Gut microbiota generation of protein-bound uremic toxins and related metabolites is not altered at different stages of chronic kidney disease

Check for updates

OPEN

Tessa Gryp[1,2,3], Kim De Paepe[4], Raymond Vanholder[1], Frederiek-Maarten Kerckhof[4], Wim Van Biesen[1], Tom Van de Wiele[4], Francis Verbeke[1], Marijn Speeckaert[1], Marie Joossens[3], Marie Madeleine Couttenye[5], Mario Vaneechoutte[2] and Griet Glorieux[1]

[1]Department of Internal Medicine and Pediatrics, Nephrology Section, Ghent University Hospital, Ghent, Belgium; [2]Department of Diagnostic Sciences, Laboratory Bacteriology Research, Ghent University, Ghent, Belgium; [3]Department of Microbiology, Immunology and Transplantation, Molecular Microbiology—Microbiome Research Lab, KU Leuven, Leuven, Belgium; [4]Department of Biotechnology, Center for Microbial Ecology and Technology, Ghent University, Ghent, Belgium; and [5]Department of Nephrology, Antwerp University Hospital, Edegem, Belgium

Chronic kidney disease (CKD) is characterized by accumulation of protein-bound uremic toxins such as *p*-cresyl sulfate, *p*-cresyl glucuronide, indoxyl sulfate and indole-3-acetic acid, which originate in the gut. Intestinal bacteria metabolize aromatic amino acids into *p*-cresol and indole, (further conjugated in the colon mucosa and liver) and indole-3-acetic acid. Here we measured fecal, plasma and urine metabolite concentrations; the contribution of gut bacterial generation to plasma protein-bound uremic toxins accumulation; and influx into the gut of circulating protein-bound uremic toxins at different stages of CKD. Feces, blood and urine were collected from 14 control individuals and 141 patients with CKD. Solutes were quantified by ultra-high performance liquid chromatography. To assess the rate of bacterial generation of *p*-cresol, indole and indole-3-acetic acid, fecal samples were cultured *ex vivo*. With CKD progression, an increase in protein-bound uremic toxins levels was observed in plasma, whereas the levels of these toxins and their precursors remained the same in feces and urine. Anaerobic culture of fecal samples showed no difference in *ex vivo p*-cresol, indole and indole-3-acetic acid generation. Therefore, differences in plasma protein-bound uremic toxins levels between different CKD stages cannot be explained by differences in bacterial generation rates in the gut, suggesting retention due to impaired kidney function as the main contributor to their increased plasma levels. Thus, as fractional clearance decreased with the progression of CKD, tubular clearance appeared to be more affected than the glomerular filtration rate, and there was no net increase in protein-bound uremic toxins influx into the gut lumen with increased plasma levels.

Correspondence: *Tessa Gryp, Nephrology Section/Laboratory Bacteriology Research, Ghent University Hospital, Department of Internal Medicine and Pediatrics/Department of Diagnostic Sciences, Corneel Heymanslaan 10, MRB II, 9000 Ghent, Belgium. E-mail: tessa.gryp@ugent.be*

Chronic kidney disease (CKD) is characterized by an accumulation of protein-bound uremic toxins (PBUTs) such as *p*-cresyl sulfate (*p*CS), *p*-cresyl glucuronide (*p*CG), indoxyl sulfate (IxS), and indole-3-acetic acid (IAA).[1–3] Each of these uremic retention solutes exerts toxic effects, and several of them have been associated with worsening outcomes in CKD patients,[4–7] in particular with cardiovascular morbidity and mortality.[7–9] All 4 PBUTs originate from the intestinal microbial metabolism of the aromatic amino acids (AAAs) tyrosine, phenylalanine, and tryptophan.[10–13] In the distal part of the colon, tyrosine and phenylalanine are converted into *p*-cresol, and tryptophan into indole and IAA. Further, *p*-cresol and indole are partly detoxified by the host through sulfation in the colon mucosa and liver into respectively the uremic toxins *p*CS and IxS, whereas a smaller fraction of *p*-cresol is detoxified through glucuronidation into *p*CG.[10,14,15] Of note, IAA is as such an intestinal bacterial fermentation metabolite of tryptophan,[16,17] but it is also considered a uremic toxin when entering the circulation.[11] Several other uremic toxins also originate from bacterial metabolization in the gut, such as hippuric acid, phenyl sulfate, trimethylamine-N-oxide, and hydrogen sulfide.[11,13,18,19]

In blood, PBUTs reversibly bind to different degrees on plasma albumin.[20] In CKD, removal of the free (glomerular filtration) as well as the bound (tubular secretion) fraction of uremic toxins is impaired, resulting in their accumulation in blood.[21–24] The highest serum PBUT levels are found in end-stage kidney disease patients on hemodialysis (HD)[21] because, in addition to a disturbed kidney function, protein binding hampers the removal of these PBUTs by dialysis.[24–26]

In CKD, gut microbial community composition and function are altered.[27–34] Several uremia-related factors are at play: (i) increased blood urea levels resulting in the influx of circulating urea into the gut, where urea is hydrolyzed by microbial ureases into ammonia. Subsequently, ammonia is converted into ammonium hydroxide, which raises the luminal pH; the remaining uremia-related factors are as follows: (ii) colonic epithelial secretion of uric acid and oxalate as an adaptive response to a decline in excretion by the kidney, (iii) dietary restrictions of fiber to prevent hyperkalemia and fluid overload, (iv) use of phosphate- and potassium-binders, (v) malnutrition, and (vi) use of antibiotics.[35] In turn, changes in the gut environment and altered intestinal microbial composition could negatively affect CKD progression. Disrupted intestinal barrier structure caused by urea-derived ammonia and ammonium hydroxide promotes translocation of bacterial products and aggravates local and systemic inflammation in CKD.[36–38] The ensuing endotoxemia is a common feature in CKD and one of the causes of systemic inflammation that affects almost all CKD patients.[35] In addition, in CKD, protein assimilation is impaired in the upper gastrointestinal tract,[39,40] which might contribute to malnutrition in CKD.[41,42] Moreover, an impaired protein assimilation will result in an elevated abundance of undigested proteins in the colon, which might lead to an increase of p-cresol, indole, and IAA generation upon microbial fermentation of AAAs.

Based on the altered microbial community composition and function, and the elevated plasma uremic toxin levels in CKD, it has been hypothesized that the generation of uremic toxin precursors could change with CKD progression.[43,44] However, to our knowledge, no comprehensive data are available about the uremic toxin precursor levels in the gut environment of CKD patients, or about the toxin-generating capacity of gut microbiota. Therefore, this study was designed to determine fecal levels of PBUTs and their precursors, in parallel to plasma and urinary concentrations of PBUTs in different stages of CKD. This allows estimating the potential contribution of p-cresol, indole, and IAA generation by intestinal microbiota to the increased plasma PBUT levels in different stages of CKD. Also, the possibility of an increased influx into the gut due to the increased circulating PBUT concentration was assessed.

RESULTS
Characteristics of the study population
Table 1 summarizes the characteristics of the study population. Plasma cholesterol levels, urinary creatinine levels, Bristol stool scale, fecal dry weight percentage, and intake of antibiotics and/or probiotics did not differ among CKD stages, whereas a positive association with CKD stage was found for age, proteinuria, serum urea, creatinine, and phosphorus, and a negative association for estimated glomerular filtration rate. Groups did not match for gender, intake of laxatives, and presence of diabetes, and plasma total protein levels were significantly higher in the healthy control group compared to all stages of CKD, but similar among CKD stages. In peritoneal dialysis (PD) patients, C-reactive protein levels were higher, and urinary phosphorus and potassium were lower, compared to CKD1 and CKD2, respectively. Fecal dry weight percentage significantly correlated with Bristol stool scale ($P < 0.001$, $r_s = -0.579$).

Fecal bacterial cell counts and intactness
The number of total, intact, and damaged bacterial cells did not differ among CKD stages, or compared to the control. In general, the number of damaged cells significantly exceeded the number of intact cells ($P < 0.05$), except for CKD3, CKD5, and PD (Figure 1).

Uremic metabolites in feces, plasma, and urine
Fecal concentrations of the AAAs and of their gut bacterial fermentation metabolites p-cresol, indole, and IAA were comparable among stages of CKD and compared to control (Figure 2; Table 2). In addition, fecal PBUT levels were similar among stages of CKD, but in a comparison of these fecal PBUT levels with the respective plasma levels for the same stages, significantly lower levels were found in feces (pCS: −97.0%; pCG: −97.9%; IxS: −99.7%; IAA: −20.7%; $P < 0.001$). Of note, molar fecal levels of tyrosine, phenylalanine, and p-cresol were significantly higher compared to tryptophan and indole, as were levels of pCS in comparison to IxS (all $P < 0.05$). Normalizing fecal metabolite concentrations to an equal number of intact bacterial cells (10^{12} cells) did not modify the results (Figure 3), except for pCS, for which significantly lower values were found in CKD stages compared to control, HD, and PD ($P < 0.05$; Figure 3).

For all quantified PBUTs, plasma levels were higher in more advanced CKD (Figure 2; Table 2). Considering all stages together, a correlation was observed between the precursors in the feces and their respective plasma PBUTs, except for indole and IxS (Supplementary Table S1). More specifically, in the total study cohort, a positive correlation was found between fecal levels of tyrosine and phenylalanine and fecal p-cresol ($r_s = 0.212$, $P = 0.008$; $r_s = 0.259$, $P = 0.001$, respectively); between fecal p-cresol and plasma pCS ($r_s = 0.354$, $P < 0.001$); and between fecal p-cresol and plasma pCG ($r_s = 0.234$, $P = 0.004$). Also, fecal levels of tryptophan correlated with fecal indole ($r_s = 0.427$, $P < 0.001$) and fecal IAA ($r_s = 0.242$, $P = 0.003$). The highest number of significant correlations was found in the CKD group not on dialysis, whereas they were less prominent in the control and the dialysis subgroups (Supplementary Table S1).

