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ORIGINAL RESEARCH

Aberrant gut microbiota alters host metabolome and impacts renal failure in humans and rodents

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ABSTRACT

Objective Patients with renal failure suffer from symptoms caused by uraemic toxins, possibly of gut microbial origin, as deduced from studies in animals. The aim of the study is to characterise relationships between the intestinal microbiome composition, uraemic toxins and renal failure symptoms in human end-stage renal disease (ESRD).

Design Characterisation of gut microbiome, serum and faecal metabolome and human phenotypes in a cohort of 223 patients with ESRD and 69 healthy controls. Multidimensional data integration to reveal links between these datasets and the use of chronic kidney disease (CKD) rodent models to test the effects of intestinal microbiome on toxin accumulation and disease severity.

Results A group of microbial species enriched in ESRD correlates tightly to patient clinical variables and encode functions involved in toxin and secondary bile acids synthesis; the relative abundance of the microbial functions correlates with the serum or faecal concentrations of these metabolites. Microbiota from patients transplanted to renal injured germ-free mice or antibiotic-treated rats induce higher production of serum uraemic toxins and aggravated renal fibrosis and oxidative stress more than microbiota from controls. Two of the species, *Eggerthella lenta* and *Fusobacterium nucleatum*, increase uraemic toxins production and promote renal disease development in a CKD rat model. A probiotic *Bifidobacterium animalis* decreases abundance of these species, reduces levels of toxins and the severity of the disease in rats.

Conclusion Aberrant gut microbiota in patients with ESRD sculpts a detrimental metabolome aggravating clinical outcomes, suggesting that the gut microbiota will be a promising target for diminishing uraemic toxicity in those patients.

Trial registration number This study was registered at ClinicalTrials.gov (NCT03010696).

Significance of this study

What is already known on this subject?

- Alterations in gut microbial composition and serum metabolome have been reported in haemodialysis patients.
- Studies in animals suggest that many uraemic toxins are of gut microbial origin.

What are the new findings?

- Faecal and serum metabolomes of the patients with end-stage renal disease (ESRD) were tightly correlated and characterised by accumulation of several uraemic toxins and secondary bile acids (SBAs).
- Gut microbiota appears to be an important determinant of the host faecal and serum metabolic landscape, especially, the enrichment of uraemic toxins and SBAs in patients with ESRD is associated with gut microbiome-mediated aromatic amino acids degradation and microbial SBA biosynthesis.
- We identified a group of ESRD-associated microbial species which appears to be the cause of the production of uraemic toxins and SBAs.
- Transplantation of ESRD microbiota from patients with ESRD induced higher production of serum uraemic toxins and aggravated renal fibrosis and oxidative stress in renal injured germ-free mice and antibiotic-treated rats.
- ESRD-enriched *Eggerthella lenta* and *Fusobacterium nucleatum* increased uraemic toxins production and promoted renal disease development in the chronic kidney disease rat model, and a probiotic *Bifidobacterium animalis* reduced levels of toxins and the severity of the disease in rats.

INTRODUCTION

End-stage renal disease (ESRD), an advanced complication of chronic kidney disease (CKD),[1] is among the leading causes of morbidity and mortality worldwide.[2 3] Currently, the cost for treating ESRD is staggering; in USA only, it is

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Significance of this study

How might it impact on clinical practice in the foreseeable future?

- An aberrant gut microbiome in patients with ESRD may contribute to disease severity.
- Therapeutic approaches involving modulation of gut microbiota as a complementary therapy for dialysis should be explored.

estimated at approximately US$34 billion annually.[4] The progression of CKD to ESRD and its complications are closely related to the accumulation of toxic metabolites in blood and other metabolic compartments.[5–7] A significant proportion of these toxins are gut microbiota derived[8 9] and often cannot be efficiently removed by dialysis.[5] Significant alterations in the gut microbiome structure in patients with CKD[10–14] and disruption of blood and faecal metabolic composition in haemodialysis patients with ESRD[15–17] suggest the existence of a microbiome-based metabolic dysregulation in CKD. However, the microbial origins of ESRD-associated metabolites such as uraemic toxins and the mechanisms underlying gut microbiota-mediated changes in ESRD metabolomes have not been fully investigated. Such investigations may yield therapeutic insights, as revealed by a recent study in which the modulation of specific gut microbes was shown to regulate concentrations of a circulating uraemic toxin, indoxyl sulphate.[18]

Herein, we performed a comprehensive study integrating multidimensional datasets of gut microbiome, serum and faecal metabolomes, and host characteristics that were based on clinical and questionnaire-derived data (referred to as 'phenome'), from a large-scale cohort of 223 haemodialysis patients and 69 healthy controls matched by age stage, body weight and dietary pattern and an independent validation cohort of 24 individuals (online supplementary table 1). Study workflow is described in the online supplementary figure 1 and data production is reported in the online supplementary table 2.

RESULTS

Serum metabolomes of patients with ESRD and healthy individuals are different

Serum samples were analysed by untargeted mass spectrometry (MS), and the abundance profiles were obtained for 180 annotated serum metabolites. ESRD and control serum metabolomes were clearly different (figure 1A); 134 of 180 metabolites had significantly different abundances (online supplementary table 3). Given the heterogeneity of the patient population relative to primary disease (protopathy) types, we stratified it into the three main types, glomerulonephritis (n=76), diabetic nephropathy (n=73) and other (n=74), and compared separately each with the control population. The metabolome differences were in each case comparable, capturing ≥97% of the serum metabolites revealed as different in the entire population, and only a small number of metabolites (<9%) had significantly different abundances in different groups (online supplementary table 4). This indicates that the ESRD serum metabolome was largely independent of protopathy. Interestingly, we found that gender exerted a moderate, but statistically significant effect on the serum metabolome of patients with ESRD ($p=0.018$, $R^2=1.7\%$). Coherently, the ESRD status was the main reason for the difference of the patient and control serum metabolomes (figure 1B), as it explained almost 11% of the variance, while other bioclinical variables (eg, gender, body weight and total blood cholesterol concentration) collectively explained additional 8.5%; the three main protopathy types did not affect the serum metabolome significantly.

The ESRD serum metabolome was characterised mainly by enrichment of nine uraemic toxins and imbalance of bile acid composition (eg, conjugated vs unconjugated bile acids and primary vs secondary bile acids (SBAs)) (figure 1C; online supplementary figure 2A,B). Accumulation of uraemic toxins in patients with ESRD was confirmed by quantification of target metabolites in a randomly selected subset of 60 individuals (ESRD patients, n=40; healthy controls, n=20, online supplementary figure 2C). Such toxins have been reported to be produced by gut microbiota via the degradation of diet-derived aromatic amino acids (AAAs) and polyphenols[19 20] (online supplementary figure 3A), except for trimethylamine N-oxide (TMAO), which is produced by bacteria from choline and carnitine.[21] Similarly, altered circulating profiles of bile acids have been linked to renal disease[22 23] and are known to be modified by the gut microbiome[24] (online supplementary figure 3B). We clustered serum metabolites[25 26] (online supplementary table 5) and examined associations of cluster abundance with the clinical variables used to evaluate the progression of CKD such as blood creatinine and estimated glomerular filtration rate (eGFR), an indicator of overall kidney function[1] levels. Importantly, clusters including uraemic toxins and bile acids were strongly associated to the relevant clinical variables accross the entire cohort; significant associations were also observed in the separate patient and control groups (online supplementary figure 4). These findings, in line with previous reports,[7 27 28] illustrate the physiological importance of these circulating metabolites for the clinical state of patients.

