Differences in Gut Microbiota Profiles and Functions Between End-stage Renal Disease and Healthy Populations

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Research

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Abstract

Background: Patients with end-stage renal disease (ESRD) have extremely high risks of mortality and morbidity, as well as altered gut microbiota and impaired intestinal barrier function. The translocation of gut-derived molecules in ESRD contributes to systemic complications. In this study, we evaluated the gut microbiome difference in ESRD patients compared to age- and gender-matched subjects without kidney disease in discovery and validation cohorts.

Results: Compared to controls with normal renal function, an increased α-diversity and distinct β-diversity were found in ESRD subjects. The increase in α-diversity was correlated with protein-bound uremic toxins, particularly hippuric acid. A higher microbial dysbiosis index (MDI) was found in ESRD patients with the following enriched genera: *Facealibacterium*, *Ruminococcus*, *Fusobacterium*, *Dorea*, *Anaerovorax*, *Sarcina*, *Akkemansia*, *Streptococcus*, and *Dysgonomonas*. MDI at the genus level demonstrated highly differentiated accuracies between ESRD and control subjects in the discovery cohort (area under the curve [AUC] of 81.9%) and between ESRD and control subjects in the validation cohort (AUC of 83.2%). On functional enrichment analysis with gut metabolic modules, ESRD subjects presented with increased saccharide and amino acid metabolism when compared with matched controls.

Conclusions: An enriched but dysbiotic gut microbiota was presented in ESRD patients, in which the bacteria that were present increase amino acid metabolism linked to the production of protein-bound uremic toxins.

Background

The gut microbiota is a complex ecosystem that has been linked to host physiology and human health [1, 2]. Disruption of the microbial equilibrium, or dysbiosis, can promote and influence intestinal and extra-intestinal diseases [3–5]. Quantitative and qualitative alterations in the microbiome are implicated in the causation and progression of disease states such as obesity, diabetes mellitus, inflammatory bowel disease, cardiovascular disease, and chronic kidney disease (CKD).[5, 6] Subjects with CKD, which is known to increase risks of mortality and morbidity, have different microbial compositions and functions compared to subjects with normal kidney function. Patients with CKD or end-stage renal disease (ESRD) present with more proteolytic microbes, leading to increased metabolism of bacteria-derived toxins such as indoxyl sulfate (IS) and p-cresyl sulfate (PCS) [7]. These toxins accumulate in CKD or ESRD patients, stimulating oxidative stress and systemic inflammation, thereby contributing to cardiovascular disease and CKD-related systemic complications [8–11].

Gut microbial dysbiosis is characterized by an imbalance in gut microbiota composition and its metabolic capacity that can promote microbiota-derived metabolites, neuroendocrine deregulation, chronic inflammation, and interruption of intestinal barrier function, all of which are representative of the pathogenesis of CKD [12, 13]. In particular, urea toxicity, gut wall edema, inflammation, and oxidative stress are major mechanisms that drive the disintegration of the intestinal barrier [13, 14]. Elevated urea level as a consequence of bacterial expansion with urease activity, lead to the increased ammonium production in gut lumen, causing gut pH change, mucosal irritation, and gut wall structural damage, resulting in the increased intestinal permeability by the alteration of tight enterocyte junctions [12, 15]. Increased permeability of the intestinal barrier in patients with CKD and ESRD leads to the translocation of gut microbial products (e.g., bacterial fragments, lipopolysaccharides, and toxins) into the systemic circulation, causing local inflammation via activated immune cells (macrophages and T lymphocyte) that release pro-inflammatory cytokines and chemokines [13]. Thus, there is gut microbiota-kidney crosstalk as CKD modifies the composition/functions of the gut microbiota and gut microbial dysbiosis aggravates the burden in CKD patients [16].

Decreases in microbial richness and diversity have been found in CKD [17] due to a strict diet of limited protein, fat, fiber, and foods with a high potassium and oxalate content. Such dietary restrictions diminish the intake of phosphate-rich (e.g., cheese and yogurt) and potassium-rich (e.g., fruits and vegetables) foods, leading to a decrease in beneficial symbiotic bacteria. However, the ESRD diet, which lacks diversity and dietary fiber, but is rich in energy, saturated fats, and animal protein [17, 18], may have a different impact on the microbiome compared to the CKD diet. Dietary intake can influence the structure and activity of the trillions of microorganisms residing in the human gut [19–21]. In general, the microbial composition reflects trade-offs between carbohydrate and protein fermentation.[20] In particular, the former favors pathways for amino acid catabolism when protein is abundant [20]. Therefore, we speculate that there are differences in gut microbial diversity and composition in ESRD patients. Some studies have evaluated microbial composition in ESRD patients compared to healthy controls and demonstrated a profusion of Firmicutes, Actinobacteria, and Proteobacteria, as well as a reduction in *Bifidobacterium* and *Lactobacillius* [22–24]. However, only a limited number of studies have investigated microbial diversity in ESRD patients and explored its possible etiology. In this study, we investigated the differences in microbial diversity and dysbiosis in ESRD patients compared to normal kidney function subjects. The relationships among microbial diversity and comorbidities, medications, and microbial-derived metabolites were explored to explain the possible mechanism(s) for these differences.

Results

Baseline characteristics of participants

There were 82 ESRD participants in the discovery cohort from Kaohsiung Medical University Hospital (KMUH) and 58 ESRD participants in the validation cohort from Taipei Tzu Chi Hospital (TTCH). Comparisons were made with age- and sex-matched control subjects (Fig. 1). Among the ESRD patients, the mean age was 59.8 ± 10.1 years in the discovery cohort and 60.5 ± 6.0 years in the validation cohort. Moreover, 57.3% and 58.6% of the subjects in the discovery and validation cohorts were male, respectively. There were higher proportions of ESRD patients with diabetes mellitus, hypertension, hyperlipidemia, and *Bacteroides* enterotype (Table 1).