In the urine, for pCG/creatinine (pCG/Crea), a higher ratio was observed in CKD3 and CKD5 compared to CKD1 (Table 2). The ratio of the other individual PBUTs/Crea, a proxy of 24-hour urinary uremic toxin excretion, which in equilibrated patients is likely to correspond to 24-hour generation, was similar among CKD stages and compared to control. Of note, in the present cohort, the ratio of urinary Urea/Crea, a proxy for protein intake, did not differ among CKD stages, or in comparison to the control (Supplementary Figure S1). With progression of CKD, the fractional kidney

Table 1 | Study population characteristics

Characteristics	Control	CKD 1	CKD 2	CKD 3	CKD 4	CKD 5	HD	PD	P value
General									
Number of subjects	14	14	23	44	23	10	16	11	N/A
Age (yr)	48 (24–84)	42 (22–69)	57 (21–83)	64 (26–86)[a]	69 (43–87)[a,b]	70 (37–87)[a,b]	74 (60–87)[a–c]	69 (50–84)[a]	<0.001[d]
Gender (M/F)	4/10	7/7	13/10	29/15	17/6	7/3	14/2	10/1	0.012[e]
Health parameters									
Body mass index (kg/m^2)	22.7 ± 3.0	25.6 ± 3.0	25.5 ± 4.2	27.9 ± 5.0[b]	26.7 ± 4.2	27.6 ± 2.2	25.5 ± 2.5	27.2 ± 3.3	0.001[f]
eGFR (ml/min per 1.73 m^2)	72.7 (62.1–88.7)	113.9 (97.7–123.2)	75.1 (65.8–79.3)	41.5 (35.7–55.1)[a,c]	22.6 (19.6–26.8)[a–c]	12.0 (10.2–13.5)[a–c,g]	6.0 (5.4–8.0)[a–c,g]	5.8 (3.5–8.8)[a–c,g]	<0.001[d]
Diabetes	0 (0)	0 (0)	6 (26.1)	15 (34.1)	5 (21.7)	4 (40.0)	9 (56.3)	2 (18.2)	0.004[e]
Cause of kidney failure									
ADPKD	N/A	3 (21.4)	2 (8.7)	5 (11.4)	1 (4.4)	2 (20.0)	1 (6.3)	2 (18.2)	N/A
Amyloidosis	N/A	1 (7.1)	0 (0)	1 (2.3)	0 (0)	0 (0)	0 (0)	0 (0)	N/A
Diabetic nephropathy	N/A	0 (0)	1 (4.4)	4 (9.1)	2 (8.7)	2 (20.0)	9 (56.3)	1 (9.1)	N/A
IgA nephropathy	N/A	2 (14.3)	1 (4.4)	1 (2.3)	4 (17.4)	1 (10.0)	1 (6.3)	1 (9.1)	N/A
Kidney cancer	N/A	0 (0)	0 (0)	5 (11.4)	0 (0)	0 (0)	0 (0)	0 (0)	N/A
Nephrotic syndrome	N/A	1 (7.1)	4 (17.4)	2 (4.6)	1 (4.4)	0 (0)	2 (12.5)	2 (18.2)	N/A
Renal infarction	N/A	0 (0)	3 (13.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	N/A
Renovascular disease	N/A	0 (0)	3 (13.0)	13 (29.6)	6 (26.1)	2 (20.0)	3 (18.8)	2 (18.2)	N/A
Others	N/A	2 (14.3)	6 (26.1)	9 (20.5)	8 (34.8)	0 (0)	1 (6.3)	3 (27.3)	N/A
Unknown	N/A	5 (35.7)	4 (17.4)	4 (9.1)	2 (8.7)	3 (30.0)	1 (6.3)	0 (0)	N/A
Blood parameters									
Cholesterol (mg/dl)	220.9 (170.8–234.8)	163.0 (141.5–197.8)	182.0 (152.8–212.0)	185.0 (160.0–220.0)	182.5 (162.3–219.8)	151.0 (137.0–187.0)	170.5 (137.3–198.0)	229.0 (129.0–231.0)	0.200[d]
C-reactive protein (mg/l)	1.6 (0.7–2.9)	0.9 (0.4–2.9)	2.3 (0.7–5.6)	2.4 (1.5–4.2)	2.4 (1.1–7.0)	1.1 (0.4–3.9)	4.2 (1.7–12.4)	6.6 (3.1–14.1)[a]	0.011[d]
Creatinine (mg/dl)	1.0 (0.9–1.1)	0.7 (0.7–0.8)	1.0 (0.9–1.2)	1.6 (1.3–1.8)[a,c]	2.6 (2.1–3.1)[a–c]	4.3 (3.8–5.3)[a–c,g]	8.1 (5.9–9.0)[a–c,g]	9.1 (6.2–11.7)[a–c,g]	<0.001[d]
Phosphorus (mmol/l)	N/A	1.1 (0.9–1.2)	1.0 (0.9–1.2)	1.0 (0.8–1.1)	1.1 (1.0–1.3)	1.4 (1.3–1.5)[c,g]	1.3 (1.0–1.5)[g]	N/A	<0.001[d]
Total protein (g/l)	82.4 ± 6.7	70.3 ± 4.8[b]	68.6 ± 3.8[b]	68.8 ± 3.7[b]	70.6 ± 4.3[b]	65.3 ± 6.1[b]	N/A	66.4 ± 7.8[b]	<0.001[f]
Urea (mg/dl)	28.1 (23.8–34.5)	28.0 (20.8–34.8)	34.5 (29.8–45.3)	56.5 (44.5–67.8)[a,b]	103.5 (79.3–114.0)[a–c,g]	136.5 (124.8–171.0)[a–c,g]	92.7 (82.7–119.7)[a–c,g]	125.0 (99.5–162.5)[a–c,g]	<0.001[d]
Urine parameters									
Creatinine (mg/dl)	84.1 (65.0–132.7)	73.5 (39.4–94.5)	99.4 (65.0–152.5)	75.6 (53.1–110.2)	70.5 (39.6–98.1)	74.8 (58.2–97.8)	N/A	46.2 (29.1–81.5)	0.173[d]
Phosphorus (mmol/l)	12.9 (6.8–29.2)	13.4 (4.9–16.2)	14.2 (9.4–18.4)	11.3 (7.1–15.2)	9.4 (7.3–15.4)	12.4 (10.3–14.2)	N/A	5.8 (3.9–9.0)[c]	0.049[d]
Potassium (mmol/l)	61.8 (33.2–98.8)	44.4 (28.9–81.9)	53.6 (40.8–81.7)	42.9 (27.1–70.6)	37.1 (21.4–43.2)	32.2 (25.2–36.9)	N/A	23.5 (14.4–38.4)[c]	0.001[d]
Total protein (g/l)	0.06 (0.03–0.07)	0.04 (0.03–0.19)	0.08 (0.06–0.13)	0.13 (0.07–0.28)	0.21 (0.07–0.70)[b]	0.48 (0.31–2.18)[a–c]	N/A	0.50 (0.16–3.17)[b,c]	<0.001[d]
Fecal parameters									
Bristol Stool Scale	4.0 (3.8–5.0)	4.5 (3.0–5.3)	4.0 (3.0–5.0)	4.0 (3.0–5.0)	4.0 (2.0–5.0)	4.0 (2.8–5.0)	3.5 (2.0–4.0)	3.0 (2.0–5.0)	0.582[d]
Dry weight (%)	0.27 ± 0.05	0.25 ± 0.10	0.26 ± 0.07	0.28 ± 0.08	0.30 ± 0.11	0.28 ± 0.08	0.29 ± 0.08	0.30 ± 0.09	0.568[f]
Medication/supplement									
Antibiotics	0 (0)	2 (14)	1 (4)	2 (5)	4 (17)	2 (20)	1 (6)	0 (0)	0.345[e]
Laxatives	0 (0)	2 (14)	2 (9)	5 (11)	3 (13)	1 (10)	2 (13)	8 (72.7)	<0.001[e]
Probiotics	2 (14)	0 (0)	2 (9)	1 (2)	2 (9)	2 (20)	1 (6)	N/A	0.462[e]

ADPKD, autosomal dominant polycystic kidney disease; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; F, female; HD, hemodialysis; PD, peritoneal dialysis; M, male; N/A, not applicable.

[a]$P \leq 0.05$ vs. CKD1.
[b]$P \leq 0.05$ vs. the control group.
[c]$P \leq 0.05$ vs. CKD2.
[d]Kruskal-Wallis test.
[e]Chi-square test; P values obtained after Bonferroni correction.
[f]One-way analysis of variance.
[g]$P \leq 0.05$ vs. CKD3.
Data are presented as mean ± SD, median (25th–75th percentile), or n (%).
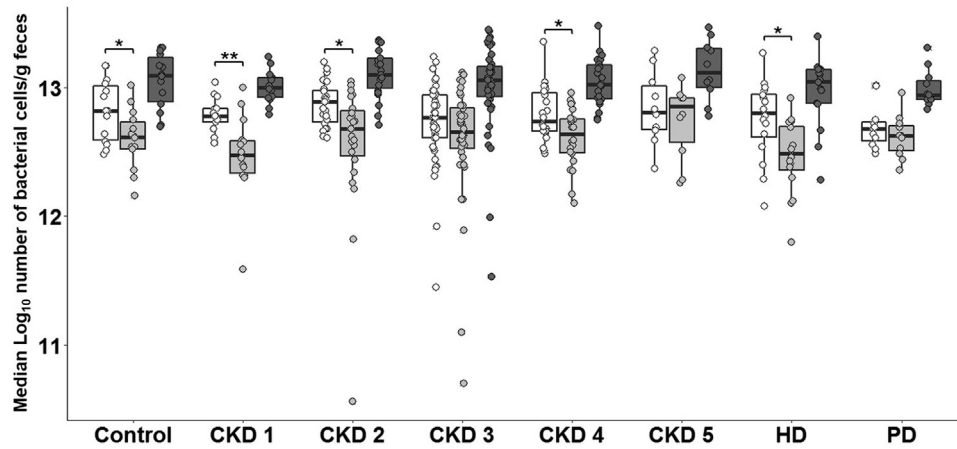
Figure 1 | Median log$_{10}$ number of the intact, damaged, and total bacterial cells in the fecal samples. Control ($n = 14$), chronic kidney disease (CKD)1 ($n = 14$), CKD2 ($n = 23$), CKD3 ($n = 44$), CKD4 ($n = 23$), CKD5 ($n = 10$), hemodialysis (HD) ($n = 16$), and peritoneal dialysis (PD) ($n = 11$). White dots: damaged cells; light gray dots: intact cells; dark gray dots: total cells. $^*P \leq 0.05$; $^{**}P \leq 0.001$. x, outlier.

clearance of *p*CS decreased. Fractional kidney clearance of *p*CG and IxS were significantly lower in CKD5 compared to CKD2-3 and CKD2, respectively. In addition, an inverse correlation was found between fractional kidney clearance and decrease of kidney function for *p*CS, *p*CG, and IxS ($P \leq$ 0.001). IxS and *p*CG had a significantly higher fractional kidney clearance compared to the other PBUTs in CKD ($P <$ 0.05; Figure 4).