Faecal metabolome of patients is altered and associated with serum metabolome

Strikingly, ESRD and control faecal metabolomes were also clearly different (figure 1D). Specifically, 98 of 255 annotated faecal metabolites had significantly different abundances between the two groups (online supplementary table 6, faecal metabolite clusters are listed in online supplementary table 7). Uraemic toxin precursors and SBAs were enriched in patient faeces, while primary bile acids, short chain fatty acids (SCFAs) and SCFA derivatives, such as methyl butanoic and methyl propanoic acid, were markedly reduced (online supplementary figure 5A,B); these compounds accounted for 49.6% of the variance of the faecal metabolome (online supplementary figure 5C). As for the faecal metabolome, two of the three protopathy groups (glomerulonephritis and other) differed from the control groups in a very similar way (≥90% of the differences seen for the entire cohort were recaptured in separate group comparisons; online supplementary table 4). Similarity was somewhat lower in diabetic nephropathy, even if a majority, 64%, of the faecal metabolites was still recaptured. The intergroup comparison confirmed the divergence of the diabetic nephropathy metabolome, as up to 23% (58 of 255) of metabolites had differential abundance (online supplementary table 4). Nevertheless, the uraemic toxin precursors or bile acids were not significantly different, with a single exception of *p*-cresol, which was more abundant in the diabetic nephropathy (online supplementary table 6). Gender showed no significant effect on faecal metabolome in the patients with ESRD ($p=0.463$. $R^2=0.43\%$), although it significantly affected serum metabolome. Notably, the ESRD status was again the main reason for the difference of the patient and control faecal metabolomes, even if it explained a lower fraction of the

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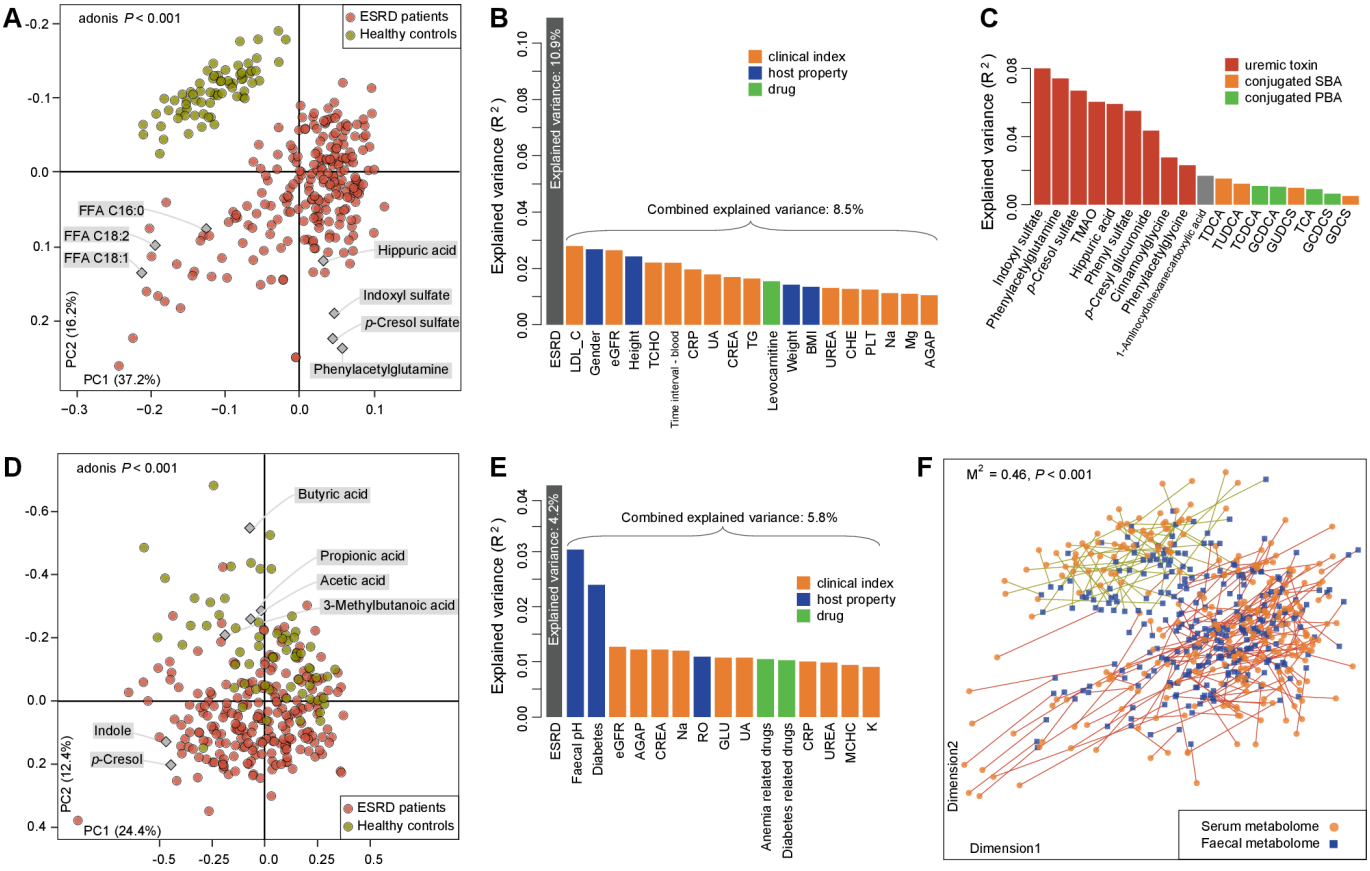


Figure 1 Distinct serum and faecal microbiome features associated with patients with ESRD. (A) Separation of serum metabolome between patients with ESRD and healthy controls, revealed by principal component analysis (PCA). The metabolites identified as the major contributors to the separation are indicated by diamonds. (B) Effect size of phenotype indexes that contribute significantly to the variance (R^2) of the serum metabolome (adonis p<0.05). This analysis was based on all subjects including patients with ESRD (n=223) and healthy controls (n=69). (C) Effect size of serum metabolites that drive the variance of serum metabolome. (D) Separation of faecal metabolome between patients with ESRD and healthy controls revealed by PCA. (E) Effect size of phenotype indexes which contribute significantly to the variance (R^2) of the faecal metabolome (adonis p<0.05). (F) Procrustes analysis of serum microbiome versus faecal microbiome. serum and faecal samples are shown as orange circles and blue squares, respectively; serum and faecal samples from the same individual are connected by red (patients with ESRD) and cyan (healthy controls) lines. AGAP, anioin gap; BMI, body mass index; CHE, cholinesterase; CREA, creatinine; CRP, C reactive protein; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; FFA, free fatty acids; LDL, low density lipids; PBA, primary bile acids; PLT, platelet; SBA, secondary bile acids; TCHO, total Cholesterol; TG, triglycerides; UA, uric acid.

variance (4.2%, figure 1E) than in the serum (10.9%). Other bioclinical variables collectively explained additional 5.8% of the variance; diabetes status was a significant contributor.

