Identification of serum metabolites associating with chronic kidney disease progression and anti-fibrotic effect of 5-methoxytryptophan

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Early detection and accurate monitoring of chronic kidney disease (CKD) could improve care and retard progression to end-stage renal disease. Here, using untargeted metabolomics in 2155 participants including patients with stage 1–5 CKD and healthy controls, we identify five metabolites, including 5-methoxytryptophan (5-MTP), whose levels strongly correlate with clinical markers of kidney disease. 5-MTP levels decrease with progression of CKD, and in mouse kidneys after unilateral ureteral obstruction (UUO). Treatment with 5-MTP ameliorates renal interstitial fibrosis, inhibits IkB/NF-κB signaling, and enhances Keap1/Nrf2 signaling in mice with UUO or ischemia/reperfusion injury, as well as in cultured human kidney cells. Overexpression of tryptophan hydroxylase-1 (TPH-1), an enzyme involved in 5-MTP synthesis, reduces renal injury by attenuating renal inflammation and fibrosis, whereas TPH-1 deficiency exacerbates renal injury and fibrosis by activating NF-κB and inhibiting Nrf2 pathways. Together, our results suggest that TPH-1 may serve as a target in the treatment of CKD.
Chronic kidney disease (CKD) results in gradual loss of kidney function leading to end-stage renal disease, requiring dialysis or renal transplantation. While the early stages of CKD, stages 1–2, have little signs or symptoms and the disease is often not detected until the later stages\(^1\), the risk of cardiovascular morbidity and mortality increases with progression of CKD.

The routine clinical assessment of CKD is based on measuring a marker of kidney damage (proteinuria) and estimate renal function (estimated Glomerular Filtration Rate, eGFR). However, estimation of eGFR using the serum creatinine may be affected by non-renal processes (e.g., muscle mass or advanced age) and as such is associated with substantial error\(^2\). At the same time, assessment of proteinuria (albuminuria) may also be affected by non-renal processes (e.g., fever, infection). Furthermore, proteinuria may persist long after the successful resolution of kidney injury in many primary renal diseases leading to unnecessary escalation of treatment for many patients\(^3,4\). Since the potential for mislabeling CKD\(^5\) status on the basis of eGFR and the assessment of kidney damage on the basis of proteinuria is very high, there is an urgent need for better assessment of renal function to improve the accuracy of the diagnosis of CKD.

Use of high-throughput and high-resolution metabolomics analyses of human plasma in large-scale epidemiologic studies has enabled metabolic phenotyping in well-characterized human cohorts\(^6–10\). Metabolomics has the potential to provide insight into the disease mechanisms by illuminating the underlying metabolic pathways and to discover potential biomarkers\(^11\). We believe that metabolomics hold the potential to improve diagnosis and management of CKD by providing stage-specific and injury detecting biomarkers.

Several recent reviews have reported instances of metabolomics applied in the identification of biomarkers for renal diseases\(^12–15\). This appears to be an exciting opportunity for this class of biomarkers relative to multivariate proteomic markers, which classify patients at high risk of progression in early CKD\(^16,17\). Clinical risk assessment of eGFR and albuminuria is inefficient in patients with early CKD, impeding the development of alternative therapies\(^18\) or even targeting patients at risk for early intervention to prevent the development of kidney damage\(^19\). We anticipate that our identified metabolites will improve on the existing clinical markers and meet two large and related unmet needs in the areas of drug development and clinical practice.

In this study we enroll 2155 subjects to identify potential early stage CKD metabolite biomarkers using ultra-performance liquid chromatography coupled with quadrupole time-of-flight SYNAPT high-definition mass spectrometry (UPLC-HDMS) which has been shown to have excellent selectivity, sensitivity and reproducibility\(^20–24\). The anti-inflammatory and anti-fibrotic effects of the most promising metabolite 5-methoxytryptophan (5-MTP) and the biological roles of its regulatory enzyme tryptophan hydroxylase-1 (TPH-1) are examined in cell and animal models. The present study demonstrates five metabolites can separate patients with early stage CKD from healthy controls and serve as accurate biomarkers of the early stage of CKD. Mechanistically, the loss of 5-MTP and its regulatory enzyme, TPH-1, contribute to kidney injury by activating NF-kB and inhibiting nuclear factor-erythroid-2-related factor 2 (Nrf2) pathways. Therefore, TPH-1 may serve as a target in the treatment of CKD.

**Results**

Demographic characteristics of the study population. Flow diagram of the overview of study design was depicted in Supplementary Fig. 1. The general clinical and demographic data on healthy controls and patients with stage 1–5 CKD for the training data set are presented in Supplementary Table 1. Age, body weight (BW), body mass index (BMI), blood pressure, eGFR, serum creatinine (CREA) and urea concentrations, proteinuria and urine protein/creatinine ratio were significantly associated with CKD stages.

The independent validation dataset contains 30 healthy controls, 30 patients with CKD1 and 30 patients with CKD2. CREA levels of each group were 64.4 ± 14.8, 66.8 ± 17.6 and 77.2 ± 20.5 μmol/l, respectively. Similarly, the urea level of healthy controls, CKD1 and CKD2 were 5.3 ± 1.4, 5.2 ± 1.5 and 6.1 ± 2.4 mmol/l, respectively. These results demonstrated that serum CREA and urea were not significantly different among the three groups.

In order to further validate the biomarkers, we used data from a longitudinal cohort study composed of 1248 participants with a normal eGFR (91.4 ± 17.0 ml/min/1.73 m\(^2\)) at baseline. In that cohort 31 participants had a decrease of eGFR (52.5 ± 6.6 ml/min/1.73 m\(^2\)) and they developed CKD during the six-year follow-up period. Potential metabolite biomarkers were also validated by drug intervention on 114 patients with CKD2.