Similar results were obtained when correlating fecal PBUT-related metabolites, plasma PBUTs levels, and the ratio of individual PBUTs/Crea with estimated glomerular filtration rate as a continuous variable. Except for IAA/Crea where a weak but significant correlation was obtained ($r_s = 0.119$; $P =$ 0.031). Based on the median values of plasma *p*CS and IxS, CKD patients were separated into 2 groups, i.e., high *p*CS and low IxS plasma levels ($n = 13$) versus low *p*CS and high IxS plasma levels ($n = 13$). Interestingly, fecal tryptophan and

indole levels were significantly higher in the patient group with low *p*CS and high IxS plasma levels. In the same group, a higher ratio of urinary IxS/Crea was observed, whereas a higher ratio of urinary *p*CS/Crea was found in the patient group with high *p*CS and low IxS serum levels (Supplementary Figure S2).

Ex vivo *p*-cresol, indole, and IAA generation in feces
Anaerobic culture of fecal samples from controls, CKD1, and CKD5 patients showed no difference in the *ex vivo* generation of *p*-cresol, indole, and IAA at different time points of incubation ($P > 0.05$; Figure 5). Tyrosine was significantly decreased after a 48-hour incubation period compared to baseline ($P < 0.05$), and metabolization of phenylalanine was slower (72 hours, $P < 0.05$). The bacterial fermentation end product, *p*-cresol, significantly increased after 48 hours ($P <$ 0.05), which was more pronounced, but not significant, in
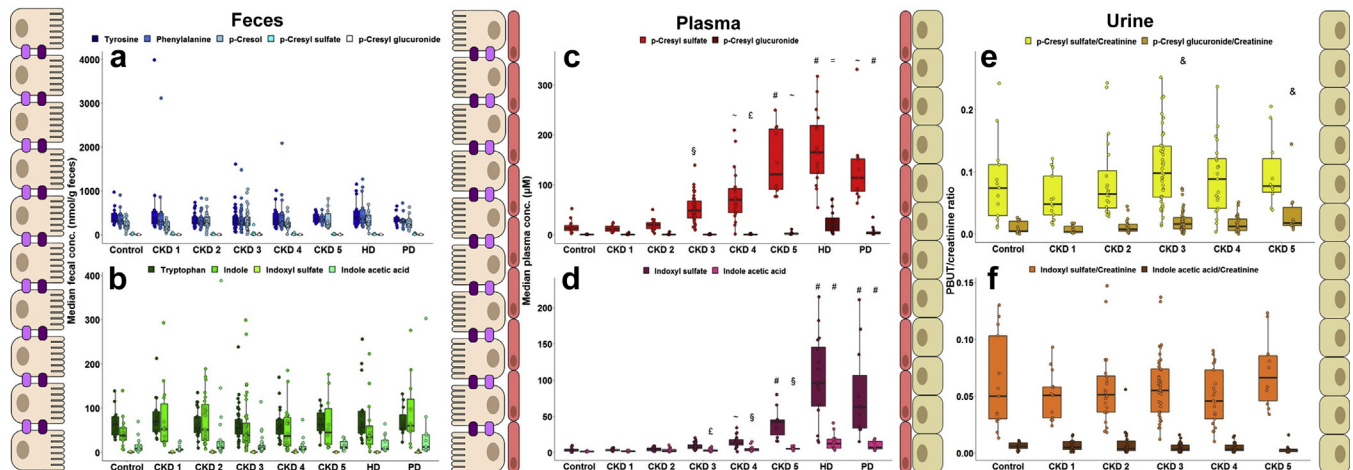

Figure 2 | Concentrations (conc.) of *p*-cresyl sulfate–, indoxyl sulfate–, and indole-3-acetic acid–related metabolites in (a,b) feces, (c,d) plasma, and (e,f) urine. Control ($n = 14$), chronic kidney disease (CKD)1 ($n = 14$), CKD2 ($n = 23$), CKD3 ($n = 44$), CKD4 ($n = 23$), CKD5 ($n = 10$), hemodialysis (HD) ($n = 16$), and peritoneal dialysis (PD) ($n = 11$). $^£P \leq 0.05$ versus control; $^\&P \leq 0.05$ versus CKD1; $^§P \leq 0.05$ versus control and CKD1; $^\sim P \leq 0.05$ versus control and CKD1–2; $^\#P \leq 0.05$ versus control, CKD1–2–3; $^=P \leq 0.05$ versus control and CKD1–2–3–4. PBUT, protein-bound uremic toxin; x, outlier.

Table 2 | Fecal, plasma, and urine metabolite concentrations in the different study groups

Metabolites	Control	CKD 1	CKD 2	CKD 3	CKD 4	CKD 5	HD	PD	P value[a]
Feces (nmol/g wet feces)									
Tyrosine	347.6 (261.5–500.3)	365.1 (223.0–536.0)	298.2 (234.1–421.1)	320.6 (215.2–428.2)	316.3 (200.7–495.2)	374.1 (263.3–489.2)	371.8 (221.7–564.4)	334.4 (269.4–397.6)	0.809
Phenylalanine	321.7 (218.8–463.4)	317.0 (190.9–494.6)	261.3 (205.3–396.4)	263.9 (205.4–397.8)	276.6 (176.1–469.9)	349.5 (260.4–443.6)	352.6 (231.6–591.8)	328.2 (228.1–345.0)	0.615
Tryptophan	63.3 (36.6–81.5)	70.1 (45.4–101.9)	54.1 (42.1–80.7)	54.9 (40.6–74.4)	53.2 (40.2–74.6)	61.8 (46.9–96.7)	57.6 (42.2–94.2)	52.7 (50.2–101.4)	0.919
p-Cresol	204.9 (129.1–342.0)	168.3 (79.9–305.0)	257.2 (191.4–429.6)	250.2 (187.9–433.2)	196.5 (164.7–288.3)	240.0 (166.8–565.1)	288.4 (190.8–469.3)	224.5 (134.2–352.9)	0.203
Indole	38.5 (23.8–58.7)	52.1 (23.1–124.9)	51.2 (25.2–115.6)	38.9 (21.1–72.7)	37.0 (12.4–82.0)	44.5 (15.9–122.3)	34.8 (16.6–67.8)	60.0 (46.1–143.5)	0.492
p-Cresyl sulfate (total)	9.89 (6.00–21.44)	7.54 (4.83–14.06)	4.99 (0.45–12.00)	6.76 (1.57–13.85)	6.18 (1.11–11.50)	5.06 (0.49–17.26)	5.44 (1.64–11.58)	6.42 (3.09–18.65)	0.544
p-Cresyl glucuronide (total)	0.19 (0.00–0.44)	0.15 (0.00–0.39)	0.09 (0.00–0.21)	0.16 (0.00–0.45)	0.19 (0.00–0.43)	0.13 (0.00–0.67)	0.00 (0.00–0.14)	0.12 (0.00–0.44)	0.322
Indoxyl sulfate (total)	0.19 (0.07–0.30)	0.08 (0.00–0.43)	0.17 (0.00–0.42)	0.25 (0.00–0.45)	0.35 (0.00–0.56)	0.23 (0.00–0.36)	0.20 (0.00–0.37)	0.00 (0.00–1.02)	0.734
Indole-3-acetic acid (total)	8.42 (3.37–21.10)	5.82 (4.17–8.83)	9.83 (7.86–25.21)	9.45 (5.02–16.15)	8.08 (4.36–14.14)	11.56 (6.48–28.16)	8.28 (4.76–33.36)	12.42 (4.90–41.87)	0.271
Plasma (µM)									
p-Cresyl sulfate (total)	13.20 (6.85–19.71)	11.61 (5.88–19.28)	19.37 (11.15–24.17)	47.97 (31.99–69.40)[b–c]	69.63 (42.57–93.92)[b–d]	121.1 (85.9–215.1)[b–e]	164.9 (116.9–229.2)[b–e]	114.0 (81.8–154.1)[b–d]	<0.001
p-Cresyl glucuronide (total)	0.06 (0.04–0.11)	0.06 (0.01–0.11)	0.10 (0.05–0.21)	0.25 (0.16–0.51)	0.50 (0.20–0.62)[b]	1.73 (0.69–3.63)[b–d]	17.92 (5.86–39.92)[b–f]	2.09 (1.30–8.60)[b–e]	<0.001
Indoxyl sulfate (total)	2.79 (2.05–5.70)	3.34 (2.37–4.11)	4.57 (2.32–6.71)	7.50 (5.28–10.74)	12.71 (9.03–17.93)[b–d]	42.51 (19.60–50.49)[b–e]	96.01 (60.45–152.1)[b–e]	62.39 (32.21–135.6)[b–e]	<0.001
Indole-3-acetic acid (total)	1.45 (1.17–1.77)	1.73 (1.24–1.91)	1.79 (1.50–4.41)	3.02 (1.93–3.70)[b]	3.69 (2.10–5.48)[b,c]	5.56 (5.17–6.60)[b,c]	12.52 (5.80–18.86)[b–e]	6.98 (4.45–16.80)[b–e]	<0.001
Urine									
[p-Cresyl sulfate]/[Crea]	0.074 (0.03–0.12)	0.048 (0.03–0.10)	0.065 (0.04–0.12)	0.098 (0.06–0.14)	0.088 (0.04–0.13)	0.077 (0.06–0.15)	N/A	N/A	0.137
[p-Cresyl glucuronide]/[Crea]	0.004 (0.00–0.02)	0.003 (0.00–0.01)	0.008 (0.00–0.02)	0.016 (0.01–0.03)[c]	0.012 (0.01–0.03)	0.018 (0.01–0.05)[c]	N/A	N/A	0.002
[Indoxyl sulfate]/[Crea]	0.050 (0.03–0.11)	0.051 (0.03–0.07)	0.052 (0.04–0.07)	0.055 (0.04–0.08)	0.046 (0.03–0.08)	0.067 (0.04–0.10)	N/A	N/A	0.534
[Indole-3-acetic acid]/[Crea]	0.006 (0.00–0.01)	0.005 (0.00–0.01)	0.004 (0.00–0.01)	0.003 (0.00–0.01)	0.003 (0.00–0.01)	0.003 (0.00–0.01)	N/A	N/A	0.238