Procrustes analysis demonstrated a strong cooperativity of the serum and faecal metabolome profiles (figure 1F). Most importantly, the serum uraemic toxins and their faecal precursors were tightly correlated (online supplementary figure 6), suggesting that the metabolic alterations in the intestinal tract of patients with ESRD contribute significantly to the accumulation of uraemic toxins in the serum.

To further validate our metabolomic results, we considered an additional independent cohort comprising 12 patients with ESRD and 12 healthy controls (online supplementary table 1). Accumulation of serum uraemic toxins in patients with ESRD was confirmed in the new cohort (online supplementary figure 7A); similarly, in patient faeces, elevation of toxin precursors accompanied with an obvious tendency of decrease of SCFAs and SCFA derivatives was observed (online supplementary figure 7B). Statistical significance (q<0.1) was reached for a majority (8/13) but not all metabolites, likely due to a limited validation cohort size.

Taxonomic and functional characterisation of ESRD microbiome

To investigate whether the gut microbiota mediates metabolome changes in patients with ESRD, we analysed the gut microbiome by metagenome shotgun sequencing, generating an average 74.7 million reads (11.2 Gb of data) per faecal sample on an Illumina HiSeq platform (online supplementary table 8). We used the sequencing data to assemble a gene catalogue of 11.4 million non-redundant genes, representing the microbiome of our cohort. The genes were annotated to 11 867 Kyoto Encyclopedia of Genes and Genomes (KEGG) functional categories and organised into 900 metagenomic species (MGSs), of which about 66% could be assigned to known genera, highlighting a considerable yet to be explored novelty (online supplementary table 9).

Using the gene catalogue, first we showed that the microbial diversity, taxonomic composition and functional potential of the ESRD microbiome markedly differed from that of healthy controls (online supplementary figure 8A–C). Some 269 MGSs were enriched in patients, while 188 were depleted (figure 2A; online supplementary figure 9A); 164 that had species-level

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Figure 2 Characterisation of ESRD microbiome and its correlation with altered metabolites in serum and faeces of patients with ESRD. (A) Gut microbiota signatures in patients with ESRD. The X-axis shows the ratio (log_2 transformed) of a species abundance in ESRD patients compared with healthy controls. The Y-axis shows the power of a species to stratify patients and healthy controls, expressed as area under the curve (AUC). Species with significant differences in abundance between the two cohorts are shown in red (ESRD enriched) and green (control enriched). Species belonging to the same genus are linked by lines. (B) The boxplot shows the prominent species that differ significantly in abundance between patients with ESRD and healthy controls. boxes represent the interqurtile range between the first and third quartiles and median (internal line). Whiskers denote the lowest and highest values within 1.5 times the range of the first and third quartiles, respectively, and circles represent outliers beyond the whiskers. (C) Correlation of the concentrations of ESRD-enriched and healthy control-enriched metabolites with microbial functions. The heatmap shows the Spearman correlation coefficients between functional modules and serum (red text, showing uraemic toxins) or faecal (green text, showing uraemic toxin precursors, bile acids and SCFAs) metabolite clusters. Black boxes highlight the ESRD-associated metabolites and their corresponding functional modules. The significance level in the correlation test is denoted as: +q<0.05; *q<0.01; **q<0.001. ESRD, end-stage renal disease; CoA, coenzyme A; GABA, gamma amino butyric acid; RF-C, replication factor C; SCFA, short chain amino acids.

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and/or genus-level taxonomic assignment were listed in online supplementary table 10. As over a half of the species present in our cohort was significantly altered (457/900 MGSs, 51%), we conclude that ESRD strongly affects the microbiome. Species that were most enriched in patients included *Eggerthella lenta*, *Flavonifractor* spp (mainly *F. plautii*), *Alistipes* spp (mainly *A. finegoldii* and *A. shahii*), *Ruminococcus* spp and *Fusobacterium* spp (figure 2B; online supplementary figure 9B). Depleted species included *Prevotella* spp (mainly *P. copri*), *Clostridium* spp and several butyrate producers[29] (*Roseburia* spp, *Faecalibacterium prausnitzii* and *Eubacterium rectale*; figure 2B; online supplementary figure 9C). The decrease of faecal SCFAs in patients with ESRD is likely due to depletion of potential SCFA-producing species (online supplementary figure 9D–F).

ESRD microbiome was significantly enriched in functions involved in oxidative stress resistance, which may be due to higher patient inflammatory tone. Functions required for amino acid biosynthesis and degradation were depleted and enriched, respectively, possibly reflecting the increased amino acid availability in patients with ESRD (online supplementary figure 10A–C). The functional modules and enzymes involved in AAA degradation and SBA biosynthesis were enriched, coherent with the enrichment of uraemic toxin precursors and SBAs in the faecal metabolome (online supplementary figure 10D). Furthermore, a number of these functions significantly covaried with the concentration of the corresponding ESRD-associated metabolites in both serum and faeces (figure 2C). We conclude that the enrichment of uraemic toxins in patients with ESRD is associated with gut microbiome-mediated AAA degradation and microbial SBA biosynthesis.

Microbiome alterations mediate metabolome changes in patients with ESRD

To further explore the links between gut microbiota and the metabolome composition, we carried out interomics correlation analysis of abundances of gut MGSs, serum and faecal metabolites. Tight connections were identified in both patients with ESRD and healthy controls (figure 3A,B). However, the ESRD correlation network was clearly different from that of healthy controls, as only ~12% of correlations were common (online supplementary figure 11A–C). Notably, uraemic toxins and bile acids accounted for 95.0% of serum metabolite correlations in patients with ESRD, substantially more than that in healthy controls (69.3%; online supplementary figure 11D,E). Nevertheless, correlations between MGSs, uraemic toxins and bile acids were conserved in the two groups, as regards both the direction (positive or negative) and strength, slightly higher in controls (online supplementary figure 11F,G), further supporting the finding that gut microbiome impacts the levels of these compounds and thus progression of the disease. Importantly and along the same lines, serum uraemic toxins and bile acids were affected significantly by both the gut microbiome and faecal metabolome in patients with ESRD (figure 3C).

