella*, *Burkholderia*, *Campylobacter*, *Enterobacter*, *Citrobacter* and *Pseudomonas* among others. All male case OTUs with > 3 were within the phylum *Proteobacteria*. Of female cases, predominant taxa included genus *Lactobacillus*, and genus *Eubacterium* (Figure 5).

Additional Subset Analyses Based on Tacrolimus Level and Ancestry

We performed subset analyses based on immunosuppression and dosing, which have been correlated with post-transplantation changes in gut microbiota³ and with allograft outcomes^{33,34}. Out of 67 patients, a total of 31 patients were on tacrolimus while the remainders were on cyclosporine (two patients were not on any calcineurin inhibitor). We grouped these patients into high (> 1.4) and low (< 1.4) tacrolimus groups based on their dose-normalized levels. There were no significant differences in Shannon diversity or beta diversity indices observed between these groups. However, by LEfSe, class *Clostridia* emerged as a significant taxon abundant in the low dose-normalized tacrolimus group. With Kruskal-Wallis one-way ANOVA testing, genera *Actinomyces* ($p=0.008$) and *Anaerococcus* ($p=0.02$) were identified as more abundant in low tacrolimus dose-normalized level group (Supplemental Figure 9).

Similar analyses were performed on all of the urinary microbiome samples based on patient ancestral origin (Caucasian vs non-Caucasian), as expression of cytochrome P450 (CYP) enzymes are linked to drug metabolism and intestinal absorption of tacrolimus³⁵ and can be differentially expressed based on ancestry^{36,37}. Only one significant taxon, genus *Ruminococcus*, emerged as significant in Caucasians. In non-Caucasian patients, phylum *Tenericutes*, and classes *Mollicutes*, *Coriobacteriia* and *Oscillatoriophyceae* emerged as significant taxa with LDA score > 2 . Kruskal-Wallis testing identified 17 significantly different taxa with $p\text{-value} < 0.005$. Of these, significant taxa in non-Caucasians were family *Lachnispiraceae* ($p=8.00 \times 10^{-6}$) and genera *Actinomyces* ($p=0.0002$) and *Megasphaera* ($p=0.0002$) (Supplemental Figure 10).

Discussion

We discovered that the urinary microbiome contains microbiologic signatures specific to patients with CAD. *Corynebacterium* was the only genus found in greater abundance in cases when compared with controls, but there were a number of OTUs that showed a trend toward different abundance in cases versus controls. Using Kruskal-Wallis testing, 21 distinct OTUs were identified, and LEfSe analysis identified 22 distinct OTUs. Within both analytic frameworks, *Corynebacterium* was highlighted as a significantly prevalent genus in the microbiome of cases. The same analyses using only female patients also showed *Corynebacterium* emerging as a significant genus in cases proving that despite the sex differences, this genus has an association with CAD. While we were unable to identify *Corynebacterium* to the species level based on our alignment, there are *Corynebacterium* species that are found in the urine in addition to the skin (including *C. urealyticum*) that have been associated with indwelling catheters and can be pathogenic in humans³⁸.