**Metabolomic analysis and five metabolite identification.** Using UPLC-HDMS, we detected 25109 variables including 16382 in positive ion mode and 8727 in negative ion mode from 703 subjects in our initial dataset. Based on least absolute shrinkage and selection operator (LASSO)-based variable selection, we identified 58 and 66 variables in the training datasets from positive and negative ion modes, respectively. Of these 124 variables, 98 metabolites including 39 in positive ion mode and 59 in negative ion mode were identified based on MS data, MS\(^2\) fragmentation, elemental compositions and available databases (Supplementary Data 1). The metabolite identification methods were presented in Methods section. A multivariate ordinal regression model was fit by using all 39 metabolites identified from the positive ion mode to determine their effect size (CKD ~ metabolites). McFadden’s pseudo \(R^2\) were computed for the positive ion mode at 95.2%, suggesting that 95.2% of the variation in the CKD stage can be explained by these 39 metabolites. The McFadden’s pseudo \(R^2\) for the 59 metabolites in negative ion mode was 94.9%. A univariate ordinal regression model was fit using these 98 metabolites (Supplementary Data 1).

To further test the classification power of these 98 metabolites, we performed random forest and support vector machine classification, and achieved an accuracy of 98.4% for random forest method, and 99.0% for support vector machine method. Metabolic pathway analysis indicated that 98 metabolites involved in Farnesoid X receptor/Retinoid X receptor (FXR/RXR) activation, aspartate biosynthesis and degradation, glutamate degradation, acetyl-CoA biosynthesis, spermine and spermidine degradation, guanosine nucleotides degradation, serotonin and melatonin biosynthesis and hypusine biosynthesis (Supplementary Fig. 2).

To further reduce the number of metabolites for clinical application and functional analysis purpose, we conducted one more round of feature reduction on these 98 metabolites plus the 17 clinical indexes. This model selected the five metabolites presented in Supplementary Table 2. Five metabolites including 5-MTP, canavaninosuccinate (CSA), acetylcarnitine, tiglylcarnitine and taurine can clearly distinguish all clinical stages of CKD (Fig. 1a–e and Supplementary Table 2). The ordinal regression LASSO model also revealed that CREA and urea that can clearly distinguish late stage CKD (Fig. 1f, g). However they are not effective at separating patients with CKD1 from healthy controls. Proteinuria also demonstrated large difference between patients
with CKD and healthy controls (Fig. 1b). Four metabolites had negative associations (5-MTP $p = 8.22 \times 10^{-13}$, CSA $p = 2.99 \times 10^{-5}$, tiglylcarnitine $p = 7.17 \times 10^{-9}$ and taurine $p = 1.63 \times 10^{-7}$), and acetylcarnitine had a positive association ($p = 3.19 \times 10^{-13}$). The changes of five metabolites are displayed in Fig. 1a–e.

The final model with the five metabolites explained 94.1% of the variation in different CKD stages. Unsupervised clustering analysis showed that five metabolites could distinguish health controls and the different stages of CKD (Fig. 2a). Supervised random forest classification using the five metabolites had a classification accuracy of 95.5%, and supervised Support Vector Machine classification showed a 92.0% accuracy (Fig. 2b–d). The receiver operating characteristic (ROC) curve showed that area under the curve (AUC) values of the five metabolites and proteinuria were equal at 0.99 (Fig. 2e), indicating that the five metabolites performed equally well as proteinuria at separating healthy controls from patients with CKD.

**External validation of five metabolites.** External validation of the five metabolites was conducted on an independent cohort with 30 healthy controls, 30 patients with CKD1 and 30 patients with CKD2. Supplementary Table 3 presents the general clinical and demographic data for this cohort. Supplementary Table 4 presents the associations between clinical variables and eGFR, all directions of associations consistent with initial findings. CKD 3–5 were not selected for external validation because our goal was focused on early stage CKD detection. Validation using unsupervised clustering, principal component analysis (PCA), supervised random forest and support vector machine revealed near perfect classification for subjects in normal healthy controls, CKD1 and CKD2 (Fig. 2f–i). The external validation results provide further evidence that five metabolites are potential biomarkers in addition to proteinuria in separating subjects with early CKD from normal healthy controls.

**Longitudinal cohort validation of five metabolites.** We further carried out a prospective incident CKD study. Among all 1248 eligible participants with an eGFR $\geq 60$ ml/min per 1.73 m$^2$ at baseline, 31 participants developed new-onset CKD (eGFR $< 60$ ml/min/1.73 m$^2$) during the course of the longitudinal study. Baseline characteristics of the longitudinal cohort sample are shown in Supplementary Table 5.

The abundance of the five metabolites was measured by UPLC-MS/MS-based targeted metabolomics. Figure 3a, b present the abundance levels of the five metabolites for the 31 subjects before and after developing CKD. The direction of change for the five metabolites agreed with the directions observed in the training dataset. PCA, orthogonal partial least squares-discriminant analysis (OPLS-DA), and unsupervised clustering showed that the five metabolites can distinguish the 31 patients with CKD by their pre and post diagnosis status (Fig. 3c–e). Sensitivity and specificity of five metabolites only were 87.1 and 96.8%, respectively (Fig. 3f). Additionally, ROC analyses showed that the five metabolites had high AUC values (Fig. 3g). Correlation between the five metabolites and eGFR (after diagnosis) was analyzed. 5-MTP, CSA, acetylcarnitine, tiglylcarnitine and taurine had a correlation coefficient of 0.91, 0.71, 0.78, 0.72 and 0.72, respectively.

