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Toxic microbiome and progression of chronic kidney disease: insights from a longitudinal CKD-Microbiome Study

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ABSTRACT

Background The gut microbiota has been linked to non-communicable diseases, including chronic kidney disease (CKD). However, the relationships between gut microbiome composition changes, uraemic toxins (UTs) accumulation, and diet on CKD severity and progression remain underexplored.

Objective To characterise relationships between gut microbiome composition and functionality, UTs diet, and CKD severity and progression, as well as assess microbial contributions to UTs accumulation through mice faecal microbiota transplantation (FMT).

Design This study profiled the gut microbiome of 240 non-dialysis patients with CKD (CKD-REIN cohort) using shotgun metagenomics, with follow-up in 103 patients after 3 years, with comparisons with healthy volunteers from the *Milieu Intérieur* cohort. A multiomics approach identifies features associated with CKD severity (and progression), with validation in an independent Belgian cohort. Experimental models used FMT to test CKD gut microbiome effects on UTs and kidney fibrosis. Changes in gut microbiome over time were evaluated, and the impact of diet on these changes was assessed.

Results Compared with matched healthy controls, patients with CKD exhibited gut microbiota alteration, with enrichment of UT precursor-producing species. Patients with severe CKD exhibited higher UT levels and greater enrichment of UT (precursor)-producing species in the microbiota than patients with moderate CKD. Over time, UT (precursor)-producing species increased, and a plant-based low protein diet appeared to mitigate these changes. FMT from patients with CKD to antibiotic-treated CKD model mice increased serum UT levels and exacerbated kidney fibrosis.

Conclusions This study highlights the role of the microbiome and UTs in CKD, suggesting a potential therapeutic target to slow disease progression.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Chronic kidney disease (CKD) is a non-communicable condition affecting approximately 9% of the global population.
⇒ Gut-derived uraemic toxins (UTs) contribute to CKD progression.
⇒ Gut microbiome composition is altered in non-dialysis patients although studies used the 16S rRNA sequencing technique, which does not allow diving into refined composition nor functionalities.
⇒ No longitudinal studies evaluating gut microbiome composition and functionality over time have been reported in non-dialysis patients.

WHAT THIS STUDY ADDS

⇒ Gut microbiome appears to be a key determinant of plasma UT levels in non-dialysis patients, as it is associated with enrichment of UT(precursor)s-producing species.
⇒ Microbiota transplantation from patients with moderate CKD led to increased production of serum UTs and exacerbated renal fibrosis in antibiotic-treated mice with kidney injury.
⇒ Gut microbiome modified progressively over time, and with CKD progression, notably with a decrease in richness and an increase of UT (precursor)-producing species and the depletion of some protective species.
⇒ A plant-based low protein diet may counteract the ratio of UT (precursor)-producing species over all species.

INTRODUCTION

Chronic kidney disease (CKD) affects ~9% of the global population and has severe health consequences, including progression to kidney failure requiring replacement therapy (KFRT), cardiovascular (CV) disease and premature death.[1–3] As there is no curative treatment, identifying factors to slow CKD progression is essential. When kidney function declines, the removal of metabolites called uraemic retention solutes is impaired. Retention

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ An altered gut microbiome in patients with CKD may play a role in worsening the disease progression.

⇒ Targeting gut microbiome and UTs in CKD, by using a diet approach, serves as an attractive avenue to slow down CKD progression.

solutes that exhibit deleterious effects on biochemical/physiological functions are referred to as uraemic toxins (UTs). Of particular interest in research on CKD are exogenous UTs such as trimethylamine-N-oxide (TMAO), p-cresyl sulfate (PCS) and indoxyl sulfate (IS).[4–6] These toxins, derived from gut microbiome metabolism of dietary components like choline, tyrosine and tryptophan, accumulate in CKD, exacerbate uraemic symptoms, and increase CV morbidity and mortality.[7] Strategies to remove UTs are limited; even haemodialysis is ineffective.[7] Therefore, it is essential to better understand their origin and find ways to limit their production.

It has been hypothesised that gut microbiota dysbiosis may play a role in the pathogenesis and progression of CKD by facilitating the conversion of dietary components into UTs, thereby contributing to their accumulation.[6] However, as summarised in recent reviews,[8 9] prior studies had limitations, including small sample sizes (<100 patients), a primary focus on dialysis-dependent CKD, potential confounding factors, and reliance on amplicon sequencing, which characterises the microbiota only at the genus level. Only one larger study (n=223) using shotgun metagenomics in haemodialysis patients linked an altered gut microbiome to changes in host metabolomes.[10] Moreover, transplanting microbiota from haemodialysis patients into germ-free CKD mice or antibiotic-treated rats increased serum UTs and aggravated kidney fibrosis.[10 11] However, it remains unclear if microbiome changes in early CKD stages impact disease progression, and longitudinal studies are lacking.

Here, we compared the gut microbiome composition of patients with CKD with that of a cohort of healthy controls (HCs) through shotgun metagenomic sequencing. We conducted a multidimensional integrated analysis of metagenomics, serum UTs levels, diet, and host data to identify associations with CKD severity (cross-sectionally) and progression (longitudinally). An independent cohort validated the findings on CKD severity. To explore causality, we performed faecal microbiota transplantation (FMT) from patients with CKD into uraemic antibiotic-treated mice, assessing the role of gut microbiota in UTs production and kidney damage. Combining clinical and preclinical investigations, we evaluated gut microbiome-related toxicity, its mechanisms in CKD progression and the potential impact of diet on this relationship.

METHODS

Study populations

Prospective CKD-Microbiome Study cohort

CKD_Microbiome is an ancillary study within the French Chronic Kidney Disease–Renal Epidemiology and Information Network (CKD-REIN) prospective cohort, which includes over 3000 patients with non-dialysis-dependent CKD stages 2–5 (estimated glomerular filtration rate (eGFR) <90 mL/min/1.73 m^2), enrolled from 40 nationally and geographically representative public or private nephrology clinics.[12 13] Faecal samples were obtained from a random subset of 240 patients among the 2804 patients who were alive and not receiving kidney replacement therapy at the 2-year follow-up of the CKD-REIN Study[2] (T0), along with demographic, clinical, biological, diet and treatment data and serum levels of 10 main UTs: TMAO, kynurenine, hippuric acid, phenylacetylglutamine (PAG), IS, kynurenic acid, p-cresyl glucuronide, PCS, indole-3-acetic acid, and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) and three UT precursors (tyrosine, tryptophan and phenylalanine).

Follow-up samples were collected 2.8±0.5 years later (T1) from 103 patients not on kidney replacement therapy (figure 1 and online supplemental figure 1). See the online supplemental methods for more details.

Milieu Intérieur cohort

As controls, we included a subset of the *Milieu Intérieur* cohort, consisting of CKD-matched healthy volunteers (HVs) recruited in France, phenotyped as described by Thomas *et al* and Patin *et al*[14 15] and whose gut microbiome was characterised as described by Byrd *et al*.[16]

Ghent microbiome cohort

The validation cohort included 79 patients with CKD with stages 2–5, and gut metagenomic data from the Ghent cohort study, a single-centre study that included 110 patients with CKD stages G1–G5 not on dialysis, from the Ghent University Hospital outpatient nephrology clinic (Belgium), between 2011 and 2014.[17]

Stool samples

For the CKD-Microbiome Study, stool samples were collected by the participants at home and sent to MetaGenoPolis, where the samples were processed according to standard operating procedures (defined in SOP 5). For the Ghent cohort, stool samples were stored on an ice pack and processed within approximately 6 hours as previously reported.[18]

Metagenomics

A full description of the sampling, sequencing and data analysis procedures is reported in the online supplemental material. Briefly, after extraction, the DNA was sequenced with an Ion Proton Sequencer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), with a minimum of 20 million high-quality 150 bp reads generated per sample. Reads were mapped to the sequences in the Integrated Gut Catalogue 2[19 20] and the human oral microbiome gene catalogue,[21] both of which were previously clustered into metagenomic species pangenomes (MSPs) with MSPminer.[22] A gene count table was generated with the Model Evaluation Tool for Environmental and Omics Responses (METEOR) software suite[23] and further processed with the R package *MetaOMineR* V.1.31[24] to yield a species abundance table.