CKD, chronic kidney disease; Crea, creatinine; HD, hemodialysis; N/A, not applicable; PD, peritoneal dialysis.
[a]Kruskal-Wallis test; P values obtained after Bonferroni correction.
[b]$P \leq 0.05$ vs. the control group.
[c]$P \leq 0.05$ vs. CKD1.
[d]$P \leq 0.05$ vs. CKD2.
[e]$P \leq 0.05$ vs. CKD3.
[f]$P \leq 0.05$ vs. CKD4.
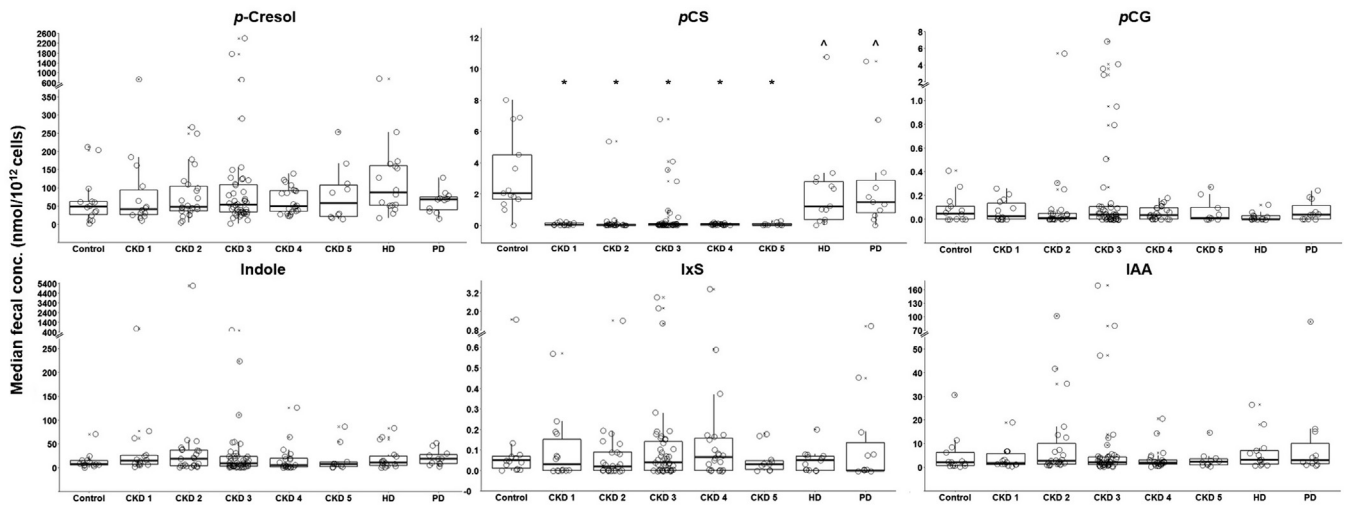Data are presented as median (25th–75th percentile).

Figure 3 | Fecal concentrations (conc.) of *p*-cresol, *p*-cresyl sulfate (*p*CS), *p*-cresyl glucuronide (*p*CG), indole, indoxyl sulfate (IxS), and indole-3-acetic acid (IAA) normalized to an equal number of intact bacterial cells. Control ($n = 14$), chronic kidney disease (CKD)1 ($n = 14$), CKD2 ($n = 23$), CKD3 ($n = 44$), CKD4 ($n = 23$), CKD5 ($n = 10$), hemodialysis (HD) ($n = 16$), and peritoneal dialysis (PD) ($n = 11$). *$P \leq 0.05$ versus control; ^$P \leq 0.05$ versus CKD1–2–3–4. x, outlier.

CKD5 compared to control and CKD1. A significant decrease of tryptophan was reached after 48 hours ($P < 0.05$), with a significant increase of indole after 24 hours and 48 hours for, respectively, the control and CKD1, and CKD5. In addition, a minor increase of IAA was found after 48 hours and 24 hours for, respectively, the control and CKD5, and CKD1 (Figure 5).

After extended incubation for 7 days, no differences in *p*-cresol, indole, or IAA generation from CKD1 and CKD5 fecal samples were observed ($P < 0.05$). However, a higher absolute amount of indole was found after 7 days versus baseline in both CKD groups ($P < 0.05$), whereas this was not significant for *p*-cresol, probably due to the high interpatient
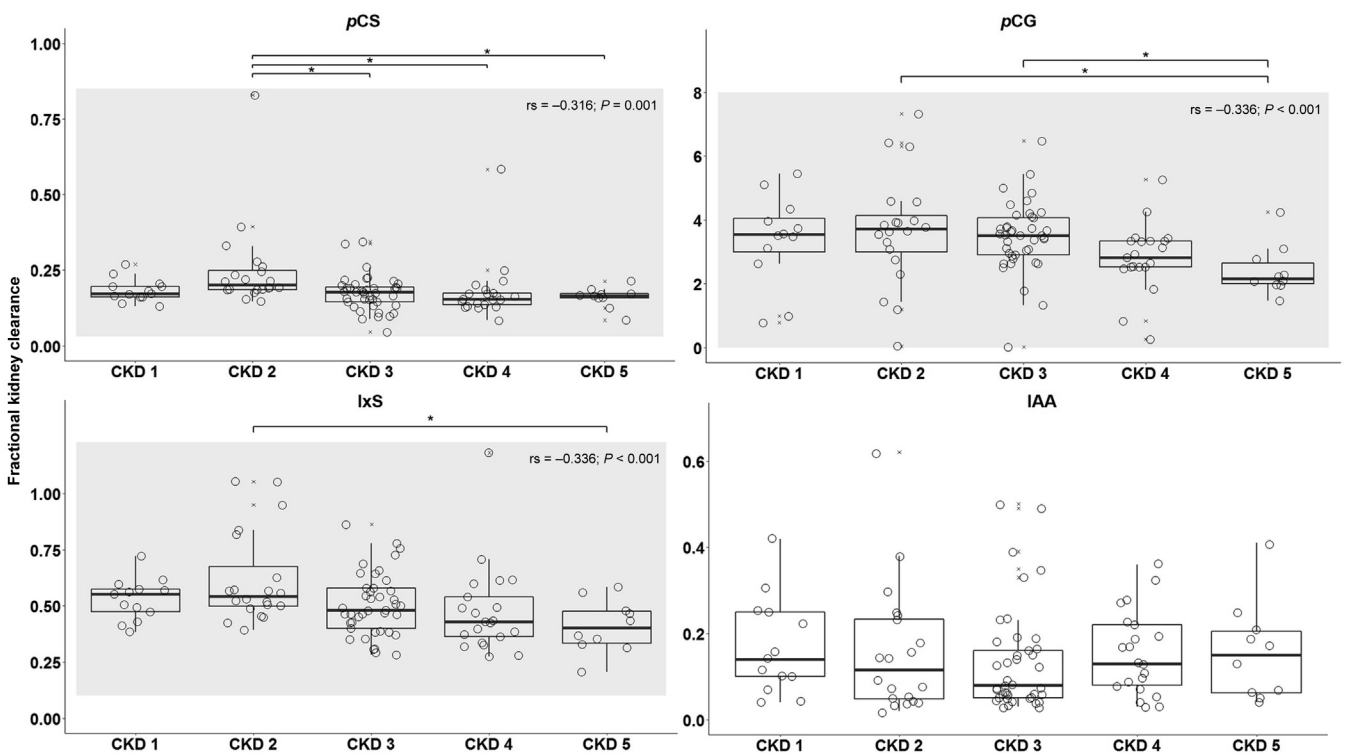

Figure 4 | Fractional kidney clearance of *p*-cresyl sulfate (*p*CS), *p*-cresyl glucuronide (*p*CG), indoxyl sulfate (IxS), and indole-3-acetic acid (IAA) per chronic kidney disease (CKD) stage. *$P \leq 0.05$. x, outlier. CKD1 ($n = 14$), CKD2 ($n = 23$), CKD3 ($n = 44$), CKD4 ($n = 23$), and CKD5 ($n = 10$). Gray square: correlation with Spearman's rank test (rs) between fractional kidney clearance and CKD. Correlation with spearman's rank test between fractional kidney clearance and estimated glomerular filtration rate: *p*CS $r_s = 0.311$, *p*CG $r_s = 0.331$, and IxS $r_s = 0.366$, all $P \leq 0.001$.
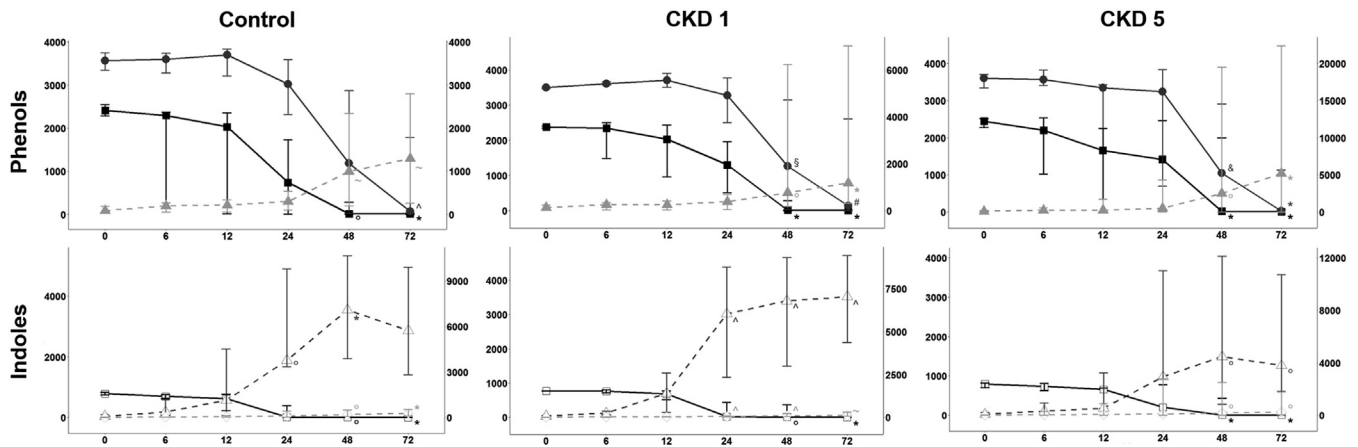
Figure 5 | *p*-Cresol, indole, and indole-3-acetic acid generation of controls, chronic kidney disease (CKD)1, and CKD5 fecal samples cultured up to 72 hours. Data are presented as median and 95% confidence interval. Right *y*-axis: median concentration in μM; left *y*-axis: median concentration in nmol/g feces. ■, tyrosine (black); ●, phenylalanine (dark gray); ▲, *p*-cresol (gray); □, tryptophan (black); Δ, indole (dark gray); ◇, indole-3-acetic acid (gray). Control (*n* = 6), CKD1 (*n* = 6), and CKD5 (*n* = 5). °$P < 0.05$ versus baseline; *$P < 0.05$ versus 0 and 6 hours of incubation; ˆ$P < 0.05$ versus 0, 6, and 12 hours of incubation; ~$P < 0.05$ versus 0, 6, 12, and 24 hours of incubation; §$P < 0.05$ versus 12 hours of incubation; #$P < 0.05$ versus 6 and 12 hours of incubation; &$P < 0.05$ versus 6 hours of incubation.

variability. For IAA, a higher absolute amount was found in CKD5 after 7 days compared to baseline ($P = 0.028$; Supplementary Figure S3).