Our research is, to our knowledge, the first to analyze the urinary microbiome of transplant recipients with early CAD, as well as the first to highlight the fungal species present in the urinary microbiome in patients with graft dysfunction. Lee et al² investigated kidney transplant recipients found to have AR but the authors examined the gut microbiome rather than urine. The work of Modena, Salomon, and colleagues¹⁶ studied the urinary microbiome as a marker for kidney allograft dysfunction, but all of these patients had already developed the irreversible changes of IFTA (scored moderate to severe by 2007 Banff criteria). Based on index biopsy data, the prevalence of moderate to severe IFTA in our cohort was less than in Salomon's cohort (see Table 2B); greater than 80% of our allograft biopsies (cases as well as controls) were scored at 0 or 1 by Banff criteria for interstitial fibrosis. Thus, changes in the urinary microbiome may precede development of IFTA and their identification may allow for earlier detection of allograft dysfunction or risk development of severe fibrosis. Of transplanted patients with IFTA in the Saloman cohort, their findings noted a non-significant decrease in female *Lactobacillus* prevalence (compared with healthy females) and a significant increase in *Streptococcus* in males with IFTA compared to healthy or unaffected transplanted males. Fricke et al¹⁵ studied various body site microbiota post-transplantation and found genera *Lactobacillus*, *Enterococcus*, *Bifidobacteriaceae*, *Pseudomonas*, and *Streptococcus* to be dominant in "healthy" subjects post-transplantation. Dominant genera outlined in each of these studies (including ours) have some overlap – namely, *Lactobacillus* in females and *Pseudomonas* as well as other *Proteobacteria* subgenera in males – but also exhibit distinct genera. We feel these variations between each of these study cohorts highlight the need for larger prospective studies designed to evaluate the urinary microbiome throughout the course of transplantation – pre-transplant, immediately post-transplant, and as CAD develops – to identify the "normal" post-transplantation urinary microbiome and assess alterations that may be associated with development of graft dysfunction.

Though numbers are small, our subanalysis based on dose-normalized tacrolimus level and by ancestry highlighted differences in urinary microbial expression, neither of which has been previously studied. Both optimizing choice of calcineurin inhibitor³³ and therapeutic serum drug level have been associated with decreasing rates of acute rejection³⁴. With respect to ancestry, race/ethnicity impacts CYP enzymatic expression, tacrolimus dose

requirements³⁷, and rates of acute rejection that disproportionately affect African-Americans^{39,40}. Rates of kidney allograft survival in African-American recipients are lower than in their Caucasian counterparts by more than 5% at 5 years (OPTN data, 2008–2015). Further investigation is needed to determine if the differences highlighted in our findings are attributable more to disparities in dose-normalized tacrolimus level based on ancestry or from differing microbiota between racial groups.

Using only female participants as controls has certain advantages. Original studies quantifying the normal human urinary microbiome have found that the female urinary tract contains both more bacteria and a more heterogeneous population than their male counterparts, owing to anatomic and hormonal differences^{12,15,41}. Using this knowledge, restricting controls to female participants minimized the risk of undersampling normal flora in male control patients. An interesting finding in our patient population was the proportion of cases with no calculated panel reactive antibodies (cPRA) at the time of transplantation compared with controls. The number of cases with 0% cPRA was higher than that of the control group, though overall numbers were small.

One limitation in the general study of the urinary microbiome is the inability to perform causal experimentation in mouse models. Human microbiota-associated (HMA) mice have been used to study gut flora, but no such model exists associating mouse urine with the human urinary microbiome. Thus, it is unclear whether changes in the urinary microbiome associated with CAD in patients are only a consequence or may also be a cause of CAD. Nevertheless, the identification of changes in the cases' urinary microbiome that occur well before development of advanced IFTA suggests that longitudinal analyses of the urine microbiome may be useful as part of a biomarker panel to detect allograft damage and predict CAD development. Our sample size is smaller and was not designed to be a prospective trial, and thus no baseline urine samples were collected prior to transplantation or in the immediate post-transplantation period. Now that we have established that there is a difference in the urinary microbiome of CAD patients, future studies will be designed prospectively with larger cohorts and baseline urine sampling to not only facilitate comparisons between groups, but even within an individual throughout the post-transplantation course. Another limitation of this study is the potential for skin and vaginal flora to contaminate our sample collection. Several *Corynebacterium* species are known skin and mucosal commensals and others are known to cause urinary tract infections, but the significant incidence increase in cases suggests their presence is due to more than simple contamination. All patients were instructed by study coordinators on proper clean-catch technique for urine sampling, and this would have minimized skin flora contamination as in other urine microbiome studies with similar outlined protocols¹⁶. Our study initially set out to identify both bacterial and fungal signatures in the cohort. However, the fungal results were not revealing, due in part to low numbers of OTUs aligning with existing mycobiome libraries, but further study with ongoing development of a more robust ITS1/2 database may shine light onto this arena. Additional studies into the mycobiome would be of interest, as we would expect opportunistic fungal organisms to be more abundant in transplant recipients. There may also be differences in the mycobiome between patients with varying levels of immunosuppression. Lastly, this study was funded as a supplement to the existing

cohort studies specifically to evaluate sex differences in post-transplantation data and thus only female participants were recruited as controls.

