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Correlation between serum trimethylamine-*N*-oxide concentration and protein energy wasting in patients on maintenance hemodialysis

Chun Hu*, Yumei Zhang*, Xiao Bi, Lu Yao, Yueling Zhou and Wei Ding

Division of Nephrology, Shanghai Ninth People's Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China

ABSTRACT

Objectives: Chronic kidney disease (CKD) is a serious health problem that is associated with several systemic changes, including protein energy wasting (PEW). However, the exact mechanism of PEW in CKD remains unclear. As one of the important intestinal flora metabolites and uremic toxins, trimethylamine-*N*-oxide (TMAO) is involved in CKD-associated mortality, which might play a role in the development of PEW in CKD patients especially in patients on maintenance hemodialysis (MHD). However, this possibility has not been investigated.

Methods: PEW was diagnosed in a group of CKD patients on MHD according to the criteria of the International Society of Renal Nutrition and Metabolism. Serum TMAO concentration was assessed by high-performance liquid chromatography and mass spectrometry. The association between TMAO concentration and PEW was assessed using linear regression and logistic analysis after adjustment for confounding factors, including basic characteristics, comorbidities, and laboratory findings.

Results: The circulating TMAO level was higher in the MHD patients than in control (healthy) individuals (5653.76 ± 2853.51 vs. 254.92 ± 197.88 ng/mL, $p < 0.001$). Further, after the MHD patients were screened for PEW, those with PEW were found to have significantly higher serum TMAO levels than those without PEW (6760.9 vs. 4016.1 ng/mL, $p < 0.001$). Further, the serum TMAO concentration exhibited a significant negative correlation with body mass index (BMI) and dietary protein intake. In the logistic regression analysis, after adjustment for confounding factors, the serum TMAO concentration was still significantly correlated with PEW occurrence.

Conclusions: The circulating TMAO level is significantly correlated with the prevalence of PEW in MHD patients. TMAO might be a potential target in the prevention and treatment of PEW in CKD especially ESRD.

Abbreviations: CKD: chronic kidney disease; PEW: protein energy wasting; TMAO: trimethylamine-*N*-oxide; MHD: maintenance hemodialysis; BMI: body mass index; CVD: cardiovascular disease; LDL: low-density lipoprotein; HDL: high-density lipoprotein; DPI: dietary protein intake; CI: confidence interval; OR: odds ratio; GFR: glomerular filtration rate

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Introduction

Chronic kidney disease (CKD) is a common outcome of abnormalities in kidney structure and function caused by various diseases. It has become an increasingly serious public health problem in the world [1]. CKD is associated with various complications that gradually affect the quality of life of patients. As an important complication of CKD, protein energy wasting (PEW) is one of the strongest predictors of poor prognosis in CKD patients. PEW is manifested as a decrease in protein and energy reserves that accompanies the progression of CKD. It is characterized by insufficient caloric intake, low body mass index (BMI), hypoalbuminemia, chronic inflammation, and progressive skeletal muscle wasting [2,3]. Patients with PEW usually exhibit obvious emaciation and inability to take care of themselves, and they also have other complications, such as serious infections and cardiovascular diseases, that significantly affect their life quality and lead to an increase in the mortality rate or other complications [4]. Previous studies have shown that more than 50% of patients on maintenance hemodialysis (MHD) have PEW symptoms [5]. Compared with CKD patients without PEW, CKD patients with PEW have a poor quality of life and an

CONTACT Wei Ding gump1015@163.com; Yueling Zhou stellabright@163.com Shanghai Ninth People's Hospital, School of Medicine, Shanghai Jiaotong University, 639 Zhizaoju Road, Shanghai 200011, China
*These authors are contributed equally to this work.

increase in mortality-associated conditions, such as infection, even when they have received effective protection and treatment [5]. Therefore, effective interventions to delay or prevent the occurrence of PEW are the top priority in the clinical management of CKD. However, the mechanisms of PEW are still unclear [6], and the causes and potential targets of PEW have been hot topics of research in recent years [7].

