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Chronic kidney disease alters intestinal microbial flora

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The population of microbes (microbiome) in the intestine is a symbiotic ecosystem conferring trophic and protective functions. Since the biochemical environment shapes the structure and function of the microbiome, we tested whether uremia and/or dietary and pharmacologic interventions in chronic kidney disease alters the microbiome. To identify different microbial populations, microbial DNA was isolated from the stools of 24 patients with end-stage renal disease (ESRD) and 12 healthy persons, and analyzed by phylogenetic microarray. There were marked differences in the abundance of 190 bacterial operational taxonomic units (OTUs) between the ESRD and control groups. OTUs from Brachybacterium, Catenibacterium, Enterobacteriaceae, Halomonadaceae, Moraxellaceae, Nesterenkonia, Polyangiaceae, Pseudomonadaceae, and Thiothrix families were markedly increased in patients with ESRD. To isolate the effect of uremia from inter-individual variations, comorbid conditions, and dietary and medicinal interventions, rats were studied 8 weeks post 5/6 nephrectomy or sham operation. This showed a significant difference in the abundance of 175 bacterial OTUs between the uremic and control animals, most notably as decreases in the Lactobacillaceae and Prevotellaceae families. Thus, uremia profoundly alters the composition of the gut microbiome. The biological impact of this phenomenon is unknown and awaits further investigation.

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The large community of microbes residing in the intestinal tract (microbiome) constitutes a dynamic and symbiotic ecosystem that is in constant interaction with the host metabolism.[1–3] Under normal conditions, the gut microbiome provides trophic[2] and protective[4] functions. In addition, the normal microbial flora influences energy metabolism[5] by facilitating absorption of complex carbohydrates and contributes to the nitrogen[6] and micronutrient homeostasis via synthesis of amino acids, such as lysine and threonine,[7] and various vitamins, such as vitamin K[6] and group B vitamins.[8]

Alteration in the functions or signaling pathways of the commensal flora contributes to the pathogenesis of diverse illnesses such as inflammatory bowel disease,[9] chronic inflammation, dyslipidemia, diabetes,[10] atopic disorders,[11] cardiovascular diseases, neoplasms,[12] and obesity.[13] The biochemical milieu has a decisive part in shaping the structure, composition, and function of the microbial flora. Uremia can profoundly modify the biochemical milieu of the gut via heavy influx of urea into the gastrointestinal tract and secretion of uric acid and oxalate by the colonic epithelium.[14–16] In addition, therapeutic interventions, including dietary restriction of fruits, vegetables, and high-fiber products to prevent hyperkalemia and oxalate overload, use of phosphate-binding agents to manage hyperphosphatemia, and administration of antibiotics to treat vascular access and other infections can modify the luminal milieu of the gut and impact its microbial flora. Alteration of microbial flora in inflammatory bowel diseases contributes to and may be exacerbated by the disruption of the gut epithelial barrier function and structure. This enables leakage of the luminal antigens and other noxious contents into the intestinal wall and the systemic circulation.[17]

Several observations suggest that uremia impairs intestinal barrier function and promotes inflammation throughout the gastrointestinal tract. This is based on the reported increase in intestinal permeability to high-molecular-weight polyethylene glycols in uremic humans and animals,[18,19] penetration of bacteria across the intestinal wall and their detection in the mesenteric lymph nodes in uremic rats,[20] the presence of endotoxemia in patients with end-stage renal disease (ESRD),[21,22] recent demonstration of the disruption of colonic epithelial tight-junction apparatus in the uremic

rats,[23] and histological evidence of chronic enterocolitis in ESRD patients maintained on dialysis.[24,25] These events can clearly contribute to systemic inflammation and oxidative stress, which are constant features of advanced chronic kidney disease (CKD) and the major mediators of cardiovascular disease, cachexia, anemia, and numerous other morbidities in this population.[26–30]

As noted above, uremia and its treatment can significantly alter the biochemical milieu of the intestinal tract and, as such, may alter the structure, composition, and function of microbial flora. This may disturb the symbiotic relationship that prevails under normal conditions and lead to the production and absorption of proinflammatory and otherwise harmful byproducts, and simultaneously limit the beneficial functions and products conferred by the normal flora. Such events can contribute to uremic toxicity, inflammation, and cardiovascular, nutritional, and other complications of CKD. The present study was designed to test the hypothesis that the biochemical modification of the gut milieu in advanced CKD can lead to significant changes in composition of the gut microbial flora.

