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Investigation of *N,N,N*-Trimethyl-L-alanyl-L-proline Betaine (TMAP) as a Biomarker of Kidney Function

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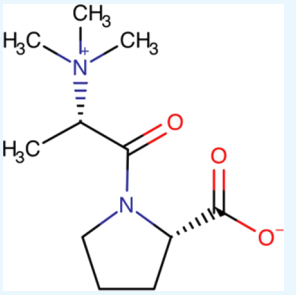
ABSTRACT: Glomerular filtration rate (GFR) is the most widely used tool for the measurement of kidney function, but endogenous biomarkers such as cystatin C and creatinine have limitations. A previous metabolomic study revealed *N,N,N*-trimethyl-L-alanyl-L-proline betaine (TMAP) to be reflective of kidney function. In this study, we developed a quantitative LCMS assay for the measurement of TMAP and evaluated TMAP as a biomarker of GFR. An assay to measure TMAP was developed using liquid chromatography-mass spectrometry. After validation of the method, we applied it to plasma samples from three distinct kidney disease patient cohorts: nondialysis chronic kidney disease (CKD) patients, patients receiving peritoneal and hemodialysis, and living kidney donors. We investigated whether TMAP was conserved in other mammalian and nonmammalian species, by analyzing plasma samples from Wistar rats with diet-induced CKD and searching for putative matches to the *m/z* for TMAP and its known fragments in the raw sample data repository "Metabolomics Workbench". The assay can measure plasma TMAP at a lower limit of quantitation (100 ng/mL) with an interday precision and accuracy of 12.8 and 12.1%, respectively. In all three patient cohorts, TMAP concentrations are significantly higher in patients with CKD than in controls with a normal GFR. Further, TMAP concentrations are also elevated in rats with CKD and TMAP is present in the sap produced from *Acer saccharum* trees. TMAP concentration is inversely related to GFR suggesting that it is a marker of kidney function. TMAP is present in nonmammalian species suggesting that it is part of a biologically conserved process.



INTRODUCTION

Chronic kidney disease (CKD) is characterized by a persistent decrease in kidney function for a minimum of 3 months, often associated with albuminuria.[1] Direct measurement of the glomerular filtration rate (GFR) is the most widely accepted index of kidney function, especially if measured using inulin clearance. Determination of measured GFR (mGFR) requires injection of an exogenous marker and timed blood and/or urine collection, thus not being routinely performed.[2] Estimated GFR (eGFR) is a proxy measurement of kidney function, relying on the measurement of an endogenous biomarker in serum.

Until recently, the standard for GFR estimation was based on the measurement of a single filtration marker, serum creatinine, and its input into an equation adjusting for age, race, and sex.[1] Recent studies present novel estimating equations which exclude race and include measurement cystatin C.[3] The limitations associated with creatinine-based GFR have been well documented.[4] In general, serum creatinine levels fail to indicate functional decline until approximately half of GFR has been lost,[5] so its use as a test of kidney function fails to detect early kidney disease. Cystatin C has improved creatinine-based GFR estimation,[6] and its inclusion improves prognostic capability for all-cause mortality, even in the case of normal range creatinine-based eGFR.[7] However, non-GFR determinants such as changes in muscle mass for creatinine and inflammation or hyperthyroidism for cystatin C limit the ubiquitous use of either marker.[8,9] Therefore, there is a need for a panel of biomarkers to provide a better estimate of true GFR.[7]

Several metabolite biomarkers have been identified as informative of CKD progression to end-stage kidney disease (ESKD), as well as being predictive of morbidity and mortality.[10,11] A metabolomics study recently identified the metabolite *N,N,N*-trimethyl-L-alanyl-L-proline betaine (TMAP) to be a candidate biomarker of kidney function.[12] This was the first study to demonstrate that TMAP is a potential biomarker of kidney filtration.[11]

The objective of this study was to evaluate TMAP as a biomarker of kidney function. We optimized a previously published TMAP synthesis protocol,[13] developed, and validated a method for quantitation of TMAP in human plasma and applied the assay to three cohorts of patients across