As CKD progresses, urine volume decreases and the excretion of metabolites is reduced. Metabolites that cannot be completely discharged from the body are referred to as uremic toxins and can have chronic adverse effects on various organs [8]. One such uremic toxin that is currently being investigated for its role in renal diseases is trimethylamine-*N*-oxide (TMAO) [9]. A number of clinical studies on TMAO have shown that the serum TMAO concentration is closely related to the progression of CKD, cardiovascular diseases, and bone metabolism disorders [10,11]. Further, it has been recently reported that TMAO can promote the formation of arterial plaques and thrombosis [12–14], and it is also considered as a potential risk factor for many chronic diseases including cardiovascular disease, type 2 diabetes mellitus, cancer, and fatty liver [15,16]. With regard to its role in CKD, metabolite studies have reported that patients with high TMAO content had significantly higher mortality: further, animal studies have shown that long-term TMAO- or choline-treated mice exhibit renal fibrosis and renal tubular injury [17]. It has been suggested that TMAO exposure worsened cardiomyocyte mechanics and intracellular handling [18,19]. Based on these previous reports, it is possible that TMAO also plays a role in the pathogenesis and mechanisms of CKD-associated PEW, but this topic has not been investigated yet.

In the present study, we investigated the potential relationship between TMAO and PEW by measuring and comparing the serum TMAO concentration in healthy individuals and MHD patients, screening MHD patients for PEW and comparing the serum TMAO concentration between those with and without PEW, and analyzing the correlation between CKD-associated PEW and serum TMAO levels. We believe that the findings give us hope that TMAO might be a potential candidate for the clinical prevention and treatment of CKD-associated PEW, although further investigated are needed.

Methods

Inclusion criteria and exclusion criteria

This cross-sectional study population comprised 50 control (healthy) individuals and 93 CKD patients who were undergoing MHD. The control group was selected from the medical examination center of the Ninth People's Hospital Affiliated to Shanghai Jiaotong University, and the MHD group was selected from among regular MHD patients at the Ninth People's Hospital Affiliated to Shanghai Jiaotong University. Any individual with full capacity between 18 and 75 years was eligible to participate in the study. Participants with no abnormalities in the routine medical examination were invited into the control group. Participants with the presence of acute kidney injury, CKD, diarrhea, malignant tumor, infectious diseases, cardiovascular and cerebrovascular disease, and women during pregnancy or lactation period were excluded from the control group. Individuals with history of autoimmune and inflammatory diseases, heart failure, cancer, chronic liver disease, acute infection, enteral or parenteral nutrition, long-term hormone or immunosuppressant treatment, and lactating or pregnant women were excluded from the MHD group. All the 93 MHD patients underwent hemodialysis three times a week (Mon/Wed/Fri or Tues/Thurs/Sat) and four hours each time. The type of HD technique included HD and HDF depend on the dialysis adequacy of each patient. All participants were fully informed about the study with written consent obtained before data collection. All procedures were performed according to the ethical standards with the Declaration of Helsinki, and the study was approved by the Ethics Committee of the Shanghai Ninth People's Hospital, School of Medicine, Shanghai Jiaotong University (SH9H-2019-T322-2).

Indicators for diagnosis of PEW

Data on the basic characteristics, primary diagnosis, comorbidities, and laboratory findings of the patients were obtained from their latest medical records. In this study, the indicators for PEW, according to the criteria of the International Society of Renal Nutrition and Metabolism [2], were as follows: (1) serum biochemical indicators: albumin <38 g/L, prealbumin level <0.3 g/L, or total cholesterol level <2.59 mmol/L; (2) unexpected body mass loss: BMI <22 kg/m^2 (over 65 years old, <23 kg/m^2), $>5\%$ weight loss in 3 months, $>10\%$ weight loss in 6 months, or $<10\%$ body fat; (3) muscle consumption: $>10\%$ reduction in mid-arm muscle circumference in 6 months; and (4) insufficient dietary protein and calorie intake: low dietary intake without intentional control for at least 2 months, <0.8 g/kg/day protein intake in dialysis patients' diet, or <25 kcal/kg/day energy intake. The dietary intake of each patient over three consecutive days, including two weekdays

Table 1. Characteristics of participants according to the presence or absence of PEW.