Table 1
Baseline characteristics between KMUH ESRD patients, TTCH ESRD patients, and control subjects

Baseline characteristics	Discovery cohort			Validation cohort		
	KMUH ESRD patients (N = 82)	Matched controls (N = 82)	*P*	TTCH ESRD patients (N = 58)	Matched controls (N = 58)	*P*
Age (years)						
Mean ± SD	59.8 ± 10.1	59.2 ± 9.5	0.720	60.5 ± 6.0	59.5 ± 5.2	0.357
Median (Q1-Q3)	59.0 (53.2–67.8)	59.0 (53.2–66.0)	0.735	62.0 (58.0–65.0)	61.5 (58.0–63.0)	0.149
Male	47 (57.3%)	47 (57.3%)	> 0.999	34 (58.6%)	34 (58.6%)	> 0.999
Comorbidities						
Diabetes mellitus	26 (31.7%)	9 (11.0%)	0.002	29 (50.0%)	10 (17.2%)	< 0.001
Hypertension	64 (78.0%)	22 (26.8%)	< 0.001	52 (89.7%)	10 (17.2%)	< 0.001
Hyperlipidemia	24 (29.3%)	6 (7.3%)	< 0.001	16 (27.6%)	5 (8.6%)	0.016
Enterotype classifier						
Bacteroides	60 (73.2%)	52 (63.4%)	0.240	42 (72.4%)	25 (43.1%)	0.002
Provetella	22 (26.8%)	30 (36.6%)	0.240	16 (27.6%)	33 (56.9%)	0.002

Biodiversity of the gut microbiota

Results of α-diversity analysis (observed operational taxonomic unit [OTU], Chao1, Shannon, Simpson, and inverse Simpson) differed significantly among ESRD participants and matched controls in the discovery and validation cohorts (observed OTU, $P < 0.001$; Chao1, $P < 0.001$; Shannon, $P < 0.001$; Simpson, $P < 0.001$; inverse Simpson $P < 0.001$; Fig. 2A). The differences in microbial composition (β-diversity) between ESRD patients and normal subjects are illustrated in the PCoA plot ($P < 0.001$; Fig. 2B). Distinct microbial compositions were found at different taxonomic levels in the discovery and validation cohorts (All $P < 0.001$; **Figure S1**).

Overall gut microbiota composition comparisons between ESRD patients and healthy controls

Gut microbiota composition and structure differed between ESRD patients and control subjects in the discovery cohort. At the phylum level, ESRD patients had lower levels of *Bacteroidetes* than controls. However, no differences were found in the levels of *Firmicutes*. Lower proportions of classes *Bacteroidia* and *Betaproteobacteria* and orders *Bacteroidales* and *Burkholderiales* were observed in ESRD patients when compared with controls. At family and genus levels, ESRD patients exhibited increased relative abundances of *Ruminococcaceae* and *Ruminococcus* but decreased *Prevotellaceae* and *Pravotella* (**Figure S2**). Other differences are presented in **Figure S2**.

Specific microbial taxa associated with ESRD patients and healthy controls

To identify the most relevant taxa responsible for the differences between ESRD patients and controls, discriminant analysis by linear discriminant analysis (LDA) effect size (LEfSe) method was performed and a cladogram was produced to represent the connections among taxa of differential abundance at different taxonomic levels (Fig. 3A). LDA scores suggested that differentially abundant taxa were potential biomarkers. Discriminating features between the two groups across different taxonomic levels were observed (**Figure S3**). In ESRD patients, predominant genera were *Facealibacterium*, *Ruminococcus*, *Fusobacterium*, *Dorea*, *Anaerovorax*, *Sarcina*, *Akkemansia*, *Streptococcus*, and *Dysgonomonas* compared to control subjects. In subjects with normal kidney function, predominant genera were *Prevotella*, *Lachnospira*, *Megamonas*, *Sutterella*, *Dialister*, *Acidaminococcus*, *Rothia*, *Megasphaera*, *5-7N15* (belonging to the family of *Bacteroidaceae*), and *Catenibacterium* compared to ESRD patients (Fig. 3B). We found increases in a total of 67 taxa in ESRD patients, including 20 species, 23 genera, 10 families, 6 orders, 5 classes, and 3 phyla (**Table S1**).

Microbial dysbiosis as ESRD marker

To illustrate the discriminative value of gut dysbiosis for ESRD, we constructed the microbial dysbiosis index (MDI) to differentiate ESRD subjects and controls. A higher MDI was found in ESRD patients (Fig. 4A). Genus-level MDI was associated with an AUC value of 81.9% in the discovery cohort and AUC value of 83.2% in the validation cohort (Fig. 4B). To evaluate the MDI at different taxonomic levels, prior discriminative values were found at the genus, species, and family levels (**Figure S4**). To consider the confounders that may influence MDI, multivariate-adjusted logistic regression was performed. After adjusting for covariates (including diabetes mellitus, hypertension, and hyperlipidemia) in the logistic regression models, ESRD patients continued to demonstrate an increased risk of microbial dysbiosis compared to matched controls (Table 2).

Table 2
The odds ratio of genus-level microbial dysbiosis index (MDI) on end-stage renal disease association in the discovery cohort

	Crude odds ratio	*P*	Adjusted odds ratio*	*P*
High microbial dysbiosis index	2.01 (1.60–2.53)	< 0.001	1.98 (1.53–2.55)	< 0.001
Low microbial dysbiosis index	Reference	–	Reference	–
*Adjusted for diabetes mellitus, hypertension, and hyperlipidemia				

Functional characterizations of the microbiome in ESRD patients and controls

Analysis of gut metabolic modules (GMMs) revealed the alteration of pathways in ESRD patients' gut microbiota in the discovery and validation cohorts. Compared to controls, ESRD patients had higher numbers of mapped genes with GMM-predicted metabolism of saccharides (lactose, arabinose, fucose, xylose, and pentose), amino acids (glutamate, alanine, cysteine, threonine, arginine, and histidine), and other metabolites (glycerol, pyruvate, lactate, mucin) (Fig. 5). The full results of GMM enrichment analysis are presented in **Table S2**. From the generated Clusters of Orthologous Groups (COGs), ESRD patients demonstrated increased gut microbial functions related to signal transduction histidine kinase, glycosyltransferase, Fe^{2+}- dicitrate sensor, and beta-galactosidase (**Figure S5**). As for the enrichment of predicted Traditional Kyoto Encyclopedia of Gene and Genomes (KEGG) Orthology (KO) groups, ESRD patients showed increased gut microbial functions associated with multiple sugar transport system, antibiotic transport system, and beta-galactosidase, as well as decreased functions of the lipoprotein-releasing system and major facilitator superfamily (MFS) transporters (**Figure S6**).

Exploration of the relationships between α-diversity and host parameters in ESRD patients

We found positive correlations between microbial-derived metabolites (hippuric acid [HA], IS, and PCS) and α-diversity in the discovery cohort. Circulating HA level was positively associated with microbial richness (r = 0.45, $P < 0.001$), Shannon index (r = 0.34, $P < 0.01$), Simpson index (r = 0.23, $P < 0.05$), and inverse Simpson index (r = 0.37, $P < 0.001$). No significant association was found between α-diversity and other clinical parameters, such as demographic data, comorbidities, and clinical laboratory data (Fig. 6). Considering that protein intake predisposes such patients to the development of protein-bound uremic toxins, we evaluated the α-diversity differences between high and low normalized protein catabolic rate (nPCR), which reflects daily dietary protein intake in stable dialysis patients. ESRD patients with nPCR value of more than1.2 g/kg per day had higher α-diversity (Shannon index, Simpson index) than ESRD patients with nPCR value of less than 1.2 g/kg per day (**Figure S7**). These findings implied that higher protein intake predisposes ESRD patients to the development of protein-bound uremic toxins that further predict microbial diversity.

