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High interindividual variability of indoxyl sulfate production identified by an oral tryptophan challenge test

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Indoxyl sulfate (IS) has been implicated in the pathogenesis of cardiovascular diseases. IS is converted from indole, a metabolite of dietary tryptophan through the action of gut microbial tryptophanase, by two hepatic enzymes: CYP2E1 and SULT1A1. We hypothesized that the effect of tryptophan intake on IS production might differ from person to person. We enrolled 72 healthy persons (33 ± 7 years; 54.2% women) to undergo an oral tryptophan challenge test (OTCT), in which 7 blood samples were collected at 0, 4, 8, 12, 24, 36, and 48 h following oral administration of L-tryptophan 2000 mg. We observed high interindividual variability of IS production in the response to an OTCT. Twenty-four subjects in the lowest tertile of the baseline-adjusted area under the curve of IS were defined as low-IS producers, whereas 24 subjects in the highest tertile were defined as high-IS producers. There was no significant difference in baseline characteristics or *CYP2E1* and *SULT1A1*-SNP genotyping distributions between the two IS-producing phenotypes. However, distinct differences in gut microbial composition were identified. In addition, the abundance of tryptophanase was significantly higher in the high-IS producers than in the low-IS producers ($P = 0.01$). The OTCT may serve as personalized dietary guidance. High-IS producers are more likely to be at greater risk of cardiovascular diseases and may benefit from consuming foods low in tryptophan. Potential clinical applications of the OTCT in precision nutrition warrant further investigation.

Patients with chronic kidney disease (CKD) have a high risk of cardiovascular diseases (CVDs)[1]. Despite advances in medical therapy, CVD remains the leading cause of morbidity and mortality among individuals with CKD[2]. Traditional cardiovascular risk factors for the general population, such as diabetes mellitus, hypertension, and dyslipidemia, are more prevalent in patients with CKD but cannot entirely account for the increased cardiovascular risk[3]. A growing number of studies suggest that uremic solutes, which accumulate in the body as kidney function declines, may play a critical role in the development and progression of CVD in CKD[4]

Indoxyl sulfate (IS), one of the most extensively studied uremic solutes, has been shown to mediate different phenotypes of CVD in patients with CKD, including atherosclerosis, arteriosclerosis, heart failure, arrhythmia, peripheral artery disease, and vascular access thrombosis[5]. A recent study found that IS, which is a uremic toxin that is generally considered to be relevant only in the setting of CKD, was also associated with incident adverse cardiovascular events during 3 years of follow-up among subjects with primarily preserved kidney function (median estimated glomerular filtration rate [eGFR] 89 [72–99] mL/min/1.73 m^2)[6], underscoring its potential relevance beyond CKD. Adding to the complexity, aside from IS, there are other microbiota-derived tryptophan metabolites. While IS has been shown to promote vascular inflammation, indole-3-propionic acid and indole-3-aldehyde exhibit protective roles[7]. Furthermore, evidence suggests that several kynurenine metabolites can differently affect cardiovascular health [8].

The synthesis of IS is initiated by a metaorganismal pathway involving gut microbial fermentation of the aromatic amino acid tryptophan into indole, which is absorbed via portal circulation and then converted within the host liver to indoxyl and IS by the sequential action of the cytochrome oxidases CYP2E1 and SULT1A1, respectively[9]. This complex pathway highlights the interplay between diet, gut microbiota, and host metabolism in determining IS levels.

Current approaches aimed at controlling blood IS levels within an acceptable range include oral adsorbents, the maintenance of gut symbiosis, and dietary protein restriction. AST-120, an orally administered charcoal

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adsorbent that binds indole, has been shown to reduce IS production in animal studies and to slow disease progression in some human studies[10]. Unfortunately, recent large-scale randomized controlled trials examining the effect of AST-120 on the progression of CKD have yielded mixed findings[11]. Furthermore, although experimental studies of modification of the gut microenvironment and microbial composition by prebiotics and/or probiotics have yielded promising results, the effectiveness was not consistent across human studies[12,13]. Dietary protein intake is a central determinant of blood IS levels. Very low-protein diet supplemented with ketoanalogs has been shown to decrease plasma IS concentrations in patients with CKD not yet on dialysis[14]. Nevertheless, an increasing number of studies have shown that daily protein intake among patients with advanced CKD is much higher than the recommended amount of 0.6–0.8 g/kg/day[15,16]. Although dietary protein restriction could be achieved by nutritional counseling in selected patients in a relatively short period[17], long-term adherence to a low-protein diet remains a challenge [18].

An increasing amount of evidence has shown that the response to a particular diet varies widely across individuals based on their genetic makeup and gut microbial composition[19]. We hypothesize that the effect of tryptophan intake on IS production may vary interindividually. To better understand this variability, the oral tryptophan challenge test (OTCT) has been developed as a method to assess individual IS production. This approach involves administering a fixed dose of tryptophan and measuring IS levels in response, thereby providing insights into the integrated contribution of diet, gut microbiota, and host metabolism. Here, we aimed to compare individual levels of IS production after fixed-dose oral tryptophan loading and to identify factors associated with interindividual variability. Understanding these mechanisms may inform personalized dietary interventions to minimize IS levels without unnecessary dietary restrictions that could impair quality of life or lead to malnutrition [20].

Results

Study population

A total of 107 participants were screened, and 35 were excluded (Supplementary Fig. 1). Thus, 72 healthy persons underwent the OTCT (Fig. 1A). The baseline characteristics of our cohort are shown in Table 1. In summary, the mean age was 33 ± 7 years; 54% were female, and 49% were vegetarians. Their mean eGFR was 99.9 ± 19.5 ml/min/1.73 m^2. Satisfactory adherence to a low-tryptophan diet during the OTCT was observed. Daily dietary tryptophan intake decreased significantly from 422 (278–577) mg before the OTCT to 216 (123–266) mg during the OTCT ($P < 0.001$).