The effect size of the gut microbiome on metabolic profiles of patients with ESRD was considerable, as it accounted for 31.3% and 39.0% of the serum and faecal metabolome variance, respectively (figure 3D). Similar effect sizes (20%–40% of the serum metabolome variance) were shown in three independent studies in obese individuals,[30] patients with cardiovascular disease[31] or diabetes[25] (online supplementary figure 12). Importantly, the effect size of the host phenome on the metabolomes was significantly smaller than that of the gut microbiome, both in patients with ESRD (15.4% and 12.5% of serum and faecal metabolome variances, respectively) and healthy controls (12.6% and 14.4% of serum and faecal metabolome variances, respectively; figure 3D,E and online supplementary table 11). These results indicate that gut microbiota, besides the kidney function, appears to be an important determinant of the host faecal and serum metabolic landscape.

Non-antibiotic drugs can affect the gut microbiome[32 33] and might thus also impact the metabolome. The treatments administered to our patients with ESRD are summarised in online supplementary table 12. Adonis-based univariate analysis suggested that most drugs had no significant effect size. Only one, levocarnitine, had a significant and expected effect on serum metabolome (figure 1B); it may relate to raised carnitine levels. Diabetes-related drugs affected the faecal metabolome and MGS composition; they may, therefore in part drive the observed effect of the diabetes status on the metabolome composition. Anaemia-related medication also affected faecal metabolome composition. In all cases, the effect size of medication was rather modest, close to 1% (figure 1B and E; online supplementary table 12). As our patients were treated, on average, with four different drugs (online supplementary figure 13), we also carried out multivariate analysis, focusing on the drugs that singly had either a significant effect ($p<0.05$) or approached significance ($p<0.1$). Results showed that the composition of these drugs had no significant effects (online supplementary table 12). Diet is yet another potential confounder of the omics differences between patients with ESRD and healthy controls.[34 35] However, in our cohort, there was little difference in the nine main food categories, deduced from food frequency questionnaire of 117 items, other than a slight excess of wheat/rice in controls ($p=0.04$, online supplementary table 13). Permutational multivariate analysis of variance analysis further revealed that the above food categories did not have significant impact on the metabolome and microbiome (online supplementary table 13). Medication and diet showed little effect on faecal metabolome or microbiome, most likely due to the fact that the predialysis levels represented an extreme intoxicated stage.

ESRD-associated bacterial species contribute to toxin accumulation in the patients and link to their clinical profiles

To further substantiate the potential role of gut microbes in the production of uraemic toxins and SBAs, we focused on the microbial genes predicted to encode enzymes critical in the main synthesis pathways of these compounds (online supplementary figure 3). We identified 5134 genes encoding the key synthetases for 6 dominant uraemic toxins and SBAs in our gene catalogue (online supplementary table 14). These genes were significantly more abundant in patients with ESRD and in ESRD-enriched microbial species (online supplementary figure 14A). Faecal concentrations of the metabolites were positively correlated to the abundance of the cognate synthetase-encoding genes (online supplementary figure 14B). Guided by these findings, we applied random forest models to determine the correlation between each toxin or SBA and the abundance of species which contain the toxin/SBA synthetase-encoding genes. Random forest models that maximised the strength of the toxins and SBAs concentration prediction in serum or faeces identified some 83 species (figure 4; online supplementary table 15). The models accounted, on average, for 26% of the variance of the target metabolite concentrations in serum or faeces, indicating that the corresponding species largely contribute toxin and SBA production. Coherently with the increase in uraemic toxins and SBAs in patients with ESRD, a majority (59%) of species were more abundant in patients than in controls (figure 4). Considering that

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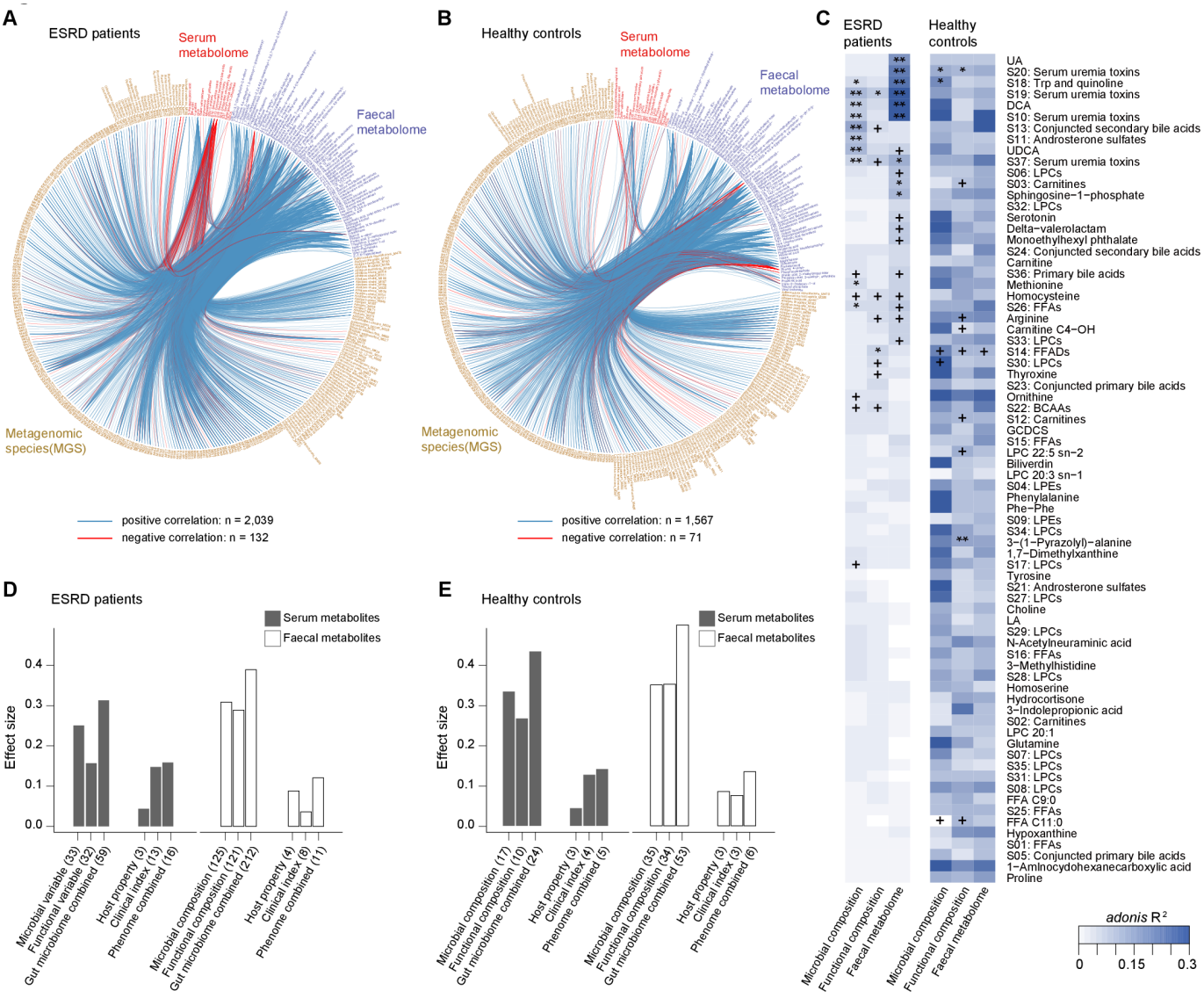