Animal experiments

For the animal FMT experiments, 10 patients with CKD and 10 HVs were selected as donors. The FMT experiments were conducted in antibiotic-treated CKD model mice.

Statistical analyses

Gut microbiome profiles were compared between patients with CKD and HCs from the Milieu–Intérieur cohort.[14–16] Individuals with diabetes or using proton pump inhibitors (PPIs) and metformin were excluded. The remaining patients with CKD (n=78) were matched to HCs (n=78) by age, sex and body mass index (BMI) (online supplemental table 1). Gut microbiome

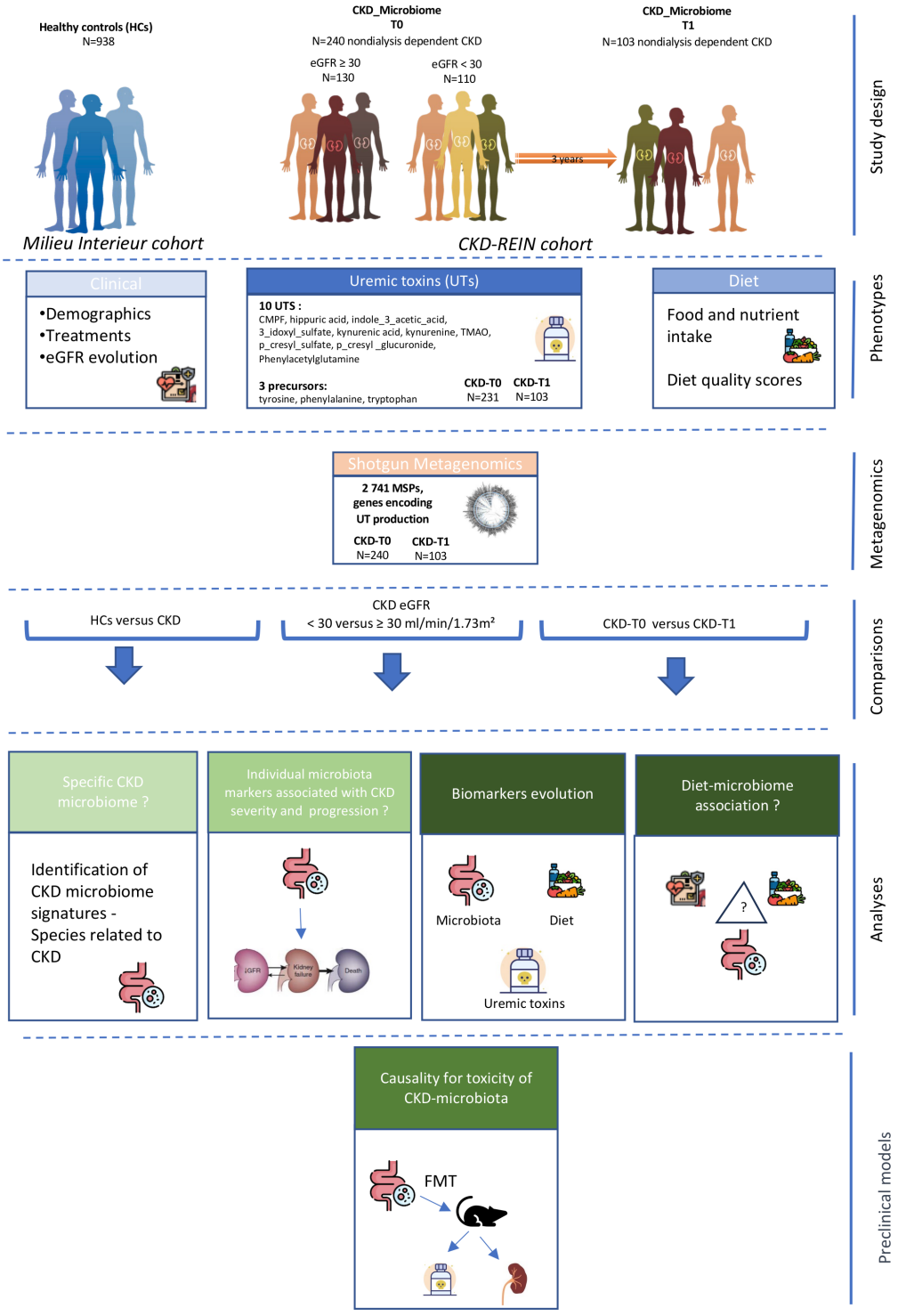


Figure 1 Overview of the study design, input data sets and analysis methods This study involved 240 non-dialysis-dependent patients with chronic kidney disease (CKD) from the Chronic Kidney Disease–Renal Epidemiology and Information Network (CKD-REIN) cohort, who underwent comprehensive bioclinical phenotyping. This detailed phenotyping provided clinical data, uraemic toxin (UTs) profiles, dietary information, and gut microbiome composition and functionality, assessed through shotgun metagenomics analyses. For 103 of these patients, the same data were collected 3 years later (T1). First, we compared the gut microbiomes of patients with CKD at baseline (T0) with healthy controls (HCs, from the *Milieu Intérieur* cohort) to identify a CKD-specific gut microbiome signature and species associated with CKD. Second, we analysed differences in the gut microbiome between patients with moderate and severe CKD, categorised by an estimated glomerular filtration rate (eGFR) <30 mL/min/1.73 m² (N=130) and ≥30 mL/min/1.73 m² (N=110). Multiomics integrative analyses were performed to identify biomarkers linked to disease severity and CKD progression. Third, we examined microbiome changes over time by comparing data from T0 and T1, assessing the impact of diet on gut microbiome composition. Finally, we conducted an experimental study to explore the causal relationships between gut microbiota, uraemic toxin production and kidney function deterioration in CKD. We performed faecal microbiota transplantation (FMT) using stool samples from patients with CKD (n=10) and healthy volunteers (HVs) (n=10) into antibiotic-treated CKD mouse models. CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; MSP, metagenomic species pangenome; TMAO, trimethylamine-N-oxide.

profiles were compared according to CKD severity by stratifying patients based on eGFR into moderate (n=130, 67≥ eGFR ≥30 mL/min/1.73 m^2) and severe (n=110, eGFR<30 mL/min/1.73 m^2), and according to CKD progressors status: fast (n=68) and slow progressors (n=172). Fast progressors were defined as a sustained 30% decline in eGFR over the 2.8-year follow-up or progression to KFRT or death. Gut microbiome profiles were compared between T0 and T1, calculating the ratio of UT precursor-producing species to total species and investigating associations with diet components.

Statistical analyses and visualisations were performed in the R environment (V.3.6.0, https://www.r-project.org/). Differences between two groups were evaluated with the non-parametrical Wilcoxon signed-rank test. To estimate correlations, we used pairwise Spearman rank correlation analysis. Corrections for multiple comparisons were performed with the false discovery rate (FDR) approach via the Benjamini–Hochberg method.

The multiomics data integration analysis included all clinical, UTs, diet and metagenomics data sets and was carried out through the *mixOmics* package V.6.28.0 via the Data Integration Analysis for Biomarker discovery using Latent cOmponents model. Details on machine learning (ML)-based metagenomics classification and data analysis are in the online supplemental material. Missing data were imputed using the multiple imputation by chained equations method (mice function) with predictive mean matching from the mice R package. When necessary, missing values were imputed; otherwise, correlation analyses were performed using the 'pairwise.complete.obs' option.

RESULTS

Study design and in-depth phenotyping

At baseline, patients (29% women) had a mean age of 68±11.6 years, a mean BMI of 27.5±5.2 kg/m^2, and a mean eGFR of 33.2±12.7 mL/min/1.73 m^2 (online supplemental table 1). During the 2.8-year follow-up, 8% of the patients died, and 24% (n=68) experienced rapid CKD progression. Using a validated food frequency questionnaire,[25] we found that daily dietary intakes were on average close to those recommended for patients with CKD:[1] 0.9±0.4 g/kg/day protein, a total energy intake of 23.6 kcal/kg/day and 19.6±8.0 g of fibre/day. Baseline characteristics of cohort patients included in CKD_Microbiome were similar to those not included, except for eGFR, which was higher, and diabetes, which was less common among those included, who also experienced slower CKD progression and better survival (online supplemental table 1).