High interpersonal variability of IS production following the OTCT

A substantial increase in plasma tryptophan concentrations occurred at 4 h, reflecting the influx of tryptophan load from the small intestine into the circulation, after which tryptophan concentrations declined over time. The shapes of the plasma concentration-time curves of tryptophan were similar in all the participants (participants 1–8 in Fig. 1B) (participants 9–72 in Supplementary Fig. 2). However, high interindividual variability in IS production was observed. The peak plasma IS concentration (Cmax) and the time when it occurs (Tmax), as well as the area under the concentration-time curve (AUC), varied markedly across individuals (participants 1–8 in Fig. 1C) (participants 9–72 in Supplementary Fig. 3). Additionally, plasma IS levels were measured in 8 participants at 0, 4, 8, 12, 24, 36, and 48 h without oral administration of L-tryptophan. The plasma concentration-time curve for IS in these participants shows minimal variation, with no significant increase in IS levels compared to baseline at any time point (Supplementary Fig. 4).

Comparison of the low- and high-IS producers: baseline characteristics

Due to the high variation in baseline IS concentrations, the incremental AUC of plasma IS concentration (baseline-adjusted AUC of IS) was

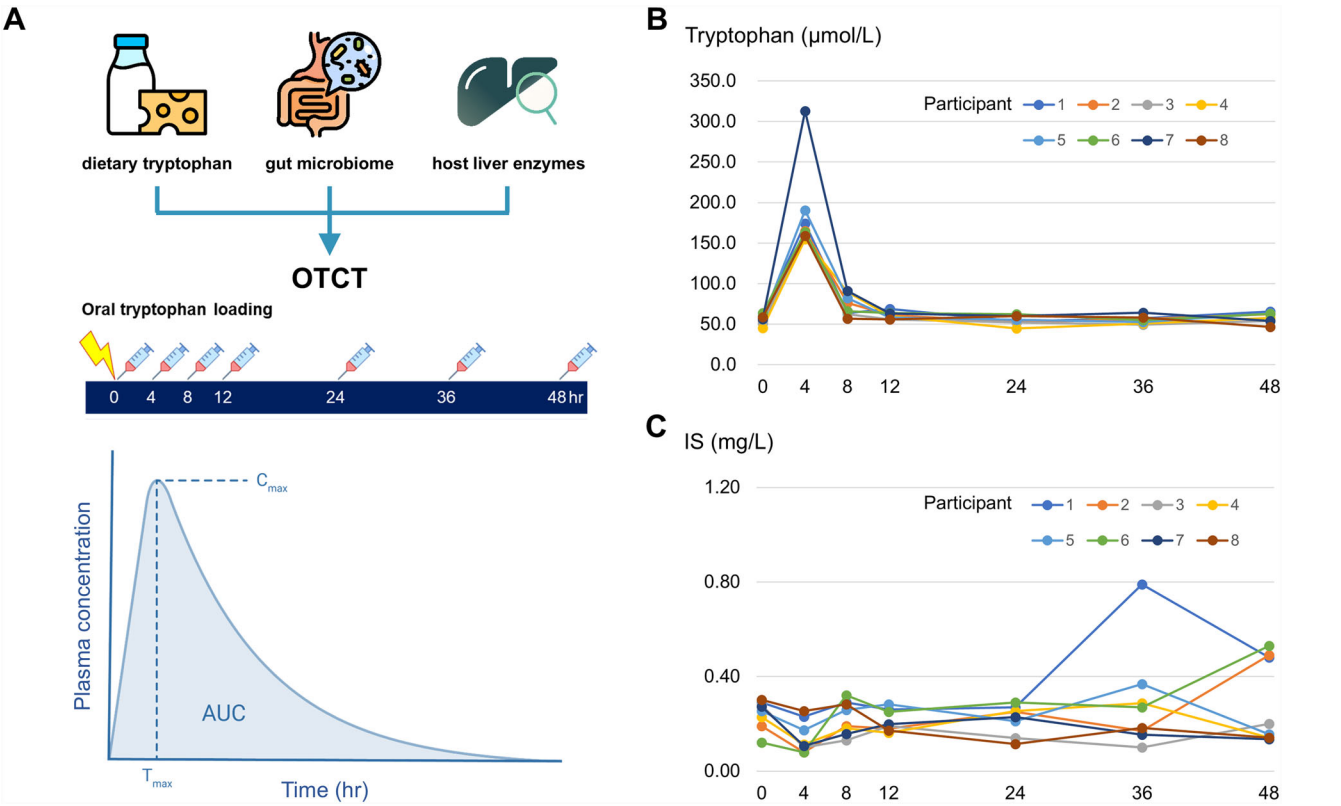

Fig. 1 | Pharmacokinetic study of OTCT. The OTCT reflects the results from crosstalk between diet, the gut microbiome, and the host and could be used to measure individual differences in IS production (**A**). Concentrations of plasma tryptophan (**B**) and IS (**C**) at various time points after the OTCT among the first 8 participants. Peaks in plasma tryptophan concentrations occurred at 4 h. Plasma IS concentrations remained at troughs for 12 h, and peaks then started to present after 24 h. AUC area under the curve, Cmax peak concentration, IS indoxyl sulfate, OTCT oral tryptophan challenge test, Tmax time to Cmax.

Table 1 | Baseline characteristics of the participants

Characteristic	All participants (n = 72)
Age (years)	33.5 ± 7.5
Men, *n* (%)	33 (45.8%)
Women, *n* (%)	39 (54.2%)
Vegetarians, *n* (%)	35 (48.6%)
BMI (kg/m^2)	21.8 ± 1.9
Genotype *CYP2E1*-SNP[a], *n* (%)	
C/C	38 (52.8%)
C/T	30 (41.7%)
T/T	4 (5.6%)
Genotype *SULT1A1*-SNP[b], *n* (%)	
C/C	35 (48.6%)
C/G	22 (30.6%)
G/G	15 (20.5%)
Fasting glucose (mg/dL)	88.1 ± 7.3
ALT (U/L)	18.9 ± 10.4
BUN (mg/dL)	12.3 ± 3.7
Creatinine (mg/dL)	0.81 ± 0.17
eGFR (ml/min per 1.73 m^2)	99.9 ± 19.5
Cholesterol (mg/dL)	164.8 ± 38.7
Triglycerides (mg/dL)	65.7 ± 31.2
LDL-C (mg/dL)	98.2 ± 35.2
Fasting IS (mg/L)	0.31 (0.22–0.56)
AUC of IS (mg/L × hour)	1.41 (0.00–5.10)