Figure 3 The gut microbiome influences host serum and faecal metabolomes in patients with ESRD. (A–B) The interomics correlation networks of all variables for the gut microbiome, serum and faecal metabolomes of patients with ESRD and healthy controls . Vertices indicate omics variables, and lines indicate a significant Spearman correlation coefficient at $|\rho|>0.35$ and $q<0.01$. (C) The permutational multivariate analysis of variance (PERMANOVA) of the covariant relationship between each serum metabolite cluster and the gut microbiome (including microbial and functional compositions) or faecal metabolome. The effect sizes between serum metabolite clusters and the gut or faecal metabolome are represented in shades of blue. Significance level: '+', permutated $p<0.05$; '*', permutated $p<0.01$; '**', permutated $p<0.001$. (D–E) The proportions of total variation in serum and faecal metabolomes of patients with ESRD (D) and healthy controls (E) explained by the gut microbiome and host phenome. To calculate the effect size, a set of non-redundant covariates was selected from the gut microbiome (including microbial and functional variables) or host phenome (including host properties and clinical indexes) by stepwise PERMANOVA analysis. The number of non-redundant covariates is shown in brackets. The detailed information for such covariates is provided in online supplementary table 11. ESRD, end-stage renal disease.

the concentration of toxins and SBAs might also be influenced independently of synthetic pathways (eg, metabolite transport), we extended the random forest models to include species that lack the synthetases and could account for an additional ~13% of variance (online supplementary figure 15; online supplementary table 16). Possibly, the gut microbiome drive of toxins and SBAs is even higher, as our models were based on the MGSs that could be constituted by the current methods; many other likely remain to be discovered.

Importantly, species linked to uraemic toxin or SBA production correlated directly and strongly to the most important ESRD clinical variables (online supplementary figure 16). In particular, a high proportion of the variance (on average, 50.8%) of eGFR, and circulating levels of creatinine, urea, C reactive protein and haemoglobin were determined by the species abundances (online supplementary table 17). We therefore hypothesise that the intestinal microbiome aggravates ESRD at least in part via uraemic toxins and SBAs.

A model based on the species discussed above provided an area under the receiver operating characteristic curve of 0.97 for patient/control stratification (online supplementary figure 17A), remarkably higher than the values reported by the gut microbiota models developed to classify metabolic and cardiovascular diseases.[31 36 37] In this model, *Eubacterium* spp, *Flavonifractor* spp and *E. lenta* were the main contributors (online supplementary figure 17B). These findings suggest that microbes involved

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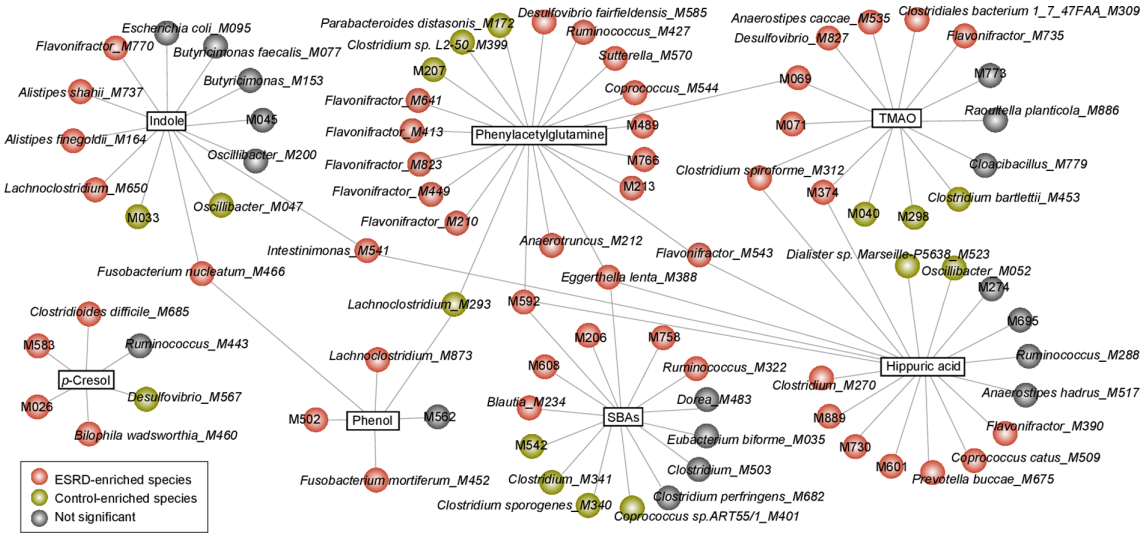


Figure 4 Alteration of the gut microbial composition in patients with ESRD contributes to uraemic toxin production and secondary bile acid biosynthesis. Network view of uraemic toxins/SBAs and MGSs. squares represent the uraemic toxins or SBAs, and the surrounding connected circles represent the species that were used in the random forest models. ESRD, end-stage renal disease; MGSs, metagenomic species; SBAs, secondary bile acids; TMAO, trimethylamine N-oxide.

in uraemic toxin and SBA production might be used as diagnostic markers for ESRD.

Modulation of the gut microbiome in CKD rodent models impacts toxin accumulation and renal disease severity

To test our hypothesis that the intestinal microbiome drives renal failure at least in part via uraemic toxins, we transplanted the fresh gut microbiota from either patients with ESRD or healthy donors into germ-free mice of adenine-induced CKD model[38] (online supplementary figure 18A). The donors were selected as having highest and lowest levels of ESRD-enriched and control-enriched species, respectively, at the first sampling; their microbiomes at the time of new sampling were similar to those at the first sampling (online supplementary figure 18B). After transplantation, the recipient mice efficiently recaptured the taxonomic features of the patient or control donors' microbiome (figure 5A; online supplementary figure 18C). Consequently, mice that received ESRD microbiota exhibited more severe renal fibrosis, glomerulosclerosis and oxidative stress, as well as increased serum level of urea and creatinine in tendency, as comparison to the recipient mice of microbiota from healthy donors (figure 5B,C; online supplementary figure 18D–H). Concomitantly, the serum levels of several uraemic toxins, including *p*-cresol sulphate, phenylacetylglycine, phenyl sulphate and indoxyl sulphate, were significantly increased in mice that received ESRD microbiota (figure 5D). Consistently, similar findings were also observed in a parallel faecal microbiota transplantation (FMT) experiment on antibiotics treated microbe-depleted CKD rats (5/6 nephrectomy model) (online supplementary figure 19). Collectively, these results demonstrate a causative contribution of the aberrant gut microbiota from patients with ESRD to renal disease development via modulation of uraemic toxins.