The composition of the gut microbiota is altered in patients with CKD compared with that in HCs

No differences in the alpha diversity indices were found between HCs and CKD (figure 2A), but beta diversity indices indicated a significant overall difference in microbial community structure between the groups (PERMANOVA, $p_{adonis}<0.05$) (figure 2B). Moreover, the Gut Microbiome Health Index (GMHI),[26] a predictive index for evaluating health status via species-level profiling, tended to be lower in patients with CKD than in HCs (figure 2C) and tended to be positively correlated with eGFR ($Rho_{Spearman}=0.15$, $p_{Spearman}=0.09$).

Further analysis identified 67 MSPs as significantly differentially abundant between the two cohorts after filtering according to an FDR $_{Wilcoxon}<0.05$ and an absolute LogFC >2 (~10% of the total microbiome). Of these, 43 species were enriched in the patients with CKD, and 24 were enriched in the HCs (online supplemental figure 2A and online supplemental table 2). The CKD-related microbiome exhibited significant enrichment of species from the genera *Enterocloster* and *Hungatella* (both members of the *Lachnospiraceae* family), and members of *Flavonifractor*, *Victivalles* and *Dysosmobacter*/*Oscillibacter*, and notable depletion of *Anaerobutyricum hallii* msp_0050, *Romboutsia timonensis* msp_0422 and *Intestinibacter bartlettii* msp_0621 (figure 2D). Among CKD-enriched MSPs, species from specific branches of the phylogenetic tree, such as the *Enterocloster* and *Hungatella* genera, showed notable abundances. Species from these genera harboured genes involved in the UT production (online supplemental figure 2B), were negatively correlated with eGFR and positively associated with C reactive protein level (online supplemental figure 2C). Collectively, these data suggest a potential role of species from this branch of the phylogenetic tree as part of the toxic and aberrant gut microbiome in CKD.

To further explore the functional role of gut microbes, we examined 3611 microbial genes predicted to encode key synthetases of UTs (online supplemental table 3).[10] Patients with CKD tended to have a greater number of MSPs harbouring these genes compared with HCs ($p_{\chi 2}=0.1$) (online supplemental table 4, figure 2D), and these MSPs correlated positively with levels of most serum UTs (online supplemental table 5).

An ML-based classification approach using random forest yielded high accuracy in distinguishing patients with CKD from HCs, with an area under the curve (AUC) of 0.97 (CI 0.91 to 1.00) (online supplemental figure 3A). The Gini coefficient further confirmed the importance of *Lachnospiraceae* family MSPs in CKD (online supplemental figure 3B).

Importantly, when analysing the full cohort (CKD n=240 and HC n=938), several species in particular from the *Enterocloster* and *Hungatella* genera remained significantly different in abundance between patients with CKD and HCs after adjusting for medication use (PPIs and metformin), diabetes status and demographic factors (age, sex, BMI), further supporting our initial findings (online supplemental figure 3C).

In summary, these data highlight the specific characteristics of the gut microbiota in patients with CKD relative to those in HCs, revealing enrichment of species from the *Enterocloster* and *Hungatella* genera and a tendency to harbour more UT-producing species among patients with CKD.

Microbiome alterations in patients with CKD are associated with exacerbated disease severity and mediate elevated serum UT levels

Given the heterogeneous aetiology of CKD, we first divided patients into eight groups based on kidney disease type (online supplemental table 1). We found no significant differences in serum UT concentrations (except for hippuric acid and CMPF), alpha diversity, beta diversity or dysbiosis indices among these groups, suggesting that serum UT levels and gut microbiota composition are independent of CKD aetiology (online supplemental table 6 and online supplemental figure 4a–C).

Next, we further analysed the association between gut microbiota species and CKD severity phenotypes (online supplemental table 1). Although species richness, beta diversity index and the GMHI were similar between the two groups (online supplemental figure 5A–C), ~6% of total species were significantly differentially abundant between them; specifically, 21 and 17 species were enriched in patients with severe and moderate CKD phenotypes, respectively ($p_{Wilcoxon}<0.05$ and a Cliff's delta (CD) >0.1) (online supplemental figure 5D, online supplemental table 7 and figure 3A).

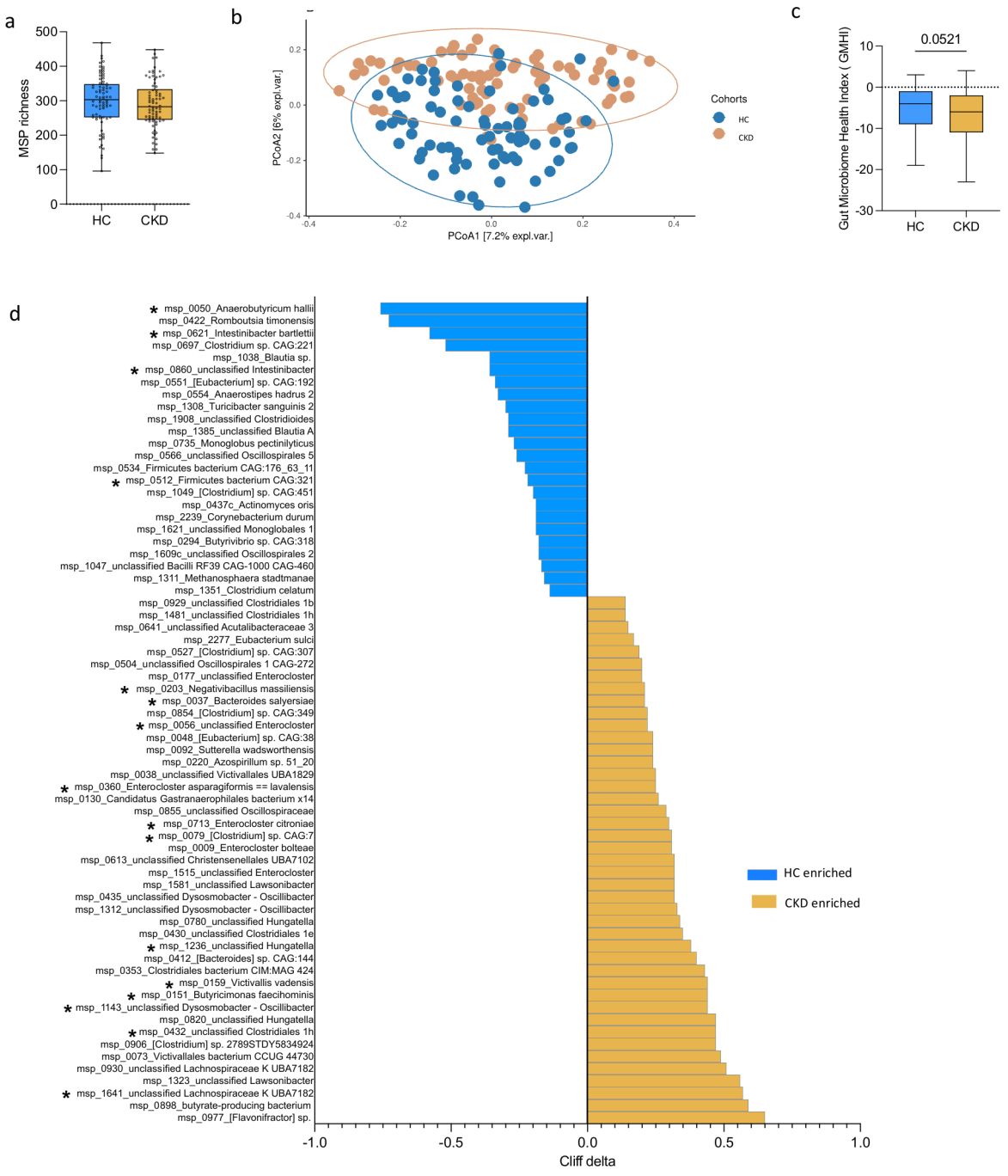


Figure 2 Alterations of gut bacterial species in patients with chronic kidney disease (CKD) compared with healthy controls (HCs) include enrichment of uraemic toxin (UT)-producing species. Box plot (line, median; box, IQR) showing (A) the richness of metagenomic species pangenomes (MSPs) between HCs and patients with CKD matched for age, sex, body mass index (BMI), diabetes, and proton pump inhibitor (PPI) and metformin use. Comparisons were performed with the two-sided Wilcoxon rank-sum test. (B) Principal coordinate analysis (PCoA) of the relative abundance of MSPs. The spatial density distribution of samples in each group is indicated with an ellipse. The x-axis and y-axis labels indicate the variance in microbial composition explained by the first two principal coordinates. (C) Box plot of the Gut Microbiome Health Index (GMHI). Comparisons were performed with the two-sided Wilcoxon rank-sum test. (D) Blot length shows effect sizes (Cliff's delta) for significantly different bacterial species between HCs and patients with CKD. Corrections were made for multiple comparisons with the Benjamini–Hochberg method (False discovery rate $(FDR)_{Wilcoxon}$<0.05 and |LogFC|>2) (see online supplemental table 2 for exact p values). Stars indicate species that carry genes encoding key enzymes involved in the main UT synthesis pathways (see online supplemental table 3 for details). Species enriched in HCs (n=78) are shown in blue, and those enriched in patients with CKD (n=78) are shown in gold.