ALT alanine aminotransferase, *AUC* area under the curve, *BMI* body mass index, *BUN* blood urea nitrogen, *eGFR* estimated glomerular filtration rate, *IS* indoxyl sulfate, *LDL-C* low-density lipoprotein cholesterol.
[a]rs2031920.
[b]rs3176926.

calculated to determine the IS-producing capacity. We divided the participating cohort into tertiles because there were no established cut-points. In addition, individuals in the lowest tertile exhibited almost no IS production [AUC of IS 0.00 (0.00–0.00)]. Individuals in the lowest tertile (Tertile 1) were classified as low-IS producers (n = 24), whereas those in the highest tertile (Tertile 3) were classified as high-IS producers (n = 24) (Fig. 2A). Baseline characteristics stratified based on the two IS producer phenotypes are shown in Table 2. There was no significant difference in age, sex, BMI, single-nucleotide polymorphisms (SNPs) of *CYP2E1* and *SULT1A1*, biochemical values, or dietary tryptophan intake between the two groups.

Comparison of the low- and high-IS producers: gut microbiome

A total of 2,763,609,396 reads (with an average of 38,383,464 reads and a range of 23,680,560–106,601,978 reads) were obtained after filtering human reads and low-complexity reads from fecal samples provided by the 72 subjects. There were a total of 36,054,666 (±14,953,055 SD) reads per low-IS producer and 33,311,597 (±13,922,843 SD) reads per high-IS producer. No significant differences in measures of α-diversity, including the number of observed species (P = 0.992), Chao1 diversity index (P = 0.992), Shannon index (P = 0.660), and Simpson index (P = 0.675) were detected between the low- and high-IS producers (Fig. 2B). The difference in microbial composition between the two phenotypes (β-diversity) was also nonsignificant (P = 0.561) (Fig. 2C). To identify the most relevant taxa responsible for the differences between the two phenotypes, the LEfSe method was applied (Fig. 2D). This analysis identified 15 discriminating taxon features between the two phenotypes across different taxonomic levels, including 1 phylum, 1 class, 1 order, 2 families, 4 genera, and 6 species. At the species level, the relative abundance of *Bacteroides salyersiae*, *Prevotella* sp. CAG:5226 and *Selenomonadales bacterium* were markedly increased in the high-IS producers, whereas *Prevotella copri*, *Prevotella* sp., and *Phocaeicola coprophilus* were dominant in the low-IS producers.

Comparison of the low- and high-IS producers: fecal microbial *tnaA* gene

To further substantiate the potential role of the gut microbiome in the capacity for IS production, we focused on the microbial genes that were predicted to encode an enzyme that is critical in the metabolic pathway transforming tryptophan into indole: the tryptophanase (EC 4.1.99.1), which is encoded by the *tnaA* gene. We found a significant difference in the relative abundance of tryptophanase between the two phenotypes (Fig. 2E). We then examined the correlation of the identified taxa with the abundance of *tnaA* among each sample. Taxa that showed a positive correlation with the sequencing number and were confirmed to possess *tnaA* genes based on the National Center for Biotechnology Information (NCBI) database were selected for further investigation. 41 TnaA protein sequences were retrieved from the 40 most correlated species with available genomes in the NCBI database (Supplementary Table 1). The amounts of *tnaA* genes belonging to each taxon were calculated after normalization to the total *tnaA* sequence number in each sample (Supplementary Table 2). We found that only the abundance of *tnaA* belonging to *Bacteroides fluxus* was significantly correlated with the baseline-adjusted AUC of IS (rs = 0.375, P < 0.001) (Supplementary Table 3). However, the *tnaA* gene of *Bacteroides fluxus* was detected in only a few samples (participants 6, 17, 61, 65, and 66), suggesting that the correlation does not adequately explain the abundance of the *tnaA* gene and its causal relationship with IS levels.

Comparison of IS production between vegetarians and omnivores

Levels of blood urea nitrogen (BUN), creatinine, total cholesterol, low-density lipoprotein cholesterol, and alanine aminotransferase were significantly lower in vegetarians than in omnivores. Data regarding nutrient intake, which was assessed by food records, are shown in Table 3. Vegetarians reported higher protein intake than omnivores. The estimated intake of energy, carbohydrates, fat, and dietary fiber was also significantly higher in vegetarians. The estimated tryptophan intake, the fasting IS levels, and the baseline-adjusted AUC of IS did not differ between groups.

High interindividual variability of indole-3-acetic acid (IAA) production following the OTCT

We further investigated whether IAA, which is another uremic toxin derived from tryptophan metabolism with known pathological roles in humans[21], exhibited similar interindividual variability as measured by the OTCT. The distributions of the baseline-adjusted AUC of IAA are shown (Fig. 3A). Individuals in tertile 1 of the baseline-adjusted AUC of IAA were classified as low-IAA producers (n = 24), whereas those in tertile 3 were classified as high-IAA producers (n = 24). We observed that the plasma concentration-time curves of IAA were quite similar across individuals. Overall, the Tmax was largely consistent earlier at 4 h, although the Cmax varied substantially (participants 1–8 in Fig. 3B) (participants 9–72 in Supplementary Fig. 5). The plasma concentration-time curves of IAA and IS according to tertiles (Tertile 3 versus Tertile 1) were compared (Fig. 4A, B). There was no correlation between the tryptophan peak and the baseline-adjusted AUC of IS so the absorption of tryptophan could not explain the difference (Tertile 3 versus Tertile 1) (Fig. 4C).