RESULTS

General data

Patients and controls. As expected, compared with the healthy control group, the ESRD patients had a significant increase in plasma concentrations of creatinine (8.6 ± 2.9 vs. 0.8 ± 0.1 mg/dl, $P<0.0001$) and urea nitrogen (70 ± 18.0 vs. 24.0 ± 9.9 mg/dl, $P<0.0001$) concentrations. All patients were treated with phosphate binders, erythropoiesis-stimulating agents, intravenous iron compounds, and multivitamin preparations. Strict dietary fluid and sodium, phosphorus, and potassium restrictions were prescribed to minimize fluid overload, hyperphosphatemia, and hyperkalemia. Patients received hemodialysis therapy for 3 h three times weekly using cellulose triacetate dialyzers. Systemic heparinization was used for anticoagulation during hemodialysis. The Kt/V in the ESRD group was 1.5 ± 0.3, reflecting adequacy of the dialysis regimen. The ethnic background of the ESRD group (9 Caucasians, 13 Hispanics, and 2 Asians) was similar to that of the control group (4 Caucasians, 7 Hispanics, and 1 Asian) Similarly, the body mass index in the ERSD group (30.4 ± 8.3) was comparable to that of the control (29.2 ± 6.1 kg/m^2, $P=0.65$).

CKD and control rats. Data are summarized in Table 1. Compared with the sham-operated control group, the CKD group exhibited significant elevation of arterial pressure, increased urinary protein excretion, elevated plasma urea and creatinine concentrations, reduced hematocrit, and lower body weight.

Microarray data

Human data. Relative richness (the number of bacterial taxa in a sample) was assessed for subfamilies found in samples in each group. Although the mean relative richness (summarized at subphylum) for ESRD and control groups was similar (Figure 1a), the relative abundances (i.e., probe intensities) of bacterial groups within the subfamilies differed significantly. Significant increases (adjusted $P<0.02$) in relative abundance were found for 190 bacterial operational taxonomic units (OTUs) in the ESRD group compared with the control group. Many (159) of the OTUs that were

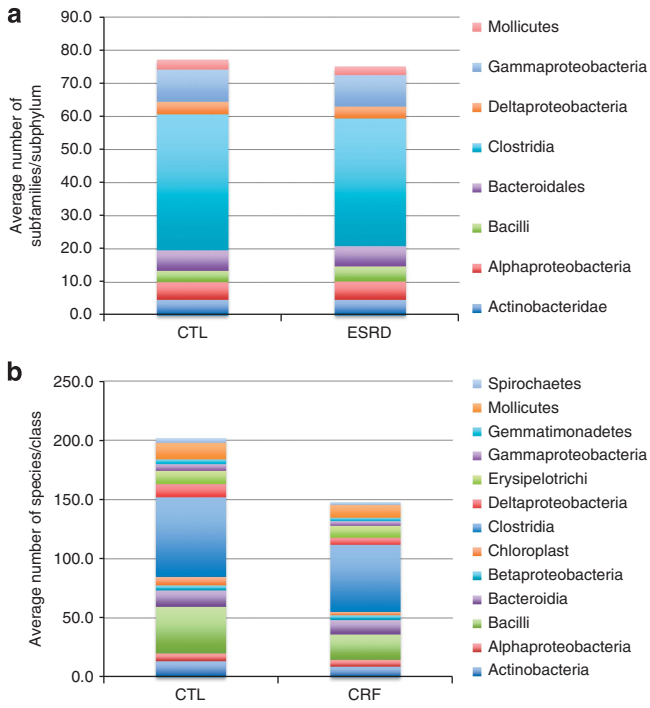

Figure 1 | Relative richness of the gut microbiome in the study groups. Relative richness comprised of the average number of (**a**) subfamilies per subphylum for control (CTL) or end-stage renal disease (ESRD) patients or (**b**) species per class for control (CTL) and chronic renal failure (CRF) rats. A subfamily or species had to be present in at least three replicates of a treatment group and also had to have an average of four subfamilies or species present in a subphylum or class to be included in the figure.

Table 1 | BW, blood pressure, Hct, serum creatinine and urea concentration, Ccr, and urinary protein excretion in normal control rats and rats with CRF

	BW (g)	BP (mm Hg)	Hematocrit (%)	Creatinine (mg/dl)	Urea (mg/dl)	Ccr (ml/min/kg)	U Protein (mg/mg Cr)
CTL	407 ± 5.9	120 ± 2.1	48 ± 1.2	0.61 ± 0.2	48 ± 3.3	8.8 ± 0.05	7.4 ± 0.5
CRF	374 ± 4.4	155 ± 2.5*	35 ± 0.7*	1.14 ± 0.2*	93 ± 7.4*	3.4 ± 0.03*	81.5 ± 5.6*

Abbreviations: BP, tail arterial pressure; BW, body weight; Ccr, creatinine clearance; Cr, plasma creatinine; CRF, chronic renal failure; CTL, control; Hct, hematocrit; U protein, urine protein excretion in the CRF and control rats.
Values are mean ± s.d.
*$P<0.05$ compared with CTL.