Discussion

In this study, we compared differences in gut microbiota between ESRD patients and age and sex-matched controls in discovery and validation cohorts. ESRD patients had higher α-diversity (within-sample diversity), distinct β-diversity (between-sample diversity), and increased gut microbial dysbiosis compared to the healthy population. In general, the gut microbiota of a healthy individual is more diverse than that of a diseased individual. However, this is not the case in ESRD patients. Diversity is fundamental to ecology as an indicator of the state of an ecosystem due to its relationships with stability, productivity, and functioning [25]. However, a higher diversity is not always better [25–27]. For example, increased α-diversity has been found in Parkinsonism [28].

No consistent reduction in diversity among patients relative to healthy individuals was found on the meta-analysis of case-control studies [29]. Host genetics, geographical region, environmental exposures (including dietary habits and drugs), and lifestyle factors are all essential to gut microbial diversity [30, 31]. In our study, microbiota diversity indices positively correlated with protein-bound uremic toxins (IS, PCS, HA) suggesting that diet drives biodiversity in the ESRD patients. The relationship between protein and microbiota diversity is further supported by higher microbial diversity in the high nPCR group. Similar finding reporting a positive correlation between urea levels (represent the protein intake) and microbiota diversity supports this claim [32]. Furthermore, a high-protein intake increases the plasma levels of protein-bound uremic toxins and urea in healthy individuals [33] and ESRD patients [34]. Thus, high-protein intake may increase gut microbiota diversity.

Recent metabolomics reports have indicated that certain protein-bound uremic toxins are strongly and positively associated with α-diversity.[35, 36] Our results demonstrated that the production of amino acid metabolites affected the composition of gut microbiota, previously identified by metabolites, such as hippurate and PCS, in non-CKD subjects [35, 36]. Our discovery of the positive relationship between IS and Shannon index agrees with previous studies, reporting a positive association between indole-containing compounds (Indolepropionate) consumption and Shannon diversity among non-CKD subjects [35]. In allogeneic stem cell transplantation patients, a positive association between the urinary level of microbiota-derived indole and microbial diversity has been found [37]. In contrast, no significant correlation has been demonstrated between IS/PCS and α-diversity in pediatric patients with ESRD [23]. Therefore, more studies are needed to explore this issue.

Individuals with CKD, especially ESRD, are often advised to follow restrictive diets based on individual nutrients such as sodium, potassium, and phosphorus to minimize circulating electrolyte imbalance and fluid retention. In the early stages of CKD, low protein intake is recommended to preserve kidney function and limit circulating nitrogenous waste. Patients that develop ESRD (complete kidney failure) and undergo dialysis are advised to increase protein consumption to more than the recommended amounts for healthy individuals and CKD patients. This is based on the need to preserve lean body mass [38, 39]. Current international recommendations for daily dietary protein intake are 0.8 g/kg for the general population [40], 0.6 to 0.8 g/kg for non-dialysis CKD patients [18, 41–43], and 1.1–1.4 g/kg for ESRD patients on dialysis [18, 43–45]. A high protein diet is recommended for ESRD patients on dialysis as reduced protein intake is associated with increased all-cause mortality [46, 47]. Insufficient protein intake in ESRD patients may lead to difficulty counteracting protein

loss and catabolism during dialysis [48]. A high protein diet, similar to the Western diet (which is rich in animal proteins and fats), stimulates the overgrowth of proteolytic bacteria, resulting in dysbiosis and accumulation of proteolytic-derived uremic toxins [49]. Also, impaired small intestine protein digestion and amino acid absorption in ESRD patients results in more proteins reaching the large intestine [50]. Prolonged colonic transit time is not only associated with high richness and diversity of the microbiota but also higher bacterial protein catabolism, facilitating increased protein fermentation by proteolytic (putrefactive) bacteria for energy metabolism [51–56]. In our study, increased microbial function related to amino acid metabolism was demonstrated in ESRD patients on GMMs enrichment analysis. However, increased α-diversity can be a double-edged sword in ESRD. Our findings of an association between protein-bound uremic toxins and microbial diversity may partly explain this paradox in ESRD patients.

In addition to the impact of protein intake and uremic toxins on α-diversity in ESRD patients, reduced consumption of fruits, vegetables, and dietary fiber to avoid potassium overload causes gut dysbiosis [56]. Other contributing factors include the rise in gastrointestinal luminal pH rise due to uremic milieu (ammonia and ammonium hydroxide) [7, 13, 57, 58] and complex drug exposure (e.g., antibiotics, phosphate binders, and iron) [59–61]. As expected, we found higher MDI in ESRD patients than in controls, which may help to differentiate ESRD patients from subjects without kidney disease. The association between high MDI and ESRD persisted even after adjusting for diabetes mellitus, hypertension, and hyperlipidemia. Dysbiosis of the gut microbiota in patients with kidney disease is characterized by a decrease in bacterial species with saccharolytic fermentation activity (e.g., *Lactobacillus* and *Prevotella*) and enrichment of bacterial strains with proteolytic fermentation activity (e.g., *Bacteroides* and *Clostridium*), which leads to the increased levels of circulating uremic toxins followed by chronic inflammation [62]. The previously reported results just correspond to the increased abundance of *Clostridium* and several *Bacteroides* strains in our ESRD patients compared to healthy controls.

In the present study, there were distinct gut microbial compositions in ESRD patients and healthy subjects. Increased abundance of several taxa has been reported [7, 22–24, 63, 64], such as the phylum *Actinobacteria*, class *Erysipelotrichi*, orders *Enterobacteriales* and *Erysipelotrichales*, families *Enterobacteriaceae*, *Verrucomicrobiaceae*, *Clostridiaceae*, and *Coriobacteriaceae*, and genera *Faecalibacterium*, *Desulfovibrio*, and *Cloacibacillus* (Table 3). In line with the findings of a previous report, microbial families that possess urease (*Enterobacteriaceae*)[7] and indole/p-cresyl-forming enzymes (*Clostridiaceae*, *Verrucomicrobiaceae*, and *Enterobacteriaceae*) [7, 65] were enriched in ESRD patients in our study. These microbial families harbor genes that encode tryptophanase–tyrosine indol-lyase, suggesting their essential roles in uremic toxins production. In an experimental study, it was confirmed that bacterial tryptophanase from *Bacteroides species* processes tryptophan to indole [66]. We also found an abundance of species *Bacteroides ovatus*, *Bacteroides uniformis, Bacteroides fragilis*, and *Bacteroides acidifaciens* in ESRD patients that might contribute to the elevation of uremic toxins. In addition, the *Ruminococcaceae* family, which can ferment tyrosine to p-cresol [67], was enriched in ESRD patients in our study, as well as, other bacterial genera reportedly increased with higher levels of protein-bound uremic toxins, such as *Akkermansia* and *Blautia* [68]. In theory, the influx of uremic toxins and urea into the gastrointestinal lumen provoke the overgrowth of bacteria that produce urease, uricase, indole, and p-cresol forming enzymes, generating a vicious cycle of inflammation and oxidative stress in ESRD patients [22]. Increased abundance of phylum *Verrucomicrobia* [63] and family *Enterobacteriaceae* [13, 64] in CKD patients has also been found. However, *Actinobacteria* phylum and *Akkermansia* genera are reduced in CKD patients [63] compared to ESRD patients. A high protein diet in experimental animal models leads to the enrichment of the *Akkermansia* genus [69]. Thus, the discrepancy regarding *Akkermansia* abundance between CKD and ESRD can be partly explained by differences in protein intake.