DISCUSSION

The main purpose of this study was to quantify fecal, plasma, and urine concentrations of PBUT-related metabolites in different stages of CKD, and to estimate the contribution of intestinal generation to increased individual plasma PBUT levels in CKD. In addition, potential influx of circulating PBUTs into the gut was assessed.

So far, intestinal generation of PBUT precursor metabolites has not been thoroughly investigated in CKD patients. In addition to impaired kidney function, an increase in intestinal generation of PBUT precursor metabolites could contribute to the increased plasma PBUT levels in CKD. In case of comparable fecal precursor metabolite levels between the different stages of CKD, next to a higher intestinal generation, an increase in gut permeability could be an assumed hypothetical explanation, but this possibility was practically ruled out by our current results. In fecal samples from our cohort, absolute levels of the AAAs and their metabolites *p*-cresol, indole, and IAA were not significantly different among CKD stages. Even when normalizing to an equal number of viable bacterial cells, no differences were observed. In plasma, the individual PBUT levels increased with progressive stages of CKD, whereas the urinary levels did not change. The urinary PBUT/Crea ratios of *p*CS, IxS, and IAA, a proxy for colonic generation,[45] did not change with progressive stages of CKD. Also *ex vivo*, bacterial generation of *p*-cresol, indole, and IAA was not different in fecal samples from controls, CKD1, and CKD5. These results indicate that, in the present CKD cohort, bacterial generation of *p*-cresol and indole does not seem to contribute to the increase in plasma PBUT levels. Moreover, the fractional kidney clearance of *p*CS, *p*CG, and IxS decreases

with CKD progression, indicating that plasma PBUT accumulation is probably mainly due to a decline in kidney function, whereby overall solute removal seems more deranged than creatinine removal, suggesting a role for tubular dysfunction on top of glomerular filtration rate. Finally, there is no net increased influx of PBUTs into the gut lumen with increasing plasma concentrations. However, the presence of very low fecal PBUT levels might be due to transport of the elevated circulating levels of PBUTs into the gut lumen via colonic epithelial transporter proteins (e.g., Mdr1a/Pgp), which are expressed at the apical surface of enterocytes. This transporter protein has been shown to cause efflux of drugs from enterocytes back into the intestinal lumen.[46] Another potential option is local intestinal production of *p*CS, as the ratio of fecal *p*CS/IxS is markedly higher than the same ratio in plasma. However, this is unlikely, given that no *p*CS could be detected in the *ex vivo* experiments next to *p*-cresol generation (data not shown).

To our knowledge, no comprehensive report in CKD patients, covering all stages of CKD, on levels of PBUTs and their precursors in feces and plasma, as well as in urine, is currently available, and the existing data diverge. Our findings are comparable to the results of Fukuuchi *et al.*, who observed no difference in fecal *p*-cresol and indole levels when comparing HD to CKD patients. In the latter study, gas chromatography was used to quantify compounds in feces.[29] Hida *et al.*, also using gas chromatography, found higher fecal *p*-cresol levels in HD compared to controls.[34] Moreover, a fecal metabolome study, using gas chromatography–mass spectrometry, also observed higher levels of *p*-cresol and indole in HD compared to controls. However, when in the latter study the fecal metabolite profiles of these HD patients were compared with those of their household contacts, who were on the same diet, no differences were observed.[14] In the same study, fecal metabolite levels of *p*-cresol and indole did

not differ in 5/6 nephrectomized rats compared to sham-operated rats,[14] whereas in another animal study, higher *p*-cresol and indole levels were observed in 5/6 nephrectomized rats versus sham-operated rats.[47] The combined results in the study of Poesen *et al.* indirectly suggested that a change in colonic microbial metabolism in CKD is largely attributable to diet and less to a loss of kidney function.[14] Similar results were found in an animal study by Mishima *et al.*, in which *p*CS and IxS were quantified by capillary electrophoresis time-of-flight mass spectrometry in germ-free (GF) and specific pathogen–free (SPF) mice with and without kidney failure.[13] Fecal and urinary concentrations of *p*CS and IxS were not different between SPF-control mice compared to SPF–kidney failure mice and were not at all detected in GF-control and GF–kidney failure mice. Unfortunately, the latter study did not report fecal levels of *p*-cresol and indole.[13] In the present study, the levels of cresolic compounds are higher in feces and plasma than those of indolic compounds, as also observed in previous studies in CKD, HD, and controls,[14,29] and for *p*CS versus IxS in SPF-control and SPF–kidney failure mice.[13]

Several studies have reported changes in gut microbial composition in CKD.[27–34] However, if this is the case, our results suggest that these changes do not affect the generation of *p*-cresol, indole, and IAA, even in more advanced stages of CKD. In addition, our *ex vivo* experiments revealed no differences in generation by fecal bacteria from controls, CKD1, and CKD5, whereas at different stages of CKD, no changes in the urinary PBUT/Crea ratios were observed, again suggesting unaltered *in vivo* colonic generation of *p*CS, IxS, and IAA in CKD.[48,49] Only for urinary *p*CG/Crea, a higher ratio was observed in patients with advanced CKD, but this likely can be attributed to increased glucuronidation in patients with advanced CKD as reported by Poesen *et al.*[45] Overall, the present results suggest that increased accumulation of PBUTs in plasma of CKD patients is not due to an increase of bacterial generation of *p*-cresol, indole, and IAA. Nevertheless, consistent with our previous findings,[50] but irrespective of kidney function, some patients do seem to generate more of some specific PBUT precursors than others, as shown by our findings comparing patients with low *p*CS and high IxS to those with high *p*CS and low IxS plasma levels.

In the present study, no differences in fecal AAA, or of the precursor metabolites *p*-cresol, indole, and IAA, were observed among different stages of CKD, likely because of an absence of major differences in dietary protein intake, as also suggested by the unaltered urinary urea/creatinine ratio over all CKD stages versus controls. Although normalization to urinary creatinine might be skewed if muscle mass is lost with progression of CKD,[51] the current approach avoids errors from incorrect 24-hour urine collection and/or dietary recall.

A decreased fractional kidney clearance was observed in the later stages of CKD (not on dialysis) for *p*CS, *p*CG, and IxS, with the highest fractional kidney clearance for IxS and *p*CG. These results correspond to those from the study of

Poesen *et al.*, in which a 3-fold higher kidney clearance was found for IxS compared to *p*CS in CKD1 to CKD5 based on a 24-hour urine collection.[52] In normal conditions, PBUTs are excreted into the urine by glomerular filtration and by organic anion and cation transporters present in the kidney tubular cells (tubular filtration).[53–55] A decreased fractional kidney clearance suggests that tubular clearance is proportionally more affected than glomerular filtration. The fact that the fractional kidney clearance of IAA did not change with CKD progression could be due to a higher transport rate compared to the other PBUTs, which has been demonstrated for erythrocytes[56] but, to our knowledge, not for tubular cells.

Flow cytometry, a single-cell enumeration technology, is an upcoming technique to quantify bacterial cells and identify the composition of bacterial populations.[57] For the first time, this technique was used to determine the total, intact, and damaged bacterial cell count in a CKD patient cohort, for which no comprehensive data on the total abundance of gastrointestinal bacterial cells are available. In the present study, a higher abundance of damaged cells in comparison with intact cells was found in controls and all CKD stages apart from CKD5. In addition, no changes among progressive CKD stages were observed. Similar results were found in a culture-based study comparing HD and controls.[34] However, in other studies, using quantitative polymerase chain reaction and culture, a decrease in total fecal bacteria was found in CKD, non-dialyzed and dialyzed patients with end-stage kidney disease compared to controls.[29,58,59]