Table 2 | Families of bacteria with the adjusted *P*-values <0.02 in the abundance of their OTUs when comparing control individuals to the ESRD patients

Phylum	Subphylum	Family	Example strain	OTU ID	Family: average intensity	
					Control	ESRD
Actinobacteria	Actinobacteridae	Brachybacterium	*Brachybacterium nesterenkovii* str. DSM 9573	537	7482	8412
		Nesterenkonia	Lake Kauhako water isolate str. K2-66	706	3492	4839
Firmicutes	Clostridia	Catabacter	Swine intestine clone p-5389-2Wb5	4419	1636	2617
		Peptostreptococcaceae	*Clostridium elmenteitii* E2SE1-B	3765	5973	6677
	Mollicutes	Catenibacterium	Midgut homogenate *Pachnoda ephippiata* larva clone PeM34	6579	11,585	11,678
Proteobacteria	Deltaproteobacteria	Polyangiaceae	Bovine fetal thymus clone EBA	4611	10,438	11,192
	Gammaproteobacteria	Alteromonas	*Alteromonas macleodii*	2019	6602	7674
		Enterobacteriales_Enterobacteriaceae	*Enterobacter* sp. str. 16-31	6063	6581	8325
		Halomonadaceae	*Halomonas* sp. str. NT N2	1163	5244	6203
		Methylococcaceae	Deep-sea hydrothermal vent clone TAG-1	1537	6697	7892
		Moraxellaceae	*Acinetobacter* sp. str. YY-5	1675	7397	7878
		Pseudomonadaceae	*Pseudomonas* sp. str. ST41	1793	8930	9631
		Thiothrix	*Thiothrix* sp. str. KRN-B2	1511	4718	5671

Abbreviations: ESRD, end-stage renal disease; OUT, operational taxonomic unit.
All significantly different OTUs in these families had higher average relative abundance in ESRD patients. A representative OTU and example sequence are shown. Average intensities for the significantly differing OTUs in the families are shown for each group.

significantly different between the study groups belonged to the Pseudomonadaceae family. Although one *Pseudomonas* sequence can trigger probes in several neighboring OTUs, the patterns of OTU intensity observed across the test samples here indicate that there were probably several related yet distinguishable members of the Pseudomonadaceae family with elevated abundances in the ESRD patients. Microbial families showing the largest increase in ESRD patients were from the Actinobacteria, Firmicutes (especially subphylum Clostridia), and Proteobacteria (primarily Gammaproteobacteria) phyla (Table 2). Four OTUs had two- to three-fold higher average abundances in the control samples than in the ESRD samples, although the differences were not statistically significant. Those OTUs belonged to the Sutterellaceae, Bacteroidaceae, and Lactobacillaceae families. To investigate diversity within and between groups, all significantly different OTUs were used to generate a non-metric multidimensional scaling plot (Figure 2). Despite overlapping distributions, the control group samples showed tighter clustering than did the ESRD samples. Significant inter-subject variability in human fecal microbiota has been noted in both healthy and diseased individuals in previous studies.[31–33] We assumed that compared with the recruited patients and controls, variability in the microbiome would be lower among genetically identical rats raised and maintained under similar conditions, in which we could precisely control the age, gender, and diet, as well as the onset, severity, and the underlying cause of CKD.