Discussion

This is the first study designed for the assessment of individual IS-producing capacity. We demonstrated high interindividual variability in IS production following the OTCT in healthy participants. Clinical demographic data, SNPs of *CYP2E1* and *SULT1A1*, dietary habits, daily macronutrient intake, and biochemical data were similar between the low- and high-IS producers. Notably, a distinct gut microbial signature differentiating the two IS-producing phenotypes was identified. Furthermore, the abundance of tryptophanase was significantly higher in the high-IS producers than in the

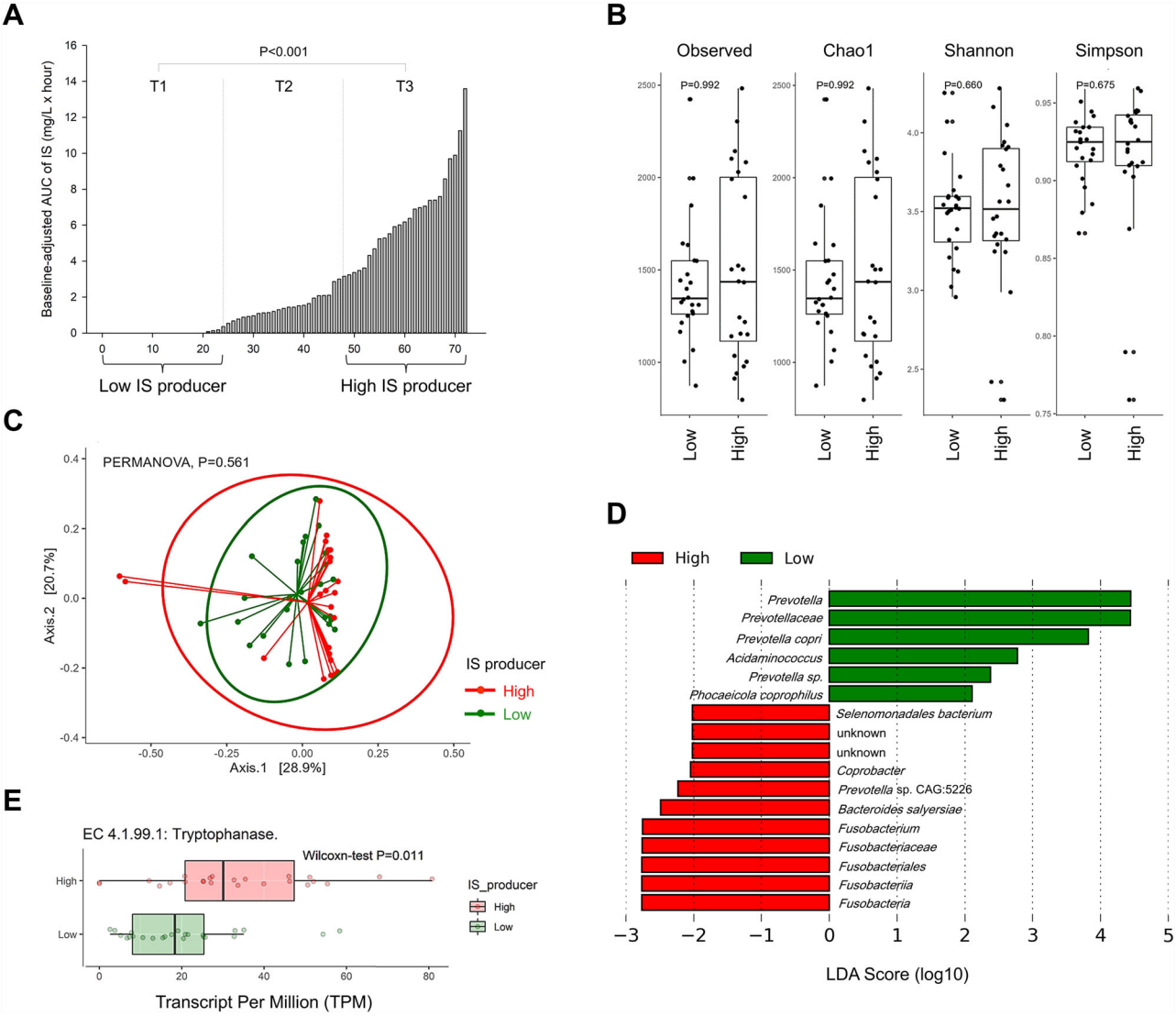

Fig. 2 | The IS-producing phenotypes grouped by OTCT were significantly associated with the differences of gut microbiome composition, features and functions. Distribution of baseline-adjusted AUC of IS among the 72 participants (**A**). Comparison of α-diversity between the low- and high-IS producers (**B**). Comparison of β-diversity between the low- and high-IS producers (**C**). The characteristic phylogenetic taxa in the low- and high-IS producers ranked by the LDA score (**D**). Comparison of tryptophanase (EC 4.1.99.1) between the low- and high-IS producers (**E**). AUC area under the curve, IS indoxyl sulfate, LDA linear discriminant analysis.

low-IS producers. Our findings suggest that the difference in IS-producing capacity might be primarily due to individual differences in gut microbial composition.

The results of our study were consistent with prior studies showing the wide variation of specific metabolites across individuals eating identical meals. Zeevi et al. measured glycemic responses to 46,898 meals in a cohort of 800 healthy persons and observed high variability of postprandial glucose levels to identical meals[19]. They further developed a model to predict postmeal glycemic responses by integrating parameters such as anthropometrics, dietary habits, physical activity, blood tests, and the gut microbiome. This model accurately predicted the postprandial glycemic responses to real-life meals and was further validated in an independent cohort of 100 healthy persons and 327 individuals without diabetes[19,22]. Recently, we have also demonstrated high variability of trimethylamine N-oxide (TMAO) production following oral carnitine challenge[23]. The TMAO producer phenotypes can be reproduced in a humanized gnotobiotic mouse model, thus revealing the role of the gut microbiome in the pathogenesis of TMAO-associated CVD. Collectively, these findings suggest that a personalized nutritional approach that takes into account an individual's unique personal and microbiome features in addition to food characteristics may be necessary to improve the long-term metabolic consequences to food consumed.

Another important finding of this study is the association of IS-producing ability with gut microbial composition. We found *Bacteroides salyersiae* to be dominant in the high-IS producers according to the results of the LEfSe analysis. Although Gryp et al. demonstrated a lack of indole production by *Bacteroides salyersiae* isolated from fecal samples of patients with CKD[24], Shagaleeva et al. showed that *Bacteroides salyersiae* was able to produce indole by species identified from the stool samples of healthy volunteers[25]. The contradictory results may be due to differences in the ethnicities of the study subjects, dietary patterns, or the status of kidney function. In addition to *Bacteroides salyersiae*, *Fusobacterium* has also been identified as a *tnaA*-encoding and indole-producing bacterium[26]. Notably, Wang et al. demonstrated that *Fusobacterium nucleatum*, which is enriched in patients with end-stage kidney disease, contributes to the increased production of uremic toxins, including IS, and promotes kidney disease progression in a rat CKD model [27].