Even if the present data indicate that the increase of plasma levels of intestinally generated PBUTs is mainly the consequence of kidney dysfunction and not of a change in fecal microbial generation, the gut bacteria remain an important potential target when considering novel therapies to prevent uremic toxin accumulation. Changes in diet could be a potential option to reduce uremic toxin levels, as shown in previous studies,[60–66] and as indirectly suggested by the study of Poesen *et al.*, which indicated that dietary intake is the most important determinant of uremic toxin precursor generation.[14] However, protein restriction also has the potential to induce protein energy wasting. On the other hand, several animal[47,67,68] and human[34,69–77] studies demonstrated favorable effects on cresol and indole metabolism by administration of pre-, pro-, and synbiotics, although most of these studies demonstrated an impact on only one of the 2 targets. Recently, our group demonstrated that, in HD, high *p*CS levels are associated with a completely different gut microbiota composition than high IxS levels.[50] The dissociation in the generation between indoles and cresols goes along with an apparent disconnection in physiologic impact. Indole, being at the origin of the toxic compound IxS, might not be unequivocally detrimental. Indole itself acts as an intercellular signal molecule and contributes to several biological functions.[78] Moreover, indole upregulates gene expression of gut epithelial cell junctions and modulates pro- and anti-inflammatory gene expression in intestinal epithelial cells, resulting in maintenance of the host-microbe homeostasis at
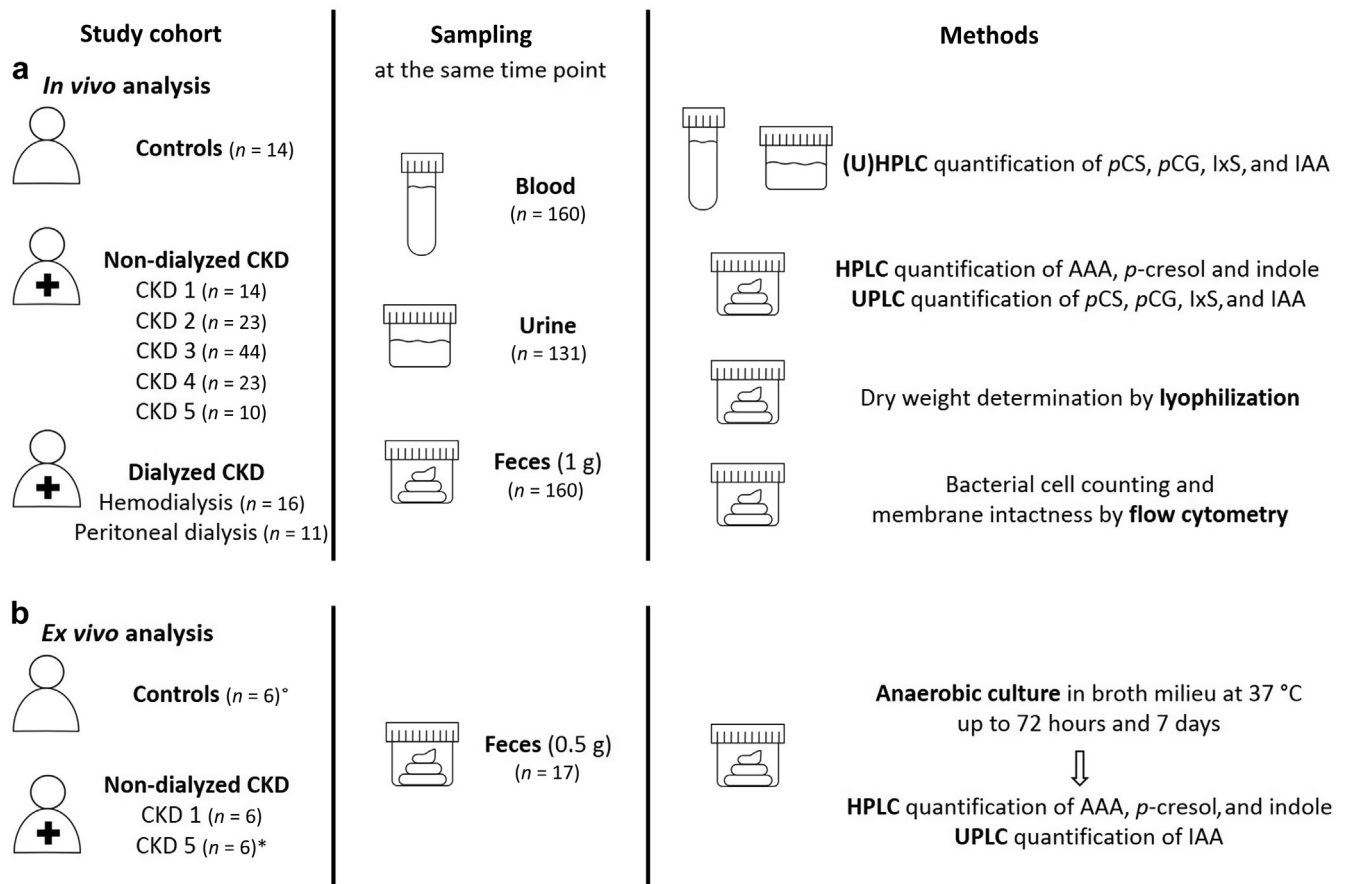
Figure 6 | Experimental overview. (a) *In vivo* analysis; **(b)** *ex vivo* analysis. °The fecal samples from control subjects were not used for *ex vivo* culture for 7 days; *the number of fecal samples from chronic kidney disease (CKD)5 patients used for *ex vivo* culture up to 72 hours was 5. AAA, aromatic amino acids; HPLC, high-performance liquid chromatography; IAA, indole-3-acetic acid; IxS, indoxyl sulfate; *p*CG, *p*-cresyl glucuronide; *p*CS, *p*-cresyl sulfate; UPLC, ultra-performance liquid chromatography.

the mucosal surface.[16,79] In contrast, no such effects have been described for the cresols, and the mother compound, *p*-cresol, is a strong inhibitor of biological functions.[80–87] Therefore, it might be necessary to reduce the toxicity of the 2 groups by different approaches, and for that matter, different bacterial origins of generation may be an asset rather than an obstacle. Primary focus on the reduction of the generation of *p*-cresol might be desirable.

A first shortcoming of this study is the lack of dietary recall and specific information on the protein intake of the CKD patients and the controls. However, neither the ratio of urinary urea over creatinine, a proxy for protein intake, nor the absolute urinary urea levels (data not shown) changed with progression of CKD, or in comparison to the control group. Second, a difference in the proportion of patients with diabetes was observed among the different stages of CKD. It is known that diabetes influences gut microbial composition and function.[88,89] However, in our dataset, no differences were found between the fecal and plasma levels and the ratio of individual urinary PBUT/Crea when comparing patients from each CKD stage with and without diabetes, assuming that the presence of diabetes has no effect on the intestinal generation capacity in CKD.

In conclusion, the present study offers a comprehensive analysis of the cresols and indoles concentrations in feces, plasma, and urine in different stages of CKD and in healthy volunteers. In this cohort, differences in plasma PBUTs levels between different stages of CKD cannot be explained by differences in bacterial generation rates of *p*-cresol, indole, and IAA in the gut. Moreover, degree of urinary PBUT excretion also did not alter with CKD progression, suggesting that retention by an impaired kidney function, of which the tubular filtration seems more affected than the glomerular filtration, is the main contributor to the plasma PBUT level increase. In addition, there is no net increase in influx of PBUTs into the gut lumen.

METHODS
Study population and sample collection
In total, 14 healthy controls, 114 non-dialyzed CKD (stage1–5), 16 HD, and 8 PD patients were recruited at the Nephrology Unit of the Ghent University Hospital, Belgium. Three PD patients were recruited from the Antwerp University Hospital, Belgium (Figure 6a). Exclusion criteria were active infection (C-reactive protein >20 mg/L), immunosuppressive therapy, body mass index > 35 kg/m², inflammatory bowel disease, active malignancy,

cardiovascular event in the past 3 months, pregnancy, transplantation, use of non-steroidal anti-inflammatory drugs within the past month, vascular access problems for HD, and age <18 years. Before inclusion, all patients and controls gave written informed consent. CKD patients were categorized into subgroups according to estimated glomerular filtration rate, using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI)–creatinine equation, as recommended by the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (KDOQI). The local ethics committee (Ref2010/033,B67020107926 and Ref2012/603,B670201214999) approved the study.

From each participant, a blood, urine and fecal sample was collected in parallel (Figure 6a). Blood from controls, CKD, and PD patients was collected in K_2EDTA tubes (Vacutainer Tubes, Becton Dickinson, Franklin Lakes, NJ) after antecubital venipuncture with a butterfly needle. Blood from HD patients was collected from the vascular access prior to the mid-week dialysis session. Plasma was obtained after 10 minutes of centrifugation at 2095 g, aliquoted, and stored at -80 °C. A spot urine sample was collected from the control and CKD1–5 subgroups. Urine was centrifuged for 10 minutes at 754 g, and the supernatant was aliquoted and stored at –80 °C. Upon collection, fecal samples were stored on an ice pack and processed within approximately 6 hours. Samples were aliquoted per 1 g after determination of the Bristol stool scale (a visual scale of the aspect of stool, from hard [1] to liquid [7][90]) and stored at –80 °C. Upon batch analysis, fecal samples were thawed and divided into two 0.5-g feces aliquots.

Fecal dry weight measurement

One fecal aliquot (0.5 g) was used to determine dry weight after lyophilization with a laboratory freeze dryer (VaCo 5, Zirbus, Bad Grund/Harz, Germany; Figure 6a). Before and after lyophilization, the weight of the vials was measured to determine dry and wet weight.

Determination of uremic metabolites in feces, plasma, and urine

Plasma and urinary creatinine (113 Da), phosphorus (31 Da), urea (60 Da), and protein content were determined by standard laboratory methods in the routine laboratory of the Ghent University Hospital, Belgium. In the fecal suspension, total concentrations of tyrosine (181 Da), phenylalanine (165 Da), tryptophan (204 Da), p-cresol (108 Da) and indole (117 Da) were measured with high-performance liquid chromatography, omitting the heat denaturation step (Supplementary Methods). In fecal suspension, plasma and urine, total concentrations of pCS (187 Da), pCG (284 Da), IxS (213 Da), and IAA (175 Da) were measured with ultra-performance liquid chromatography [(Supplementary Methods), and for HD patients as previously described.[91,92]

The individual PBUT concentrations in the spot urine samples were normalized to creatinine concentration (formula 1). The ratio of urinary urea over creatinine was calculated, and considered as a proxy for protein intake (formula 2). Fractional kidney clearance of PBUTs was determined according to formula 3:

$$\frac{[\text{urinary PBUT}]}{[\text{urinary creatinine}]}; \tag{1}$$

$$\frac{[\text{urinary urea}]}{[\text{urinary creatinine}]}; \tag{2}$$

$$\frac{[\text{urinary PBUT}] \times [\text{serum creatinine}]}{[\text{urinary creatinine}] \times [\text{plasma PBUT}]}. \tag{3}$$

Fecal bacterial cell counts and intactness

To determine the total, intact, and damaged bacterial cell count, flow cytometric analysis was performed on the fecal suspension supernatant (Supplementary Methods). Samples were 1000 times diluted with filtered phosphate-buffered saline, intact/damaged stained, and incubated for 13 minutes at 37 °C (Supplementary Methods). Samples were analyzed with a 3-laser BD FACSVerse flow cytometer (Becton Dickinson, San Jose, CA), equipped with a flow sensor for volumetric counting as previously described.[93,94]

Ex vivo p-cresol, indole, and IAA generation

From the collected fecal samples, samples were randomly selected from the controls, CKD1, and CKD5 patients. These samples were used for *ex vivo* anaerobic culture at 37 °C up to 72 hours and for 7 days in a Yeast Casitone Fatty Acid Glucose (YCFAG) broth medium,[95] supplemented with AAAs (Figure 6b; Supplementary Methods). Concentrations of AAAs, p-cresol, indole, and IAA were quantified with ultra/high-performance liquid chromatography before and after 6, 12, 24, 48, and 72 hours and 7 days of incubation. Prior to ultra/high-performance liquid chromatography analysis, broth medium was centrifuged for 30 minutes at 10,000 g, sterilized with a 0.22-μm filter (Millex-GV syringe filter unit with polyvinylidene diflouride membrane, Millipore Merck, Darmstadt, Germany) and stored at –80 °C.

AUTHOR CONTRIBUTIONS

GG and TG conceived the original idea and designed the study. TG, KDP, F-MK, and GG collected the data for the study, which were analyzed by TG and F-MK. The data interpretation and manuscript drafting were performed by TG, GG, RV, F-MK, KDP, and WVB. TG, KDP, RV, F-MK, TVdW, WVB, FV, MS, MJ, MMC, MV, and GG reviewed and edited the manuscript and gave the final approval for submission.

SUPPLEMENTARY MATERIAL

Supplementary File (Word)

Supplementary Methods. (U)HPLC specifications, YCFAG broth medium composition, fecal suspension preparation, flow cytometry solutions compositions, and statistical analysis.

Table S1. Correlations between protein-bound uremic toxin-related metabolites. Data are presented as Spearman's rho correlation. CKD, chronic kidney disease; HD, hemodialysis; IAA, indole-3-acetic acid; IxS, indoxyl sulfate; N/A, not applicable; pCG, p-cresyl glucuronide; pCS, p-cresyl sulfate; PD, peritoneal dialysis; Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan. Gray: $P \leq 0.05$; $^+P = 0.051$.