Consistent with previous studies,[10 27] we observed that patients with severe CKD had higher UT levels and a distinct serum UT profile compared with those with moderate CKD (online supplemental table 6 and figure 3B). To further investigate the association between the gut microbiome and UT production, we conducted a correlation analysis between the abundances of MSPs that differed between moderate and severe CKD and the concentrations of serum UTs and their precursors (figure 3A).

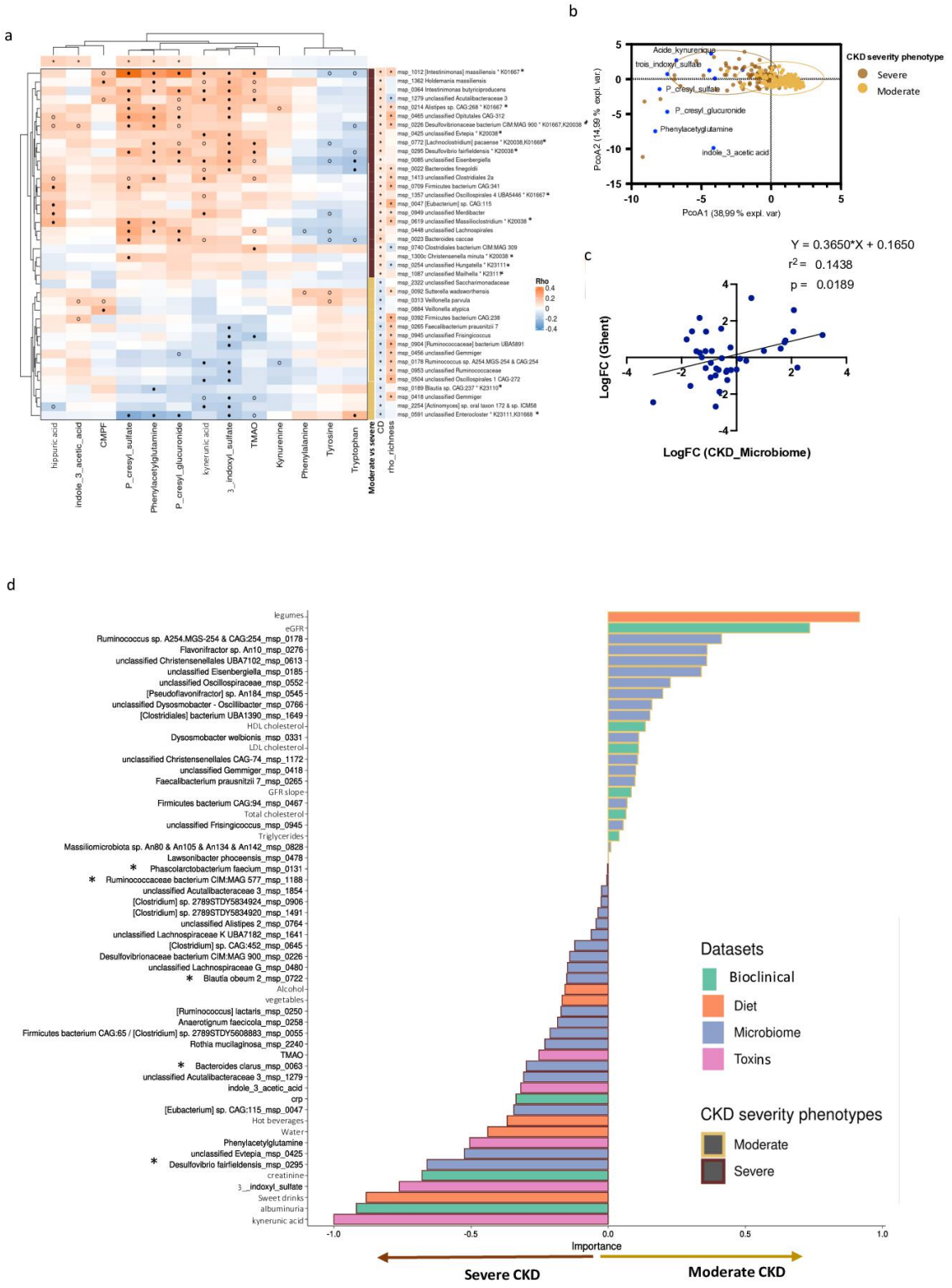


Figure 3 Microbiome signature associated with severity of chronic kidney disease (CKD). (A) Heatmap showing correlations between the abundances of metagenomic species pangenomes (MSPs) that differed between patients with severe and moderate CKD and the levels of serum uraemic toxins (UTs) and precursors. Stars indicate species whose genes encode key enzymes involved in the main UT synthesis pathways. White circles, p<0.01; black circles, p<0.05. CD refers to Cliff's delta, species more abundant in moderate (in blue) and severe (in red) associated with UTs. Rho richness refers to the richness of identified species in patients with CKD. (B) Separation of serum UTs and precursors between patients with severe and moderate CKD according to principal coordinate analysis (PCoA). (C) For the gut microbial and plasma metabolome features common to both CKD-Microbiome and Ghent cohorts, a Spearman correlation analysis was conducted between the LogFC effect size for moderate versus severe CKD comparison in each study after recalculating LogFC in the Ghent population. (D) Data Integration Analysis for Biomarker discovery using Latent cOmponents (DIABLO) variable loading plot displaying vectors that contributed the most to the difference between severe and moderate CKD patient groups on the basis of microbiome, bioclinical, UTs and diet data, reported as a bar plot. Stars indicate species whose genes encode key enzymes involved in the main UTs synthesis pathways. CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; TMAO, trimethylamine-N-oxide.

Interestingly, the number of UT precursor-producing MSPs tended to be greater in the severe CKD group compared with the moderate group ($p_{\chi 2}$=0.06) (online supplemental table 4). Moreover, MSPs enriched in severe patients with CKD showed positive correlation with UT levels and negative correlation with UT precursors, while the opposite pattern was observed for MSPs enriched in patients with moderate CKD (figure 3A).

Notably, the relative abundances of severe CKD-related MSPs, such as *Desulfovibrio fairfieldensis* msp_0295, *Lachnoclostridium pacaense* msp_0772 and *Intestinimonas massiliensis* msp_1012, were positively correlated with the levels of indole-derived and phenol-derived UT, as previously reported in haemodialysis patients.[10] Among the bacterial species whose abundances were inversely correlated with the levels of blood UT, we identified *Faecalibacterium prausnitzii* msp_0265, which has been associated with protecting kidney function in CKD model mice and patients with CKD.[28] These data support the hypothesis that UTs being present at high levels in patients with severe CKD might be associated with aromatic amino acid degradation by the gut microbiota.