We next tested the hypothesis that presence of species capable of producing uraemic toxin precursors in an otherwise unaltered gut microbiome can aggravate renal disease progression. For that, we used two species, *E. lenta,* predicted to participate in production of hippuric acid and phenylacetylglutamine and *Fusobacterium nucleatum*, in production of indole and phenol (figure 4). In 5/6 nephrectomy CKD rat model, gavage with *E. lenta* or *F. nucleatum* (for study design, online supplementary figure 20A) increased their abundances in faeces significantly, but had no significant effect on the overall microbial composition (online supplementary figure 21A–C). Importantly, the serum levels of the uraemic toxins increased significantly relative to sham-fed rats (figure 5E), concomitantly with increased severity of oxidative stress, glomerulosclerosis, renal fibrosis and increased serum levels of creatinine and/or urea (figure 5F,G; online supplementary figure 20B–F). Furthermore, the relative abundances of *E. lenta* or *F. nucleatum* correlated directly with the concentration of their corresponding serum uraemic toxins (online supplementary figure 21D,E). These findings are consistent with the effects of uraemic toxin gavage of CKD rats in previous studies.[39 40] These results indicate that uramic toxin production by bacterial species could aggravate kidney disease.

A corollary of the direct role of gut bacterial species in kidney disease is that decrease of the driver species abundance should attenuate the severity of the disease. Consequently, we gavaged the CKD rats with *Bifidobacterium animalis* A6, a health-promoting probiotic,[41 42] and found that, even if the overall composition of the microbiome was not altered significantly (online supplementary figure 22A–C), the abundances of two toxin-driving species, *E. lenta* and *Fusobacterium* spp, were significantly decreased (online supplementary figure 22D). In parallel, serum concentrations of uraemic toxins, creatinine and urea were significantly decreased and renal fibrosis and glomerulosclerosis were reduced (online supplementary figure 23). These results suggest that treatment with this probiotic strain can alleviate renal disease development in CKD rat model via modulation of toxin-driving species.

DISCUSSION

In this work, we demonstrate that a severely aberrant gut microbiota in individuals with renal failure had a functional potential for accelerated biosynthesis of a number of toxic compounds, leading to elevated plasma concentrations of uraemic toxins and aggravated kidney disease (figure 6). The majority of toxin-producing bacterial species were ESRD-enriched and highly correlated with

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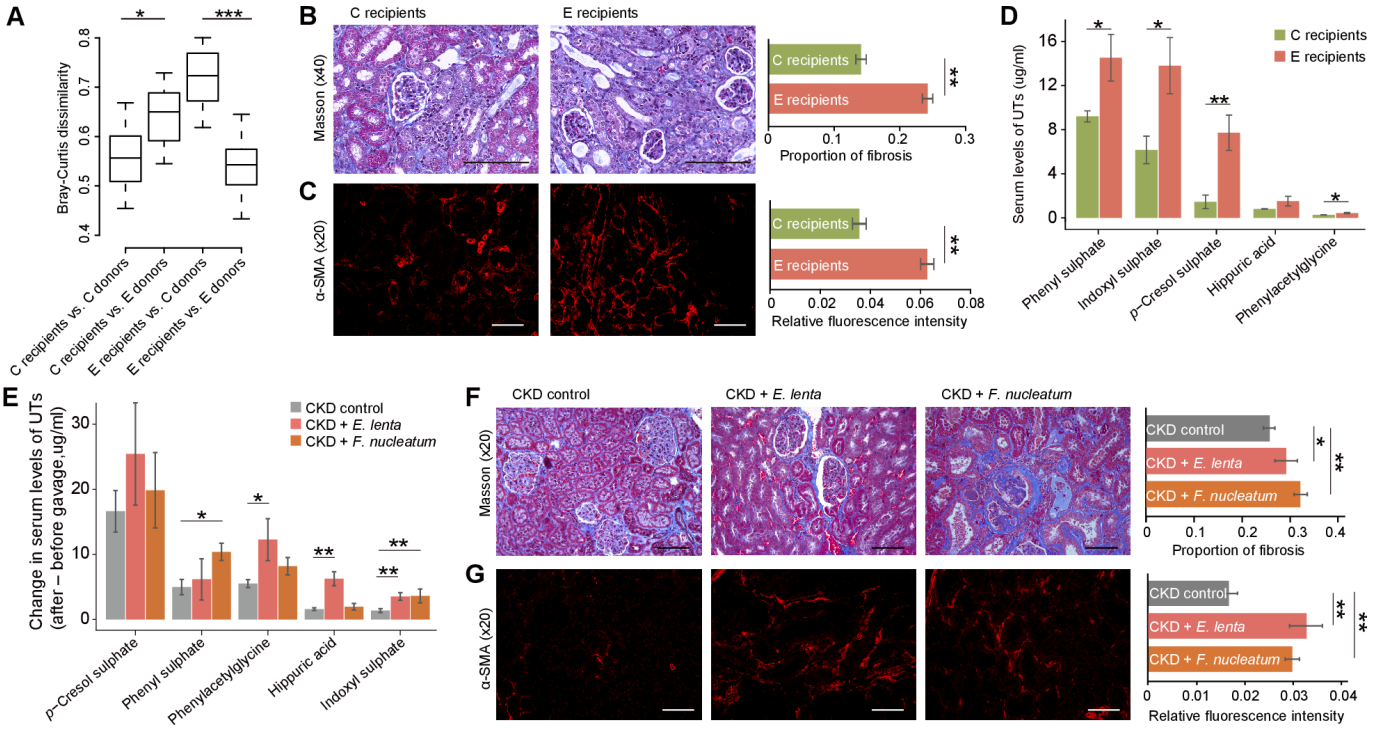


Figure 5 Animal experiments validate the role of ESRD microbiota and two species, *Eggerthella lenta* and *Fusobacterium nucleatum*, in producing serum uraemic toxins and aggravating renal disease development. (A) Bray-Curtis dissimilarity between recipients and donors. compositions of gut microbial taxa of ESRD donors (pool of n=13), control donors (pool of n=13), ESRD-microbiota recipients (abbreviated as E recipients, n=6) and control-microbiota recipients (abbreviated as C recipients, n=6) were determined by 16S rRNA sequencing. (B) Masson's trichrome staining and quantification of the proportion of the fibrotic area in the renal cortex of recipient mice after faecal transplantation for 2 weeks. (C) Immunofluorescence of α-SMA and quantification of the relative fluorescence intensity of the α-SMA$^+$ area in renal tubular of recipient mice after faecal transplantation for 2 weeks. (D) Concentration of serum uraemic toxin levels in recipient mice after faecal transplantation for 2 weeks. (E) Changes of serum uraemic toxin levels in CKD rats after 8 weeks gavage-feeding *E. lenta* or *F. nucleatum*. for (D, E), the serum concentrations of phenylacetylglycine (a major adduct of phenylacetate in rodents) were evaluated in mice and rats. (F) Masson's trichrome staining and quantification of the proportion of the fibrotic area in the renal cortex of CKD rats after 8 week gavage-feeding *E. lenta* or *F. nucleatum*. (G) Immunofluorescence of α-SMA and quantification of the relative fluorescence intensity of the α-SMA$^+$ area in renal tubular of CKD rats after 8 weeks gavage-feeding *E. lenta* or *F. nucleatum*. Data are shown as mean±s.e.m. *p<0.05; **p<0.01. CKD, chronic kidney disease; ESRD, end-stage renal disease.