**Validation of drug treatment.** Next, we examined how pharmacological therapies with enalapril, prednisone and Wulingsan (also called Oryeongsan) affected the five metabolites' abundance levels in a cohort of 114 patients with CKD2. The eGFR value was not significantly different between pre-treatment (68.7 ± 13.4 ml/
min/1.73 m²) and post-treatment (71.4 ± 15.4 ml/min/1.73 m²) by enalapril treatment. However, proteinuria was significantly improved after the treatment with enalapril (Fig. 4a, b), indicating therapeutic benefit of enalapril treatment. Except for taurine, the other four potential metabolites were significantly restored after patients received an enalapril treatment. Although eGFR did not change significantly during the 6-month treatment study, the five metabolites were significantly altered, which indicated the higher sensitivity of five metabolites than eGFR in early stage of CKD. All five metabolites exhibited abundance level shifts in directions concordant with results from training dataset (Fig. 4a, b). PCA, OPLS-DA, and heatmap showed that post-treatment patients with CKD2 could be separated from pre-treatment patients with CKD2 by the combination of proteinuria and the five metabolites (Fig. 4c–e) with sensitivity and specificity of being 90.0 and 80.0%, respectively (Fig. 4f). Similar results were also observed by using only the five metabolites (Fig. 4g–j). ROC analyses showed that all five metabolites and proteinuria had high AUC (Fig. 4k).
Fig. 2 Multivariate statistical analysis of the metabolites and clinical indexes and external validation. a Heatmap and unsupervised cluster constructed using the five metabolites and two clinical parameters. Strong cluster separation can be observed among CKD stages. All normal healthy controls were clustered together. Subjects with early CKD stages clearly separated from subjects with late CKD stages. Darker color was low abundance, light color was high abundance. b Bar plot demonstrates the accuracy and fitness of the all models tested. Three evaluation parameters were plotted: Pseudo $R^2$, random forest accuracy and support vector machine accuracy. The models with best fitness and accuracy are the models with the 98 metabolites. We further chose the model of five metabolites with two clinical parameters as our final model. c Confusion matrix of random forest evaluation on the final model. The accuracy was 95.5%. d Confusion matrix of support vector machine evaluation on the final model. The accuracy was 92.0%. e ROC curves of five metabolites and proteinuria in normal healthy controls and all patients with CKD. f Unsupervised cluster results show all 30 normal healthy controls clustered together and separated from patients with CKD. Two outliers were observed between CKD1 and CKD2. Darker color was low abundance, while light color was high abundance. g Scatter plot of PC1 vs PC2, clear separation can be observed between normal healthy controls and patients with CKD. h Random forest method was able to classify subjects to correct CKD stages. i Support vector machine method was able to classify subjects to correct CKD stages. AUC area under the curve, RF random forest, SVM support vector machine

Fig. 3 The five metabolites were further validated by a longitudinal cohort study. a Dot plots of levels of five metabolites including 5-MTP, CSA, acetylcarnitine, tiglylcarnitine and taurine in serum of normal healthy controls and CKD case. They were determined by UPLC-HDMS method. Mean values are presented by horizontal bars. Upper and lower lines indicate standard deviation values. b Dots and lines showing changes of five metabolites in each individual from normal status to CKD case status. c PCA of two components of five metabolites from normal healthy controls and CKD case. d OPLS-DA of five metabolites from normal healthy controls and CKD case. e Heatmap of five metabolites from normal healthy controls and CKD case. f Diagnostic performances of five metabolites from normal healthy controls and CKD case based on the PLS-DA model. The black dots with red squares or black circles with blue squares are for the incorrectly predicted samples in CKD case and normal controls. 27 Out of the 31 CKD case were located in CKD case area (87.1% sensitivity) and 30 out of the 31 normal healthy controls were correctly grouped (96.8% specificity). These results demonstrated that the five metabolites show high prediction class probabilities. g Analysis of OPLS-DA based ROC curves of five metabolites in normal healthy controls and CKD case from 31 individuals. The associated AUC, 95% confidence interval (CI), sensitivity and specificity values were indicated. Student's $t$ test was used for the significance of difference between two groups. OPLS-DA orthogonal partial least squares-discriminant analysis, PCA principal component analysis
**Fig. 4** Five metabolites were further validated in pre- and post-treatment by enalapril. **a** Box plots of levels of five metabolites including 5-MTP, CSA, acetylcarnitine, tiglylcarnitine and taurine in patients with CKD2 pre- and post-treatment by enalapril. Mean values are presented by horizontal bars. The whiskers indicate the maximum and minimum points. **b** Dots and lines showing changes of five metabolites in each individual from patients with CKD2 pre- and post-treatment by enalapril. **c** PCA of two components of proteinuria and five biomarkers. **d** OPLS-DA of two components of proteinuria and five biomarkers from patients with CKD2 pre- and post-treatment by enalapril. **e** Heatmap of two components of proteinuria and five biomarkers. **f** Diagnostic performances of two components of proteinuria and five biomarkers based on the OPLS-DA model. The black dots with red squares or black circles with blue squares are for the incorrectly predicted samples in pre- and post-treatment. **g** Out of the 30 patients with CKD2 pre-treatment were correctly grouped (96.7% sensitivity) and 27 out of the 30 patients with CKD2 of post-treatment were correctly grouped (96.7% sensitivity). These results demonstrated that the five biomarkers show high prediction class probabilities. **k** Analysis of PLS-DA based ROC curves of proteinuria and five metabolites in patients with CKD2 pre- and post-treatment by enalapril. The associated AUC, 95% confidence interval (CI), sensitivity and specificity values were indicated. Student’s t test was used for the significance of difference between two groups.
Significantly increased proteinuria was observed after the treatment with prednisone. Except for tiglylcarnitine, the other four potential biomarkers were significantly restored after patients received a prednisone treatment (Supplementary Fig. 5a, b). PCA, OPLS-DA, and heatmap showed that post-treatment patients with CKD2 could be separated from pre-treatment patients with CKD2 by the combination of proteinuria and five biomarkers (Supplementary Fig. 3c–e). Sensitivity and specificity of the combination of proteinuria and five biomarkers are 80.0 and 83.3%, respectively (Supplementary Fig. 3f). Similar results are also observed by only five biomarkers (Supplementary Fig. 3g–i). ROC analyses show 5-MTP, CSA and acetylcarnitine have a high AUC values compared with the proteinuria (Supplementary Fig. 3k).