Table 3. The relationship between the gut microbial change in patients with kidney disease compared to relative healthy subjects and the reference which reported about the effects of kidney disease

			Increased abundance of taxa at a different level				
Study and subject sample	Analyzing tools	Microbial diversity	Phylum	Class	Order	Family	Genus
ESRD with dialysis							
Current study ESRD with HD (n=82) Control (n=82)	16s RNA Sequencing	Increased	Fusobacteria Verrucomicrobia **Actinobacteria**	Fusobacteriia Verrucomicrobiae Bacilli **Erysipelotrichi** Coriobacteriia	Fusobacteriales **Enterobacteriales** Verrucomicrobiales Lactobacillales **Erysipelotrichales** Coriobacteriales	Ruminococcaceae Fusobacteriaceae **Enterobacteriaceae** **Verrucomicrobiaceae** Gracilibacteraceae Streptococcaceae Erysipelotrichaceae Acidaminobacteraceae **Clostridiaceae** **Coriobacteriaceae**	**Faecalibacterium** Ruminococcaceae Fusobacterium Dorea Anaerovorax Sarcina Akkermansia Streptococcus Lachnospiraceae_ Dysgonomonas Cetobacterium Blautia CF231 **Desulfovibrio** Lachnobacterium Plesiomonas Eggerthella Epulopiscium Eubacterium Clostridium **Cloacibacillus** Adlercreutzia Lactococcus
Vaziri et al.[22] Wong et al.[7] ESRD with HD (n=24) Control (n=12)	16s RNA Microarray	No report				Beutenbergiaceae Cellulomonadaceae Dermabacteraceae Micrococcaceae Catabacteriaceae Clostridiaceae Coprobacillaceae Polyangiaceae Alteromonadaceae OM60 Enterobacteriaceae Methylococcaceae Halomonadaceae SUP05 Moraxellaceae Pseudomonadaceae Thiotrichaceae	

						Xanthomonadaceae Verrucomicrobiaceae	
Jiang et al.[24] ESRD (n=27) Control (n=26)	16s RNA sequencing	No difference		Deltaproteobacteria	Desulfovibrionales	Bacteroidaceae Desulfovibrionaceae	Bacteroidetes Parabacteroides Parasporobacteriu Marvinbryantia Cloacibacillus Anaerotruncus Paraprevotella
Crespo-Salgado et al.[23] (Pediatric) ESRD with HD (n=8) Control (n=13)	16s RNA sequencing	No difference					Bacteroidetes
CKD without dialysis							
Li et al. [63] CKD (n=50) Control (n=22)	16s RNA sequencing	Decreased	Actinobacteria	Actinobacteria	Actinomycetales Coriobacteriales	Methylobacteriaceae Clostridiaceae 1 Coriobacteriaceae	Methylobacterium Clostridum sensu s Desulfovibrio Paraprevotella Alloprevotella Clostrium IV
Wu et al. [64] CKD (n=92) Control (n=30)	16s RNA sequencing	Decreased	Proteobacteria	Negativicutes Erysipelotrichia Gammaproteobacteria	Selenomonadales Erysipelotrichales Enterobacteriales	Veillonellaceae Lactobacillaceae CFT112H7 Erysipelotrichaceae Enterobacteriaceae	Pseudobutyrivibric Megamonas Lactobacillus Faecalibacterium

There were several limitations to this study. For example, the cross-sectional design led to the inability to prove causality, and the sequencing of the 16S rRNA gene limited the analysis to the strain level. In addition, subjects were Asian ESRD patients. Their genetics, epigenetics, environmental factors, and diet may differ from those of other populations. For instance, the phylum *Bacteroidetes* has been reported to be most dominant in healthy Chinese adults [70, 71]. The switch from *Prevotella* enterotype to *Bacteroides* enterotype in ESRD patients [24] could only be observed in participants from the validation cohort but not from the discovery cohort. Lastly, our targeted protein-bound uremic toxin approach allowed for precise metabolite measurements but was limited to predefined target metabolites. In terms of its strengths, a large number of ESRD participants were enrolled in this discovery and validation study to analyze 16S rRNA amplicon profiling, enabling us to adequately investigate microbial diversity, gut dysbiosis, and microbial function.

Conclusions

ESRD patients have a distinct microbial composition, increased microbial diversity, predicted gut dysbiosis, and increased microbial function related to amino acid metabolism compared to healthy controls. Circulating protein-bound uremic toxins, especially HA, explained the increased microbial diversity in ESRD patients. Our findings demonstrated an association between gut microbiota and kidney failure treatment strategy, especially in terms of dietary recommendations.

Methods

Study participants

From August 2017 to February 2018, 194 ESRD patients from the dialysis units of KMUH and TTCH, Taiwan, were enrolled. Eligible participants received regular hemodialysis three times per week, 3.5-4 hours each time with high-flux dialyzers. Control participants without kidney disease were enrolled from the health management center at Taichung Veterans General Hospital, Taiwan, from January 2015 to December 2015. Participants with previous malignancies, prior gastric surgery, or who had received antibiotics within three months before enrollment were not included. Experiments were carried out following the protocols approved by the Ethics Committee of KMUH (KMUHIRB-E(I)-20160095 and KMUHIRB-E(I)-20180118), TTCH (07-X01-002), and Taichung Veterans General Hospital (07-X01-002). All participants signed an informed consent form. In the present study, KMUH ESRD patients (n = 82) served as the discovery cohort, and TTCH ESRD patients (n = 58) served as the validation cohort with 1:1 age- and sex-matching to investigate differences in gut microbiota.