the clinical parameters in the patients. *E. lenta* was one of the most enriched species in the patients with ESRD and was predicted to be associated with the production of several toxins including hippuric acid, phenylacetylglutamine and SBAs. Consistently, *E. lenta* was involved in the degradation of polyphenols into benzoic acid or 4-hydroxybenzoic acid, which are precursors of hippuric acid,[43] and possesses enzymes for deactivating the cardiac drug digoxin.[44] Another important toxin-producing species, *F. nucleatum* was identified as participating in the production of indole and phenol. It is worth noting that *F. nucleatum* has been identified as a proinflammatory autochthonous bacterium in human colorectal cancer.[45] Among the ESRD-enriched bacteria, *Alistipes shahii* was an indole-positive bacterium,[46] and *Clostridium difficile* was the major producer of *p*-cresol.[47] Meanwhile, we also identified the driver species for toxins including phenylacetylglutamine, phenyl sulphate and hippuric acid, which have not been fully studied. Furthermore, this work illustrates that the metabolic alterations in the intestinal tract of patients with ESRD contributed significantly to the accumulation of uraemic toxins in the serum. Altogether, the ESRD-enriched toxin-producing species account for the accumulation of gut-derived uraemic toxins in the patients with ESRD.

This work helps establish a causative relationship between an aberrant gut microbiota and kidney disease progression in human subjects. Some of the intestinal microbiota-derived uraemic toxins, including indoxyl sulphate, *p*-cresol sulphate, phenylacetylglutamine and TMAO were elevated in both CKD[19 48 49] and patients with ESRD, and associated with the CKD progression, mortality, major adverse cardiovascular events and other severe clinical end points.[14 50] Toxins such as IS and PCS have been experimentally proved to induce renal fibrosis and cause significant renal tubular damage in CKD rats.[50] In agreement, gavage of *E. lenta* or *F. nucleatum* enhanced their corresponding toxins in serum and aggravated renal fibrosis in 5/6 nephrectomy rat model. These findings support the importance of ESRD-enriched toxin-producing species in the disease development, and reinforce the model that increase of gut-derived uraemic toxins can aggravate the progression of kidney disease.

Intestinal barrier is often impaired in patients with ESRD,[51] promoting the penetrance of uraemic toxin precursors into the circulation system. This would contribute to the accumulation of serum toxins. It has been shown that the prevailing systemic inflammation in patients with uraemia may partially be attributed to impairment of their intestinal barrier function.[52] Our recent findings have demonstrated that microbiota from patients with ESRD could disrupt the intestinal barrier by producing excessive phenol.[53] With the anticipated impairment or disruption of the intestinal barrier, more uraemic toxins can penetrate into the circulation of patients with ESRD, aggravating the development of kidney disease and its complications.

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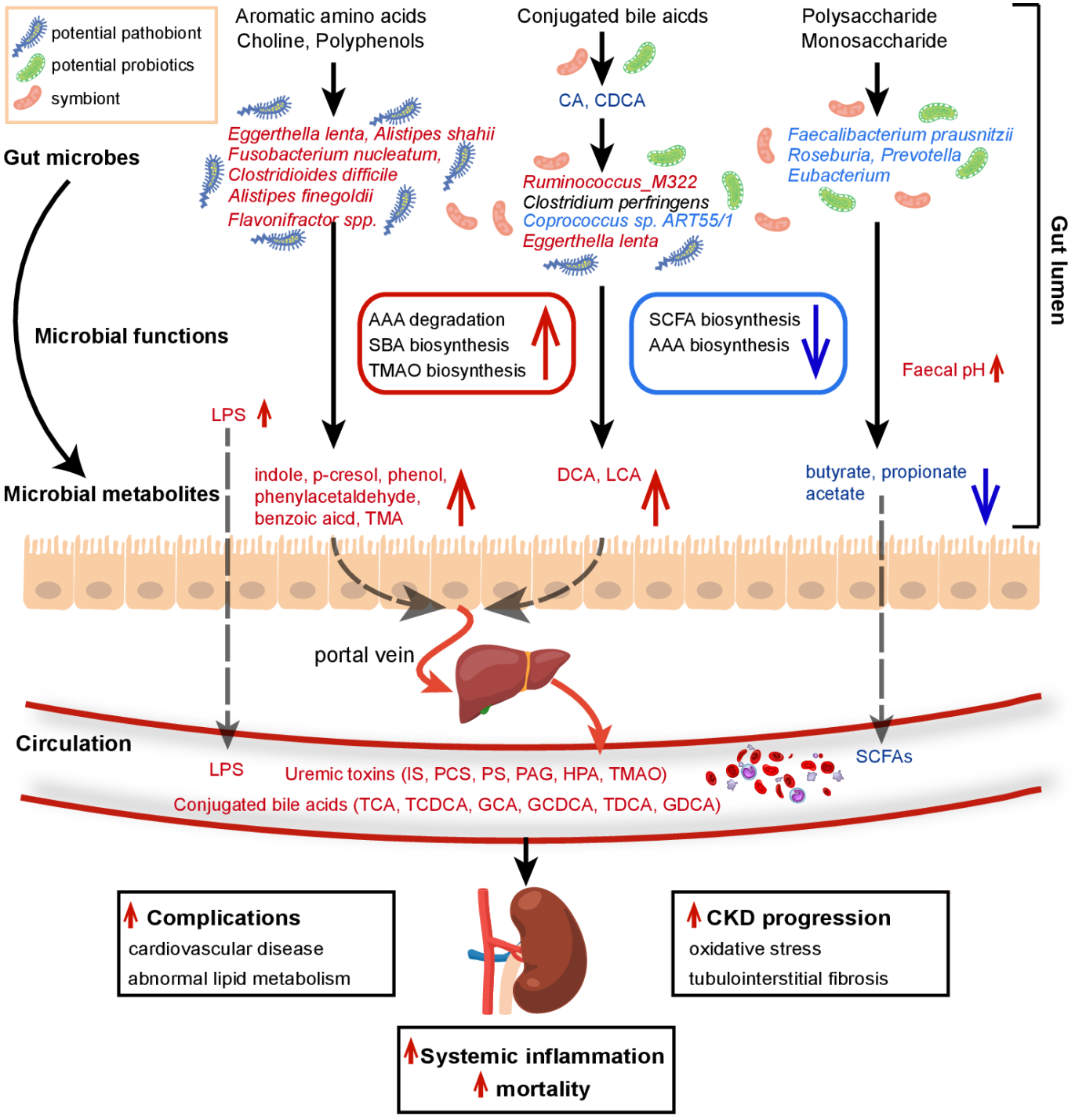