Based on our study, in addition to other cited works, there is evidence that alterations in the microbiota of transplant recipients is correlated with allograft dysfunction, IFTA, and acute rejection. It is thought that alterations in microbiota are both reflections of and drivers for changes conferred by allotransplantation and immunosuppression, and there is increasing understanding of the alterations in adaptive immune responses conferred by changes in the microbiota following immunosuppression^{7,9,42–44}. How individual microbial communities interplay with the immune system to modulate the risk of various diseases is only starting to be investigated, but it is becoming increasingly clear that dysbiosis may be associated with chronic inflammatory conditions. For example, there are multiple animal models in which immune tolerance to transplanted organs is prevented by inflammatory stimuli from bacteria^{45–47,45–47}. Such infections appear to stimulate the innate immune system that enhances alloreactivity in a bystander manner and switches the balance from regulatory to effector immune responses^{7,48–51}. Moreover, certain intestinal commensal communities, but not others, can enhance alloreactivity and accelerate skin graft rejection when transferred orally into germ-free mice⁵¹, demonstrating a different impact on allograft outcome of different commensal community structures. The microbiome is clearly not merely a passive marker of an underlying process, though how the urinary microbiome may affect local or systemic immune responses is not yet known. In summary, the urinary microbiome holds significant potential in the ongoing quest to identify a microbial signature associated with CAD in transplant recipients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CAD	Chronic Allograft Dysfunction
KRV	kernel RV coefficient
OTU	Operational Taxonomic Unit
SCr	Serum Creatinine
IFTA	Interstitial Fibrosis and Tubular Atrophy
DeKAF	Deterioration of Kidney Allograft Function (DeKAF) Genomics

LDA**Linear Discriminant Analysis****References**

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Figure 1:

Significant OTUs Determined by LEfSe Analysis comparing cases (all patients with CAD) vs controls (all patients without CAD). Statistically significant OTUs were identified based on comparing linear discriminant analysis (LDA) score, which is a technique to identify clusters of variables that separate into distinct classes or groupings. In this analysis, a threshold LDA score of >2 was used to identify significant OTUs. Higher LDA scores correlate with more “separate” or distinct groupings or unique microbial signatures. Taxonomy nomenclature: p = phylum, c = class, o = order, f = family, g = genus, s = species. All denoted OTUs are within kingdom *Bacteria*.

Figure 2:

Cladogram of LefSe Analysis Results comparing cases (all patients with CAD) versus controls (all patients without CAD). A cladogram organizes individual data points or species by biological hierarchy. In this set, the circular rings represent levels of taxonomy; the outer ring is the Genus level, the ring within this is Family, then Order, then Class, with the innermost intact ring representing Phylum. Linear spokes are used to connect phylogenetic relationships tracing back to higher levels in grouping. For instance, highlighted in red are numerous significant OTUs at the outermost genus level that can all be traced back to a unified class-level association within *Actinobacteria*. Similarly, there are significant groupings under the larger umbrella of both order *Bacillales* and class *Cytophagia*. Taxonomy nomenclature: p = phylum, c = class, o = order, f = family, g = genus, s = species. All denoted OTUs are within kingdom *Bacteria*.

Figure 3:

Cladogram of LEfSe analysis results comparing female cases versus controls (all female).

Taxonomy nomenclature: p = phylum, c = class, o = order, f = family, g = genus, s = species.

All denoted OTUs are within kingdom *Bacteria*.