To assess the robustness of these microbiota signatures for CKD severity, we performed a validation study in an independent cohort of 79 patients with eGFR <67.3 mL/min/1.73 m^2 from Ghent, Belgium.[29] As in our cohort, we found that UTs and albuminuria discriminate patients with severe versus moderate CKD (online supplemental table 8). Although individual identified species were not significantly correlated with eGFR in the Ghent cohort, comparing the LogFC of discriminating biomarkers in both cohorts (present study vs Ghent cohort) showed a correlation suggesting that a part of the microbiome evolving with CKD severity follows a similar trend in the Ghent cohort (online supplemental tables 8 and 9, figure 3C).

We next conducted a multiomics data integration analysis using supervised models of the gut microbiome, UT producers, bioclinical parameters and diet to identify combined biomarkers differentiating the CKD severity phenotypes. This identified 57 biomarkers that distinguish CKD severity with a classification error rate of 0.25 (online supplemental figure 6; figure 3D). In the severe CKD group, species such as *Desulfovibrio fairfieldensis* msp_0295, *Bacteroides clarus* msp_0063 and *Blautia obeum* msp_0722, which harbour genes essential for UT precursors production, were enriched. Patients with severe CKD also had higher water, alcohol and hot drink consumption, inflammation markers, and elevated serum levels of UTs like kynurenic acid, IS and PAG.

Conversely, the MSPs associated with the moderate group, such as *Faecalibacterium prausnitzii 7* msp_0265, do not carry genes for UT precursors production. Interestingly, high legume intake is associated with less severe disease.

Overall, these observations underscore the interplay among diet, gut microbiome modifications, UT accumulation and clinical status, highlighting the complex dynamics within the gut microbial ecosystem in patients with CKD.

Kidney fibrosis worsens following CKD-related faecal microbiota transplantation in animal models

To investigate causal relationships between gut microbiota, UT production and the worsening of kidney function in CKD, we performed FMT using stool samples from patients with CKD (n=10) and HVs (n=10) into antibiotic-treated model CKD mice induced by adenine diet (n=26) (figure 4A). Characteristics of the selected patients are summarised in online supplemental table 10. After 2 weeks, compared with those transplanted with stool from HVs, the mice transplanted with stool from patients with CKD exhibit greater areas of kidney fibrosis, as visualised by Sirius Red staining. Glomerular area and glomerulosclerosis score were measured at 2 weeks and showed no significant differences between the two groups (data not shown). At 6 weeks, we observed the same phenotype, with a trend towards increased fibrosis (figure 4B,C).

After 2 weeks, we further investigated whether differences in gut microbiota were maintained in the FMT recipient mice by performing 16S rRNA gene sequencing on caecal contents. The gut microbiota composition differed between the transplant groups according to the beta diversity index (PERMANOVA, p_{adonis}<0.05) (figure 4D), and in both groups, the recipient mice exhibited the taxonomic features of the corresponding donor microbiomes (figure 4E). At 2 weeks, serum levels of several UTs, including IS, PCS and PCG, were significantly higher in CKD mice that received CKD patient stool compared with those that received HV stool (PERMANOVA, p_{adonis}<0.05). These differences persist after 6 weeks (figure 4F–H).

At the species level, *Faecalibacterium prausnitzii* was notably less abundant in CKD stool-recipient mice than in HV stool-recipient mice (online supplemental table 11), and its abundance was strongly negatively correlated with kidney fibrosis area and serum UT levels (figure 4I), underscoring its potential protective role in kidney function preservation.

Collectively, these results demonstrate that the altered gut microbiome of patients with non-dialysis CKD contributes to kidney function deterioration and is associated with increased UT production.

Microbiome signatures associated with CKD progression

We then aimed to predict CKD progression and identify baseline prognostic biomarkers by stratifying patients. Using various ML strategies,[30] we classified fast and slow progressors with models built on (1) Routine clinical markers such as baseline eGFR, the presence of albuminuria, age, and sex; (2) Diet; (3) Overall microbiome composition; (4) The toxin-producing microbial species profile; and (5) The levels of serum UTs identified in the current study. All models trained with gut microbiome markers yielded AUC values lower than 0.7 in all cases and consistently underperformed compared with those trained with clinical markers or UTs, regardless of the chosen algorithm (figure 5A). The levels of UTs and their precursors demonstrated good predictive power in classifying disease progression across all tested models. However, combining clinical markers with UTs did not improve classification accuracy, suggesting that gut microbiota-derived metabolites may independently predict CKD progression.

Next, we identified features specifically associated with fast CKD progression ($p_{Wilcoxon}$<0.05 and and CD >0.1). Several UT-producing species, such as *Desulfovibrionaceae bacterium* msp_0226 and *Alistipes putredinis* msp_0090, were significantly more abundant in fast progressors, while another *Faecalibacterium prausnitzii* 4 msp_0389 was associated with slower progression (figure 5B). Interestingly, five species were associated with severe CKD and fast progressors, of which only *Desulfovibrionaceae bacterium* msp_0226 is a toxin precursor-producing species (online supplemental table 5). After adjusting for baseline eGFR and albuminuria, some microbial species remained significantly associated with fast CKD progression, in particular msp_0075 and msp_0124 (FDR<0.1) and the toxins producer *unclassified Christensenellales CAG.74* msp_0622(FDR<0.2). Conversely, msp_0389 *Faecalibacterium prausnitzii* 4 remained negatively