Clinical and biochemical values	PEW group ($n = 48$)	Non-PEW group ($n = 45$)	p Value
Age (years)	60.67 ± 13.97	56.26 ± 13.26	NS
Gender (male)	55.10% (26/48)	63.04% (28/45)	NS
BMI (kg/m^2)	21.23 ± 2.49	24.89 ± 1.89	<0.001
Hypertension	73.47% (35/48)	69.57% (31/45)	NS
Diabetes	26.53% (13/48)	32.61% (15/45)	NS
CVD	18.37% (9/48)	13.04% (6/45)	NS
Triglyceride (mmol/L)	1.94 (1.08–3.54)	1.92 (1.37–3.04)	NS
Cholesterol (mmol/L)	3.52 (3.11–4.26)	3.51 (3.15–4.29)	NS
LDL (mmol/L)	2.02 ± 0.68	2.09 ± 0.83	NS
HDL (mmol/L)	0.91 ± 0.26	0.93 ± 0.29	NS
Albumin (g/L)	36.97 ± 2.32	38.45 ± 2.52	0.001
Pre-albumin (g/L)	0.28 ± 0.070	0.34 ± 0.094	0.004
Ferritin (ng/mL)	252.71 (51.72–483.49)	192.89 (55.34–476.19)	NS
Hemoglobin (g/L)	100.06 ± 16.40	102.16 ± 18.34	NS
PTH (pg/mL)	293.0 (127.5–616.1)	227.6 (102.2–606.4)	NS
DPI (g/kg/day)	0.69 ± 0.12	1.05 ± 0.24	<0.001
Dialysis vintage (months)	43.2 (17.1–86.5)	41.3 (15.3–83.6)	NS
Kt/V	1.32 ± 0.25	1.34 ± 0.29	NS
TMAO (ng/mL)	6760.9 (5495.7–8618.1)	4016.1 (2875.3–5415.5)	<0.001

Mean ± SD values are presented for variables with normal distribution, while median (IQR) values are presented for variables with abnormal distribution. Values were missing for ferritin ($n = 1$) and PTH ($n = 3$).
BMI: body mass index; CVD: cardiovascular disease; LDL: low-density lipoprotein; HDL: high-density lipoprotein; DPI: dietary protein intake.

and one weekend, was recorded to determine the dietary protein intake (DPI) of each patient. Individuals in the MHD group who have at least three out of the above four indicators and at least one test satisfied in each of the selected indicator are diagnosed with PEW.

TMAO detection

TMAO levels were detected in fasting blood samples that were drawn before hemodialysis in the MHD group. The serum samples were analyzed using an liquid chromatography-tandem mass spectrometry method that was developed and validated by our lab. The linearity range of TMAO was 3–10,000 ng/mL, and the correlations coefficients (r^2) were above 0.99. Briefly, the serum sample (50 μL) was mixed with 150 μL of acetonitrile and vortexed for 2 min. After centrifugation at 12,000 rpm for 5 min, 10 μL of the supernatant was diluted with 190 μL of 0.1% formic acid-aqueous solution and 5 μL of the diluted sample was injected into the ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system for analysis. Chromatographic separation was conducted at 40 °C on an Ultimate 3000 UPLC system (Thermo Fisher Scientific, Waltham, MA, USA) using a Waters Acquity UPLC BEH C18 column (column dimensions: 2.1 × 100 mm, pore diameter: 1.7 μm). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (methanol) at a v/v ratio of 95:5, and the flow rate was set at 0.25 mL/min. The total run time was 2 min. Mass spectrometry was performed on a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Fisher Scientific, MA, USA) with an electrospray ionization interface. TMAO was quantified in the selected reaction monitoring mode. The precursor and product positive ion had m/z (mass-to-charge ratio) values of 76.08 and 58.15, respectively, with a collision energy of 20.2 V.