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Table 1. Patient Demographics by Cohort[a]

cohort	patient group	number	age (years)	sex	body mass index (BMI)	serum creatinine (μM)	CKD-EPI eGFR (mL/min/1.73 m^2)
LHSC cohort	control	8	52 (34−66)	3M, 5F	25.1 (21.2−28.6)	69 (57−87)	95 (70−111)
	CKD	23	60 (25−87)	11M, 12F	25.7 (17.3−33.3)	418 (120−1017)	18 (4−54)
	PD	8	70 (41−86)	3M, 5F	28.3 (26.0−34.2)	684 (240−989)	6 (3−15)
	HD	9	65 (46−87)	3M, 6F	28.6 (22.4−33.3)	703 (199−823)	0
RDH cohort	RV	25	57 (42−72)	20M, 5F	28.7 (24.7−32.7)	163 (95−233)	41 (18−68)
	YR1	19	58 (43−73)	15M, 4F	28.4 (24.4−32.4)	160 (83−241)	43 (23−80)
living kidney donor cohort	control RV	10	38 (22−62)	3M, 7F	27.1 (21.5−40.0)	77 (60−103)	95 (81−112)
	control YR1		39 (23−63)			76 (60−110)	96 (74−111)
	donor RV	10	47 (32−60)	1M, 9F	27.1 (20.6−33.7)	75 (65−91)	87 (68−109)
	donor YR1		48 (33−61)			102 (77−134)	60 (48−77)

[a]Characteristics are presented as mean with a range of values. CKD, chronic kidney disease; HD, hemodialysis; LHSC, London Health Science Center; PD, peritoneal dialysis; RDH, Royal Derby Hospital; RV, recruitment visit; YR1, 1 year follow-up.

various stages of the disease. Finally, we put forth the hypothesis that TMAP may be indicative of a highly conserved evolutionary process, as it was also quantitated in plasma samples from a CKD rat model, and its presence was confirmed in tree xylem samples.

MATERIALS AND METHODS

Synthesis. TMAP synthesis was optimized from a previously published protocol[13] to increase product purity. To limit a side reaction forming a TMAP methyl ester, half the volume of iodomethane (and d3-iodomethane for d9-TMAP production) was added into the reaction vessel with L-alanyl-L-proline and silver oxide, and the reaction vessel was incubated on ice overnight. Water (1.0 mL) was added to the residue that remained after evaporation and the product underwent solid phase extraction (SPE) with a Strata reverse phase C18-T (55 μm, 140 Å) column (Phenomenex). Following SPE, the purified mixture was transferred into a 15 mL conical tube and partially sealed. The sample underwent 3 rounds of overnight lyophilization at −60 °C and a vacuum of 200 mTorr. Identity of the product was confirmed via proton NMR analysis.

Sample Preparation. Standards were prepared in human double charcoal stripped plasma (BioIVT LLC) by the addition of TMAP from a 50 μg/mL aqueous stock solution. Standards were prepared at 0, 25, 50, 100, 300, 500, 1000, 1500, 2000, 2500, and 3000 ng/mL. Quality control (QC) samples were prepared at 100, 1000, and 2000 ng/mL. Ice-cold acetonitrile (150 μL) containing d9-TMAP (200 ng/mL) as an internal standard was added to the samples (50 μL) to precipitate plasma proteins. Samples were incubated at −20 °C for 20 min and centrifuged at 14,000 g for 10 min. The clear supernatant was transferred to a glass tube and evaporated to dryness. The supernatant was reconstituted to half its volume (60 μL) in Nanopure water and analyzed by liquid chromatography-mass spectrometry (LCMS).

Liquid Chromatography-Mass Spectrometry. Using a Waters Acquity I-class UPLC system (Milford, Massachusetts, USA), 10 μL of the sample was injected onto a Waters HSS T3 column (1.8 μm particle size, 100 × 2.1 mm) maintained at 45 °C, and a flow rate of 0.45 mL/min. Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. The gradient consisted of 0% solvent B to 30% B over 0.4 min, followed by 30% solvent B to 80% solvent B over 0.6 min, and held at 80% B for 1 min.

Samples were analyzed using a Waters Xevo-G2S Quadrupole-Time of Flight (QTOF) mass spectrometer with electrospray ionization. The source and desolvation temperatures were maintained at 150 and 500 °C, respectively throughout analyses. Cone and desolvation gases were 50 and 1000 L/h, respectively. Capillary and cone voltage were maintained at 2 kV and 40 V, respectively. Experiments were conducted in positive ionization mode and analyzer mode was set to resolution. Ions in the mass range 50−1200 Da were acquired at a scan time of 0.1 s, with target enhancement at m/z 229.1549. The reference lockmass was leucine-enkephalin (1 ng/μL).