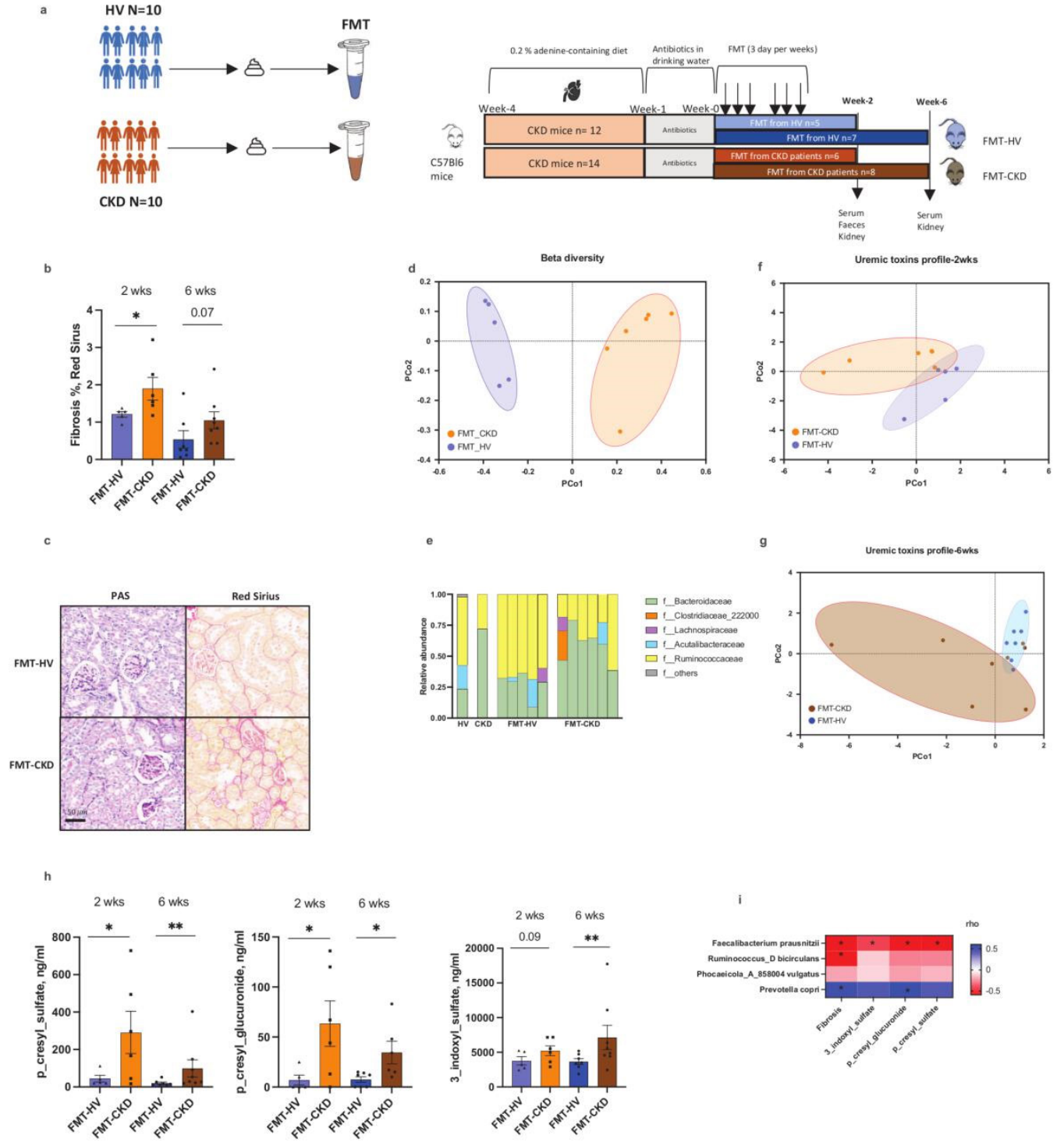
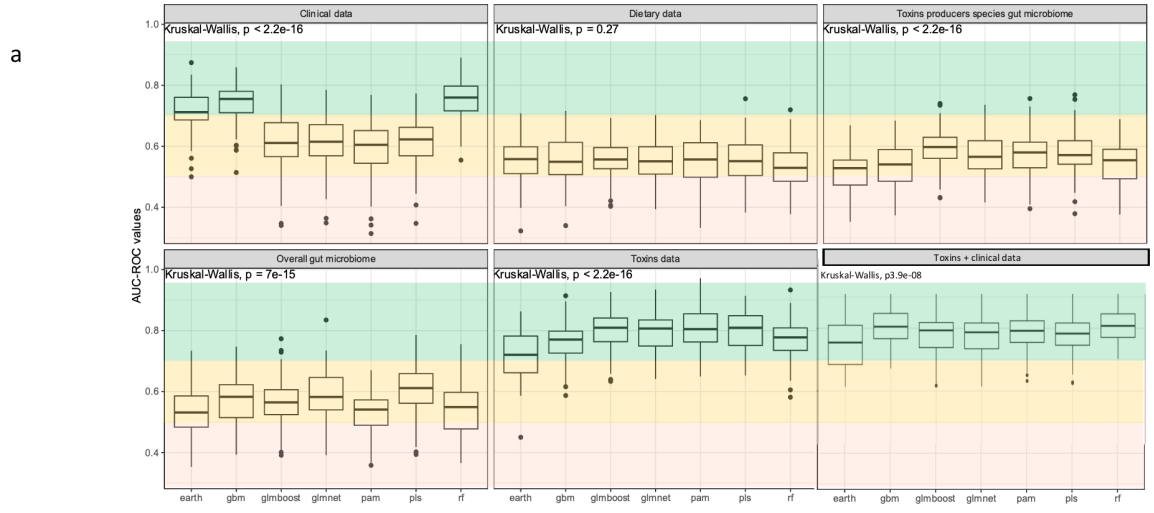
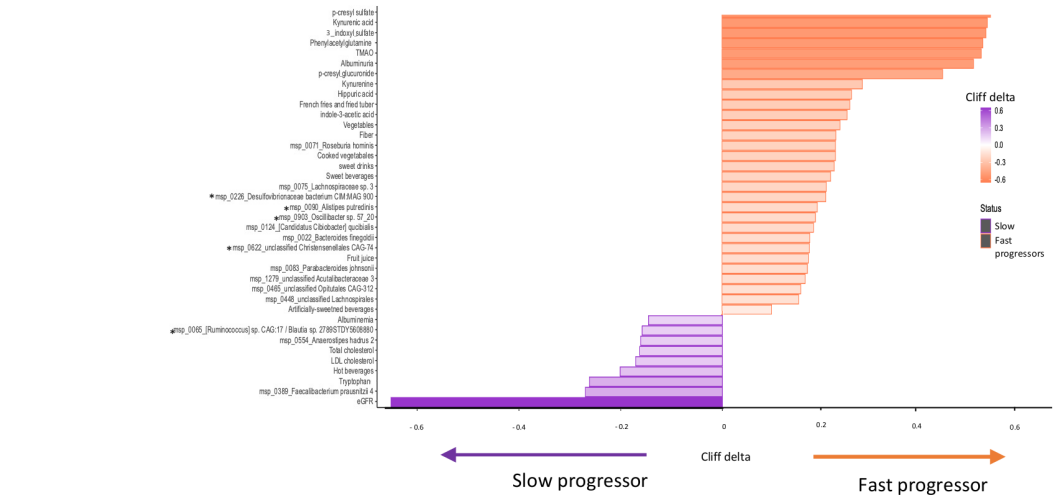


Figure 4 Impact of faecal microbiota transplantation (FMT) of stool from patients with CKD or healthy volunteers (HVs) on kidney function in mice. (A) Chronogram of the experiment. (B) Bar plots show the percentage of fibrosis of the kidney interstitial according to Sirius Red staining at 2 weeks and 6 weeks. (C) Representative images of Sirius Red staining (scale bar=50 µm) at 2 weeks. (D) Principal coordinates analysis (PCoA) according to Bray–Curtis dissimilarities among samples at 2 weeks, performed on the basis of species abundances. The samples are colour-coded and shape-coded by group. The spatial density distribution of samples in each group is indicated separately with an ellipse. The x-axis and y-axis labels indicate the microbial compositional variance explained by the first two principal components. (E) Taxonomic overview at the family level per sample at 2 weeks. Bar plots display the relative abundance of the taxa. PcoA of the relative abundance of plasmatic uraemic toxins (UT) and precursors. The spatial density distribution of samples in each group is indicated separately with an ellipse. The x-axis and y-axis labels indicate the metabolic compositional variance explained by the first two principal coordinates (F) at 2 weeks and (G) at 6 weeks. (H) Concentration of microbiota-derived UTs in the serum at 2 weeks and 6 weeks (n=5–8). The data are presented as the means±SEMs. *p<0.05 and **p<0.01 represents a significant difference between groups. One-way Student's t-test was performed. (i) Heatmap of Spearman rank correlations between the abundance of the most differentially abundant species between FMT-CKD and FMT-HVs mice and kidney parameters and UTs at 2 weeks (*p < 0.05; Spearman correlation). FMT, faecal microbiota transplantation.





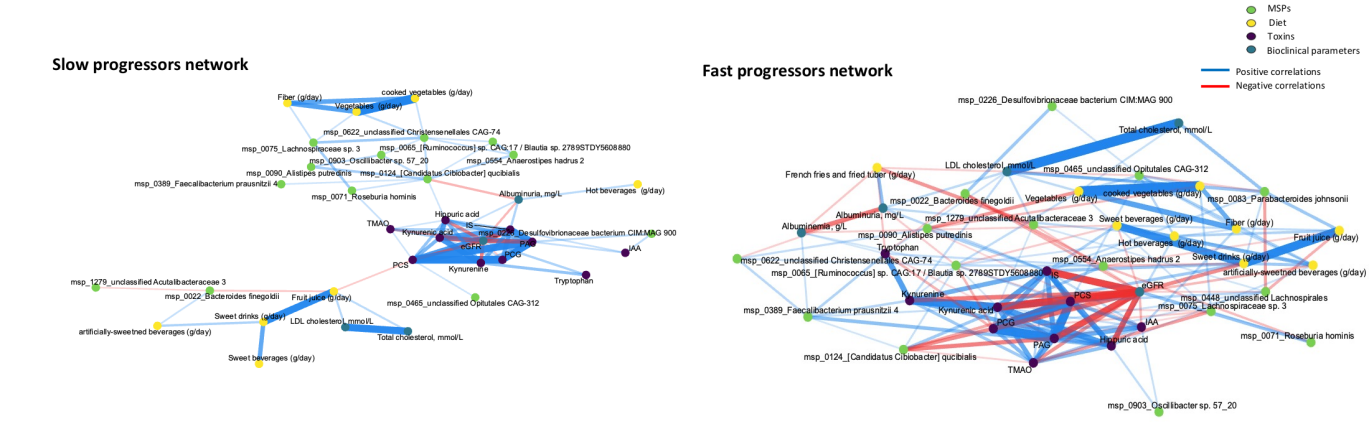


Figure 5 Differences in CKD-related omics data between fast and slow progressors. (A) Boxplots (line, median; box, IQR) represent the distribution of areas under the receiver operating characteristic curve (AUROCs) derived from different machine learning (ML) algorithms on the basis of different omics sets to classify progressors (n=68) versus non-progressors (NP) (n=172). EARTH, enhanced adaptive regression through hashing; GBM, gradient boosting machine; GLMBOOST, generalised linear model boosting; GLMNET, generalised linear model via penalised maximum likelihood; PAM, partitioning around medoids; PLS, partial least squares; RF, random forest. (B) Absolute values of Cliff's delta (CD) values of microbiome, bioclinical, diet and uraemic toxins (UTs) features linked to CKD progression identified as significantly different between fast and slow progressors ($p_{Wilcoxon}$<0.05 and and CD > 0.1). Stars indicate species harbouring genes encoding key enzymes involved in the main UTs synthesis pathways. (C) Networks of correlations for significantly different features between fast and slow progressors, comprising metagenomic species pangenomes (MSPs) (green), bioclinical characteristics (blue), UTs (black) and diet (yellow). The red and blue edges indicate significant negative and positive correlations, respectively. Line thickness represents the magnitude of the correlation coefficient; only correlations with a coefficient magnitude above 0.4 are shown. CKD, chronic kidney disease.