Statistical analysis

The count data were expressed as number and percentages (%), and the measurement data were expressed as mean ± standard deviation values or median and interquartile ranges, according to their distribution. The Student's t-test or Mann–Whitney U-test were used to analyze differences between groups, and the Fisher exact test was used for comparison of categorical data. Correlation analysis of serum TMAO concentration and PEW prevalence was carried out by stepwise regression analysis. For logistic analysis of PEW, odds ratio (OR) and 95% confidence interval (CI) for every 100 ng/mL increase in TMAO concentration were determined. The two groups were further divided into high and low TMAO groups based on the median TMAO value. Model 1 was adjusted for age and gender. Model 2 was adjusted for model 1 variables, hypertension, diabetes, and cardiovascular disease. Model 3 was adjusted for model 2 variables and hemoglobin, ferritin, and PTH levels. All statistical analyses were performed using SPSS Version 20.0 (IBM, New York, USA). Statistical significance was defined by p values <0.05.

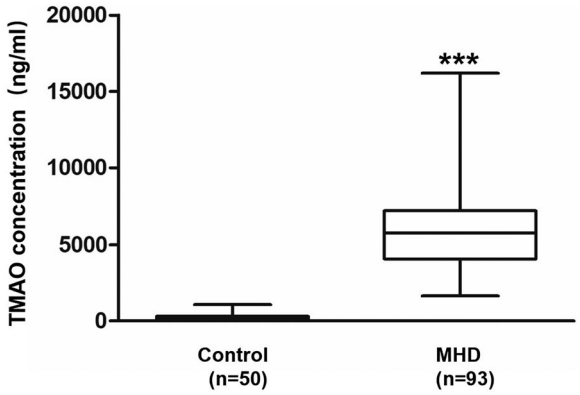

Figure 1. Serum TMAO levels in the CKD ($n = 93$) and control groups ($n = 50$). The serum TMAO levels were significantly higher in the CKD patients on MHD. $^{***}p < 0.001$.

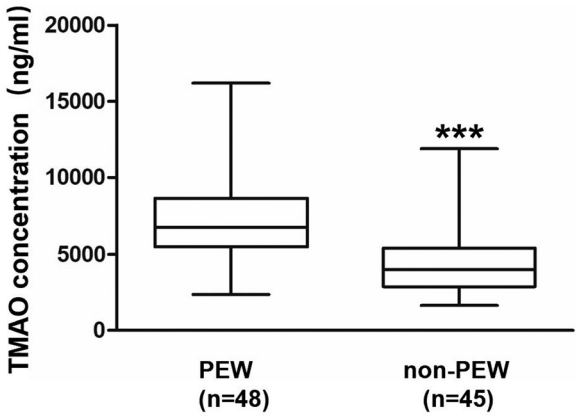

Figure 2. Serum TMAO levels in CKD patients with ($n = 48$) and without PEW ($n = 45$). The serum TMAO levels were significantly higher in the CKD patients with PEW than in the CKD patients without PEW. $^{***}p < 0.001$.

Results

Diagnosis and features of PEW in the MHD group

The MHD group ($n = 93$) was divided into PEW and non-PEW group based on previously established criteria with the 24-h dietary recall method, which included measurement of DPI, biochemical indices, and body weight. The results showed that 48 patients (26 males and 22 females; age, 60.67 ± 13.97 years) had PEW and 45 patients (28 males and 17 females; age, 56.26 ± 13.26 years) did not have PEW. The demographic and clinical characteristics of the PEW and non-PEW group are shown in Table 1. No significant difference was found between the PEW and non-PEW group with regard to age, gender, cause of CKD, LDL, HDL, cholesterol, ferritin, hemoglobin, and PTH. However, the values of the PEW indicators, mainly BMI, albumin, prealbumin, and DPI, were obviously lower in the PEW group than in the non-PEW group.

Serum TMAO levels in MHD patients with and without PEW and in the control patients

The results of HPLC/MS analysis showed that circulatory TMAO levels in the MHD group (5653.76 ± 2853.51 ng/mL) was significantly higher than those in the control group (254.92 ± 197.88 ng/mL) ($p < 0.001$, Figure 1). Further, within the MHD group, the median value of serum TMAO in the PEW patients was 6760.9 ng/mL, and it was significantly lower at 4016.1 ng/mL in the non-PEW patients ($p < 0.001$, Figure 2).