Figure S1. Urinary [urea]/[creatinine] per CKD stage. x: outlier; control ($n = 14$); CKD1 ($n = 14$); CKD2 ($n = 23$); CKD3 ($n = 44$); CKD4 ($n = 23$); CKD5 ($n = 10$); CKD, chronic kidney disease.

Figure S2. (**A**) Fecal PBUT precursor metabolite levels and (**B**) ratio of urinary protein-bound uremic toxins to creatinine, in patients with high p-cresyl sulfate and low indoxyl sulfate, and low p-cresyl sulfate and high indoxyl sulfate serum levels. Crea, creatinine; Ind, indole; IxS, indoxyl sulfate; PBUT, protein-bound uremic toxin; pC, p-cresol; pCS, p-cresyl sulfate; Phe, phenylanine; Trp, tryptophan; Tyr, tyrosine. pCShigh/IxSlow ($n = 13$); pCSlow/IxShigh ($n = 13$); $^*P < 0.05$; x: outlier.

Figure S3. p-Cresol, indole, and IAA generation of CKD1 and CKD5 fecal samples cultured for 7 days. Data presented as median and 95% CI. Right *y*-axis: median concentration in μM; left *y*-axis: median concentration in nmol/g feces. 0: measurement before incubation; 7: measurement after 7 days of anaerobic incubation. ■: tyrosine (black); ●: phenylalanine (dark gray); ▲: p-cresol (gray); □: tryptophan (black); Δ: indole (dark gray); ◇: Indole-3-acetic acid (gray); CKD1 ($n = 6$); CKD5 ($n = 6$); $^*P < 0.05$ versus baseline. CKD, chronic kidney disease.

Supplementary References.

REFERENCES

1. Duranton F, Cohen G, De Smet R, et al. Normal and pathologic concentrations of uremic toxins. *J Am Soc Nephrol*. 2012;23:1258–1270.
2. Vanholder R, De Smet R, Glorieux G, et al. Review on uremic toxins: classification, concentration, and interindividual variability. *Kidney int*. 2003;63:1934–1943.
3. Vanholder R, Glorieux G, De Smet R, et al. New insights in uremic toxins. *Kidney Int*. 2003;63(suppl 84):S6–10.
4. Liabeuf S, Barreto DV, Barreto FC, et al. Free p-cresyl sulphate is a predictor of mortality in patients at different stages of chronic kidney disease. *Nephrol Dial Transplant*. 2010;25:1183–1191.
5. Liabeuf S, Glorieux G, Lenglet A, et al. Does p-cresyl glucuronide have the same impact on mortality as other protein-bound uremic toxins? *PLoS One*. 2013;8:e67168.
6. Barreto FC, Barreto DV, Liabeuf S, et al. Serum indoxyl sulfate is associated with vascular disease and mortality in chronic kidney disease patients. *Clin J Am Soc Nephrol*. 2009;4:1551–1558.
7. Dou L, Sallee M, Cerini C, et al. The cardiovascular effect of the uremic solute indole-3 acetic acid. *J Am Soc Nephrol*. 2015;26:876–887.
8. Bammens B, Evenepoel P, Keuleers H, et al. Free serum concentrations of the protein-bound retention solute p-cresol predict mortality in hemodialysis patients. *Kidney Int*. 2006;69:1081–1087.
9. Lin CJ, Chuang CK, Jayakumar T, et al. Serum p-cresyl sulfate predicts cardiovascular disease and mortality in elderly hemodialysis patients. *Arch Med Sci*. 2013;9:662–668.
10. Gryp T, Vanholder R, Vaneechoutte M, et al. p-Cresyl sulfate. *Toxins*. 2017;9.
11. Mair RD, Sirich TL, Plummer NS, et al. Characteristics of colon-derived uremic solutes. *Clin J Am Soc Nephrol*. 2018;13:1398–1404.
12. Evenepoel P, Meijers BK, Bammens BR, et al. Uremic toxins originating from colonic microbial metabolism. *Kidney Int*. 2009;76(Ssuppl 114):S12–19.
13. Mishima E, Fukuda S, Mukawa C, et al. Evaluation of the impact of gut microbiota on uremic solute accumulation by a CE-TOFMS-based metabolomics approach. *Kidney Int*. 2017;92:634–645.
14. Poesen R, Windey K, Neven E, et al. The Influence of CKD on colonic microbial metabolism. *J Am Soc Nephrol*. 2016;27:1389–1399.
15. de Loor H, Bammens B, Evenepoel P, et al. Gas chromatographic-mass spectrometric analysis for measurement of p-cresol and its conjugated metabolites in uremic and normal serum. *Clin Chem*. 2005;51:1535–1538.
16. Zhang LS, Davies SS. Microbial metabolism of dietary components to bioactive metabolites: opportunities for new therapeutic interventions. *Genome Med*. 2016;8:46.
17. Zelante T, Iannitti RG, Cunha C, et al. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. *Immunity*. 2013;39:372–385.
18. Jankowski J, Westhof T, Vaziri ND, et al. Gases as uremic toxins: is there something in the air? *Semin Nephrol*. 2014;34:135–150.
19. Perna AF, Glorieux G, Zacchia M, et al. The role of the intestinal microbiota in uremic solute accumulation: a focus on sulfur compounds. *J Nephrol*. 2019;32:733–740.
20. Deltombe O, Van Biesen W, Glorieux G, et al. Exploring protein binding of uremic toxins in patients with different stages of chronic kidney disease and during hemodialysis. *Toxins*. 2015;7:3933–3946.
21. Lin CJ, Chen HH, Pan CF, et al. p-Cresylsulfate and indoxyl sulfate level at different stages of chronic kidney disease. *J Clin Lab Anal*. 2011;25:191–197.
22. Pretorius CJ, McWhinney BC, Sipinkoski B, et al. Reference ranges and biological variation of free and total serum indoxyl- and p-cresyl sulphate measured with a rapid UPLC fluorescence detection method. *Clin Chim Acta*. 2013;419:122–126.
23. Wu IW, Hsu KH, Lee CC, et al. p-Cresyl sulphate and indoxyl sulphate predict progression of chronic kidney disease. *Nephrol Dial Transplant*. 2011;26:938–947.
24. Meijers BK, De Loor H, Bammens B, et al. p-Cresyl sulfate and indoxyl sulfate in hemodialysis patients. *Clin J Am Soc Nephrol*. 2009;4:1932–1938.
25. Martinez AW, Recht NS, Hostetter TH, et al. Removal of p-cresol sulfate by hemodialysis. *J Am Soc Nephrol*. 2005;16:3430–3436.
26. Lee CT, Kuo CC, Chen YM, et al. Factors associated with blood concentrations of indoxyl sulfate and p-cresol in patients undergoing peritoneal dialysis. *Perit Dial Int*. 2010;30:456–463.
27. Vaziri ND, Wong J, Pahl M, et al. Chronic kidney disease alters intestinal microbial flora. *Kidney Int*. 2013;83:308–315.
28. Shi K, Wang F, Jiang H, et al. Gut bacterial translocation may aggravate microinflammation in hemodialysis patients. *Dig Dis Sci*. 2014;59:2109–2117.
29. Fukuuchi F, Hida M, Aiba Y, et al. Intestinal bacteria-derived putrefactants in chronic renal failure. *Clin Exp Nephrol*. 2002;6:99–104.
30. Strid H, Simren M, Stotzer PO, et al. Patients with chronic renal failure have abnormal small intestinal motility and a high prevalence of small intestinal bacterial overgrowth. *Digestion*. 2003;67:129–137.
31. Simenhoff ML, Saukkonen JJ, Burke JF, et al. Bacterial populations of the small intestine in uremia. *Nephron*. 1978;22:63–68.
32. Wang F, Zhang P, Jiang H, et al. Gut bacterial translocation contributes to microinflammation in experimental uremia. *Dig Dis Sci*. 2012;57:2856–2862.
33. Wong J, Piceno YM, Desantis TZ, et al. Expansion of urease- and uricase-containing, indole- and p-cresol-forming and contraction of short-chain fatty acid-producing intestinal microbiota in ESRD. *Am J Nephrol*. 2014;39:230–237.
34. Hida M, Aiba Y, Sawamura S, et al. Inhibition of the accumulation of uremic toxins in the blood and their precursors in the feces after oral administration of Lebenin, a lactic acid bacteria preparation, to uremic patients undergoing hemodialysis. *Nephron*. 1996;74:349–355.
35. Vaziri ND, Zhao YY, Pahl MV. Altered intestinal microbial flora and impaired epithelial barrier structure and function in CKD: the nature, mechanisms, consequences and potential treatment. *Nephrol Dial Transplant*. 2016;31:737–746.
36. Vaziri ND, Yuan J, Nazertehrani S, et al. Chronic kidney disease causes disruption of gastric and small intestinal epithelial tight junction. *Am J Nephrol*. 2013;38:99–103.
37. Vaziri ND, Yuan J, Rahimi A, et al. Disintegration of colonic epithelial tight junction in uremia: a likely cause of CKD-associated inflammation. *Nephrol Dial Transplant*. 2012;27:2686–2693.
38. Vaziri ND, Yuan J, Norris K. Role of urea in intestinal barrier dysfunction and disruption of epithelial tight junction in chronic kidney disease. *Am J Nephrol*. 2013;37:1–6.

39. Bammens B, Verbeke K, Vanrenterghem Y, et al. Evidence for impaired assimilation of protein in chronic renal failure. *Kidney Int*. 2003;64:2196–2203.

40. Bammens B, Evenepoel P, Verbeke K, et al. Impairment of small intestinal protein assimilation in patients with end-stage renal disease: extending the malnutrition-inflammation-atherosclerosis concept. *Am J Clin Nutr*. 2004;80:1536–1543.

41. Qureshi AR, Alvestrand A, Danielsson A, et al. Factors predicting malnutrition in hemodialysis patients: a cross-sectional study. *Kidney Int*. 1998;53:773–782.

42. Cianciaruso B, Brunori G, Kopple JD, et al. Cross-sectional comparison of malnutrition in continuous ambulatory peritoneal dialysis and hemodialysis patients. *Am J Kidney Dis*. 1995;26:475–486.

43. Pahl MV, Vaziri ND. The chronic kidney disease—colonic axis. *Semin Dial*. 2015;28:459–463.

44. Armani RG, Ramezani A, Yasir A, et al. Gut microbiome in chronic kidney disease. *Curr Hypertens Rep*. 2017;19:29.

45. Poesen R, Evenepoel P, de Loor H, et al. Metabolism, protein binding, and renal clearance of microbiota-derived *p*-cresol in patients with CKD. *Clin J Am Soc Nephrol*. 2016;11:1136–1144.