Figure 4:

Significant OTUs by LEfSe analysis comparing male vs female cases. Given the large number of significant OTUs when linear discriminant analysis (LDA) score was set to >2 , a higher threshold of >3 was used for this analysis, resulting in even higher significance. Taxonomy nomenclature: p = phylum, c = class, o = order, f = family, g = genus, s = species. All denoted OTUs are within kingdom *Bacteria*.

Figure 5:

Cladogram of LefSe analysis results comparing male vs female cases. Cladogram organization is described in the legend of Figure 4. The highlighted male cases all are phylogenetically grouped together under phylum *Proteobacteria*, which contains a wide variety of Gram negative bacilli including genera *Escherichia*, *Burkholderia*, *Pseudomonas*, *Citrobacter*, among others. Taxonomy nomenclature: p = phylum, c = class, o = order, f = family, g = genus, s = species. All denoted OTUs are within kingdom *Bacteria*.

Table 1:

Baseline characteristics of the study participants. By study design, the control cohort included only female participants.

Characteristic	Control (N=32)	Case (N=35)	P-value [†]
Race [‡] :			0.55
White	28 (87.5%)	30 (85.7%)	
Black or African American	3 (9.4%)	3 (8.6%)	
Asian	0 (0%)	2 (5.7%)	
Multiracial	0 (0%)	0 (0%)	
Native American/Aleutian Islander	1 (3.1%)	0 (0%)	
Hispanic/Latino	0 (0%)	0 (0%)	-
Male	0 (0%)	19 (54.3%)	<.0001
Age at transplant, mean (SD)	51.3 (12.8)	48.0 (15.2)	0.34
Serum creatinine (mg/dL), mean (SD)	1.0 (0.2)	2.0 (0.7)	<0.001
Cause of End Stage Kidney Disease [§] :			0.23
Diabetes	4 (12.5%)	9 (26.5%)	
Glomerular disease	4 (12.5%)	8 (23.5%)	
Hypertension	3 (9.4%)	3 (8.8%)	
Polycystic kidney disease	9 (28.1%)	3 (8.8%)	
Other	11 (34.4%)	9 (26.5%)	
Unknown	1 (3.1%)	2 (5.9%)	
Living donor transplant [§]	16 (50.0%)	25 (73.5%)	0.075
Donor age, mean (SD)	37.4 (14.3)	44.8 (13.5)	0.035
Male donor ^c	17 (53.1%)	19 (55.9%)	1.0
Cold ischemia time >24 h [§]	3 (9.4%)	1 (2.9%)	0.35
Prior kidney transplant [§]	4 (12.5%)	4 (11.8%)	1.0
Need for dialysis in the first 14 days post-transplant	1 (3.1%)	3 (8.6%)	0.62
Zero % Panel Reactive Antibodies [§]	8 (25.0%)	18 (52.9%)	0.025
T or B Cross-match positive [¶]	0 (0%)	0 (0%)	-
Plasmapheresis prior to transplants ^{††}	0 (0%)	2 (6.5%)	0.49
Zero HLA mismatches [§]	5 (15.6%)	7 (20.6%)	0.75
Antibody Induction [§] :			0.77
IL-2 blockers	6 (18.8%)	4 (11.8%)	
None	2 (6.3%)	1 (2.9%)	
Combination	1 (3.1%)	1 (2.9%)	
Polyclonal	23 (71.9%)	28 (82.4%)	