Fecal *tnaA*, which encodes tryptophanase, is a useful biomarker in identifying the IS producer phenotype according to our study.

Table 2 | Baseline characteristics of participants according to IS-producing phenotypes

Characteristic	Low-IS producer(n = 24)	High-IS producer (n = 24)	P-value
Age (years)	32.6 ± 6.7	34.0 ± 8.7	0.554
Sex			
Men, n (%)	12 (50.0%)	9 (37.5%)	0.383
Women, n (%)	12 (50.0%)	15 (62.5%)	
Vegetarians, n (%)	11 (45.8%)	12 (50.0%)	0.773
BMI (kg/m^2)	21.9 ± 1.8	21.7 ± 1.8	0.701
Genotype *CYP2E1*-SNP[a], n (%)			0.336
C/C	12 (50.0%)	12 (50.0%)	
C/T	12 (50.0%)	10 (41.7%)	
T/T	0 (0.0%)	2 (8.3%)	
Genotype *SULT1A1*-SNP[b], n(%)			0.738
C/C	12 (50.0%)	13 (54.2%)	
C/G	7 (29.2%)	8 (33.3%)	
G/G	5 (20.8%)	3 (12.5%)	
Fasting glucose (mg/dL)	87.0 ± 9.9	89.0 ± 5.3	0.380
ALT (U/L)	20.0 ± 11.7	18.2 ± 8.1	0.541
BUN (mg/dL)	11.9 ± 3.2	11.3 ± 3.5	0.501
Creatinine (mg/dL)	0.81 ± 0.16	0.78 ± 0.16	0.524
eGFR (ml/min per 1.73 m^2)	100.0 ± 18.4	102.1 ± 18.1	0.695
Cholesterol (mg/dL)	162.9 ± 26.7	160.3 ± 41.6	0.799
Triglycerides (mg/dL)	69.1 ± 31.4	60.7 ± 29.7	0.343
LDL-C (mg/dL)	96.0 ± 25.6	94.1 ± 37.6	0.834
Fasting IS (mg/L)	0.42 (0.27–0.60)	0.27 (0.15–0.58)	0.081
AUC of IS (mg/L × hour)	0.00 (0.00–0.00)	6.28 (4.82–7.55)	<0.001
Dietary nutrient intake			
Energy (kcal)	2074 (1765–2883)	1939 (1289–2448)	0.224
Protein (g)	75 (56–108)	71 (49–95)	0.741
Tryptophan (mg)	570 (354–834)	511 (410–786)	0.885
Carbohydrate (g)	310 (206–397)	224 (163–324)	0.038
Fat (g)	73 (45–89)	61 (46–103)	0.934
Fiber (g)	22 (15–31)	20 (12–32)	0.789

ALT alanine aminotransferase, *AUC* area under the curve, *BMI* body mass index, *BUN* blood urea nitrogen, *eGFR* estimated glomerular filtration rate, *IS* indoxyl sulfate, *LDL-C* low-density lipoprotein cholesterol.
[a]rs2031920.
[b]rs3176926.

Table 3 | Baseline characteristics of participants according to dietary habits

Characteristic	Vegetarian (n = 35)	Omnivore (n = 37)	P-value
Age (years)	34.5 ± 8.2	32.5 ± 6.7	0.239
Sex			
Men, n (%)	15 (42.9%)	18 (48.6%)	0.622
Women, n (%)	20 (57.1%)	19 (51.4%)	
BMI (kg/m^2)	21.5 ± 1.9	22.1 ± 1.9	0.166
Genotype *CYP2E1*-SNP[a], n (%)			0.553
C/C	18 (51.4%)	20 (54.1%)	
C/T	16 (45.7%)	14 (37.8%)	
T/T	1 (2.9%)	3 (8.1%)	
Genotype *SULT1A1*-SNP[b], n (%)			0.681
C/C	18 (51.4%)	17 (45.9%)	
C/G	9 (25.7%)	13 (35.1%)	
G/G	8 (22.9%)	7 (18.9%)	
Fasting glucose (mg/dL)	86.7 ± 7.5	89.4 ± 7.0	0.119
ALT (U/L)	16.4 ± 6.8	21.2 ± 12.5	0.048
BUN (mg/dL)	11.2 ± 3.7	13.4 ± 3.2	0.008
Creatinine (mg/dL)	0.76 ± 0.16	0.85 ± 0.17	0.030
eGFR (ml/min per 1.73 m^2)	105.2 ± 16.3	94.9 ± 21.0	0.024
Cholesterol (mg/dL)	148.7 ± 33.2	180.0 ± 37.9	<0.001
Triglycerides (mg/dL)	62.9 ± 20.4	68.3 ± 38.8	0.468
LDL-C (mg/dL)	85.6 ± 31.0	110.2 ± 35.0	0.002
Fasting IS (mg/L)	0.41 (0.22–0.58)	0.30 (0.22–0.42)	0.295
AUC of IS (mg/L × hour)	1.44 (0.00–4.32)	1.37 (0.00–5.72)	0.661
Dietary nutrient intake			
Energy (kcal)	2417 (2047–3159)	1706 (1366–2014)	<0.001
Protein (g)	90 (67–115)	59 (49–90)	0.011
Tryptophan (mg)	557 (415–800)	529 (415–729)	0.524
Carbohydrate (g)	335 (242–413)	223 (242–413)	<0.001
Fat (g)	75 (51–109)	52 (42–84)	0.023
Fiber (g)	31 (23–44)	15 (12–24)	<0.001

ALT alanine aminotransferase, *AUC* area under the curve, *BMI* body mass index, *BUN* blood urea nitrogen, *eGFR* estimated glomerular filtration rate, *IS* indoxyl sulfate, *LDL-C* low-density lipoprotein cholesterol.
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[b]rs3176926.