Figure 6 Proposed mechanism of ESRD aggravation by the altered gut microbiome. schematic summary illustrating the flux of metabolites from gut microbiota to faeces and then to serum, affecting the clinical status of the patient. ESRD-enriched and depleted microbial species, functions and faecal/serum metabolites are labelled in red and blue, respectively. The enrichment of species such as *Eggerthella lenta*, *Fusobacterium nucleatum* and *Alistipes shahii* lead to increased AAA degradation, SBA and TMAO biosynthesis in the gut, resulting in higher levels of uraemic toxins and SBAs in faeces and blood of patients with ESRD. Simultaneously, the depletion of species such as *Faecalibacterium prausnitzii*, *Roseburia* and *Prevotella* lead to decreased gut microbial SCFA biosynthesis. Such gut microbiota-driven adverse metabolism may aggravate CKD progression, induce complications and systemic inflammation, and increase mortality of patients with ESRD. AAA, aromatic amino acid; ESRD, end-stage renal disease; SBA, secondary bile acid; SCFA, short chain fatty acid; TMAO, trimethylamine N-oxide.

Individuals with kidney failure, who are regularly treated with dialysis, as those examined in the current study, suffer from the toxin-induced residual uraemic syndrome,[54] a condition that severely compromises their life quality. One reason for toxins accumulation in patients with ESRD is that some gut microbiota-derived uraemic toxins cannot be efficiently removed by traditional dialysis. Therefore, there is a major need in future clinically controlled trials of patients with varying degrees of impaired kidney function to explore if modulation of the gut microbiota by diet, probiotics or other therapeutic means can alleviate the devastating symptoms

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of the residual syndrome, improve survival and life quality or postpone the need of resource demanding dialysis treatment or kidney transplantation.

METHODS

Study design and sample collection

A total of 223 haemodialysis patients were recruited from four haemodialysis centres in Beijing, China. All participants were diagnosed with ESRD according to the Kidney Disease: Improving Global Outcomes Clinical Practice guidelines and were undergoing stable haemodialysis (1–3 times per week). The control group included 69 healthy volunteers. The validation cohort consisted of 12 patients with ESRD and 12 gender-matched and age-matched healthy controls. The exclusion criteria for controls included hypertension, diabetes, obesity, metabolic syndrome, inflammatory bowel disease, cancer, abnormal liver or kidney function, dyslipidaemia. Individuals were excluded if they had taken antibiotics medication within 30 days or probiotic products within 14 days.

Blood samples of patients were collected immediately before haemodialysis in hospital, while healthy control samples were collected during physical examination. Serum was isolated and stored at −80°C. Fresh faecal samples were obtained at the same period as blood collection; each sample was divided into two parts, one part was stored in DNA protection solution at 4°C, DNA extractions were performed in the sample within 2 months. The other was frozen at −80°C, which is used for metabolomics analysis within 2 months. Clinical chemistry analyses of blood samples, measurements of basic anthropometric and faecal pH, and questionnaire survey were carried out (online supplementary table 1).

Metabolome profiling of serum and faecal samples

Serum metabolic profiling was analysed using ultra-high-performance liquid chromatograph (HPLC) (Waters, USA) coupled with a tripleTOF 5600 plus (Applied Biosystems, USA) mass spectrometer. A total of 6600 metabolite peaks were measured, and 180 out of them were structurally identified and annotated according to an in-house LC-MS/MS database. Among them, five metabolites were further quantified in sixty randomly chosen serum samples by external calibration curves using Shimadzu nexera x2 ultra HPLC (Shimadzu, Japan) coupled with triple quadrupole mass spectrometer (Shimadzu 8050, Japan).

Faecal volatile organic compounds were analysed using a headspace solid phase microextraction–gas chromatography-MS (GC-MS) method. A total of 582 faecal metabolite peaks were measured based on the NIST database, and 255 out of them were annotated and left after further filtering by excluding compounds showing mass spectra similarity factor <700. In addition, 16 kinds of bile acids were quantified by external calibration curves using ultra HPLC coupled with triple quadrupole mass spectrometer (Shimadzu 8050, Japan).

Clusters of coabundant metabolites were identified with the dynamic hybrid tree-cutting algorithm (R package: *dynamicTreeCut*),[55] using *deepSplit* of 4 and a minimum cluster size of 2. Metabolites that did not fit the clustering criteria were regarded as singletons. Finally, the metabolites in some clusters were manually separated based on their physiological and biochemical characteristics.

Details of the sample preparation and metabolome profiling of serum and faecal samples are available in online supplementary materials.

Faecal DNA extraction and metagenomic sequencing

Total faecal DNA was extracted using standard methods.[37] The fresh genome DNA samples were mechanically fragmented to ~400 bp with Bioruptor Pico (Diagenode, Belgium). A magnetic beads-based method was used for DNA fragments selection following a standard protocol (Agencourt AMPure XP). Libraries were prepared by using the NEBnext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs). The Illumina HiSeq X Ten platform was then used for 2×150 bp paired-end whole-metagenome sequencing. Low quality or human genomic DNA reads were removed.

Bioinformatic and statistical analyses

All methods were detailed in online supplementary materials, and the statistical scripts were available at https://github.com/lish2/omics.

Animal experiments

All animal studies were approved by Ethical Committee of Experimental Animal Care of China Agricultural University. For animal FMT experiments, 13 ESRD patients and 13 healthy controls from the original 292 subjects were selected as donors. The FMT experiments were conducted in germ-free mice and antibiotics-treated rats. For single strain gavage experiments, two independent animal studies were performed to test the role of the *E. lenta* or *F. nucleatum* (study 1) and probiotics (study 2, *Bifidobacterium animalis* A6) in renal disease.

Details of animal studies, including sample collection, bacteria culture and bacterial suspension preparation, DNA extraction and sequencing, real-time quantitative PCR, quantitation of serum uraemic toxins, as well as histological and immunohistochemical analyses, are available in online supplementary materials.

Data availability

The shotgun sequencing data for all metagenomic samples have been deposited into the European Bioinformatic Institute database under the BioProject accession code PRJNA449784. The serum and faecal metabolomes datasets reported in this article are available at the MetaboLights database (http://www.ebi.ac.uk/metabolights/) with accession number MTBLS700. The authors declare that all other data supporting the findings of the study are available in the paper and online supplementary materials, or from the corresponding authors on request.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not required.

Ethics approval The study protocol was approved by the Ethics Committees of China Agricultural University (No. 2015035) and Beijing Aerospace Center Hospital (No. 20151230-YW-01).

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Data availability statement Data are available in a public, open access repository. The shotgun sequencing data for all metagenomic samples have been deposited into the European Bioinformatic Institute (EBI) database under the BioProject accession code PRJNA449784. The serum and faecal metabolomes datasets reported in this article are available at the MetaboLights database (http://www.ebi.ac.uk/metabolights/) with accession number MTBLS700. The authors declare that all other data supporting the findings of the study are available in the paper and supplementary materials, or from the corresponding authors on request.

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