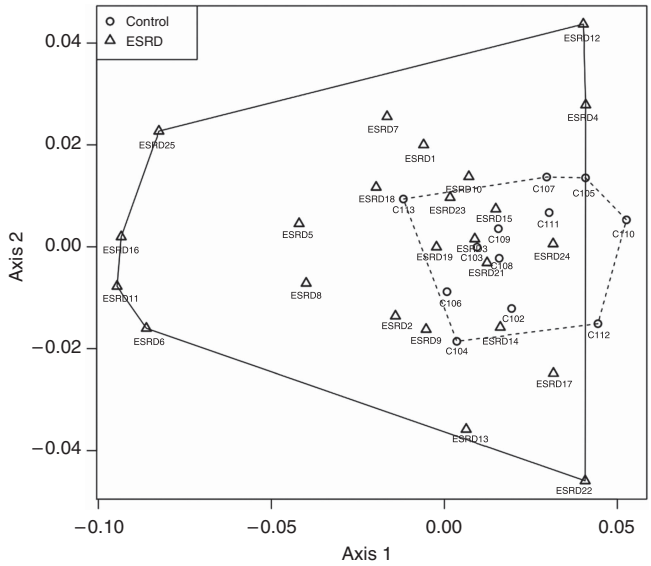

Figure 2 | Two-dimensional representation (plane of view) of non-metric multidimensional scaling analysis of standardized operational taxonomic unit (OTU) intensities (hybridized probe sets) for the dynamic subset of the microbial community consisting of 190 PhyloChip OTUs from controls or end-stage renal disease (ESRD) patients. Stress = 0.10. Each point is a human fecal community from control (C) or ESRD individuals. The model is based on a Bray–Curtis similarity matrix. Connecting lines were used to aid in the visualization of the distribution of all members of a group.

Animal data. In confirmation of other studies,[34,35] Firmicutes (e.g., class Clostridia, Bacilli, Mollicutes), Bacteroidetes (e.g., class Bacteroidia), Actinobacteria, and Proteobacteria were among the taxa with the greatest number of species in the fecal samples of the rats used in the present study. With the exception of the Betaproteobacteria, which had a greater number of OTUs in the chronic renal failure (CRF) rats (36 OTUs) than control rats (33 OTUs), total richness was significantly greater (Wilcoxon test, $P = 0.0086$) in the control group compared with the CRF group, and an ANOSIM (analysis of similarity) test of the number of species per class included in Figure 1b indicated weak differences between groups ($R = 0.356$), with class Bacilli contributing the most (19%) to the difference between groups (SIMPER data not shown, though see Supplementary Figure S1).

Bacterial community structure was distinctive between normal and CRF rats as shown by non-metric multidimensional scaling analysis based on standardized intensities of the hybridized probe sets of the bacterial communities (Figure 3) and supported by an Adonis test ($P = 0.002$). There were 175 OTUs that were significantly different

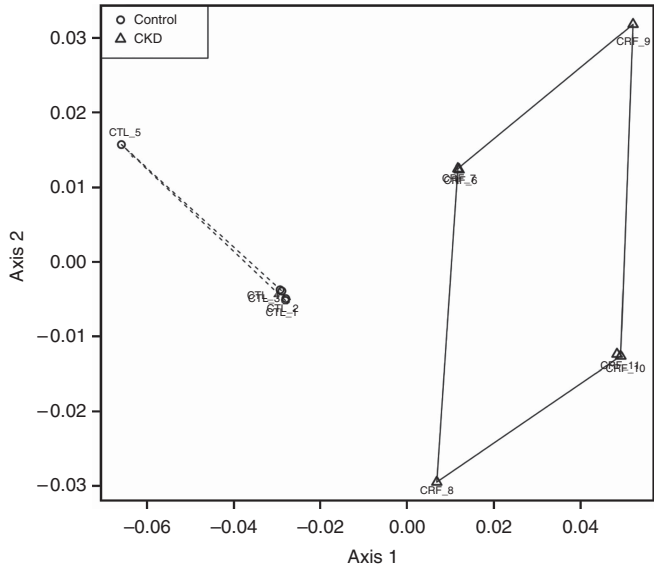

Figure 3 | Two-dimensional representation of non-metric multidimensional scaling analysis of standardized operational taxonomic unit (OTU) intensities (hybridized probe-sets) in bacterial communities of rat fecal microbiota. Stress = 0.02. The model is based on a Bray–Curtis similarity matrix. Connecting lines were used to aid in the visualization of the distribution of all members of a group. CKD, chronic kidney disease; CRF, chronic renal failure; CTL, control.