Comorbidities, laboratory data, and clinical variables

Sociodemographic data, age, sex, medical history, and prescribed medications were obtained for all participants from electronic health care records. Diabetes was defined as HbA1C of 6.5% or higher or the use of oral antidiabetic agents or insulin. Hypertension was defined as blood pressure of 140/90 mmHg or higher or the use of blood pressure-lowering drugs. Hyperlipidemia was defined according to physician diagnosis or the use of lipid-lowering medications. Blood samples were obtained from patients after overnight fast through arteriovenous access before a scheduled midweek hemodialysis session. Biochemical data included serum values for hemoglobin, albumin, fasting glucose, total cholesterol, triglyceride, low-density lipoprotein, blood urea nitrogen, creatinine, sodium potassium, total calcium, phosphate, parathyroid hormone, alkaline phosphatase, aluminum, iron, full iron-binding capacity, ferritin, transferrin saturation, uric acid, high sensitivity C-reactive protein, single pool Kt/V, and normalized protein catabolic rate (nPCR), obtained within 30 days of fecal microbiota testing.

Fecal sample collection and DNA extraction

All participants provided a stool sample that was immediately frozen after home collection and delivered by using cooler bags within 24 hours via commercial transport to the laboratory (Germark Biotechnology, Taichung, Taiwan). DNA was extracted from fecal samples using a QIAamp DNA Stool Mini Kit (Qiagen, MD, USA). The isolated DNA aliquot was stored at − 80 °C before 16S rRNA gene sequencing (at least 500 ng per sample). The DNA quality and concentration of each sample were measured by agarose gel electrophoresis and NanoDrop ND-1000 (Thermo, DE, USA) with standards of ≥ 500 ng, 260/280 ratio of 1.7–1.8 and 260/230 ratio of 1.8–2.2 before downstream processing.

16S ribosomal RNA gene sequencing and data processing

Metagenomic DNA was amplified using the hypervariable region V3–V4 of bacterial 16S rRNA genes by bar-coded universal primers 341F (F, forward primer; 5-CCTACgggNggCWgCAg-3′) and 805R (R, reverse primer; 5′-gACTACHCgggTATCTAATCC-3′) with Illumina adapter overhang.[72] Library construction and sequencing of amplicon DNA samples were achieved with Genomics BioScience (Taipei, Taiwan). Sequencing was performed using MiSeq Reagent v3 Kit (Illumina, Wilmington, DE, USA) with a pair-end library (insert size of 465 bp for each sample) and high-throughput sequencing was performed using Illumina MiSeq 2000 platform (Illumina). All samples were sequenced at the same time in the same laboratory (Germark Biotechnology, Taichung, Taiwan).

Bioinformatics analysis of the 16S rRNA amplicon was conducted as previously described [73]. Briefly, on a per-sample basis, pair-end reads were merged using USEARCH (v8.0.1623), with 8 bp minimum overlap of reading pairs [74]. Merged sequences were processed using Mothur (v1.35.1) to remove reads shorter than 450 bp or longer than 550 bp, as well as any read not meeting the minimum quality score of 27 [75]. Reads containing ambiguous base or homopolymer exceeding 8 bp were discarded. Chimeric sequences were identified and removed by USEARCH (reference mode and 3% minimum divergence). Quality-filtered and non-chimeric reads were analyzed (UPARSE pipeline [76]) to generate OTUs per sample at a 97% similarity cutoff. The identified OTU representative sequences were then aligned using USEARCH to determine the corresponding taxonomy from the Greengenes reference database (version 13.5) [76, 77]. OTUs without a hit or with only a weak hit were excluded from the following analysis.

Analysis of richness and biodiversity

The α-diversity measures the richness and evenness of taxa within each sample. The β-diversity compares the taxa between pairs of individual samples. The α-diversity indices were estimated with the R package “phyloseq”. The species richness indices (Observed, Chao 1) and species diversity (Shannon, Simpson, Inv Simpson) were tested by Student’s t-test [78]. The β-diversity was assessed by computing the Bray-Curtis distance and displayed via Principal Coordinates Analysis (PCoA) to evaluate microbial compositional similarities between ESRD patients and controls [79]. Between-class analysis of inertia percentage was performed by the R package “ade4” using Monte-Carlo test with 10,000 permutations to assess the statistical significance between groups [80].

Bacterial community comparisons between ESRD and control subjects

A pseudo-count of 0.0001 was added to the relative abundance (in percentage) before the logarithmic transformation [81]. Differential abundance analysis was performed using the Wilcoxon rank-sum test. Only taxa with average abundance > 0.2% and sample coverage > 10% were included in the differential analysis. LEfSe was applied to determine the taxa of significantly differential abundance between the ESRD patients and controls, evaluated with α of 0.05 (Kruskal-Wallis and Wilcoxon tests) and effect size threshold of 2 using the stand-alone implementation [82]. The results were plotted on a cladogram based on phylogenetic relationships among taxa.

Microbial dysbiosis index analysis

MDI is an index that contrasts the abundance of enriched taxa in case and control cohorts [83]. In the present study, MDI was determined by the logarithm of accumulated relative abundance of enriched taxa (LEfSe [82]) in ESRD and control subjects. The 10% of globally non-zero minimal relative abundance was used in the logarithm if the sum was zero. MDI was analyzed at six taxonomic levels from phylum to species, and its distribution was visualized. The empirical probability of ESRD was predicted by the proportion of ESRD over all subjects (i.e., control and ESRD) in the corresponding MDI. Logistic regression was applied to model MDI versus ESRD probability. The receiver operating characteristic curve was generated from all samples, and the most significant area determined the best taxonomic level for classification under curve. MDI of best sensitivity and specificity was used for validation of predicted performance.

Gut metabolic module analysis

Functional analysis is a critical advantage of shotgun sequencing data. KEGG annotation methods hold redundant information and are not suitable for the interaction between host and microorganism. Thus, we investigated GMMs [84] to show the functional changes in ESRD patients. GMMs are bacterial and archaeal metabolic pathways associated with the human gut, mainly anaerobic fermentation processes. Each module is composed of prokaryotic and archaeal KEGG Orthology (KO) groups to describe an enzymatic process that converts the input compound to output metabolite. GMMs were first grouped by their position in the gut metabolic map (i.e. input, central, and output; module numbers 75, 11, and 17, respectively), then by 10 metabolic categories and 30 subcategories. There were 103 GMMs in total. To infer the GMM profile, the KO profile was first predicted by PICRUSt [85], then subjected to Omixer-RPM [86] with default settings except for output format, which was set to 2. To identify GMMs with differential abundance, enrichment analysis was performed by two-tailed Wilcoxon test with Benjamini-Hochberg false discovery rate correction for multiple testing.