associated with progression (FDR<0.1) (online supplemental table 13). Consistent with previous studies, serum UT levels and albuminuria were also associated with CKD progression.[31–33]

To further explore the associations between CKD progression and these potential biomarkers, we carried out a correlation network analysis involving gut species, dietary, UTs and bioclinical data. Although the number of potential biomarkers differed between groups, the fast progressor correlation network clearly differed from that of slow progressors, featuring substantially more connections between omics features (figure 5C). However, strong interactions among UTs were observed for both fast and slow progressors and were negatively associated with the eGFR. Notably, unlike in the slow progressor network, the correlations between serum UT levels and the values of clinical parameters significantly contributed (20.4%) to the total $\chi 2$ score in the fast progressor network ($p_{\chi 2}=0.04$), corroborating the importance of the interplay among different kinds of omics data in differentiating between fast and slow disease progression (online supplemental table 14, figure 5C).

Adherence to a plant-based low-protein diet potentially counteracts the toxic modifications of the gut microbiota over time in patients with CKD

To date, the temporal relationships among diet, CKD status and the gut microbiota have not been explored; therefore, we performed a detailed analysis of 103 patients with CKD who were alive, did not require KFRT, and had been followed up (mean follow-up time: 2.8 years). In these patients, we observed an average decrease in their eGFR of 1.4 mL/min/1.73 m^2 per year (figure 6A). After 2.8 years, their microbiome composition was altered, demonstrating a loss of species richness (figure 6B,C). Interestingly, the ratio of UT-producing species increased over time, as quantified by the abundance of UT-producing species to the total number of species, hereafter referred to as the 'toxic species ratio' (figure 6D), as well as an increase in the serum levels of several UTs (online supplemental table 6).

Adherence to healthy dietary patterns is associated with slower CKD progression.[34] In our cohort, we found an overall decrease in the Dietary Variety Score (DVS) after 2.8 years (figure 6E). Interestingly, a greater decline in eGFR (median: −6.1 ml/min/1.73 m^2) was observed in participants whose DVS decreased over time, whereas a slight improvement in eGFR (median: +1.8 mL/min/1.73 m^2) was noted in those whose DVS increased over time (figure 6F). We combined these findings with those of our previous analyses to investigate the potential role of diet in gut microbiome alterations during CKD progression. We observed a significant reduction (26.9 vs 27.5 at T0 and T1, respectively; p=0.03) in the toxic species ratio (ie, species with the ability to produce UT precursors) over time in patients. Besides, the toxic species ratio decreased in patients who exhibited increased vegetable intake, which are dietary precursors of potentially nephroprotective compounds (p=0.0065, figure 6G,H) or reduced protein intake, which are dietary uraemic nephrotoxic precursors (p=0.018, figure 6I). Although we did not have specific information about the strain combination, patients with a stable consumption of probiotics (either as probiotic-enriched yoghurt-type dairy products or as dietary supplements) over the 3 years of follow-up period exhibited a significant reduction in the toxic species ratio (p=0.025, figure 6J). On the contrary, the ratio of toxic species increased in patients with a decreased fibre intake (p=0.029, figure 6H). Collectively, these data illustrate the interconnection between plant-based low-protein diet and gut microbiome composition, in particular UT-producing species.

DISCUSSION

We demonstrated that an aberrant gut microbiota of non-dialysis patients with CKD accelerates UT biosynthesis, contributing to raising serum UT levels and CKD progression. Our findings, drawn from a large, well-phenotyped patient cohort and validated in an external cohort regarding CKD severity, reveal that some gut microbiome signatures and UT profiles previously observed in dialysis patients[8 10 35 36] are already present in earlier CKD stages.

The functional role of the gut microbiome in CKD remains underexplored. Here, we investigated microbiome-related pathways relevant to UT production, showing that genes encoding key enzymes for UT precursor biosynthesis were enriched in patients with more severe CKD. Bacterial species producing UTs were abundant in patients with CKD, and their levels strongly correlated with serum UT concentrations. Notably, species belonging to the *Enterocloster* and *Hungatella* genera (*Lachnospiraceae* family), harbouring genes for UT production, were enriched in patients with CKD compared with HCs and negatively correlated with eGFR. This highlights the potential role of this branch of the phylogenetic tree, predominantly consisting of species that generate phenolic compounds,[37] in CKD-associated dysbiosis.

Several studies[10 35 36 38] have consistently reported an enrichment of *Desulfovibrio* species, particularly *Desulfovibrionaceae bacterium msp_0226*, in patients with severe CKD. These bacteria produce UT precursors like indole and trimethylamine,[10 39] exacerbating disease progression. We confirmed their association with CKD severity and progression. Additionally, species such as *Alistipes CAG268*[40] and *Intestinimonas massiliensis*,[41] known indole producers, were highly enriched in severe CKD.

Conversely, *Faecalibacterium prausnitzii,* a butyrate-producing bacterium with anti-inflammatory properties, was depleted in patients with severe CKD, and fast progressors. Its levels were inversely correlated with serum UT concentrations, consistent with its enrichment in healthy individuals.[28 42] Previous studies demonstrated the protective role of *Faecalibacterium prausnitzii*[28] or butyrate supplementation in CKD murine models, where their administration improved kidney outcomes.[28 43 44]

This study supports a causative link between gut microbiota dysbiosis and CKD progression. UTs like IS and PCS induce kidney fibrosis and tubular damage in CKD rodents.[45] CKD mice transplanted with stools from patients with CKD exhibited higher serum UT levels and larger areas of kidney fibrosis than those receiving healthy stool transplants. These findings emphasise the role of UT precursor-producing species enriched in CKD in the development of the disease and support the hypothesis that an increase in the levels of gut-derived UTs exacerbates CKD progression.

Our study identified microbiome features differentiating patients with CKD based on severity and status, with serum UT levels emerging as strong predictors of CKD progression. Notably, the top five features were all UTs, highlighting their pathophysiological significance. The importance of gut-derived metabolites is likely amplified in CKD because of unique circumstances where their concentration is dependent on a combination of factors: impaired kidney function leading to reduced clearance, increased de novo production by the intestinal microbiota and increased intestinal permeability.[46]