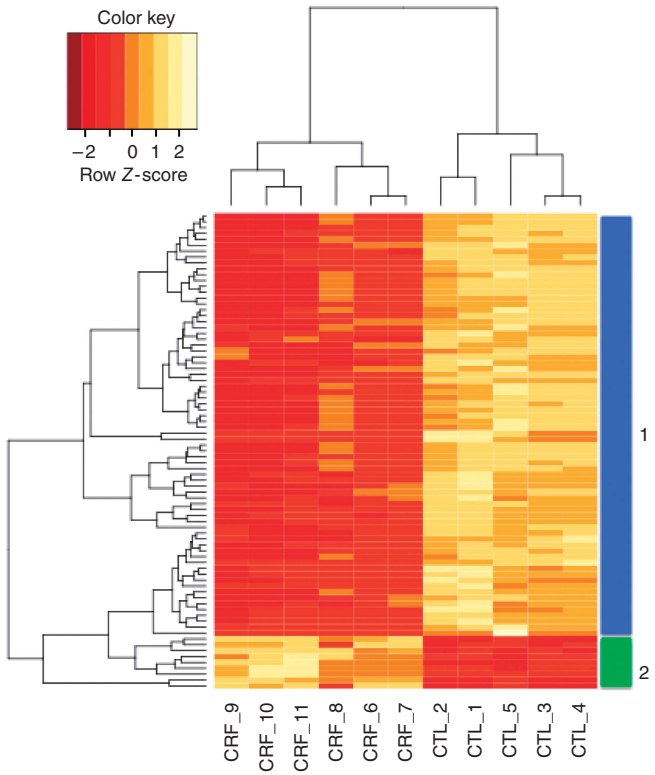

Figure 4 | Hierarchical clustering of probe sets representing significantly different operational taxonomic units (OTUs) between groups with a minimum two-fold change in untransformed intensities between samples to show relative abundance. Yellow indicates increased abundance and red indicates decreased abundance relative to the mean for each OTU. Columns represent rat fecal microbiota composition for control (CTL) and chronic renal failure (CRF) individual rats. Rows are OTUs with mean fold change among samples and cluster based on the similarity of their abundance profiles across the data set, with similar OTUs connected at the hierarchical tree on the left. Bars on the right represent (1) OTUs from the Firmicutes (especially Lactobacillaceae and a few Coprococcus within the Lachnospiraceae), Bacteroidetes (Prevotellaceae, two Rikenellaceae OTUs) that had higher abundances in CTL samples, or (2) OTUs from the Firmicutes (unclassified Lachnospiraceae) and other Rikenellaceae (in Bacteroides) that have higher abundances in CRF samples.

(adjusted $P<0.05$) between groups, of which certain families in the Bacteroidetes and Firmicutes were less prevalent in the CRF rats, especially Lactobacillaceae and Prevotellaceae. Of the significantly different OTUs, 81 had at least a two-fold change in average probe intensity. A heat map shows that a majority of these OTUs decreased in relative abundance in the CRF compared with the control rats (Figure 4). Previous experiments used to validate G3 PhyloChips correlating defined concentrations of target OTUs with hybridization intensity found that, to a rough approximation, as the intensity doubled, the relative abundance increased over four-fold (unpublished data). A table summarizing families with the greatest differences between CKD and control rat samples is included in the Supplementary Table S1 online.

As expected, there was much less variability among samples within each group for rats compared with humans (Supplementary Table S2 online). The percent coefficient of variation for OTU intensities was 14.2% for control individuals and 18.6% for ESRD patients compared with 7% for control rats and 14.4% for CKD rats.

DISCUSSION

The selection pressures on the part of the host and the microbes shapes the structure, composition, and function of the microbial flora. In this context, advanced renal failure can profoundly alter the biochemical milieu of the gastrointestinal tract by several mechanisms: (1) elevation of urea concentration in the intracellular and extracellular fluids leads to its massive influx into the gastrointestinal tract via passive diffusion and incorporation in glandular secretions. Hydrolysis of urea by urease, which is expressed in some microbial species in the gut flora, results in the formation of large quantities of ammonia [$CO(NH_2)2 + H_2O \rightarrow CO_2 + 2 NH_3$]. This phenomenon leads to modification of the luminal pH and causes uremic enterocolitis.[24,25] (2) Uric acid, the end product of purine metabolism in humans, is normally excreted in the urine via a complex interplay of glomerular filtration and tubular reabsorption and secretion processes. However, in advanced renal failure, the colon replaces the kidney as the primary site of uric acid excretion. This process is mediated by an adaptive rise in the secretory flux of uric acid[14,15] and accounts for the relatively minor increase in plasma uric acid despite total or near-total loss of renal function. (3) As with uric acid, the colon has a major