Additionally, significantly increased proteinuria was improved after the treatment with Wulingsan. Except for tiglylcarnitine, the other four potential biomarkers were significantly restored after patients received a Oryeongsan treatment (Fig. 5a, b). PCA, OPLS-DA, and heatmap showed that post-treatment patients with CKD2 could be separated from pre-treatment patients with CKD2 by the combination of proteinuria and five biomarkers (Fig. 5c–e). Sensitivity and specificity of the combination of proteinuria and five biomarkers are 93.3 and 86.6%, respectively (Fig. 5f). Similar results are also observed by only five biomarkers (Fig. 5g–i). Interestingly, ROC analyses show proteinuria has a higher AUC values than other five biomarkers by pre- and post-treatment of Wulingsan (Fig. 5k). This results might indicate that Wulingsan show a more beneficial effect on proteinuria improvement in patients with early stage CKD than enalapril or prednisone. These results demonstrated that five metabolites could be considered as biomarkers for diagnosis of early CKD.

The renoprotective effects of 5-MTP. Among the five metabolites, 5-MTP was one of the metabolites identified in progressive CKD and strongly associated with eGFR which was consistent with previous finding.10 Furthermore, the protective effects of 5-MTP were demonstrated in vascular injury,25,26 which were strongly associated with CKD. The anti-inflammatory effects of 5-MTP have been documented by several studies,27 but have not been demonstrated in animals with kidney injury or renal tubular epithelial cells. Thus, we examined the renal protective effects of 5-MTP in vitro and in vivo. To confirm the tissues specificity of 5-MTP, we measured the 5-MTP level in multiple organs. The results demonstrated that 5-MTP was more significantly decrease in the kidney than in other organs such as heart, liver, lung, spleen, prostate and brain in unilateral ureteral obstruction (UUO) mice (Fig. 6a).

Next, UUO mice were employed to demonstrate the anti-inflammatory and anti-fibrotic effects of 5-MTP. 5-MTP was administrated at doses of 10 and 100 mg/kg per day by intraperitoneal injection for one week. Hematoxylin and eosin (H&E) and Masson staining indicated that renal inflammation and fibrosis were attenuated by 5-MTP in UUO mice (Fig. 6b). This was associated with increased Nr2f, heme oxygenase-1 (HO-1), NAD(P)H quinone dehydrogenase 1 (NQO-1), inhibitor of kappa B alpha (IkBa) and decreased phosphorylated-IkBα (p-IkBα), nuclear factor kappa B p65 (NF-κB-p65), monocyte chemoattractant protein-1 (MCP-1) and cyclooxygenase-2 (COX-2) pointing to attenuation of inflammation in UUO by 5-MTP (Fig. 6c, d). The numbers of CD3+ and CD68+ cells in the renal interstitium were significantly decreased by 5-MTP (Fig. 6e, f). Similarly, immunohistochemical examination revealed that expressions of fibronectin and vimentin were attenuated in renal interstitium by 5-MTP (Fig. 6g). The pro-fibrotic proteins collagen I, fibronectin, alpha-smooth muscle actin (α-SMA) were downregulated after treatment with 5-MTP (Fig. 6h, i).

The protective effects of TPH-1 on renal disease. We hypothesized that upregulation of endogenous 5-MTP by TPH-1 exerts favorable therapeutic effects on renal diseases. TPH-1 is a key enzyme in the synthesis of 5-MTP from L-tryptophan, and knock-down of TPH-1 has been linked to the downregulation of 5-MTP.28 TPH-1 expression was reduced after kidney injury caused by TGF-β1 and LPS stimulation (Fig. 8a). Knock down of TPH-1 suppressed expression of epithelial cell markers and increased expression of pro-fibrotic markers (Fig. 8b–d). We further investigated the expression of TPH-1 in UUO and renal ischemia/reperfusion injury (IRI) mice and found that TPH-1 expression was significantly downregulated in both UUO and IRI mice (Fig. 8e and Supplementary Fig. 4). TPH-1 overexpression resulted in significant upregulation of 5-MTP in mice (Fig. 8f, g). TPH-1 overexpression decreased expression of pro-inflammatory and pro-fibrotic markers in UUO mice (Fig. 8h–k). In contrast, TPH-1 deficiency exacerbated inflammation and fibrosis in UUO mice (Fig. 8l–o). Taken together, these results identified TPH-1 as a potential therapeutic target for treatment of CKD.

Discussion

Through analysis of high-throughput metabolomics data and multi-step validations, we identified 5-MTP, CSA, acetylcarnitine, tiglylcarnitine and taurine which can serve as biomarkers for early stage CKD in addition to proteinuria. The analyses were conducted primarily using advanced bioinformatics approach that combines machine learning techniques and statistic feature reduction method. While the study identified five promising metabolites, other potential metabolites have also been identified. A feature may show very strong effect on the outcome in the univariate analysis but not selected by the LASSO, and vice versa. Univariate analysis is the simplest form of statistical analysis which involves only one variable. The significance of the relationship between any given variable with the outcome is based solely on that variable and does not consider the presence of other variables. While in reality, it is always important not only to evaluate the effect of that variable alone, but also in combination with other variables, some of which may be correlated. In LASSO, when a number of highly correlated variables are associated with the outcome, LASSO tends to select those that are more explanatory among the correlated group. With the presence of the five
selected metabolites, other metabolites [DG(38:1), DG(42:3), etc.] significant by univariable analysis were not needed to make a robust final model. However, they could also be important markers of altered kidney function, and their role deserves further exploration in future studies.