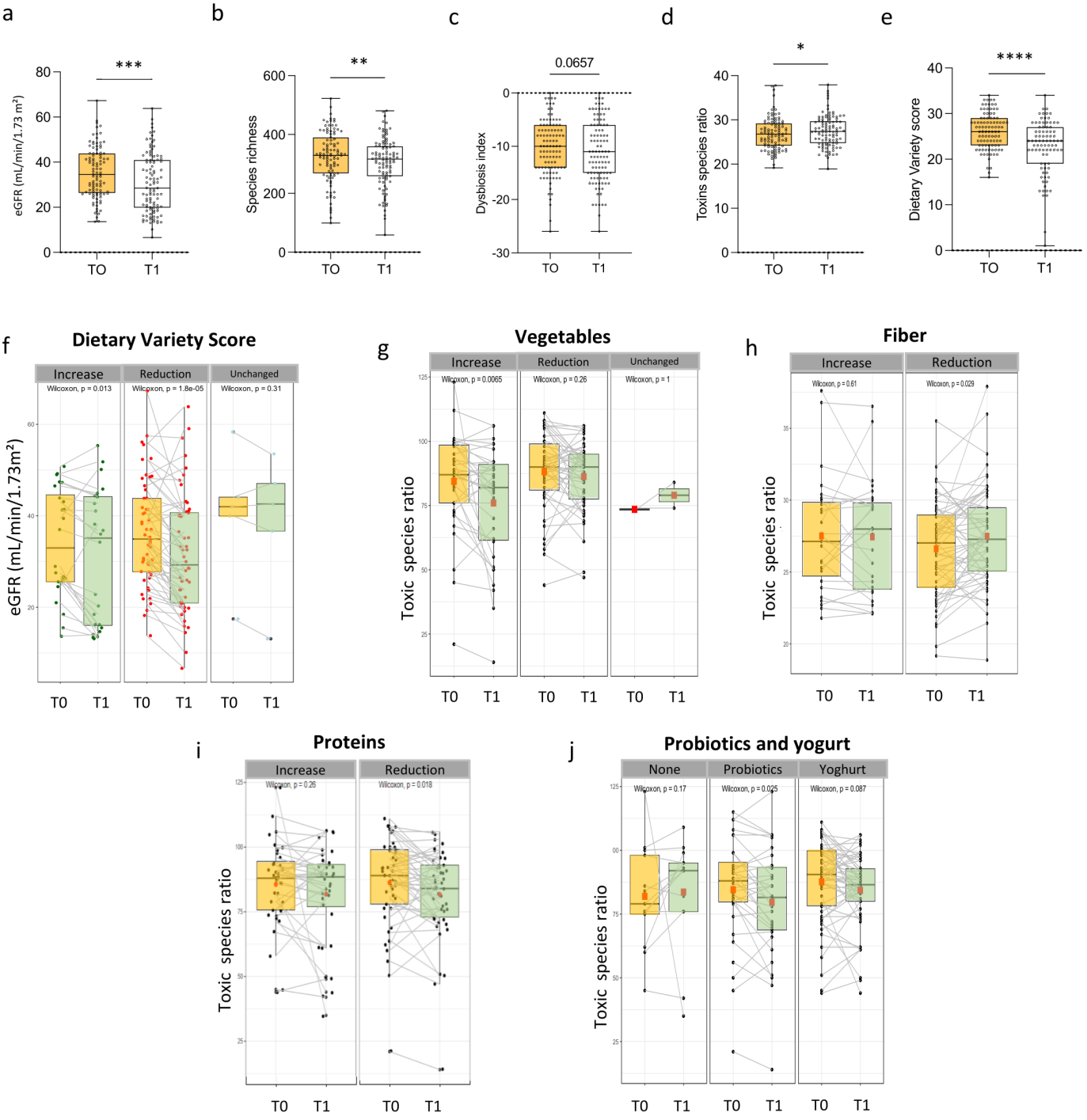


Figure 6 Gut microbiome modification in patients with CKD over 3 years is associated with estimated glomerular filtration rate (eGFR) decline and influenced by changes in diet. Box plot (line, median; box, IQR) showing (A) eGFR; (B) metagenomic species pangenomes (MSPs) richness; (C) the Gut Microbiome Health Index (Dysbiosis Index); (D) toxic species ratio; and (E) Dietary Variety Score (DVS) at inclusion (yellow, n=103) and 3 years later (white, n=103), (F) eGFR depending on the variation in DVS. Toxic species ratio depending on the vegetables (G), fiber (H), protein (I) and probiotics and yoghurt (J) dietary modifications during the follow-up. Comparisons performed with the two-sided Wilcoxon rank-sum test. *p<0.05; **p<0.01; ***p<0.001; ****p< 0.0001.

The present study reported declining microbial richness and enrichment of UT-producing species over time in non-dialysis patients with CKD. Given that greater microbial diversity is strongly linked to better health outcomes,[47] reduced dietary diversity and a high ratio of toxic species in CKD underscore the potential benefit of dietary and nutritional interventions. Our findings suggest that reducing protein intake, increasing vegetable intake, and using food or dietary supplements rich in probiotics over time may beneficially modulate the gut microbiota, reducing the abundances of UT-producing species throughout the follow-up period. This could partly explain the kidney-protective effects observed in large CKD populations consuming diets low in protein, high in plant-based foods.[34 48 49] The beneficial effects of increased vegetable intake on gut microbiota composition may be attributed to their fibre content.[50 51] Higher fibre intake modulates gut microbiota composition by

promoting the growth of some species such as *Bifidobacterium* or *Lactobacillus*, and more broadly, species involved in the production of short-chain fatty acids (SCFAs).[51] However, in our study, patients who increased their fibre intake did not exhibit a significantly different toxic species ratio between T0 and T1, but inversely, patients who decreased fibre intake had an increase in the ratio. The type of fibre consumed may lead to different microbial effects. Dietary intervention involving fructans and galacto-oligosaccharides led to higher abundance of *Bifidobacterium* and *Lactobacillus* spp while those involving polydextrose and resistant starch led to a higher abundance of *Bifidobacterium* but not *Lactobacillus* spp.[52] Other bioactive compounds found in vegetables, such as polyphenols or glucoraphanin, may also contribute to gut health similarly to fibre by promoting the growth of SCFA-producing bacteria notably.[53 54]

Interestingly, in our study, decreasing protein intake (mean decrease of 38%) was beneficial in reducing the abundances of UT-producing species throughout the follow-up period. Previous randomised clinical trials have shown that a very low protein diet can modulate gut microbiota, notably by increasing the abundance of butyrate-forming species.[55] Surprisingly, increasing protein intake (mean increase of 26%) did not lead to an increase in the toxic species ratio, suggesting a ceiling effect. Moving forward, targeted, controlled dietary intervention studies are essential to determine the clinical efficacy of these strategies and to refine personalised nutritional recommendations for gut microbiota modulation.

Despite its strengths, our study has limitations. While we collected extensive bioclinical, dietary and treatment data, absolute microbiome quantification methods, such as those described by Vandeputte *et al*,[56] could enhance the interpretation of microbiome changes. A portion of the observed difference between both cohorts may also arise from batch effects due to differences in sample collection and extraction methods; the perfect overlap between case/control status and those batch effects prevents direct statistical correction. Additionally, controlling for confounding factors like intestinal transit time would refine insights into microbiome dynamics.[8] While our findings suggest several potential mechanistic links such as the UT precursor production capacity of species from the genera *Enterocloster* and *Hungatella*, additional experiments such as monoculture inoculations with single target bacterial species are required to further refine and validate the hypotheses generated by this study.

In conclusion, this multiomic study revealed significant gut microbiome disruptions in non-dialysis patients with CKD, leading to functional changes and elevated UT levels strongly associated with CKD stage. These disruptions worsened as CKD progressed. Dietary modifications or probiotic supplements hold potential for future targeted placebo-controlled studies and clinical trials to investigate their clinical efficacy to modulate gut dysbiosis, reduce UT accumulation and slow down CKD progression.

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Correction notice This article has been corrected since it published Online First. The author's name, Geert RB Huys, has been updated.

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Patient and public involvement Patients and the public were not involved in the design or conduct or reporting or dissemination of the present study.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants. All legal authorisations were obtained, including those from the Comité consultatif sur le traitement de l'information en matière de recherche dans le domaine de la santé (CCTIRS N°12.360), the Commission nationale de l'informatique et des libertés (CNIL N°DR-2012-469), and the Kremlin-Bicêtre Comité de protection des personnes (CPP N°IDRCB 2012-A00902-41). The CKD-REIN biological collection is registered in the management application of the COnservation D'Eléments du COrps Humain (CODECOCH N°-2012-1624). The Institut national de la santé et de la recherche médical (Inserm) Institutional Review Board approved the study protocol (IRB00003888). ClinicalTrials.gov Identifier: NCT03381950. For the Ghent cohort, this study was approved by the local ethical committee at the Ghent University Hospital (2010/033; B67020107926). Written informed consent was obtained from all participants before participating in the study.

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