role in excretion of oxalate in renal failure.[16] (4) Diet has a major role in shaping the gut microbial flora. Strict dietary restrictions intended to prevent severe hyperkalemia and oxalate overload in patients with advanced CKD severely limit consumption of fruits, vegetables, and high-fiber products, which are rich in potassium and oxalate. These products normally contain most of the indigestible dietary complex carbohydrates that serve as the primary source of nutrients for the gut microbiota. Therefore, these dietary restrictions could affect the makeup and/or metabolism of the gut flora. (5) Patients with advanced CKD are invariably instructed to take large quantities of phosphate-binding agents (calcium acetate, calcium carbonate, aluminum hydroxide, and anion-exchange resins) with each meal, to control hyperphosphatemia by limiting phosphate absorption. Long-term consumption of these agents can modify the luminal milieu of the gut and affect the resident microbial flora. (6) Common use of antibiotics to treat vascular access and other infections is yet another factor that is well known to modify the composition of the enteric commensal organisms in such patients. Together, these events may alter the composition and, most likely, the function of the gut microbial flora in patients with advanced renal disease. This supposition was confirmed by the results of the present study, which showed significant differences in the abundance of 190 microbial OTUs between the ESRD and the normal control individuals. These OTUs were classified mostly in the families containing aerobic and facultative anaerobic bacteria. These findings are consistent with the results of two previous studies using the microbial culture technique. In a placebo-controlled clinical trial of a probiotic product, Rangnathan *et al.*[36] found a trend toward reduction of the numbers of culturable anaerobic bacteria in patients with CKD stages 3–4. In another study, Fukuuchi *et al*[37] showed a significant increase in the percentage of culturable aerobic bacteria from 0.12% in controls to 4.29% in patients with CRF. In fact, similar to the findings of Fukuuchi *et al.*,[37] we found increased abundance of the Enterobacteriaceae, particularly the OTUs containing certain clusters of *E. coli* sequences in our ESRD patients.

Together, these observations point to a significant difference in the composition of the gut microbiome between the ESRD patients and healthy control individuals included in this study. However, in addition to uremia, differences in the underlying systemic diseases and therapeutic and, particularly, dietary interventions as opposed to uremia, *per se*, could have modified the structure and function of the gut microbiome in the ESRD patients. It is nearly impossible to dissect the partial contribution of each of these factors to the observed changes in the gut microbiome in the ESRD patients. In an attempt to isolate the effect of renal failure, *per se*, we examined the microbial flora in male Sprague–Dawley rats randomly assigned to undergo subtotal nephrectomy or sham operation. The study revealed substantially lower species richness as measured by the number of OTUs in the CRF rats. The impressive impact of uremia on the composition of the gut microbiome was evidenced by the differences in relative abundance of 175 microbial OTUs between the animals with CRF and the control rats. Of the 81 statistically significant OTUs that had at least a two-fold difference in intensity values, most were decreased in abundance in CRF rats (Figure 4). The composition of these most variable OTUs was limited primarily to a small number of families within the Bacteroidales, Lactobacillales, and Clostridiales. Only 9 of these 81 OTUs were increased in CKD rats, primarily in the Rickenellaceae and Lachnospiraceae families. Thus, the transition in the intestinal environment from healthy to CRF resulted in a shift of the microbiota from a more evenly distributed and complex community to one that was simpler and apparently more dominated. The use of genetically identical rats that were highly controlled for all factors other than the disease state effectively reduced the microbial community variability, and thus a clear difference was observed. As the two groups differed only in the presence or absence of CKD, this experiment provided irrefutable evidence for the impact of the advanced CKD on the gut microbiome. The observed differences between the normal and CKD rats suggest that the profound alteration in the microbial flora found in the ESRD patients is, at least in part, due to uremia. However, given the critical role of the diet in shaping the structure and function of the gut microbiome, the strict dietary restrictions that ESRD patients were under must have also contributed to the observed changes in their microbial flora. Further studies are needed to dissect the effect of the dietary restrictions from those of uremia, *per se*, in this population.

It is noteworthy that the changes in composition of the gut microbial flora in the CRF rats observed in the present study are accompanied by severe disruption of the colonic epithelial tight junction, recently demonstrated by our group in this model.[23] The latter study showed massive depletion of the transcellular (occludin and claudin 1) and intracellular (ZO1) protein constituents of the tight-junction complex. This was accompanied by accumulation of inflammatory cells in the lamina propria and evidence of systemic inflammation. The alteration of the microbial flora observed in the present study may be, in part, a cause or consequence of the disruption of the intestinal barrier function and structure, and the associated inflammation.

Together, the observed changes in the composition of the gut microbiome and disruption of its barrier structure/function may result in the production and absorption of noxious by-products that can contribute to uremic toxicity, inflammation, malnutrition, and other morbidities in the uremic patients and animals. This supposition is supported by the results of a study recently published by Aronov *et al.*[38] By using high-resolution mass spectrometry, they provided irrefutable evidence for the colonic origin of a number of known uremic toxins and many as-yet-unidentified products in the plasma of patients with ESRD by comparing data obtained from ESRD patients who had undergone colonic

resection with those found in ESRD patients with intact colon and normal control subjects. Together, the observed disruption of the intestinal barrier structure shown in our recent study[23] and profound changes in composition of the gut microbiome shown here validate the concept and pathophysiological relevance of the 'intestinal-renal syndrome' recently introduced by Professor Ritz.[39]

The limitations of the present study include the cross-sectional nature and the relatively small size of the study population. Longitudinal studies are planned to explore the evolution in the composition of the gut microbiome during the course of CKD progression to ESRD and restoration of kidney function with renal transplantation.