Interday accuracy and precision of the assay were based on the mean of four replicate samples for each QC (100, 1000, and 2000 ng/mL), over 6 different days, for a total of n = 24 at each QC level. Intraday accuracy and precision were based on the mean of 4 replicates for each QC level in 1 day. QC samples were prepared the same day as analysis. Precision was calculated as percent relative standard deviation (% RSD), and accuracy was calculated as percent error.

Clinical Study Participants. The assay was applied to three distinct cohorts of adult human plasma samples (Table 1). The first cohort from the London Health Sciences Center (LHSC cohort) was recruited from the Southwestern Ontario Regional Renal Program between 2010 and 2015 and was comprised of normal kidney function (n = 8), nondialysis-dependent CKD (n = 23), and ESKD patients receiving standard thrice-weekly dialysis treatment via peritoneal dialysis (n = 8) and hemodialysis (n = 9), as previously described.[12,14] The Royal Derby Hospital (RDH) cohort was recruited between 2015 and 2016 as part of the Renal Magnetic Resonance Feasibility in Renal Disease (REMIND) study (NCT03578523) at the University of Nottingham.[15] This cohort was comprised of 25 CKD patients with mGFR determined by iohexol clearance at a recruitment visit, with 19 patients undergoing repeated sampling 1 year later. The living kidney donor cohort was recruited from LHSC[12] and included control patients with healthy kidney function (n = 10) and living kidney donors (n = 9). Patients attended a recruitment visit where blood samples were collected. Nondonor controls returned 1 year later to provide additional blood samples. Living kidney donors returned 1 year following kidney donation to provide a blood sample.

All study protocols were approved by the Health Sciences Research Ethics Board at Western University or East Midlands Research Ethics Committee at RDH and subjects provided

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written informed consent. Where enough plasma remained, cystatin C was quantitated by particle-enhanced turbidimetric immunoassay (PETIA) using the Roche Diagnostics Tina-quant Cystatin C Generation 2 assay by the Clinical Biochemistry Laboratory at LHSC-Victoria Campus.

Animal Model of Kidney Disease. Animal studies were approved by Western University's animal care committee and conform to the standards of the Canadian Council on Animal Care. The TMAP assay was applied to plasma samples from a rat model of CKD, induced via supplementation of adenine in the diet. Male Wistar rats (250 grams) were randomly divided into control, and two levels of CKD (n = 8 in each group, n = 24 in total). Animals in the two CKD groups received a diet supplemented with either 0.5 or 0.7% adenine for 5 weeks, followed by 3 weeks of control diet, as previously described.[16] Control animals were pair-fed standard rat chow.

Data Processing and Statistical Analyses. A standard curve was generated by subtracting the baseline TMAP in blank charcoal-stripped plasma from each standard. Each nominal standard concentration is correlated to its respective relative response. The relative response is calculated as the TMAP peak area divided by the d9-TMAP peak area.

Comparison of TMAP concentrations in the LHSC cohort (control, CKD, PD, HD) was conducted via a multiple comparisons Kruskal–Wallis one-way ANOVA (α = 0.05), with Dunn's post hoc test. A linear regression model was used to test the relationship between eGFR with inversely transformed TMAP, creatinine, and cystatin C concentrations. For the RDH Cohort linear regression model analysis, recruitment visit and year 1 measurements for TMAP, creatinine, and cystatin C were treated as unpaired measurements, allowing for the inclusion of samples from patients that did not return for repeated measurements. In the living kidney donor cohort, a paired t-test was conducted to analyze differences in TMAP concentration from recruitment visit to the visit 1 year later (α = 0.05).

For rat plasma samples, a comparison of TMAP concentrations among the experimental groups was conducted via a multiple comparisons one-way ANOVA (α = 0.05), with Tukey's post hoc test.

MS Database Search for TMAP in Nonmammalian Samples. MS databases for nonmammalian samples on "The Metabolomics Workbench, https://www.metabolomicsworkbench.org/" were searched for TMAP and its fragments[12] by m/z = 70.0651 and 142.0863 ±5 ppm mass error. Putative matches were confirmed with an analytical standard where possible. Namely, fresh xylem samples from both *Acer saccharum* and a related tree *Acer rubrum* were prepared by diluting sap (200 μL) twofold with 30 mM ammonium formate, 0.15% formic acid in acetonitrile. Samples were vortexed, centrifuged at 10,000 rcf for 10 min at 4 °C to pellet precipitated material, and supernatant (200 μL) was transferred to an HPLC vial. Samples were analyzed using a 1290 Agilent HPLC coupled to a Thermo Q-Exactive Orbitrap mass spectrometer. Sample (5 μL) was injected onto a Zorbax Eclipse Plus RRHD C18 column maintained at 35 °C (2.1 × 50 mm, 1.8 μm; Agilent). Mobile phase A (0.1% formic acid in water) began at 100% and was held for 1.25 min. Mobile phase B (0.1% formic acid in acetonitrile) was then increased to 50% over 1.75 min, and 100% over 0.5 min. Mobile phase B was maintained at 100% for 1.5 min and returned to 0% over 0.5 min.