However, the limited correlation between fecal microbial *tnaA* gene abundance and plasma IS levels observed in our study likely arises from a combination of methodological and biological factors. First, variations in sampling techniques, timing, and assay sensitivity may introduce inconsistencies in measuring microbial gene abundance. Moreover, fecal samples predominantly represent colonic microbial communities and may not fully capture microbial activity along the entire gastrointestinal tract. Spatial heterogeneity, particularly in regions such as the small intestine, where microbial density and activity differ significantly, could contribute to the observed discrepancies. Second, tryptophan metabolism involves multiple pathways, leading to the production of diverse metabolites beyond IS. For example, tryptophan can also be metabolized into compounds such as indole-3-lactic acid and indole-3-propionic acid, which may compete with IS production[28]. Third, dietary sulfur-containing amino acids have been shown to posttranslationally modify microbial tryptophanase activity in a mouse model of CKD[29]. However, we did not assess fecal quantitative proteomics or perform activity-based protein profiling, which could provide deeper insights into the functional capacity of the gut microbiota.

Interestingly, we observed that vegetarians had significantly higher daily protein consumption but lower BUN levels than omnivores. Our findings are in line with a prior study by Berryman et al.[30]. Using data from the NHANES from 2007 to 2010, they found that BUN concentrations were positively correlated with animal and dairy protein intake. In contrast, plant protein intake was inversely correlated with BUN levels after adjusting for demographic factors, physical activity, poverty-to-income ratio, BMI, and individual intake for each of the other two protein sources. However, after further adjusting for macronutrients, plant protein intake was no longer correlated with BUN levels, thus suggesting that other nutrients in foods high in plant protein may account for the relation. In addition, increased dietary fiber consumption has also been shown to decrease BUN levels in patients with CKD[31]. It remains unclear whether a lower tryptophan-to-protein ratio

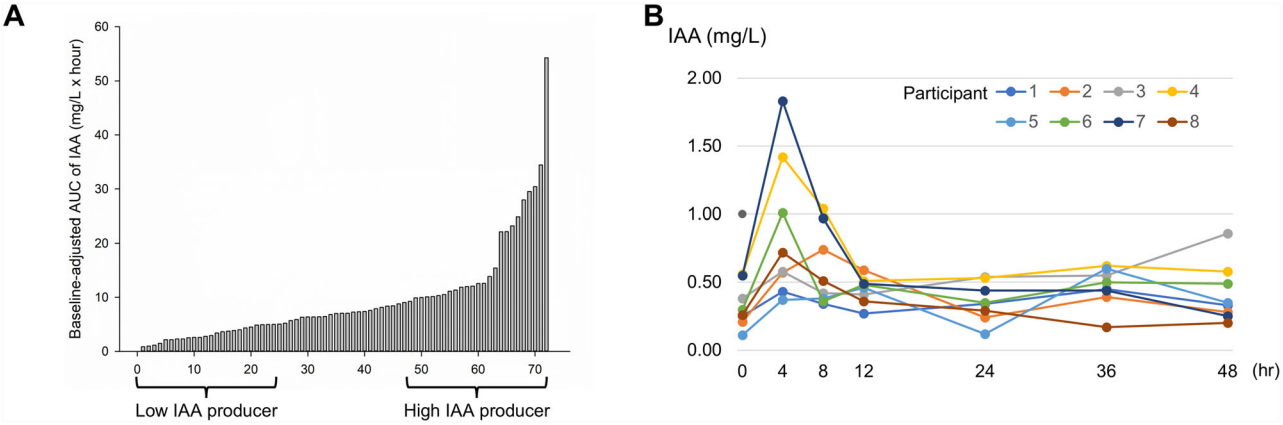

Fig. 3 | The IAA-producing phenotypes grouped by OTCT. Distribution of the baseline-adjusted AUC of IAA among the 72 participants (**A**). Concentrations of plasma IAA (**B**) at various time points after the OTCT among the first 8 participants. Peaks in plasma IAA concentrations occurred at 4 h. AUC area under the curve, IAA indole-3-acetic acid, OTCT oral tryptophan challenge test.

in a vegetarian diet contributes to lower IS production when the protein intake remains constant.

Gut transit time and tryptophan metabolism pathways are major determinants of plasma concentrations of IAA and IS. In healthy populations, the transit time varies throughout the gastrointestinal tract, with a median of approximately 1 h in gastric emptying time, 5 h in small intestinal transit time, and 21 h in colonic transit time[32]. The lag time and late Tmax of IS can be explained by the microbial degradation of tryptophan into indole in the large intestine. In contrast, the early Tmax of IAA suggests that it might be produced not only in the colon but also via microbial activity in the small intestine, where tryptophan availability is high following oral loading. This interpretation aligns with conflicting findings from prior studies. Aronov et al. reported that plasma IAA levels in colectomy patients compared to those with intact colons showed a relatively modest reduction (ratio of 0.57), suggesting that a significant portion of IAA production may be independent of the colon microbiota[33]. Conversely, Mair et al. demonstrated that the ratio of IAA in the urine of colectomy patients compared to controls was 0.11, implying that urinary IAA is largely dependent on the colon microbiota[34]. Taken together, these findings suggest a more complex scenario, where IAA production may involve contributions from both the small and large intestinal microbiota, with host pathways potentially playing a supplementary role.

Several limitations of our study should be acknowledged. First, IS is a classic substrate of organic anion transporters 1 and 3 on the basolateral membrane of the proximal renal tubule[35], but we did not estimate tubular secretory function. Indeed, there are no gold standard measures for renal tubular secretory clearance, and there is still no definitive method to prove that a substance is cleared exclusively by renal tubular secretion[36]. Second, we conducted the OTCT among healthy participants to minimize potential confounding factors, such as comorbidities and medications, that could influence the association between IS-producing capacity and the gut microbiome in patients with CKD. Additionally, previous studies have shown that elevated IS levels can be detrimental even in individuals without impaired kidney function[6]. Therefore, healthy participants were selected for this proof-of-concept study. However, this study design limits the generalizability of our findings to the CKD population. Lastly, the IS-producing functional phenotypes determined using the OTCT were based on complex interactions among diet, gut microbiota, and host factors. While this study provides an initial framework for identifying IS producer phenotypes, further experimental validation is needed to confirm the mechanisms underlying these phenotypes. For example, fecal microbiota transplantation could be performed using feces from high-IS and low-IS producers to demonstrate the transmissibility of IS-producing phenotypes to germ-free mice. Such studies would offer direct evidence of the causal role of gut microbiota in IS production and strengthen the link between microbial gene abundance and metabolite levels.