Our study is first to identify 5-MTP as the most promising biomarker metabolite for detection of early stage CKD. 5-MTP, an endogenous tryptophan metabolite, decreased in serum but increased in urine in CKD. Few studies reported the changes of 5-MTP in CKD. However, notably, tryptophan show the same trend with 5-MTP in CKD[10,29,30], which may result from reduced reabsorption of tryptophan and 5-MTP in the kidney. 5-MTP is converted by TPH-1[31] and possess anti-inflammatory activity[32]. Furthermore, by suppressing activation of NF-kB and consequent

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**Fig. 5** Five potential biomarkers were further validated in pre- and post-treatment by Wulingsan. **a** Box plots of levels of five potential biomarkers including 5-MTP, CSA, acetylcarnitine, tiglylcarnitine and taurine in patients with CKD2 pre- and post-treatment by Wulingsan. Mean values are presented by horizontal bars. The whiskers indicate the maximum and minimum points. **b** Dots and lines showing changes of five potential biomarkers. **c** PCA of two components of proteinuria and five biomarkers. **d** OPLS-DA of two components of proteinuria and five biomarkers. **e** Heatmap of two components of proteinuria and five biomarkers. **f** Diagnostic performances of two components of proteinuria and five biomarkers based on the PLS-DA model. The black dots with red squares or black circles with blue squares are for the incorrectly predicted samples in pre- and post-treatment. 26 Out of the 30 patients with CKD2 of pre-treatment were located in pre-treatment area (86.6% specificity) and all the 30 patients with CKD2 of post-treatment were correctly grouped (100.0% sensitivity). These results demonstrated that the five biomarkers show high prediction class probabilities. **g** PCA of two components of five biomarkers. **h** OPLS-DA of five biomarkers. **i** Heatmap of five biomarkers. **j** Diagnostic performances of five biomarkers based on the PLS-DA model. The black dots with red squares and or black circles with blue squares are for the incorrectly predicted samples in pre- and post-treatment. 26 Out of the 30 patients with CKD2 of pre-treatment were located in pre-treatment area (86.6% specificity) and all the 30 patients with CKD2 of post-treatment were correctly grouped (100.0% sensitivity). These results demonstrated that the five biomarkers show high prediction class probabilities. **k** Analysis of PLS-DA based ROC curves of proteinuria and five potential biomarkers. The associated AUC, 95% confidence interval (CI), sensitivity and specificity values were indicated. Student’s t test was used for the significance of difference between two groups.
inhibition of transcripational activation of COX-2, 5-MTP attenuates production of inflammatory cytokines, thereby alleviates inflammation and tissue injury\textsuperscript{33}. In addition, it has been recently suggested that 5-MTP can protect cardiomyocytes against \textit{H}_{2}\textit{O}_{2}-induced oxidative injury, post-myocardial infarction cardiac injury and ventricular remodeling\textsuperscript{32,34}.

Our investigation of 5-MTP’s biological roles using UUO mice and HK-2, HMC cells demonstrated that 5-MTP attenuates the pro-inflammatory factor NF-\kappa B p65, and expression of its target gene products, HO-1 and NQO-1, as well as Nrf2 and its downstream gene products, COX-2, MCP-1, as well as Nrf2 and its downstream gene products, HO-1 and NQO-1, in indicated groups.\textsuperscript{32}\textsuperscript{33}.

The anti-inflammatory and anti-fibrotic effects of 5-MTP in vivo.\textsuperscript{32}\textsuperscript{33} The level of 5-MTP in the different tissues in UUO mice. \textsuperscript{32}\textsuperscript{33}b &c &d &e &f &g &h &i

\textbf{Fig. 6} The anti-inflammatory and anti-fibrotic effects of 5-MTP in vivo. \textbf{a} The level of 5-MTP in the different tissues in UUO mice. \textsuperscript{*}P < 0.05, \textsuperscript{**}P < 0.01 compared with sham group (n = 6). \textbf{b} H&E and Masson trichrome staining of kidney tissues of UUO mice. Representative micrographs showed kidney inflammation and fibrotic lesions in indicated groups. Paraffin sections were used for H&E and Masson trichrome staining. Scale bar, 55 \textmu m. \textbf{c}, \textbf{d} The protein expression and relative quantitative data of ikB\alpha, p-ikB\alpha, NF-\kappa B, and its downstream gene products, COX-2, MCP-1, as well as Nrf2 and its downstream gene products, HO-1 and NQO-1, in indicated groups. \textsuperscript{*}P < 0.05, \textsuperscript{**}P < 0.01 compared with sham group (n = 6). \textbf{e}, \textbf{f} Immunohistochemical staining of CD3 and CD68 and relative quantitative data in indicated groups. Scale bar, 50 \textmu m. \textsuperscript{32}\textsuperscript{33}g, \textbf{h} The protein expression and relative quantitative data of collagen I, fibronectin, \alpha-SMA and E-cadherin in different groups as indicated. \textsuperscript{*}P < 0.05, \textsuperscript{**}P < 0.01 compared with sham group (n = 6). \textbf{i} Immunohistochemical staining of fibronectin and vimentin in indicated groups. Scale bar, 50 \textmu m.\textsuperscript{32}\textsuperscript{33}

UUO, unilateral ureteral obstruction. Dot presents the single data results in bar graph. Data are presented as means \pm SD. Student’s \textit{t} test was used for the significance of difference between two groups; one-way ANOVA followed by Dunnett’s post hoc test for multiple comparisons was used for three or more groups.

CSA was another important identified metabolite. CSA is synthesized from ureidohomoserine and aspartate by arginino-succinate synthetas\textsuperscript{35,36}. Both serum\textsuperscript{36} and urinary\textsuperscript{37} CSA have been shown to have high sensitivity and specificity as biomarkers in discrimination of hepatocellular carcinoma from liver cirrhosis. In the present study, CSA was identified as one of the major predictive metabolite for early CKD detection. Decreased CSA in patients with early stage CKD was restored by the treatments of enalapril, prednisone and Wulingsan.