The samples were analyzed by MS/MS at a resolution of 17.5 k, automatic gain control of 5 × 10^6, max injection time of 64 ms, isolation window of 1.2 m/z units, and normalized collision energies of 20, 30, 40, 50 NCE.

RESULTS

TMAP Synthesis. Following the previously published protocol[11,12] with described modifications, synthesis was scaled up twofold to yield 13.1 mg of pure TMAP. After lyophilization, the product had a crystallized powder appearance. Identity of the substance was confirmed to be TMAP via proton NMR (Supplemental Figure 1).

Assay Performance. TMAP and d_9TMAP formed a single peak at a retention time of 1.06 min (Supplemental Figure 2A). TMAP had m/z = 229.1552 and d_9TMAP had m/z = 238.2120. The standard curve was generated with 11 data points ranging in concentration from 0 to 3000 ng/mL. The curve shows linearity in this range, with a coefficient of determination r^2 = 0.993 (Supplemental Figure 2B).

The intra-day accuracy was 8.3, 7.0, and 7.8% and the intraday precision was 8.1, 9.4, and 9.9% for low, middle, and high QCs, respectively. Interday accuracy for low, middle, and high QC levels were 12.1, 7.0, and 8.7%, respectively, and interday precision was 12.8, 7.5, and 10.8% for the same QC levels.

TMAP Concentration in Study Participants. Mean TMAP concentrations for control, CKD, PD, and HD patients from the LHSC cohort were 146 ±21.7, 1045 ±712, 1703 ±572, and 1766 ±735 ng/mL, respectively (Figure 1A). There

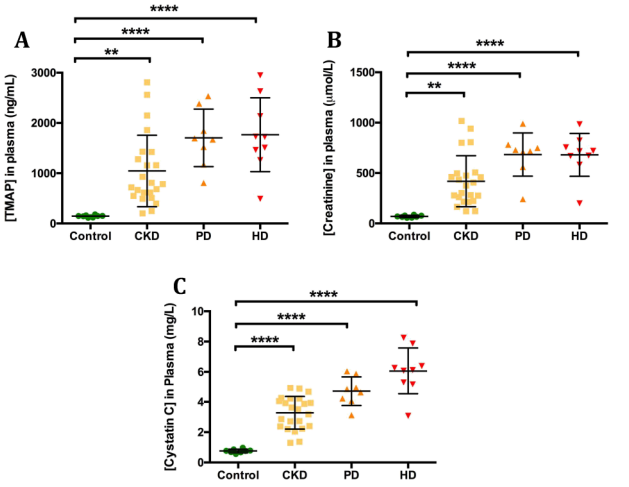

Figure 1. Concentration of TMAP (ng/mL), creatinine (μmol/L), and cystatin C (mg/L) in human control, CKD, and ESKD patients from LHSC. (A and B) ** p < 0.01, *** p < 0.001 for multiple comparisons one-way Kruskal–Wallis ANOVA with Dunn's post-hoc test. (C) ****p < 0.0001 for multiple comparisons one-way ANOVA with Tukey's post-hoc test. Error bars represent mean ± SD.

was a significant difference in plasma TMAP between controls compared to CKD (p < 0.01) and dialysis modalities (p < 0.001). TMAP concentrations were inversely transformed and plotted against the patients' eGFR. The linear equation used to model the relationship between eGFR and TMAP concentrations had r^2 = 0.9464 (Figure 2A).