Characteristic	Control (N=32)	Case (N=35)	P-value [†]
Smoking status:			1.0
Never	19 (59.4%)	20 (57.1%)	
Past	11 (34.4%)	13 (37.1%)	
Current	2 (6.3%)	2 (5.7%)	
Pre-emptive transplant [§]	17 (53.1%)	11 (32.4%)	0.13
Steroid withdrawal by day 14 post-transplant ^{††}	19 (63.3%)	23 (67.7%)	0.79
Calcineurin Inhibitor type ^{††}			0.25
Cyclosporine	11 (36.7%)	17 (50.0%)	
Tacrolimus	17 (56.7%)	17 (50.0%)	
None	2 (6.7%)	0 (0%)	
Simultaneous Pancreas Kidney Transplant [§]	2 (6.3%)	4 (11.8%)	0.67
Prior Non-kidney Transplants [§]	2 (6.3%)	7 (20.6%)	0.15
Cytomegalovirus Recipient/Donor Status ^{§§}			0.65
Recipient (-)/ Donor (-)	8 (25.8%)	11 (33.3%)	
Recipient (+)	19 (61.3%)	16 (48.5%)	
Recipient (-)/ Donor (+)	4 (12.9%)	6 (18.2%)	
Years after tx to urine collection, mean (SD)	5.8 (3.2)	6.7 (3)	0.21
Years after tx to biopsy, mean (SD) ^{¶¶}	0.27(0.24)	1.50(1.32)	0.0008

[†]P values were calculated using chi-square test for categorical variables and t-test for continuous variables

[‡]Race was self-reported

[§]One case is missing data

[¶]1 control and 3 cases are missing data

^{††}2 controls and 5 cases are missing data

^{†††}2 controls and 1 case are missing data

^{§§}1 control and 2 cases are missing data

^{¶¶}N for cases = 19 and N for controls = 11

Table 2A:

Description of all for cause allograft biopsies performed prior to urinary microbiome sample collection.

	Case (%) N=35	Control (%) N=32
Allograft biopsy [†]	19 (54.3)	11 (34.4)
Index biopsy [‡]	11 (31.4)	1 (3.1)
Acute rejection biopsy	4 (11.4)	2 (2.6)

[†] All biopsies noted above were obtained prior to urine sample collection

[‡] Index biopsy is defined as the first allograft biopsy obtained for a persistent elevation in SCr ≥ 25% above 3 month post-transplant baseline.

Table 2B:

The distribution of pathology scores based on Banff criteria for antibody-mediated rejection for the first (if more than one per patient available) kidney allograft biopsies obtained prior to urinary microbiome sample collection described in Table 2A.

	Case (N=19)				Control (N=11)			
Biopsy Score	0	1	2	3	0	1	2	3
i (interstitial inflammation)	83%	6%	6%	6%	91%	9%	0%	0%
t (tubulitis)	72%	11%	6%	11%	82%	9%	9%	0%
ci (interstitial fibrosis)	50%	33%	17%	0%	73%	18%	9%	0%
ct (tubular atrophy)	44%	39%	17%	0%	64%	36%	0%	0%
cv (vascular fibrosis)	67%	17%	17%	0%	82%	18%	0%	0%
cg (glomerulopathy)	94%	6%	0%	0%	100%	0%	0%	0%
ah (arteriolar hyaline thickening)	83%	17%	0%	0%	73%	27%	0%	0%
v (intimal arteritis)	100%	0%	0%	0%	91%	9%	0%	0%
g (glomerulitis)	100%	0%	0%	0%	100%	0%	0%	0%
Other Laboratory Data								
	Positive	Negative	Not Done		Positive	Negative	Not Done	
C4d staining	0%	42%	58%		0%	64%	36%	

Table 2C:

Donor specific antibodies at time of biopsies

	Cases (N=19)				Controls (N=11)		
	Positive	Negative	Not Done		Positive	Negative	Not Done
DSA	5.3%	42.1%	52.6%		9.1%	54.5%	36.4%

Table 3:

Statistically significant OTUs by taxonomy using Kruskal-Wallis testing comparing cases (all patients with CAD) versus controls (all patients without CAD). The false discovery rate (FDR) of the set is 0.67. FDR is the expected proportion of type 1 errors when conducting multiple comparisons.