\textbf{Table 2} The level of CSA in different tissues in UUO mice. \textsuperscript{138}\textsuperscript{138}
Acetylcarnitine could facilitate movement of acetyl-CoA into the matrices of the mitochondria during fatty acid oxidation. Several studies have shown elevation of serum acetylcarnitine in humans and animals with different kidney diseases. Accumulation of acetylcarnitine in patients with CKD may lead to the inhibition of carnitine acetyltransferase activity and mitochondrial dysfunction in skeletal muscles by shifting there is action in the opposite direction. Our current study demonstrated that the serum acetylcarnitine level was significantly increased with reduction of renal function in patients with CKD. Elevated serum
acetylcarnitine levels in patients with CKD may lead to accumulation of acetyl-CoA and depletion of CoA in mitochondria by stimulating the reverse carnitine acetyltransferase reaction. Besides our current study was the first to identify tiglylcarnitine deficiency as a potential biomarker in patients with CKD. However, based on the data collected from Caucasians, Goek et al. and Sekula et al. found that high levels of tiglylcarnitine are linked to low eGFR in patients with CKD\textsuperscript{43,44}. It should be noted that the serum samples tested in the Goek et al. and Sekula et al. study were from Caucasians which are different from our samples that were from Han Chinese. The observed difference may be due to differences in the diets and ethnic backgrounds of the study populations. In addition, Liu et al. study was also different from the present study\textsuperscript{45}. In that it studied predominantly diabetic kidney disease (DKD); furthermore, most short to medium chain acylcarnitines differed among the DKD and nonDKD cohorts analyzed in the aforementioned study. We interpret these discrepancies between the cohorts analyzed by Liu et al. and our findings as indicative of a more specific role of tiglylcarnitine in DKD vs non-diabetic forms of CKD. Future studies should explore this relationship more fully, since it may lead to a non-invasive marker that differentiates diabetic from non-diabetic forms of CKD in patients.

Taurine is an important endogenous metabolite, which has been shown to be significantly affected in patients with CKD\textsuperscript{46,47}. For example, a large population-based study identified changes in...
taurine level in patients with early stage CKD. Another small cross-sectional study identified taurine deficiency as an important biomarker distinguishing healthy controls from patients with CKD1 to CKD4. Recently, two metabolomics studies have demonstrated a significant reduction of parenchymal taurine in the kidney allograft-form-individuals with low eGFR. The mechanism of CKD-induced taurine deficiency is not clear. Oxidative stress contributes to the pro-inflammatory state in CKD through the NF-κB activation. The improvement in oxidative stress and inflammation in CKD in response to therapy might account for the observed restoration of the taurine level.

Our current study has several limitations. We used eGFR to represent kidney function instead of measuring GFR by inulin clearance method which is impractical for a large general population-based study. In addition, the five metabolites were not validated from populations of other races. However, the positive results from cell and animal models suggested possible generalization of 5-MTP in a different population.

In summary, the present study uncovered five metabolites which can separate patients with early stage CKD from healthy control and serve as accurate biomarkers of the early stage kidney disease. These biomarkers can serve as useful supplements to the existing biochemical indicators of kidney disease. And our results have shown that these five metabolites can separate patients with early stage CKD from healthy controls. Mechanistically, the loss of 5-MTP and its regulatory enzyme, TPH-1, contribute to kidney injury by activating NF-κB and inhibiting Nrf2 pathways. Therefore, targeting TPH-1 may serve as a strategy for the treatment of CKD.

**Validation cohort.** An additional 90 serum samples including 30 healthy controls, 30 CKD1 and 30 CKD2 were collected as the external validation cohort. The inclusion-exclusion criteria for external validation cohort are the same as mentioned in the previous paragraph. Patients with acute kidney injury, liver disease, active vasculitis, gastrointestinal pathology or cancer were excluded from the study.

**Longitudinal cohort.** We utilized data from a longitudinal cohort study. A total of 1248 participants were registered in the physical examination center of the four hospitals mentioned above between 2011 and 2016. The participants visited the hospitals once a year. CKD was defined as an eGFR < 60 ml/min per 1.73 m² (CKD stage 3 or worse). Proteinuria was defined as trace or higher levels by dipstick proteinuria in this study. Individuals were excluded from this study if they had CKD at the baseline visit (first examination). Among all 1248 eligible participants with an eGFR ≥ 60 ml/min per 1.73 m² at baseline, 31 participants developed new-onset chronic renal injuries (eGFR < 60 ml/min/1.73 m²) during the course of the longitudinal study and these individuals were considered as cases.

Of the 1217 participants had an eGFR ≥ 60 ml/min/1.73 m² and did not develop chronic renal injuries.

**Drug intervention cohort.** Based on eGFR and 24-hour proteinuria results, an additional 114 patients with CKD2 were studied for further metabolite validation. Forty and thirty-six patients with proteinuria between 0.5 and 1.0 g/day were treated by enalapril (10 mg/d) and Wulingsan (0.15 g/kg/d, a renoprotective natural medicine) for six months, respectively. Wulingsan is one of the compund medicines, composed of 5 major ingredients, and has been used for the treatment of various renal diseases.

Out of these patients, 31, 27 and 18 patients had a kidney biopsy consistent with chronic glomerulonephritis, idiopathic membranous nephropathy and hypertensive nephropathy, respectively. An additional thirty-eight patients had biopsy proven IgA nephropathy. According to the Kidney Disease Improving Global Outcomes (KDIGO) guideline, patients with proteinuria >10 g/day were treated by decreasing dosages of prednisone 0.8 mg/kg/d in the first two months, 0.6 mg/kg/d during second two months and 0.4 mg/kg/d during the third two months. A total of 228 serum samples were collected from patients with CKD before and after drug treatments.