For the RDH cohort, the mean GFR values at the recruitment visit were 43.79 and 43.74 mL/min/1.73 m^2 1 year later (p = 0.9671). The mean TMAP concentration for

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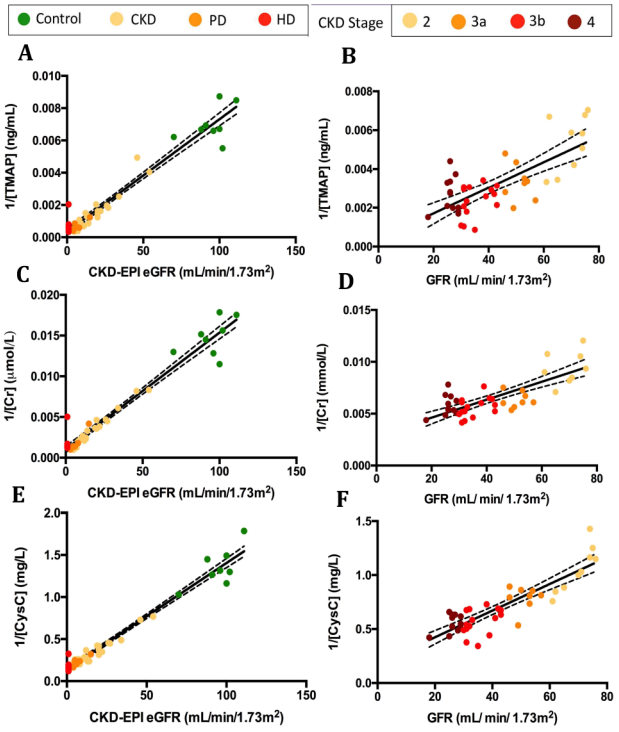

Figure 2. Linear regression for inversely transformed TMAP (A, B), creatinine (C, D), and cystatin C (E, F) measurements against eGFR for the LHSC and mGFR for the RDH cohort, respectively, has a coefficient of correlation r^2 = 0.9464 (A) and r^2 = 0.5308 (B), r^2 = 0.9526 (C) and r^2 = 0.6087 (D), r^2 = 0.9692 (E) and r^2 = 0.7506 (F), respectively. Dashed lines represent the 95% confidence interval.

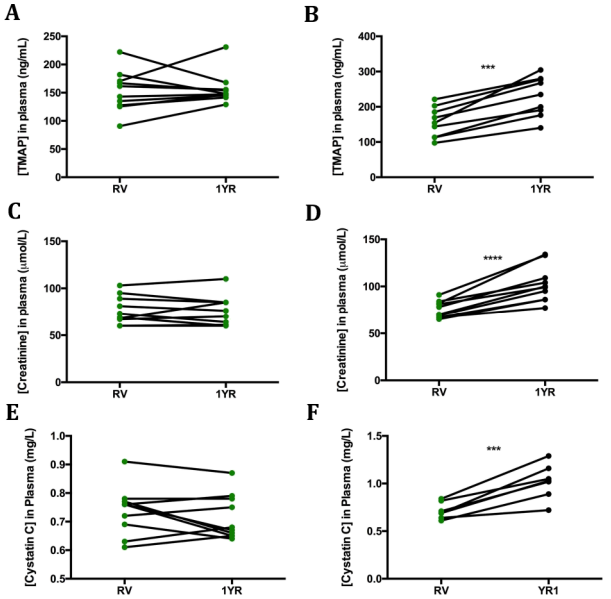

Figure 3. TMAP (A, B), creatinine (C, D), and cystatin C (E and F) concentrations in plasma from recruitment visit to 1 year later in control patients with normal GFR (A, C, E) and living kidney donors (D, E, F). A paired t-test shows a significant difference between timepoints for creatinine (n = 10), TMAP (n = 9), and cystatin C (n = 7) concentrations in living kidney donors ***p < 0.001, ****p < 0.0001.

the recruitment visit and year 1 visit was 361 ±188 and 360 ±188 ng/mL, respectively (p = 0.9560). The linear equation used to model the relationship between patient eGFR and TMAP concentrations had r^2 = 0.5308 (Figure 2B).

For the living kidney donor cohort, control patients had mean TMAP plasma concentration at the recruitment visit and 1 year later of 153.0 ±36.4 and 157.0 ±28.0 ng/mL, respectively (p = 0.7100) (Figure 3A). Kidney donors had a mean TMAP plasma concentration of 156.0 ±42.9 ng/mL at recruitment visit, prior to kidney donation. One year following kidney donation, these patients had a mean TMAP plasma concentration of 230.0 ±56.1 ng/mL (p < 0.0001) (Figure 3B).