In conclusion, we found high interindividual variability of IS production by the OTCT, suggesting that universal dietary recommendations of a low-protein diet for patients with CKD may have limited utility. Successful implementation of a restricted diet requires a highly personalized, holistic care approach. The OTCT exhibits better efficacy than fasting plasma IS in terms of identifying different IS producer phenotypes, which may serve as personalized dietary guidance tailored to individual needs. Further studies are warranted to investigate the potential clinical applications of OTCT in precision nutrition and to validate its use in patients with CKD.

Methods

Study design and participants

Healthy individuals aged 20 years or older were eligible for inclusion. Participants were recruited from September 2019 to July 2023 via advertisements in the hospital. The initial screening was conducted by an in-person interview to collect anthropometric and medical data. Additionally, laboratory testing was conducted. The exclusion criteria were as follows: a body mass index (BMI) < 18.5 or ≥25 kg/m^2; gastrointestinal diseases or recent gastrointestinal discomfort (such as abdominal pain, constipation, or diarrhea); a history of any chronic disease; abnormal blood and urine chemistry values indicative of organ dysfunctions; pregnancy or breastfeeding; and the use of antibiotics or probiotics within 3 months of recruitment. We a priori planned to enroll vegetarians and omnivores at a 1:1 ratio because the results from a prior study have shown that IS production was markedly lower in vegetarians than in omnivores[37]. Vegetarians were defined as individuals who reported not having eaten any meat or seafood products for at least 2 years. This study was conducted in accordance with the ethical principles of the Declaration of Helsinki. All participants gave informed consent to protocols that were approved by the Institutional Review Board at Taipei Tzu Chi Hospital (08-P-055). This study was registered with ClinicalTrials.gov, NCT04117191.

Oral tryptophan challenge test

We performed an OTCT to simulate postprandial IS changes. We assumed that IS formation in blood occurred at 24–48 h after tryptophan intake based on the time needed for bowel transit, microbial metabolism, and the hepatic enzymatic conversion of ingested indole into plasma IS. The OTCT, which controls for the amount of tryptophan intake, is a comprehensive approach to assess an individual's IS-producing capacity, which reflects the crosstalk among diet, gut microbiome, and the host. One day prior to and during the study period with the OTCT, participants were instructed to avoid consuming high-tryptophan foods such as nuts, soy products, cheese, eggs,

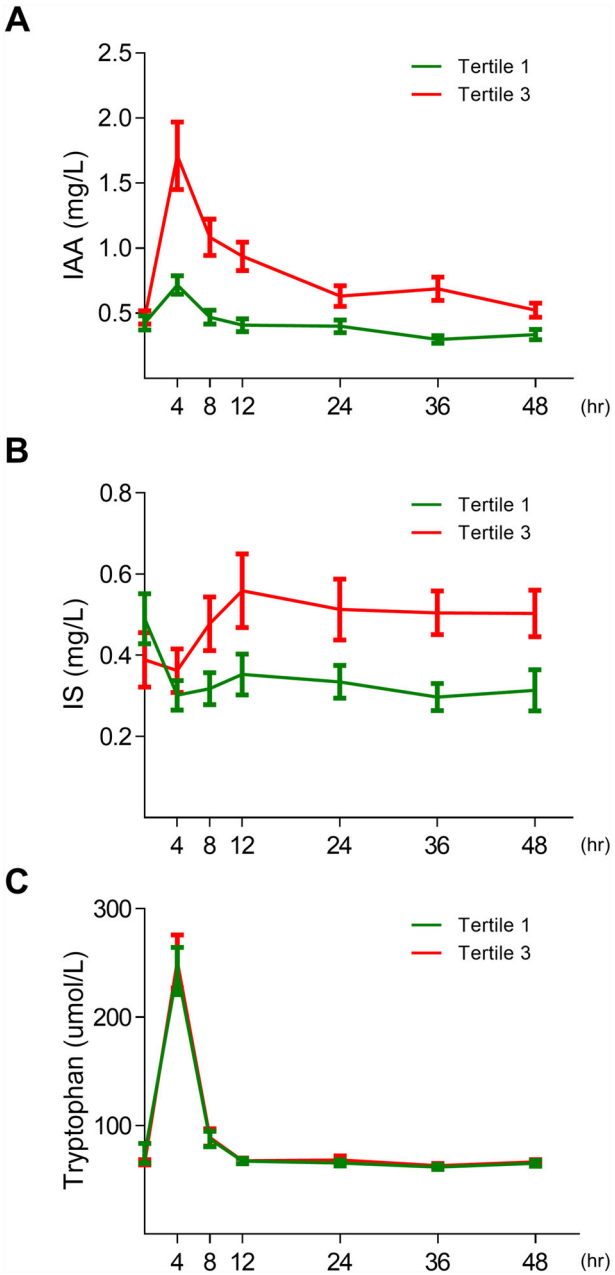

Fig. 4 | Comparison of plasma IAA, IS and tryptophan during the OTCT. The differences in plasma IAA concentrations between tertile 3 and tertile 1 of the baseline-adjusted AUC of IAA. Data are expressed as the mean ± SEM (**A**). The differences in plasma IS concentrations between tertile 3 and tertile 1 of the baseline-adjusted AUC of IS. Data are expressed as the mean ± SEM (**B**). The differences in plasma tryptophan concentrations between tertile 3 and tertile 1 of the baseline-adjusted AUC of IS. Data are expressed as the mean ± SEM (**C**). AUC area under the curve, IAA indole-3-acetic acid, IS indoxyl sulfate, OTCT oral tryptophan challenge test, SEM standard error of the mean.

poultry, deep-sea fish, and red meat. Participants were also instructed to avoid using any medication. After overnight fasting (>8 h), blood was drawn as a baseline. Four tablets of L-tryptophan (500 mg × 4 = 2000 mg) from Doctor's Best® were then administered orally to the participants. Subsequently, the participants underwent blood sampling at 4, 8, 12, 24, 36, and 48 h after tryptophan loading. The L-tryptophan tablets were examined using high-performance liquid chromatography (HPLC) to verify the amount of tryptophan used in the OTCT. The results demonstrated that the mean dose of tryptophan in each tablet was 447 mg.