In conclusion, ESRD significantly modifies the composition of gut microbiome in humans. The presence of an equally significant difference in the intestinal microbial flora between the uremic and control rats, which were otherwise identical, helped to substantiate the impact of uremia *per se* on the composition of the gut microbial flora. In addition to uremia, the strict dietary restrictions that ESRD patients were under must have contributed to the observed changes in their microbial flora. Further studies are needed to dissect the effect of the dietary restrictions from those of uremia, *per se*, in this population.

MATERIALS AND METHODS

Patients and controls

Twenty-four stable patients (6 men and 18 women, aged 57 ± 14 years) with ESRD on maintenance hemodialysis for a minimum of 3 months were recruited in the study. The underlying causes of ESRD in the study population included diabetic nephropathy in 15 patients, hypertensive nephrosclerosis in 4, polycystic kidney disease in 2, lithium nephropathy in 1, and glomerulonephritis in 2 patients. Hemodialysis blood access consisted of arteriovenous fistulas in 14 patients, polytetrafluoroethylene grafts in 7 patients, and tunneled, dual lumen central catheters in 3 patients. Patients with acute intercurrent illnesses and infections, and those who used antibiotics or immunosuppressive drugs within 3 months before enrollment, were excluded.

A group of 12 normal subjects (4 men and 8 women, aged 51 ± 12 years) served as controls. Individuals with intercurrent illnesses, acute or chronic infections, and those with a history of antibiotic therapy during the prior 3 months were excluded. The study protocol was approved by Human Subjects Institutional Review Board of the University of California Irvine (HS number 2007-5572) and completed with the assistance of the University Of California Irvine Institute Of Clinical Translational Science.

Animals

Male Sprague–Dawley rats with an average body weight of 225–250 g (Harlan Sprague-Dawley, Indianapolis, IL) were used in this study. Animals were housed in a climate-controlled vivarium with 12-h light and dark cycles, and were fed a standard laboratory diet (Purina Mills, Brentwood, MO) and water *ad libitum*. The animals were randomly assigned to the CRF and sham-operated control groups. The CRF group (6 animals) underwent 5/6 nephrectomy by surgical resection of the upper and lower thirds of left kidney, followed by right nephrectomy 7 days later. The control group (five animals) underwent sham operation. The procedures were carried out under general anesthesia (intraperitoneal injection of ketamine/xylazine) using strict hemostasis and aseptic techniques. The animals were observed for 8 weeks. Timed urine collections were obtained using metabolic cages. Urine protein and creatinine concentrations were measured using standard techniques. At the end of the observation period, animals were anesthetized and killed by exsanguination using cardiac puncture. Animals' colons were immediately excised and fecal material was harvested for microbial DNA extraction. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of California (Irvine, CA).

Fecal collection and bacterial DNA isolation procedures

DNA of microbial flora contained in the stool samples was isolated using the PSP Spin Stool DNA *PLUS* Kit following the manufacturer's specifications (Invitek Biotechnology and Biodesign, Berlin-Buch, Germany). The kit provides stool collection containers and DNA stabilization, isolation, and purification components.

PCR amplification and sample preparation for 16S rRNA gene PhyloChip analysis

All 36 individually processed human fecal gDNA extractions were PCR amplified. Primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) were used to amplify the 16S rRNA gene regions for bacteria. Each PCR reaction contained 1× Ex Taq buffer (Takara Bio, Otsu, Japan), 0.025 units/μl *Ex Taq* polymerase, 0.8 mmol/l dNTP mixture, 1.0 μg/μl bovine serum albumin, 300 pM of each primer, and 10–30 ng gDNA as template. For the PhyloChip assay[40] (Second Genome, San Francisco, CA), each sample was amplified in 8 replicate 25-μl reactions spanning a temperature range of 48–56 °C and 25 cycles for the annealing step. Amplicons from each temperature gradient were pooled and concentrated to 40 μl or less final volume using Microcon YM-100 filters (Millipore, Billerica, MA).

Eleven individually processed rat fecal gDNA extractions were amplified as above with the exception of using 5× GoTaq buffer (Promega, Madison, WI), 5 units/μl GoTaq polymerase, 200 pM of each primer, a dNTP mixture (2.5 mmol/l dATP, dCTP, dGTP; 1.67 mmol/l dTTP; 0.83 mmol/l dUTP) for the amplification step to provide uracil incorporation, and 2 ng of template per annealing temperature. PCR was run for 30 cycles. In addition, the pooled products were concentrated and purified using a Qiagen MinElute PCR kit (Qiagen, Hilden, Germany) and eluted in 20 μl of elution buffer. A volume of 1 μl of concentrated PCR product was quantified on a 2% agarose E-gel using the Low Range Quantitative DNA Ladder (Invitrogen, Carlsbad, CA).