Creatinine and Cystatin C Concentrations in Study Participants. Mean creatinine concentrations for control (n = 8), CKD (n = 23), PD (n = 8), and HD (n = 9) patient groups from the first LHSC cohort were 69.3 ± 10.8, 418.0 ± 253.0, 684.0 ±215.0, and 680.0 ±213.0 μmol/L. There was a significant difference in concentration between control and CKD groups (p < 0.01), as well as between control to PD and HD groups (p < 0.0001) (Figure 1B). Cystatin C concentrations were determined for the same patient plasma samples, where a sufficient sample was available. Mean cystatin C concentrations for the patient groups in this cohort were 0.76 ±0.13, 3.28 ±1.08, 4.72 ±0.96, and 6.05 ±1.52 mg/L respectively. There was a significant difference (p < 0.05) when comparing CKD and PD groups (Figure 1C). There was also a significant difference (p < 0.0001) in mean concentrations between control and every other patient group, as well as between CKD and HD groups. Linear regression was used to model the relationship between eGFR and inversely transformed creatinine and cystatin C concentrations, which had an r^2 = 0.9526 (Figure 2C) and r^2 = 0.9692 (Figure 2E), respectively.

For the RDH cohort, the mean creatinine concentrations at the recruitment visit and 1 year later were 156.0 ±30.4 and 160.0 ±41.9 μmol/L (p < 0.4762), respectively, and the mean cystatin C concentrations were 1.52 ±0.48 and 1.48 ±0.39 mg/L, respectively (p < 0.4844). The linear regression fit to model the relationship between mGFR and inversely transformed creatinine and cystatin C concentrations had r^2 = 0.6087 (Figure 2D) and r^2 = 0.7506 (Figure 2F), respectively.

For the living kidney donor cohort, control patients had mean creatinine plasma concentration at the recruitment visit and 1 year later of 76.5 ±14.9 and 75.6 ±16.0 μmol/L, respectively (p = 0.7440) (Figure 3C). Kidney donors had a mean creatinine concentration of 75.4 ±8.9 μmol/L at the recruitment visit and 1 year later, after kidney donation, the concentration was 102.0 ±19.0 μmol/L (p < 0.0001) (Figure 3D). For cystatin C, plasma concentrations at the recruitment visit and 1 year later were 0.74 ±0.08 and 0.71 ±0.08 mg/L (p < 0.2431), respectively, for controls (Figure 3E), and 0.71 ±0.09 and 1.02 ±0.18 mg/L (p = 0.0009), respectively, for kidney donors (Figure 3F).

TMAP Concentration in a Rat Model of CKD. Mean TMAP concentration for control, 0.5% adenine CKD, and 0.7% adenine CKD rat groups were 92.7 ± 22.3, 195.0 ±80.2, and 516.0 ±234.0 ng/mL, respectively (Figure 4). A significant difference in mean TMAP was detected between the control and 0.7% adenine CKD groups (p < 0.001) and between the 0.5 and 0.7% adenine CKD groups (p < 0.0001).

MS Database Search for TMAP in Nonmammalian Samples. TMAP was putatively detected in deposited *A. saccharum* datasets of project ST001435 in metabolomics workbench, (https://www.metabolomicsworkbench.org/).

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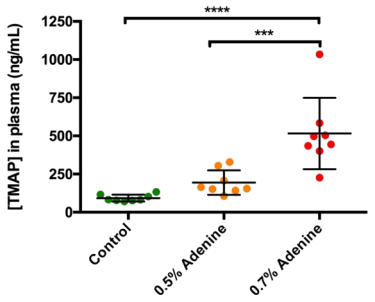

Figure 4. Concentration of TMAP (ng/mL) in male Wistar rats with adenine-induced CKD, in comparison to controls. Control (n = 8), 0.5% adenine CKD (n = 8), and 0.7% adenine CKD (n = 8). Error bars represent mean ± SD. ***p < 0.001, ****p < 0.0001 for multiple comparisons one-way ANOVA with Tukey's post-hoc test.

The retention time of the putative TMAP in the sap was compared to an authentic TMAP standard, as was the product ion spectra showing excellent matches in both cases (Figure 5). Another putative match was identified for the potential presence of TMAP in soybean root, based on MS/MS data,[17] although not confirmed by analytical standard.

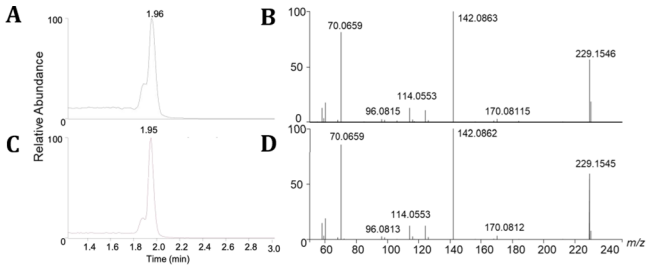

Figure 5. Extracted ion chromatogram of 142.0862 (5 ppm) from MS/MS of 229.1 at 50 NCE for a 5 μg/mL TMAP standard (A) and a sap extract from *A. saccharum* (C). The product ion spectra of the TMAP standard (B) and a sap extract from *A. saccharum* (D) show the presence of additional product ions at the same relative ion ratios supporting that TMAP is present within this plant-based sample.