Laboratory measurements

Complete blood count, urinalysis, and routine biochemistry parameters were determined using an autoanalyzer with standard procedures. Plasma concentrations of tryptophan, IS, and IAA were analyzed with HPLC.

Dietary assessments

Eligible persons were evaluated by a registered dietitian. Dietary information was obtained using a semiquantitative Food Frequency Questionnaire (FFQ). The FFQ used in this study has been validated, and it has been shown to have reliability and validity for identifying major nutrients in the diets of Taiwanese vegetarians and omnivores[38]. To ensure adherence to a low-tryptophan diet during the study period, 24-h dietary records were collected on one weekday, one weekend day before, and one weekday during the OTCT from the first 63 participants. The nutrient analysis program used to analyze the results of the FFQ and dietary records was based on Taiwan's Food Composition Database.

Single-nucleotide polymorphism genotyping, gut microbiome sampling, and metagenomics sequencing

SNPs of *CYP2E1* (rs2031920[39]) and *SULT1A1* (rs3176926[40]) were genotyped by TaqMan SNP genotyping assays (Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA was extracted from peripheral blood using the QIAamp DNA Blood Maxi Kit (Qiagen, Hilden, Germany). PCR amplifications were conducted in a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions, and StepOne™ Software was used for allelic discrimination. In the experiment, 10% of DNA samples with good quality and quantity were randomly selected for repeated experiments to verify the accuracy of the results. The consistency of genotyping results in all repeated samples was 100%.

Fecal samples were collected by the participants at home, within 3 days before the OTCT and delivered to the laboratory (Germark Biotechnology, Taichung, Taiwan) within 24 h by refrigerated (4 °C) transportation. Samples were subsequently aliquoted, and a 200-mg subsample was immediately kept in InhibitEx buffer (Qiagen, Valencia, CA) for downstream DNA extraction using the QIAamp Fast DNA Stool Mini Kit (Qiagen, MD, CA). The concentration of bacterial DNA was measured using a NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE). The DNA was stored at −80 °C prior to library construction and sequencing.

Extracted DNA (approximately 250 ng) was fragmented to 350 bp by the Covaris S2 system (Covaris, Inc., Woburn, MA) and then subjected to library construction with the TruSeq DNA Nano Library Prep Kit (Illumina, San Diego, CA). Sequencing was performed using the Illumina HiSeq 4000 sequencer, resulting in paired-end reads 150 bp in length. On a per-sample basis, sequence reads were processed with the KneadData pipeline (https://github.com/biobakery/kneaddata), which uses Trimmomatic (v0.39) and Bowtie2 (v2.3.4.1) for the removal of low-quality read bases and human and PhiX genomes, respectively[41,42]. Taxonomic profiling was performed using the MetaPhlAn2 (v2.7.7)[43] phylogenetic clade identification pipeline based on a database (mpa_v20_m200) of clade-specific marker genes. Functional profiling was analyzed using HUMAnN2 (v2.8.1)[44], in which the reads were nucleotide searched against ChocoPhlAn pangenomes by using Bowtie2, and the unmapped reads were further searched in translation based on the UniRef90 protein database[45] using DIAMOND[46]. The generated gene lists were collapsed into functional profiles based on the Pfam[47] and MetaCyc databases [48].

We further sequenced *tnaA* genes based on sequence similarity greater than 30% to *tnaA* genes from *Bacteroides thetaiotaomicron* (*Bt*) and *Bacteroides ovatus* (*Bo*), which are the two species known to commonly harbor this gene[49]. Thus, amino acid sequences of open reading frames (ORFs) in metagenome were searched against *Bt* and *Bo* TnaA proteins (i.e., WP_011107821.1 from *Bt* and WP_004300638.1 from *Bo*) by using mmseqs2 to estimate the *tnaA* abundance[50]. For ORFs best hit *Bt* or *Bo* TnaA proteins with an *e*-value < 10^{-5} and identity >30%, their sequencing coverage (in transcript per million, TPM) was summed as the abundance of

tnaA per sample. To identify TnaA proteins in gut species beyond *Bt* and *Bo*, we examined the correlation between *tnaA* abundance and the abundance of bacterial species with an average relative abundance >0.01% and a prevalence >30% in our metagenomes.

Bioinformatic analysis

Within-sample diversity, including the observed, Chao1, Shannon, and Simpson diversity indices on the species level data for each group was calculated by using the R package phyloseq. Between-sample diversity was calculated based on Bray–Curtis distances on the species-level data, and the dissimilarity matrix was then used for the calculation of principal coordinate analysis (PCoA) with the R package ade4. Linear discriminant analysis (LDA) effect size (LEfSe) was applied to determine the significantly differential taxa between groups. Only taxa with an LDA score >2 and a significance of $\alpha < 0.05$ are shown [51].

Statistical analysis

Categorical data are presented as frequencies and percentages and were compared by the χ^2 test. Continuous data with a normal distribution are expressed as the means ± standard deviations and were compared by Student's *t*-test. Continuous data without a normal distribution are expressed as medians and interquartile ranges and were compared by the Mann–Whitney *U*-test. In the box-and-whisker plot, the upper and lower boundaries of the box represent the 25th and 75th percentiles, the median is marked by a horizontal line inside the box, and the whiskers represent the 10th and 90th percentiles. Correlations between paired samples were determined by Pearson's correlation analysis. A 2-tailed *P*-value < 0.05 was considered statistically significant. Statistical analyses were performed using the computer software SPSS version 20.0 (SPSS Inc., Chicago, IL).

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files. The raw sequence data reported in this study can be found in NCBI with accession code PRJNA1098353.

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Author contributions

T.Y.L., W.K.W., and S.C.H. designed research. T.Y.L. and S.C.H. conducted research. T.Y.L. and W.K.W. analyzed data, and T.Y.L. and S.C.H. wrote the paper. T.Y.L. and S.C.H. had primary responsibility for the final content. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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