OTU count		P-value	Taxonomy [†]
Case (mean)	Control (mean)		
195.69	3.91	0.0008	p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Corynebacteriaceae; g__Corynebacterium ; s__
122.51	2.34	0.0087	p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Nocardiaceae; g__Rhodococcus ; s__
0.00	1.13	0.0078	p__Actinobacteria; c__Coriobacteriia; o__Coriobacteriales; f__Coriobacteriaceae; g__Adlercreutzia ; s__
0.00	0.50	0.0018	p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides ; s__
5.57	0.16	0.0026	p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Parabacteroides ; s__
12.71	12.13	0.0014	p__Firmicutes; c__Bacilli; o__Bacillales; f__Staphylococcaceae; g__Staphylococcus ; s__
3.69	3.19	0.0086	p__Firmicutes; c__Bacilli; o__Bacillales; f__Staphylococcaceae; g__Staphylococcus ; s__
368.89	307.47	0.0028	p__Firmicutes; c__Bacilli; o__Bacillales; f__Staphylococcaceae; g__Staphylococcus ; s__aureus
5.63	6.69	0.0063	p__Firmicutes; c__Bacilli; o__Bacillales; f__Planococcaceae ; g__; s__
30.34	23.88	0.0083	p__Firmicutes; c__Bacilli; o__Bacillales; f__Planococcaceae ; g__; s__
141.54	74.94	0.0029	p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Aerococcaceae; g__Facklamia ; s__
0.00	2.16	0.0037	p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus ; s__
1.69	0.06	0.0039	p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus ; s__
5.80	0.31	0.0091	p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus ; s__
32.60	2.41	0.0052	p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus ; s__
0.03	2.81	0.0032	p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae ; g__; s__
0.00	5.06	0.0078	p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae ; g__; s__
0.00	4.69	0.0078	p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae ; g__Dorea ; s__
0.00	0.34	0.0078	p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae ; g__Ruminococcus ; s__
0.54	1.91	0.0104	p__Firmicutes; c__Clostridia; o__Clostridiales; f__Tissierellaceae; g__Helcococcus ; s__
0.11	7.75	0.0046	p__Fusobacteria; c__Fusobacteriia; o__Fusobacteriales; f__Fusobacteriaceae; g__Fusobacterium ; s__

[†]Taxonomy nomenclature: p = phylum, c = class, o = order, f = family, g = genus, s = species. All denoted OTUs are within kingdom *Bacteria*. The narrow taxonomic rank in each significant OTU is in bolded text.

Table 4:

Statistically significant OTUs by taxonomy using Kruskal-Wallis testing comparing cases (female patients with CAD) versus controls (female patients without CAD).

OTU count		P-value	Taxonomy [†]
Case (mean)	Control (mean)		
6.4	1.5	0.01	p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Corynebacteriaceae; g__Corynebacterium ; s__
3	0	0.01	p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella ; s__
0.8	0	0.01	p__Firmicutes; c__Bacilli; o__Bacillales; f__Planococcaceae ; g__; s__
15.7	12.1	0.004	p__Firmicutes; c__Bacilli; o__Bacillales; f__Staphylococcaceae; g__Staphylococcus ; s__
0.5	0	0.01	p__Firmicutes; c__Bacilli; o__Bacillales; f__Staphylococcaceae; g__Staphylococcus ; s__ sequorum
0	48.9	0.01	p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Aerococcaceae; g__Aerococcus ; s__
1.1	0	0.004	p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus ; s__
55.7	0	0.005	p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus ; s__
0.3	0	0.01	p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus ; s__
0.4	0	0.004	p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus ; s__
0.5	0	0.004	p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus ; s__
5.6	2.7	0.002	p__Firmicutes; c__Clostridia; o__Clostridiales; f__[Tissierellaceae]; g__Anaerococcus ; s__
79.3	0	0.01	p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae ; g__; s__
0.8	0	0.01	p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Dialister ; s__
0	0.5	0.009	p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae ; g__; s__

[†] Taxonomy nomenclature: p = phylum, c = class, o = order, f = family, g = genus, s = species. All denoted OTUs are within kingdom *Bacteria*. The narrow taxonomic rank in each significant OTU is in bolded text.