**Untargeted metabolomics and measurement of metabolites.** The samples were analyzed by a 2.1 × 100 mm ACQUITY 1.8 μm HSS T3 using a Waters Acquity UPLC system equipped with a Waters Xevo TQG2 QToF MS (Milford, MA, USA). The metabolomic procedure including sample preparation, metabolite separation and detection, data preprocessing and statistical analysis for metabolite identifications was performed following previous protocols with minor modifications. The methods of chromatographic separation and mass spectrometry were described in detail as follow:

The UPLC analysis was performed with a Waters Acquity Ultra Performance LC system (Waters Corporation, Milford, MA, USA) equipped with a Waters Xevo TQG2 QToF MS (Waters MS Technologies, Manchester, UK). Chromatographic separation was carried out at 40 °C on an ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm, UK). The mobile phase consisted of water (A) and acetonitrile (B), each containing 0.1% formic acid. The optimized UPLC elution conditions were as follow:

- 0–1.0 min, 1.0% B; 1.0–12.0 min, 1.0–99.0% B; 12.0–14.0 min, 99.0–1.0% B and 14.0–15.0 min, 1.0% B. The flow rate was 0.40 ml/min. The autosampler was maintained at 4 °C. Every 1 µl sample solution was injected for each run. Mass spectrometry was performed on a Xevo TQ (Waters MS Technologies, Manchester, UK). The scan range was from 50 to 1200 m/z. For positive electrospray mode, the capillary and cone voltage were set at 3 kV and 30 V, respectively. MS/MS was used. The cone gas was set to 60 l/h at a temperature of 450 °C. The scan mass range was 600–2000 Da.
range extension. The data acquisition rate was set to 0.1 s, with a 0.014 s interscan delay. The collision energy was set to 1.0 kV and 30 V, respectively. Source and desolvation temperatures were 145.2 for tiglylcarnitine, and m/z 126.0 for taurine with a scan time of 0.52 and 2.9%, respectively. This method showed the good repeatability and precision in this study. Data were log2 transformed and normalized to total signal per pixel.

Targeted metabolomics and measurement of serum biomarkers. Targeted metabolomics was performed on a Waters Acquity UPLC™ BEH C18 column (2.1 × 50 mm, 1.7 µm) using a Waters Acquity™ UPLC system. The mobile phase and UPLC gradient elution program of untargeted metabolomics was used for determination of the potential biomarkers. The flow rate was 0.3 ml/min. The sample temperature and column compartment were set at 4 and 40 °C, respectively. The injection volume was 10 µL.

After the chromatographic separation, tandem mass spectrometry (MS/MS) analysis and data acquisition were carried out on a Waters Micromass® Quattro micro™ mass-spectrometer equipped with an ion interface. The five potential biomarkers including 5-MTP, CSA, acetylcarnitine, tiglylcarnitine and taurine were identified in positive ion mode using untargeted metabolomics, so targeted data detection was performed in the multiple-reaction-monitoring (MRM) in positive ion mode. The reference standards of 5-MTP, CSA, acetylcarnitine, carnitine and taurine were used for identification of the biomarkers and for quantification of the potential biomarkers. The isotopic pattern of the elemental composition matches a cluster of peaks in the MS spectra, increasing confidence in identified metabolites and simplifying results. Chemical elements and atom amounts including carbon (0–50), hydrogen (0–500), nitrogen (0–50), sulphur (0–80), helium (0–15), bromine (0–15) and phosphorous (0–10) were used to match m/z data. The lower the i-FT value, the better the fit. When element compositions were calculated, three possible element compositions of C₄H₆NO₄S, C₅H₁₀NO₄S₂ and C₃H₆N₃O₆S were obtained. Third, the elemental composition was converted to exact m/z values by using all possible element compositions. The repeatability and precision were tested by six replicate injections of pure standards. The repeatability RSD% of retention times and peak areas were calculated from tested quality control samples. The above-mentioned procedure was performed every day.

Animal models and treatment strategies. Male BALB/c mice, weighing 20–22 g, were used to establish UUO model by an established protocol. After general anesthesia, complete UUO was carried out by double-ligating the left ureter by 4–0 silk following the dorsal incision. The ureters of sham operated mice were exposed, but not ligated. Mice were randomly assigned to four groups (n = 6): (1) sham control, (2) UUO, (3) UUO + 10 mg/kg-5 MTP, and (4) UUO + 100 mg/kg-5 MTP at doses of 100 µg/kg and 1,000 µg/kg, respectively. The mice were injected intragastric administration. The four group mice were sacrificed at the 7th day. The ligated kidney were immediately frozen and saved in liquid nitrogen for Western blot and immunohistochemical staining.

Male BALB/c mice, weighing 20–22 g, were used for renal IRI model with 30- min ischemia time. Briefly, under general anesthesia, a dorsal incision was made, and bilateral renal pedicles were clamped for 30 min using bulldog clamp. All procedures followed the ARRIVE Guidelines for Reporting Animal Research.

Knock-in and knock-down of TPH1 in vivo. Lentinus express whole-length mouse Tph1 cDNA (TPH-1 over) and lentinus containing empty plasmids (vector) were constructed by Sangon (Shanghai, China). Besides, lentinus carrying shRNA against Tph1 and lentinus containing nonspecific shRNA (scramble) were also constructed by Sangon. After anesthesia and surgery, mice were laparotomized and injected with recombinant adenovirus vector by a 23 g needle at the lower pole of kidney parallel to the long axis. The 100 µl of saline or lentinus vector (1 × 10⁵ IU/µl) were injected into kidney. No toxic effects were founded after treatment with lentiviral vector.
Cell culture and treatment. Human kidney proximal epithelial cells (HK-2) and human mesangial cells (HMC) were obtained from the American Type Culture Collection (Manassas, VA, USA). HK-2 and HMC cells were cultured in DMEM/F-12 and DMEM, respectively, supplemented with 10% fetal bovine serum at 37 °C with 5% CO₂. HK-2 was treated with 1 μM recombinant human TGF-β1 protein (R&D system, USA), while HMC were treated with 10 μg/ml LPS (L2630, Sigma, USA). The concentrations of 5- MTP (M4001, Sigma, USA) for HK-2 and HMC were 5 and 10 μM for 24 h. After 24 h culture, cells were harvested for next experiments.