46. Klaassen CD, Aleksunes LM. Xenobiotic, bile acid, and cholesterol transporters: function and regulation. *Pharmacol Rev*. 2010;62:1–96.

47. Yoshifuji A, Wakino S, Irie J, et al. Gut *Lactobacillus* protects against the progression of renal damage by modulating the gut environment in rats. *Nephrol Dial Transplant*. 2016;31:401–412.

48. Evenepoel P, Claus D, Geypens B, et al. Evidence for impaired assimilation and increased colonic fermentation of protein, related to gastric acid suppression therapy. *Aliment Pharmacol Ther*. 1998;12:1011–1019.

49. Evenepoel P, Claus D, Geypens B, et al. Amount and fate of egg protein escaping assimilation in the small intestine of humans. *Am J Physiol*. 1999;277:G935–943.

50. Joossens M, Faust K, Gryp T, et al. Gut microbiota dynamics and uraemic toxins: one size does not fit all. *Gut*. 2019;68:2257–2260.

51. John SG, Sigrist MK, Taal MW, et al. Natural history of skeletal muscle mass changes in chronic kidney disease stage 4 and 5 patients: an observational study. *PLoS One*. 2013;8:e65372.

52. Poesen R, Viaene L, Verbeke K, et al. Renal clearance and intestinal generation of *p*-cresyl sulfate and indoxyl sulfate in CKD. *Clin J Am Soc Nephrol*. 2013;8:1508–1514.

53. Suchy-Dicey AM, Laha T, Hoofnagle A, et al. Tubular secretion in CKD. *J Am Soc Nephrol*. 2016;27:2148–2155.

54. Masereeuw R, Mutsaers HA, Toyohara T, et al. The kidney and uremic toxin removal: glomerulus or tubule? *Semin Nephrol*. 2014;34:191–208.

55. Takada T, Yamamoto T, Matsuo H, et al. Identification of ABCG2 as an exporter of uremic toxin indoxyl sulfate in mice and as a crucial factor influencing CKD progression. *Sci Rep*. 2018;8:11147.

56. Deltombe O, Glorieux G, Marzouki S, et al. Selective transport of protein-bound uremic toxins in erythrocytes. *Toxins*. 2019;11.

57. Buysschaert B, Kerckhof FM, Vandamme P, et al. Flow cytometric fingerprinting for microbial strain discrimination and physiological characterization. *Cytometry A*. 2018;93:201–212.

58. Jiang S, Xie S, Lv D, et al. Alteration of the gut microbiota in Chinese population with chronic kidney disease. *Sci Rep*. 2017;7:2870.

59. Wang IK, Lai HC, Yu CJ, et al. Real-time PCR analysis of the intestinal microbiotas in peritoneal dialysis patients. *Appl Environ Microbiol*. 2012;78:1107–1112.

60. Cummings JH, Hill MJ, Bone ES, et al. The effect of meat protein and dietary fiber on colonic function and metabolism. II. Bacterial metabolites in feces and urine. *Am J Clin Nutr*. 1979;32:2094–2101.

61. Ling WH, Hanninen O. Shifting from a conventional diet to an uncooked vegan diet reversibly alters fecal hydrolytic activities in humans. *J Nutr*. 1992;122:924–930.

62. Patel KP, Luo FJ, Plummer NS, et al. The production of *p*-cresol sulfate and indoxyl sulfate in vegetarians versus omnivores. *Clin J Am Soc Nephrol*. 2012;7:982–988.

63. Birkett A, Muir J, Phillips J, et al. Resistant starch lowers fecal concentrations of ammonia and phenols in humans. *Am J Clin Nutr*. 1996;63:766–772.

64. Rossi M, Johnson DW, Xu H, et al. Dietary protein-fiber ratio associates with circulating levels of indoxyl sulfate and p-cresyl sulfate in chronic kidney disease patients. *Nutr Metab Cardiovasc Dis*. 2015;25:860–865.

65. Sirich TL, Plummer NS, Gardner CD, et al. Effect of increasing dietary fiber on plasma levels of colon-derived solutes in hemodialysis patients. *Clin J Am Soc Nephrol*. 2014;9:1603–1610.

66. Poesen R, Mutsaers HA, Windey K, et al. The influence of dietary protein intake on mammalian tryptophan and phenolic metabolites. *PLoS One*. 2015;10:e0140820.

67. Tohyama K, Kobayashi Y, Kan T, et al. Effect of lactobacilli on urinary indican excretion in gnotobiotic rats and in man. *Microbiol Immunol*. 1981;25:101–112.

68. Kawakami K, Makino I, Asahara T, et al. Dietary galacto-oligosaccharides mixture can suppress serum phenol and *p*-cresol levels in rats fed tyrosine diet. *J Nutr Sci Vitaminol (Tokyo)*. 2005;51:182–186.

69. Rossi M, Johnson DW, Morrison M, et al. Synbiotics easing renal failure by improving gut microbiology (SYNERGY): a randomized trial. *Clin J Am Soc Nephrol*. 2016;11:223–231.

70. De Preter V, Vanhoutte T, Huys G, et al. Effects of *Lactobacillus casei* Shirota, *Bifidobacterium breve*, and oligofructose-enriched inulin on colonic nitrogen-protein metabolism in healthy humans. *Am J Physiol Gastrointest Liver Physiol*. 2007;292:G358–368.

71. de Preter V, Vanhoutte T, Huys G, et al. Baseline microbiota activity and initial bifidobacteria counts influence responses to prebiotic dosing in healthy subjects. *Aliment Pharmacol Ther*. 2008;27:504–513.

72. Fujiwara S, Seto Y, Kimura A, et al. Establishment of orally-administered *Lactobacillus gasseri* SBT2055SR in the gastrointestinal tract of humans and its influence on intestinal microflora and metabolism. *J Appl Microbiol*. 2001;90:343–352.

73. Meijers BK, De Preter V, Verbeke K, et al. *p*-Cresyl sulfate serum concentrations in haemodialysis patients are reduced by the prebiotic oligofructose-enriched inulin. *Nephrol Dial Transplant*. 2010;25:219–224.

74. Poesen R, Evenepoel P, de Loor H, et al. The influence of prebiotic arabinoxylan oligosaccharides on microbiota-derived uremic retention solutes in patients with chronic kidney disease: a randomized controlled trial. *PLoS One*. 2016;11:e0153893.

75. Nakabayashi I, Nakamura M, Kawakami K, et al. Effects of synbiotic treatment on serum level of *p*-cresol in haemodialysis patients: a preliminary study. *Nephrol Dial Transplant*. 2011;26:1094–1098.

76. Guida B, Germano R, Trio R, et al. Effect of short-term synbiotic treatment on plasma p-cresol levels in patients with chronic renal failure: a randomized clinical trial. *Nutr Metab Cardiovasc Dis*. 2014;24:1043–1049.

77. Evenepoel P, Bammens B, Verbeke K, et al. Acarbose treatment lowers generation and serum concentrations of the protein-bound solute *p*-cresol: a pilot study. *Kidney Int*. 2006;70:192–198.

78. Lee JH, Lee J. Indole as an intercellular signal in microbial communities. *FEMS Microbiol Rev*. 2010;34:426–444.

79. Shimada Y, Kinoshita M, Harada K, et al. Commensal bacteria-dependent indole production enhances epithelial barrier function in the colon. *PLoS One*. 2013;8:e80604.

80. Vanholder R, De Smet R, Lesaffer G. *p*-cresol: a toxin revealing many neglected but relevant aspects of uraemic toxicity. *Nephrol Dial Transplant*. 1999;14:2813–2815.

81. Vanholder R, De Smet R, Waterloos MA, et al. Mechanisms of uremic inhibition of phagocyte reactive species production: characterization of the role of p-cresol. *Kidney Int*. 1995;47:510–517.

82. Cerini C, Dou L, Anfosso F, et al. P-cresol, a uremic retention solute, alters the endothelial barrier function *in vitro*. *Thromb Haemost*. 2004;92:140–150.

83. Yan Z, Zhong HM, Maher N, et al. Bioactivation of 4-methylphenol (*p*-cresol) via cytochrome P450-mediated aromatic oxidation in human liver microsomes. *Drug Metab Dispos*. 2005;33:1867–1876.

84. Dou L, Cerini C, Brunet P, et al. P-cresol, a uremic toxin, decreases endothelial cell response to inflammatory cytokines. *Kidney Int*. 2002;62:1999–2009.

85. Dou L, Bertrand E, Cerini C, et al. The uremic solutes p-cresol and indoxyl sulfate inhibit endothelial proliferation and wound repair. *Kidney Int*. 2004;65:442–451.

86. Meijers BK, Bammens B, De Moor B, et al. Free *p*-cresol is associated with cardiovascular disease in hemodialysis patients. *Kidney Int*. 2008;73:1174–1180.

87. Faure V, Dou L, Sabatier F, et al. Elevation of circulating endothelial microparticles in patients with chronic renal failure. *J Thromb Haemost*. 2006;4:566–573.

88. Dunne JL, Triplett EW, Gevers D, et al. The intestinal microbiome in type 1 diabetes. *Clin Exp Immunol*. 2014;177:30–37.

89. Qin J, Li Y, Cai Z, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature*. 2012;490:55–60.

90. Lewis SJ, Heaton KW. Stool form scale as a useful guide to intestinal transit time. *Scand J Gastroenterol*. 1997;32:920–924.

91. Fagugli RM, De Smet R, Buoncristiani U, et al. Behavior of non-protein-bound and protein-bound uremic solutes during daily hemodialysis. *Am J Kidney Dis*. 2002;40:339–347.

92. Meert N, Schepers E, Glorieux G, et al. Novel method for simultaneous determination of *p*-cresylsulphate and *p*-cresylglucuronide: clinical data and pathophysiological implications. *Nephrol Dial Transplant*. 2012;27:2388–2396.

93. Van Nevel S, Koetzsch S, Weilenmann HU, et al. Routine bacterial analysis with automated flow cytometry. *J Microbiol Methods*. 2013;94:73–76.

94. De Roy K, Clement L, Thas O, et al. Flow cytometry for fast microbial community fingerprinting. *Water Res*. 2012;46:907–919.

95. Duncan SH, Hold GL, Harmsen HJ, et al. Growth requirements and fermentation products of *Fusobacterium prausnitzii*, and a proposal to reclassify it as *Faecalibacterium prausnitzii* gen. nov., comb. nov. *Int J Syst Evol Microbiol*. 2002;52:2141–2146.