Bacterially derived protein-bound uremic toxin profiling

Circulating free form IS, PCS, and HA were measured by mass spectrometry as previously described. Briefly, each ESRD patient's serum sample (300μL) was packed into a centrifugal filter device (Amico Ultra 3K, MerckMillipore). After centrifugation, the supernatant was evaporated and re-dissolved with 100 uL 30% acetonitrile (MeCN) and 0.1% formic acid. Subsequently, 10 μL IS-d4 (internal standard purchased from Sigma–Aldrich, 1000 ng/mL) were added to the sample and filtrated with 0.22 μm polytetrafluoroethylene (PTFE) filters for mass spectrometer analysis. The tandem mass spectrometry system was equipped with Micro electrospray ionization (ESI) ion source, coupled with Acella 1250 Ultra-high performance liquid chromatography (UHPLC) analytical system (Thermo Fisher Scientific Inc., Waltham, MA, USA) with 3.0 kV setting. The samples were separated using a Shiseido HPLC CAPCELL PAK C18 MGII column (150 mm × 1.5 mm, 3.0 μm, Tokyo, Japan) after injection into the UHPLC system through Acella 1250 autosampler. Quantification was carried out by survey scan mode multiple reaction monitoring (MRM) transitions 212 > 80 and 212 > 132 for IS and 187 > 80 and 187 > 107 for PCS. MS spectra and mass spectrometer access were achieved with Xcalibur software (version 2.2, Thermo-Finnigan Inc., San Jose, CA, USA).

For HA measurement, we prepared calibration standards solution by dissolving HA (Sigma–Aldrich, Catalog # 112003, St Louis, MO, USA) in ethanol using a contained mixture at 8 serial concentrations from 2 to 250 μg/ml. HA levels were measured with Agilent 1200 HPLC (Agilent Technologies, Palo Alto, CA) coupled with API 4000Q triple-quadrupole mass spectrometer (API 4000QTrap, Applied Biosystems/MDS SCIEX, Concord, Canada) and electrospray ionization (ESI) source in negative ion mode. Chromatographic separation was conducted with the Kinetex Phenomenex C8 column (250 mm × 4.6 mm × 5 μm) at room temperature in the mobile phase. MRM mode was used with characteristic fragmentation transitions. The mass transition pairs for quantification and qualification of HA were m/z 177.9 fragmented in MS-MS with major product ion at m/z 133.8 and second abundant ion at m/z 76.9. Data acquisition and quantitative processing were accomplished using Analyst (1.4.2) software (Applied Biosystems).

Statistical analyses

Continuous data with or without a normal distribution are expressed as mean ± SD or median and interquartile range. Comparisons were conducted using Student's t-test or Mann–Whitney U test. Categorical data are expressed as frequencies and percentages. Comparisons were conducted using Chi-squared analysis. The correlation measures among demographic data (age, sex, ESRD etiology, comorbidities, medications, clinical laboratory data, and uremic toxins) and alpha diversity were tested by Spearman correlation or Point Biserial correlation as appropriate. Considering the confounding factors, logistic regression models were used to identify associations between MDI and ESRD, adjusting for diabetes mellitus, hypertension, and hyperlipidemia. P values < 0.05 were considered statistically significant. All statistical analyses were performed using R software (version 3.5.1).

List Of Abbreviations

ESRD, end-stage renal disease; CKD, chronic kidney disease; IS, indoxyl sulfate; PCS, p-cresyl sulfate; HA, hippuric acid; OTU, operational taxonomic unit; MDI, microbial dysbiosis index; linear discriminant analysis, LDA; linear discriminant analysis effect size, LEfSe; GMM, gut metabolic module; normalized protein catabolic rate, nPCR

Declarations

Ethics approval and consent to participate

The subjects gave informed consent, and the study protocol was approved by the Ethics Committee of KMUH (KMUHIRB-E(I)-20160095 and KMUHIRB-E(I)-20180118), TTCH (07-X01-002), and Taichung Veterans General Hospital (07-X01-002).

Consent for publication

Not applicable

Availability of data and materials

Raw FASTQ data files of 140 samples from ESRD patients and 140 samples from control subjects have been deposited in NCBI Sequence Read Archive database under BioProject accession number PRJNA648014.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Dr. Ping-Hsun Wu, Dr. Ting-Yun Lin, Dr. Hsiu J. Ho, Dr. Ching-Hung Tseng, and Prof. Chun-Ying Wu take responsibility for the research design, data, research process, analyses, and interpretation. Prof. Shih-Shin Liang and Mrs. Hei-Hwa Lee take responsibility for the measurement of uremic toxins by tandem mass spectrometry. Dr. Yi-Ting Lin, Dr. Mei-Chuan Kuo, Dr. Szu-Chun Hung, and Dr. Yi-Wen Chiu critically revised the manuscript for relevant intellectual content. All authors have read and approved the manuscript.