PEW prevalence in MHD patients according to TMAO levels

According to the median level of serum TMAO, the MHD patients were divided into two groups: the high TMAO group and the low TMAO group. The characteristics of the patients according to their TMAO levels are shown in Table 2. Patients with higher serum levels of TMAO tended to have lower BMI, triglyceride levels, and DPI ($p < 0.001$). Further, 85% of MHD patients in the high TMAO group had PEW, while only 34% of MHD patients in the low TMAO group had PEW. Accordingly, linear regression analysis showed that patients with higher serum TMAO levels tended to have lower BMI and DPI, which are the major PEW markers (Figure 3).

Correlation of serum TMAO concentration with the prevalence of PEW in MHD patients

Multivariate logistic regression was used to further analyze the relationship between the prevalence of PEW and serum TMAO levels, with the occurrence of PEW as the dependent variable and gender, age, hypertension, diabetes, and cardiovascular complications as independent variables. The results showed that after adjusting for the above confounding factors, the prevalence of PEW increased with every 100 ng/mL increase in the TMAO level in model 3 (OR = 1.09; 95% CI = 1.05–1.13; $p = 0.001$; Table 3). This association was strengthened by the results of analysis in the TMAO group: the high TMAO group contributes to the prevalence of PEW compared with the low TMAO group (model 1: OR = 10.80, 95% CI = 3.95–29.52; model 2: OR = 10.80, 95% CI = 3.95–29.52; model 3: OR = 12.33, 95% CI = 4.26–35.68; Table 3).

Discussion

To our knowledge, this is the first study on the relationship between TMAO and PEW in MHD patients. Our

Table 2. Characteristics of participants according to their serum TMAO concentration.

Clinical and biochemical values	TMAO higher ($n = 46$)	TMAO lower ($n = 47$)	p Value
Age (years)	58.34 ± 13.18	59.35 ± 14.33	NS
Gender (male)	48.9% (22/46)	54.2% (25/47)	NS
BMI (kg/m^2)	21.78 ± 2.64	23.67 ± 2.75	0.001
Hypertension	66.0% (30/46)	77.1% (36/47)	NS
Diabetes	25.5% (12/46)	20.8% (10/47)	NS
CVD	19.1% (9/46)	12.5% (6/47)	NS
Triglyceride (mmol/L)	1.94 (1.31–3.33)	1.90 (1.16–3.51)	0.021
Cholesterol (mmol/L)	3.49 (2.95–4.23)	3.58 (3.20–4.30)	NS
LDL (mmol/L)	1.93 ± 0.70	2.16 ± 0.77	NS
HDL (mmol/L)	0.87 ± 0.25	0.96 ± 0.28	NS
Albumin (g/L)	37.70 ± 2.67	37.45 ± 2.34	NS
Pre-albumin (g/L)	0.31 ± 0.087	0.31 ± 0.084	NS
Ferritin (ng/mL)	282.18 (80.02–473.45)	175.10 (39.10–583.58)	NS
Hemoglobin (g/L)	100.35 ± 16.60	102.22 ± 17.52	NS
PTH (pg/mL)	257.7 (84.4–616.10)	293.0 (114.55–609.95)	NS
DPI (g/kg/day)	0.72 ± 0.15	0.94 ± 0.29	<0.001
Dialysis vintage (months)	42.5 (16.8–85.4)	42.8 (15.3–86.6)	NS
Kt/V	1.31 ± 0.28	1.35 ± 0.30	NS
TMAO (ng/mL)	7206.0 (6469.6–9165.9)	4080.9 (2965.5–4979.6)	<0.001
PEW	39 (85%)	16 (34%)	<0.001

Mean ± SD values are presented for variables with normal distribution, while median (IQR) values are presented for variables with abnormal distribution. Values were missing for ferritin ($n = 1$) and PTH ($n = 4$). BMI: body mass index; CVD: cardiovascular disease; LDL: low-density lipoprotein; HDL: high-density lipoprotein; DPI: dietary protein intake.