Microarray processing

Microarray analysis was performed using the PhyloChip,[41] an Affymetrix-platform microarray (Affymetrix, Santa Clara, CA). For each sample, 500 ng of PCR-amplified 16S rRNA product, a series of defined quantitative standards for internal controls ranging from 4.10×10^{8} to 8.87×10^{10} copies, and 2 μl of Affymetrix control oligo B2 were prepared for PhyloChip hybridization. Human samples were analyzed on the second-generation (G2) PhyloChip containing approximately 500,000 twenty-five-mer oligonucleotide probes targeting 8364 bacterial OTUs.[40] Laboratory rat samples were analyzed at a later date, by which time the third-generation (G3) PhyloChip

containing approximately 1,100,000 twenty-five-mer oligonucleotide probes targeting 58,000 OTUs[42] was available. There is no one-to-one correspondence of bacterial OTUs from the G2 and G3 versions of the PhyloChips, and thus the results were analyzed separately. Processing of amplified PCR product was previously reported[40,42] for the G2 and G3 chips, respectively. Briefly, DNA amplicons for the G2 chip analysis were fragmented with DNAse (Invitrogen), biotin labeled, denatured, and hybridized to the DNA microarray at 48 °C overnight (>16 h). Amplicons for the G3 chips were prepared using the GeneChip WT Double-Stranded DNA Terminal Labeling Kit, ControlOligo B2, and the GeneChip Hybridization, Wash, and Stain Kit (Affymetrix) as per the manufacturer's instructions, with optimizations for the custom microarray as detailed in the Supplementary Information. The fragmentation mixture was incubated at 99 °C for 5 min to denature the DNA strands, and then held at 48 °C for 5 min before being transferred to a prehybridized array also being held at 48 °C.

Arrays were hybridized at 48 °C and 60 r.p.m. overnight and then washed and stained using procedures recommended by Affymetrix. Details of washing, staining, image capturing, and initial data processing have been described previously.[40,42] Fluorescence intensities of the internal standards were used to normalize total array intensities among samples.

The CEL files obtained from the Affymetrix software that produced information about the fluorescence intensity of each probe were analyzed. The detailed criteria for scoring the probe fluorescence intensities were described elsewhere[40,42] and detailed in the Supplementary Information. OTUs and higher-level taxa selection for G2 and G3 data analysis followed the procedure by Hazen *et al.*,[42] although for G2 arrays, PhyCA parameters (Stage1 and Stage2) were selected to yield similar results to the previously used CEL analysis pf $\geqslant 0.9$ parameter for scoring an OTU present. OTUs within passing subfamilies or species were used for abundance comparisons.

Statistical analyses

Probe intensity data corresponding to OTU called present were used to perform Student's *t*-test (Excel, two-tailed distribution, unequal variance) between groups. The Benjamini–Hochberg procedure for adjusting *P*-values for multiple comparisons was used to identify taxa with significantly different abundances (adjusted *P*-value <0.05, R 'stats' package[43]). The OTU intensities of the significantly differing OTUs were used to visualize sample ordination using non-metric multidimensional scaling.[44]

Total relative richness between control and CRF groups was tested for significance in R using the nonparametric Wilcoxon's test[43] because of the small sample sizes. A nonparametric (permutational) multivariate analysis of variance was performed using adonis (R 'vegan' package[44]) to test for differences in community profiles between control and CRF rat groups. The OTU intensities were used to analyze the samples and plot them using non-metric multidimensional scaling.[44] Student's *t*-tests with adjusted *P*-values were performed to identify significantly differing OTUs, as described above. OTUs differing significantly and having at least a two-fold difference in intensity were used to make a heat map (R 'gplots' package[45]). ANOSIM[46] was used to test for differences in total richness between sample groups. SIMPER analysis[46] was used to obtain a list of OTUs contributing to a summed total of 20% difference between groups.

All data are presented as mean ± s.d.

DISCLOSURE

TZD is employed by Second Genome. All other authors declared no competing interests.

SUPPLEMENTARY MATERIAL

Table S1. Families of operational taxonomic units (OTU) differing the most between Control (CTL) and Chronic Renal Failure (CRF) rats' intestinal microbiota determined by SIMPER analysis (using OTU contributing an accumulated total of 20% difference between groups).

Table S2. Measures of within-group dissimilarity for bacterial abundances (larger numbers indicate greater variability within a group).

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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