DISCUSSION

This study demonstrates that TMAP is a biomarker of kidney function, which has not previously been described. We present a strategy for improved TMAP synthesis, developed an LCMS assay for the quantitation of TMAP in plasma, and applied this method to plasma samples from three distinct patient cohorts. We compared TMAP to creatinine and cystatin C concentrations within the same samples to explore its potential utility, advantages, and limitations. Further, TMAP was measured in a rodent model of CKD, to seek its presence in other mammalian species and examine changes in circulating levels in models of kidney damage. Last, we discovered that TMAP is present in plants, namely, *A. saccharum*. The presence of TMAP in this species suggests that it may play a role in a highly conserved biological process.

We report an accurate and reproducible method to measure TMAP in human plasma. Accuracy and precision of the assay were determined through the 6-time repeated measurement of each level of QC standards (low, middle, and high). For the purposes of TMAP quantitation in adults, where control patients consistently have measurements above 100 ng/mL, an LLOQ QC level was omitted. However, this level may become warranted in the analysis of pediatric or neonatal patient samples if concentrations are lower than adults. Since the accuracy and precision of our assay were less than 15% of the nominal concentration, these parameters are acceptable by FDA bioanalytical method validation standards.

We applied our plasma TMAP assay to three distinct cohorts of kidney disease to characterize the robustness of TMAP as a biomarker of reduced kidney function in various clinical settings. The LHSC cohort included normal kidney function control patients, nondialysis CKD patients, and two groups of ESKD patients: PD and HD treatment groups. All patients receiving PD retained some residual renal filtration, whereas HD patients were anuric. Since known kidney biomarkers have an inverse relationship with GFR, we evaluated the relationship between TMAP with eGFR. We observed increasing concentrations of TMAP with reduced renal filtration, similar to established kidney biomarkers creatinine and cystatin C. Creatinine, TMAP, and cystatin C concentrations were all significantly greater in CKD, PD, and HD patient groups when compared to controls. When inversely transformed concentrations are plotted against eGFR, a near identical pattern is observed: creatinine, cystatin C, and TMAP values are highly correlated to eGFR and show excellent separation between control and CKD patient groups. Quantitation of TMAP in this cohort reveals some larger interindividual variability than creatinine and cystatin C measurements. While it is unclear what the cause of this variability is, it highlights that TMAP concentrations in patients with normal kidney function are consistently low, and impaired kidney function results in profound elevation of plasma TMAP. Future studies investigating the pharmacokinetics of TMAP will be required to provide insight into why TMAP appears to have higher variability in patients with kidney disease than in some other kidney function markers.

The RDH cohort validated our findings of TMAP as a kidney function biomarker in a distinct patient cohort. Similar to the LHSC cohort, there was a clear positive correlation between GFR and inverse plasma TMAP levels. The patients in the RDH cohort had GFR measured by iohexol clearance at a recruitment visit and again 1 year later. Measured GFR in these patients did not differ 1 year following the recruitment visit. Similarly, plasma TMAP, serum creatinine, and cystatin C did not differ in this time frame. This is further evidence of the utility of TMAP as a novel biomarker of GFR. When inverse TMAP, creatinine, and cystatin C were evaluated using linear regression models, cystatin C showed the best correlation with mGFR although TMAP and creatinine were also correlated with mGFR.

The living kidney donor cohort was comprised of normal GFR control patients and prospective living kidney donors. Notably, kidney donors had measurements taken before kidney donation, and 1 year post kidney donation. Control patients showed no significant difference in kidney function over 1 year, whereas a significant increase in creatinine, TMAP, and cystatin C concentrations was measured when comparing patient concentrations before and after kidney donation. TMAP consistently reflects the same trends as validated markers of GFR.

Studies in rodents are often used to model clinical conditions such as CKD or acute kidney injury. We induced CKD using the dietary adenine model in Wistar rats.[16] Analysis of rat plasma revealed a significant increase in TMAP concentration between control and 0.7% adenine diet CKD

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rats and between 0.5 and 0.7% adenine diet CKD rats. These data show that the production of TMAP in nonhuman mammals is also reflective of kidney function, suggesting it is a robust marker.