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Figures

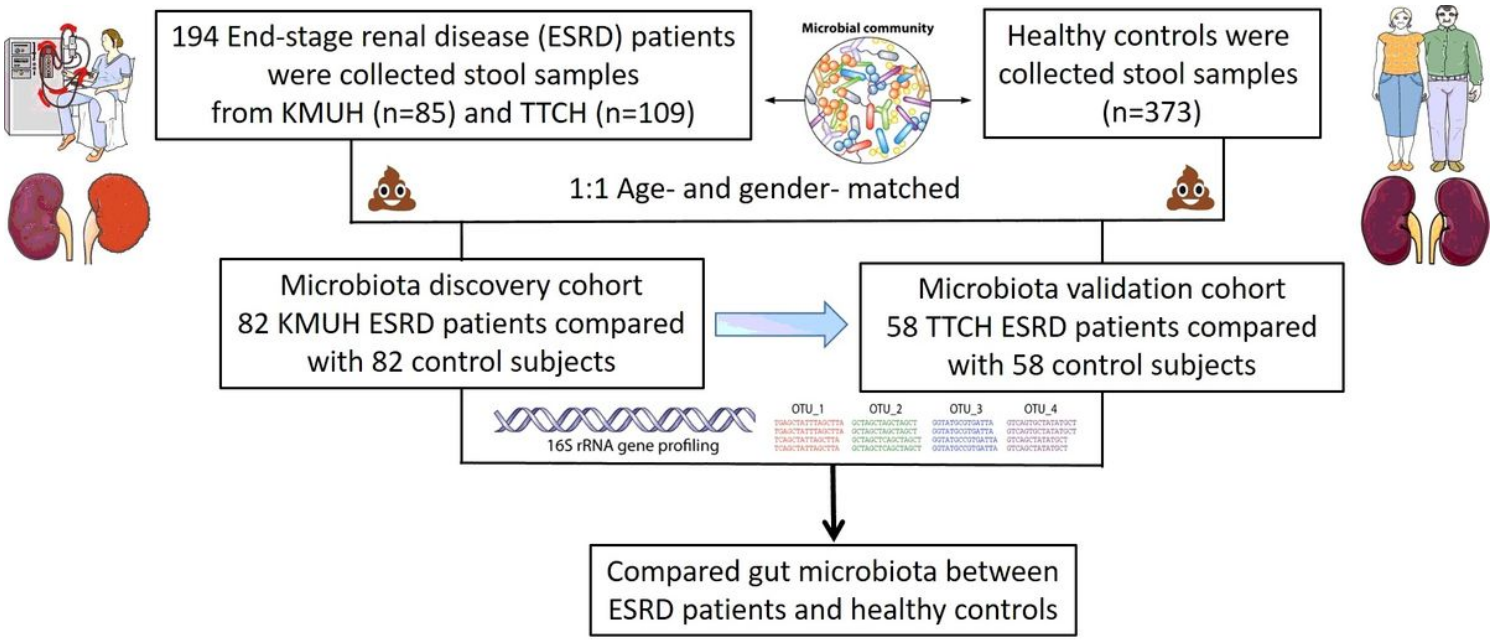

Figure 1

Study design

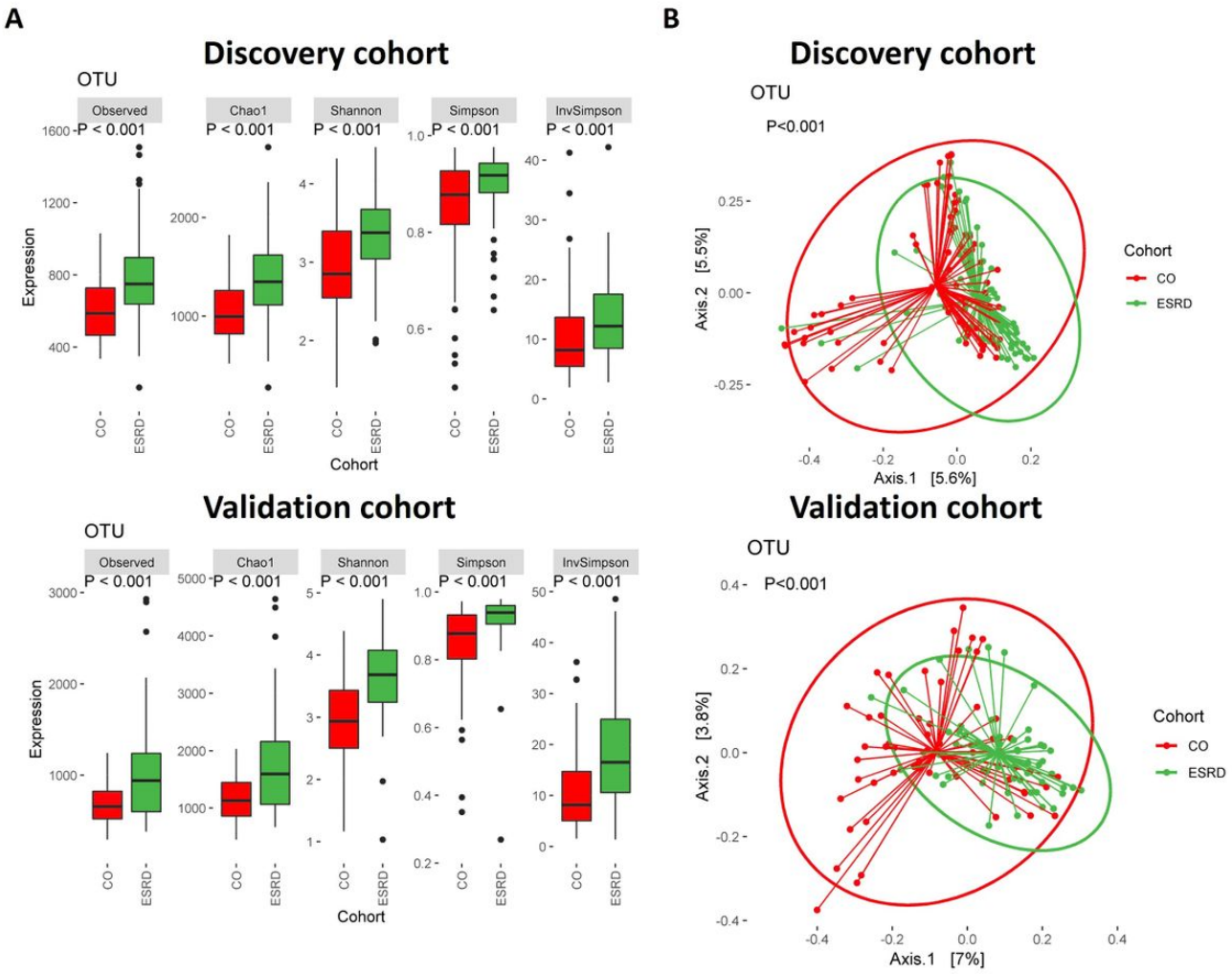

Figure 2

ESRD patients had a higher α diversity (observed, Chao 1 index, Shannon index, Simpson index, and inverse Simpson index) and different β diversity (Bray-Curtis distance metrics) compared to the general population in the discovery cohort and validation cohort

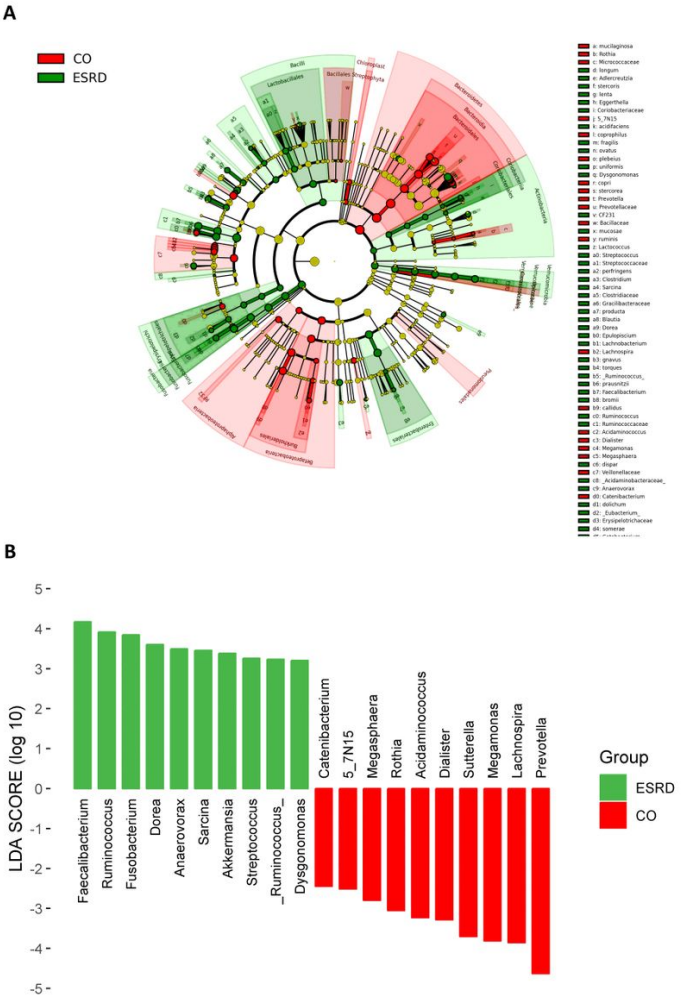

Figure 3

Linear discriminant analysis Effect Size (LEfSe) showing microbiome differences between ESRD and control subjects at various taxonomic levels. (A) Cladogram demonstrating microbiome differences at various phylogenic levels, (B) LEfSe analysis with linear discriminant analysis (LDA) score representing statistical and biological differences between groups at the genus level in the discovery cohort. Green indicates taxa enriched in the ESRD group and red indicates taxa enriched in the control group