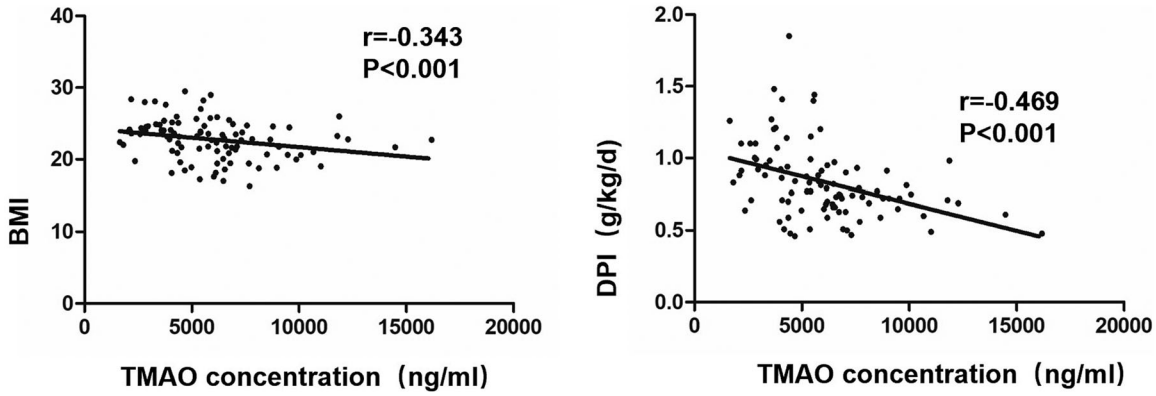

Figure 3. Correlation of serum TMAO concentration with BMI and DPI in CKD patients. The serum TMAO concentration was negatively correlated with BMI and DPI, which are indicators of PEW.

Table 3. Adjusted odds ratio for PEW according to serum TMAO levels in 93 hemodialysis patients.

Model 1[a]		Model 2[b]		Model 3[c]	
OR (95% CI)	p Value	OR (95% CI)	p Value	OR (95% CI)	p Value
Continuous: TMAO each 100 ng/mL increase					
1.07 (1.04–1.11)	<0.001	1.07 (1.04–1.11)	<0.001	1.09 (1.05–1.13)	0.001
Categorical: TMAO lower group reference TMAO higher group					
10.80 (3.95–29.51)	<0.001	10.80 (3.95–29.51)	<0.001	12.33 (4.26–35.68)	<0.001

[a]Model 1 adjusted for age and gender.
[b]Model 2 adjusted for model 1 variables, hypertension, diabetes, and cardiovascular disease.
[c]Model 3 adjusted for model 2 variables, hemoglobin, ferritin, and PTH.

findings indicate that the serum TMAO levels of patients undergoing MHD were significantly higher than those of the healthy control group. This confirms previous findings that have reported an association between high TMAO levels and CKD progression [11]. Moreover, we also found that MHD patients with PEW were found to have significantly higher serum TMAO levels than those without PEW. These findings indicate that elevated serum TMAO concentration may contribute to the development of PEW in MHD patients.

PEW is highly prevalent in ESRD patients, with a prevalence of 30–75% among patients on hemodialysis [20]. Several signaling pathways and humoral factors have been discovered to be involved in CKD-induced PEW, including the ubiquitin proteasome system (UPS), myostatin/activin pathway, endogenous

glucocorticoids, insulin-like growth factor 1 (IGF-1) pathway, metabolic acidosis, and persistent inflammation [21–23]. In our previous study, we also showed that mitochondrial dysfunction/NLRP3 inflammasome axis may contribute to angiotensin II-induced skeletal muscle wasting, the use of siRNA or genetic depletion of NLRP3, mitochondria-targeted antioxidant, or PPAR-γ agonists significantly normalized muscle function and the protein energy balance [24,25]. The factors associated with these pathways lead to decreased anabolism and/or increased catabolism, which increases resting energy expenditure and causes hypermetabolism, eventually leading to progressive muscle wasting. Uremic toxins, which are organic solutes normally metabolized and excreted by the kidneys, often accumulated in the presence of impaired kidney function, causing body toxicity [26]. However, to date, there are few published reports on gut microbiota-dependent metabolite TMAO in patients with CKD, and the association between TMAO concentrations and PEW/muscle wasting has not been investigated.