The presence of TMAP in both human and rat plasma led to the inquiry into its presence in nonmammalian species. To investigate the possible presence of TMAP in other species, mass spectrometry-based datasets deposited into metabolomics repositories were queried for the mass of intact TMAP. When possible, datasets with MS/MS information were evaluated for characteristic TMAP fragments (m/z = 70.0651 and 142.0863) to make a putative match. The m/z = 70.0651 fragment could also correspond to a proline immonium ion, as is the case in the fragmentation of the dipeptide leucyl-proline. Leucyl-proline is isobaric to TMAP and also has an m/z = 229.1547; therefore, its fragment m/z = 86.0970 was also searched to ensure a putative match of native TMAP was not leucyl-proline.

In higher plant species with xylem and phloem vascular systems, nitrogen metabolism yields amino acid metabolites.[18] These amino acids contribute to key metabolic pathways, generating peptide metabolites.[19] To determine if TMAP was present in plants, we investigated sap samples from *A. saccharum*, commonly known as the sugar maple tree, as well as the related red maple tree, *A. rubrum*. Samples were collected from trees over the entire production season in 2019 by members of the Ontario Maple Syrup Producers Association, as previously described.[20] The presence of TMAP in xylem samples was confirmed and suggests that TMAP is highly conserved. While the biological function of TMAP is unknown, levels increased toward the end of the maple syrup-producing season. It has been well documented that an increase in proline biosynthesis occurs in response to both biotic and abiotic stresses.[21] Therefore, it is possible that TMAP is produced in response to stress although this requires further experimentation.

While our study describes the potential utility of TMAP as a biomarker of kidney function for the first time, there are some important limitations. While our LC-MS method yielded accurate and precise determination of TMAP concentrations in human plasma, a more detailed validation would be required should TMAP become of interest for routine diagnostics. For example, the stability (long-term storage, freeze-thaw stability) of TMAP would need to be assessed and increasing the QC concentrations from three to four levels to meet the guidelines set forth by the FDA and EMEA would be necessary. In addition, should TMAP be used for routine laboratory diagnostics, LC−MS would likely not be the ideal method for high throughput analysis. In the context of further exploring the function and relevance of TMAP experimentally, our assay was validated with human plasma and future studies should examine matrix effects of other fluids such as urine and rodent tissue and biofluids.

Collectively, our study demonstrates that TMAP is a robust and reproducible marker of reduced kidney function. The most common method of estimating kidney function is to use serum creatinine in eGFR equations. Creatinine undergoes significant secretion resulting in the overestimation of renal filtration. The ideal filtration marker is freely filtered at the glomerulus, does not undergo extrarenal clearance, is not metabolized by the kidney, and does not undergo secretion or reabsorption.[2] Although TMAP correlates with GFR, it is currently unknown whether it is secreted or reabsorbed. A broader understanding of the pharmacokinetics of TMAP is required to fully determine its suitability as a kidney function biomarker. These studies should include the determination of TMAP clearance, fractional excretion, tubular transport, and plasma protein binding. Previous metabolomic work conducted in our laboratory shows that TMAP undergoes high dialytic clearance,[12] suggesting low plasma protein binding. Beyond this, the pharmacokinetic properties of TMAP remain to be elucidated. It is likely that a panel of metabolites will more accurately estimate eGFR than a single analyte. TMAP may carry utility in a panel of metabolites to improve estimations of GFR. A fascinating finding in our study is the conservation of TMAP in plant species. This suggests that TMAP plays an important biological role that is conserved across species. Investigation into the biological role of TMAP in plants and animals is required to elucidate its potential relevance in the development of disease and response to stress.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c00153.

Proton NMR spectrum of synthesized TMAP; TMAP chromatogram and standard curve (PDF)

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Author Contributions

N.A.S. and B.L.U. conceived the study. T.J.V. identified the TMAP compound, and G.A.L. and N.A.S. synthesized the compound. N.A.S. performed the experiments, analyzed the data, and drafted the initial version of the manuscript. A.A.H., M.A.W., B.K.A.T., A.X.G., R.N., N.S., and M.T. are responsible for patient recruitment and sample collection of respective studies. M.J.K. is responsible for all measurements of sample cystatin C. J.B.R. and T.M. identified and confirmed TMAP in plant species samples. G.A.L., G.F., and R.G.T. provided guidance and advice throughout the study. All authors have reviewed, edited, and given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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