The knock-down of TPH-1 were operated by siRNA. TPH-1 siRNA and negative control siRNA, constructed by Sangon (Shanghai, China), were transfected into cells by Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s guide.

Immunohistochemical staining. Paraflin-embedded mice kidney sections (5-μm thickness) were prepared as a routine procedure. The sections were deparaffinized by three xylene washes, hydrated by alcohol and washed with distilled water. The sections were added with three percent hydrogen peroxide to block endogenous peroxidase activity. Antigen retrieval was performed by a microwave oven for 15 min in the citrate buffer (10 mM, pH 6.0). Five percent BSA were incubated for 30 min at room temperature. Next, the sections were incubated with the primary antibody at 4 °C overnight. Negative controls were incubated without primary antibodies. After being washed, the sections were incubated with the secondary antibody at room temperature for 2 h. DAB were added to visualize and diaminobenzidine was used to counterstain, then the sections were mounted by neutral gum. Image analysis was done by using Image-Pro Plus 6.0 software.

Immunofluorescence staining and confocal microscopy. Cells cultured on coverslips were fixed with 4% paraformaldehyde for 10 min Ten percent goat serum was employed to block nonspecific sites. The slides were incubated with primary antibodies at 4 °C overnight. After three times washed by PBS, the slides were incubated with secondary antibody at room temperature for 2 h and 10 min with 4,6-diamidino-2-phenylindole (DAPI). The slides were mounted with 80% glycerinum in PBS. The slides were stored at −20 °C or examined by a laser-scanning confocal microscope (FV1000, Olympus, Japan) equipped with FV10-ASW 4.0 VIEW (Olympus).

Western blot analysis. Protein expression was analyzed by western blot analysis. Protein concentration was measured by Pierce™ BCA Protein Assay Kit (23227, Thermo Scientific, USA). The 20–30 μg of total protein was fractioned by Tris-Glycine resolving gel and transferred to a 0.45 μm polyvinylidene difluoride membrane (10600023, Amersham™ HybondTM, GE Healthcare, USA). After incubated for 1 h in 5% non-fat milk blocking buffer, the membranes were incubated overnight at 4 °C with primary antibody. The secondary antibodies of goat anti-rabbit (1:5000, ab6721, Abcam, USA), goat anti-mouse (1:5000, A21100, Abbkine, USA) or rabbit anti-goat (1:5000, A21110, Abbkine, USA) were incubated with 2 h at room temperature. The membrane was then visualized by enhanced chemiluminescence western blotting detection reagent. Signal intensities from immunoblots were quantified using ImageJ software (version 1.48v, NIH, Bethesda, MD, USA). Band densities were normalized by β-actin or GAPDH expression levels. The uncropped blots were shown in Supplementary Figs 5–13.

The following primary antibodies were employed (dilution): collagen I (1:5000, ab34710, Abcam, USA), α-SMA (1:300, ab7817, Abcam, USA), fibronectin (1:1000, ab2413, Abcam, USA), vinculin (1:1000, ab9254, Abcam, USA), E-cadherin (1:500, ab76055, Abcam, USA), p-IκBα (1:2000, 2857, Cell Signaling Technology, USA), IkBα (1:2000, 4812, Cell Signaling Technology, USA), NF-κB p65 (1:1000, ab65520, Abcam, USA), MCP-1 (1:1000, ab7202, Abcam, USA), COX-2 (1:1000, ab22331, Abcam, USA), Nrf2 (1:1000, ab31163, Abcam, USA), HO-1 (1:1000, ab4177, Abcam, USA), NQO-1 (1:1000, ab32947, Abcam, USA) and TPH-1 (1:500, ab25954, Abcam, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:5000, 10494-1-AP) and α-tubulin (1:1000, 11224-1-AP) were purchased from Proteintech Company (Wuhan, China).

Statistics. The number of replicates was 6 per group for each data set and results were presented as mean ± standard deviation (SD) unless stated otherwise. GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for statistical analysis of the data. The significance of the differences between two groups was analysed using unpaired Student’s t test, and multiple comparisons was performed by one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test. All tests were two-tailed. P < 0.05 was considered statistically significant.

Study approval. The part of patient study was approved by the Ethical Committee and all patients provided informed consent prior to entering the study, and all clinical investigation have been conducted according to the principles expressed in the Declaration of Helsinki. The present study has complied with all relevant ethical regulations. The sample collection was approved Shaxian Traditional Chinese Medicine Hospital (Permit Number: SYXK 2010-004). All surgery was performed under urethane anesthesia, and all efforts were made to minimize suffering. All procedures and care of the rats were in accordance with the institutional guidelines for animal use in research.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Code availability

Majority of the data analyses were performed using R x64 3.4.2. All R codes written for this manuscript are available from the corresponding author upon request.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions
Y.-Y.Z., Y.G. and D.Q.C. conceived and designed the project, and managed the study. W.S., I.Z. and X.-H.C. made clinical diagnosis, recruited subjects and performed intervention. X.Y.Y., J.R.M., S.X.M., J.Z., Y.Z.Y., Y.Q.S. and M.X.M. collected samples and clinical phenotypes. Y.G., C.P.A., H.Y., D.C.S., S.L.Z., H.K. and F.Y. performed bioinformatics analyses. Y.-Y.Z., D.-Q.C., G.C., H.C. and X.-R.L. performed metabolomics profiling and data analysis. D.Q.C., L.C., M.W. and D.L. conducted animal and cell experiments. Y.-Y.Z., Y.G., D.-Q.C. and N.D.V. wrote the manuscript. Y.-Y.Z., Y.G., D.-Q.C., N.D.V., S.G.Z. and G.P.B. contributed to text revision and discussion.

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