Gut microbiota-dependent metabolite TMAO is formed enzymatically by flavin-containing monooxygenase-3 in the liver from trimethylamine (TMA), which is produced by the action of gut microbiota from phosphatidylcholine and choline [27]. Under normal physiologic conditions, circulating TMAO is rapidly cleared almost exclusively by urinary excretion; TMAO and choline levels are increased with decreasing renal function [9]. TMAO has recently been involved in the pathogenesis of CKD. In animal models, Tang *et al.* demonstrated that choline or TMAO treated mice showed collagen deposition, phosphorylation of Smad3 and tubulointerstitial fibrosis [28]. Dietary supplementation of mice with choline, TMAO or betaine promoted upregulation of multiple macrophage scavenger receptors links to atherosclerosis, and eventually promoted atherosclerosis [29]. Evidence from human studies also showed that circulating TMAO concentrations were increased in patients with CKD and predicted increased long-time mortality independently of traditional risk factors [30]. Zhang *et al.* indicated that high plasma TMAO level was significantly and independently associated with cardiovascular and all-cause mortality in HD patients [31]. Consistent with these previous studies, in the present study, we observed that the serum TMAO concentrations of patients undergoing MHD were significantly higher than those of the healthy control group. MHD patients with PEW had significantly higher serum TMAO levels than MHD patients without PEW. Importantly, MHD patients with high TMAO levels had a higher prevalence of PEW than MHD patients with low TMAO levels. In accordance with this finding, regression analysis showed that the serum TMAO level was significantly correlated with PEW occurrence in MHD patients. Thus, these findings first indicate that serum TMAO may contribute to the occurrence of PEW in MHD patients. However, this study has several limitations: (1) because this study had a cross-sectional design, causal relationships among TMAO concentrations and PEW in MHD patients can only be hypothesized, but need to be confirmed and (2) the number of investigated patients was limited, which may have caused limited generalizability.

Gut microbiota have recently become an exciting new topic due to the important roles played in digestion, metabolism and immune function [32], and gut microbial profile is altered in patients with CKD. Dysbiosis in CKD has been touted to be due to slow intestinal transit time, iron therapy, decreased dietary fiber intake and frequent use of proton pump inhibitors and antibiotics. Abundant evidence indicates that gut microbiome is the source of a number of retention solutes in patients with CKD [33]. Gut microbiota can metabolize certain substrates to form TMAO, the kidneys filter out TMAO from the blood and excrete it through the urine in order to maintain low levels of circulating TMAO. Previous studies have indicated that interventions that target the intestinal flora could affect the amount of gut-derived uremic toxins such as *p*-cresyl sulfate and indoxyl sulfate. Lin *et al.* also demonstrated that in hemodialysis patients, PEW assessed with the subjective global assessment was associated with gut dysbiosis [34]. Based on these findings, future therapies for PEW in CKD patients could specifically target the intestinal microbiota that plays a role in TMAO formation.

Conclusions

To the best of our knowledge, this is the first study to evaluate the association between the prevalence of PEW and serum TMAO levels in MHD patients. Our study shows that the serum TMAO level is correlated with CKD-associated PEW, but the mechanism of PEW in CKD is still unclear, especially with regard to the TMAO-specific mechanisms. Since the limitation of cross-sectional study, we could not give causal relationship between TMAO and PEW in MHD patients yet. However, the findings indeed give us full of hope that TMAO might be a potential candidate which is associated with PEW in MHD. Based on the background, we put forward a possible research direction that whether we could reduce the TMAO production by regulating

intestinal flora in order to meliorate or prevent PEW. Nonetheless, these findings shed new light on the pathogenesis of PEW and present a new target for its clinical prevention and treatment.

Author contribution

W.D. and Y.Z. designed the research; C.H., Y.Z., X.B., and L.Y. performed the experiments; W.D. and C.H. analyzed the data; C.H. and Y.Z. prepared figures and wrote the article. W.D. and Y.Z. revised the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

The datasets used/analyzed during the current study are available from the corresponding author on reasonable request.

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