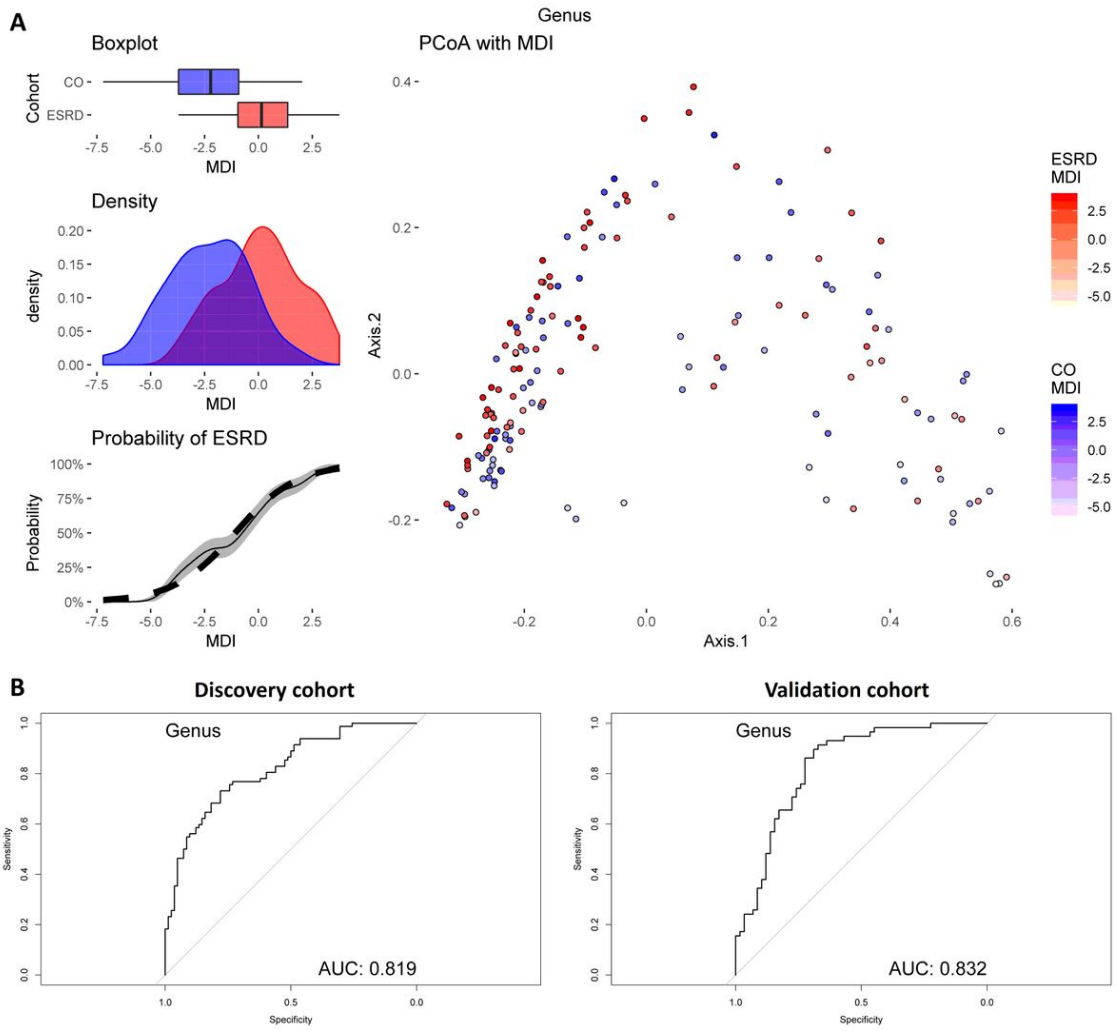

Figure 4

The microbial dysbiosis index (MDI) between ESRD patients and control subjects at genus levels in the discovery cohort and validation cohort. (A) Higher MDI in ESRD patients compared to control. (B) MDI has high differentiated accuracy between ESRD and control subjects in the discovery cohort and validation cohort

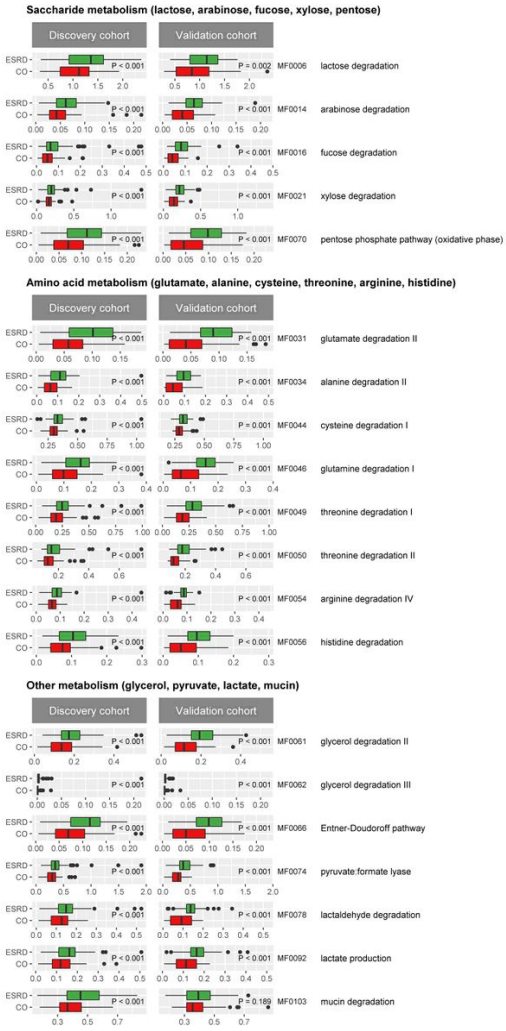

Figure 5

Enrichment analysis was performed to identify gene functions of differential abundances in gut microbiota between ESRD patients and control subjects. Functional classification of the predicted metagenome content of the microbiota of ESRD using the gut metabolic module (GMM) in the discovery cohort and validation cohort. Significance was considered for an adjusted P<0.05

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Figure 6

Explore the demographic data, comorbidities, clinical laboratory data, protein-bound uremic toxins, and alpha diversity relationship in the discovery cohort. The correlation measures between two continuous variables were tested by Spearman correlation. The correlation measures between categorical and continuous variables were tested by the Point Biserial correlation

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Allsupplementaryfiguresandtables20200919.docx