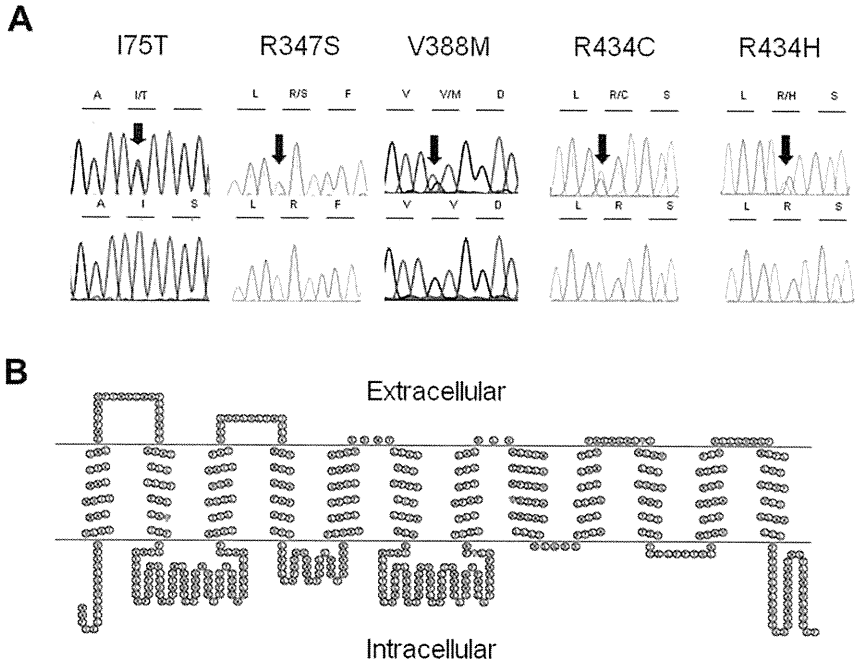

Figure 1. Identification of mutations within SLC22A12 encoding URAT1. A. Chromatograms showing sequence data and translated amino acids. These demonstrate heterozygous missense variants (above, arrowed) and normal controls (below). B. SLC22A12 encodes URAT1, a 553 amino acid protein with a predicted 12 Transmembrane domains (TMPred). It has an intracellular N- and C- terminus. Amino acid residues implicated in hypouricaemia and modelled in the present study are highlighted in red and include Isoleucine, I at position 75, arginine, R at position 347, Valine V at position 388 and Arginine, R at position 434.
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31%) and hyperphosphaturia (FE_{PO4} varied between 21–33%). Ultrasound examination revealed bilateral nephrocalcinosis and a solitary cyst in the left kidney measuring 10 mm. Following alkali therapy, metabolic compensation was achieved and her growth pattern improved. Following correction of the metabolic acidosis, the proximal defects of aminoaciduria and low molecular proteinuria resolved. Serum electrolytes also normalized, but there was persistent hypouricaemia during the two year observational period (1.24–1.36 mg/dl). Since the hypouricaemia was associated with an elevated FE_{urate}, we undertook mutational analysis of *SLC22A12* which revealed a heterozygous missense mutation, leading to amino acid change R434C. The mother of patient SK-3 (R434C) was also heterozygous for the R434C variant in *SLC22A12*. Biochemical evaluation confirmed she had a low normal serum uric acid level (2.3 mg/dl) with fractional excretion of urate of 19.3%. She had a normal renal ultrasound, but a past medical history of renal colic and passage of a single calculus 10 years ago.

Patient SK-4: This 8 year old boy presented with recurrent attacks of visible hematuria and sensorineural hearing loss. A percutaneous renal biopsy was performed which demonstrated minimal glomerular abnormalities on light microscopy. Immunofluorescence studies did not reveal any immune deposits and electron microscopic analysis was not available. Given a clinical suspicion of Alport syndrome, genetic studies were performed, identifying a mutation in the *COL4A5* gene. Following another attack of visible hematuria with very severe colicky pain, serum biochemistry revealed a low uric acid level. Repeated examinations of the uric acid confirmed persistent hypouricaemia, with high FE_{urate}. Mutational analysis of *SLC22A12* revealed a heterozygous missense mutation, leading to the amino acid change R347S. Genetic analysis confirmed that the mother of patient SK-4 was also heterozygous for the sequence variant R347S.

Patient SK-5: A 7 year old female presented with vitiligo, prompting a screen for autoimmune diseases. She was found to have compensated hypothyroidism, with typical ultrasound changes of the thyroid gland and increased antithyroid antibodies, suggesting Hashimoto thyroiditis. She was also found to have hypouricaemia with significant hyperuricosuria. She underwent mutation analysis of *SLC22A12* which revealed a heterozygous missense mutation, p.R434H.

Patient NC-1: A 41 year old female presented with recurrent renal colic, with 2 episodes in less than 12 months. She received lithotripsy treatment for a left renal calculus (struvite stone). Her past medical history was noteworthy for type 1 diabetes mellitus, since 10 years of age, complicated by peripheral neuropathy and urinary tract infections. She also had treated hypothyroidism. At presentation, serum electrolyte abnormalities revealed a borderline low uric acid level and a serum creatinine of 95 μmol/L. A FE_{urate} was transiently raised at 16%. Repeat serum biochemistry revealed a serum uric acid level of 3.86 mg/dl and a normalised FE_{urate}. Following successful lithotripsy she has remained stone free for 5 years, following advice regarding increased fluid intake. Genetic analysis of *SLC22A12* revealed a heterozygous missense mutation, p.V388M.

Patient NC-2: A 45 year old lady presented to the regional lithotripsy unit for treatment. She had recurrent renal stone disease (calcium oxalate) for 7 years, requiring both lithotripsy and a right pyeloplasty. She was on no medications. Serum electrolytes revealed a normal serum creatinine (75 μmol/l) with a low serum uric acid (2.01mg/dl). FE_{urate} was not available for this patient. We performed genetic analysis of *SLC22A12*, which revealed a heterozygous missense mutation, p.I75T.

In silico analysis of mutations

Following the identification of variants within the *SLC22A12* gene encoding URAT1 (Figure 1A,B) we used online databases

and mutation prediction software to attempt to score the pathogenicity of each variant. Within the URAT1 amino acid sequence, residues R347, V388 and R434 are highly conserved, whilst I75 is not well conserved (Table 2). SIFT and Snps3d analysis predicted all 5 sequence variants to be either not tolerated (SIFT) or deleterious (Snps3D). Polyphen analysis predicted that 3 out of the 5 missense changes to be "probably damaging", with V388M and I75T predicted to be benign (Table 2).

Functional studies of URAT1 variants

Using mammalian cells, we evaluated the urate transport function of URAT1 sequence variants found in this study. Urate uptake was measured in transiently transfected HEK293 cells, comparing wild-type transporter activity to sequence variants I75T, R347S, R434C and R434H.

Following transient transfection of HEK293 cells with FLAG-tagged URAT1 constructs, we demonstrate strong plasma membrane expression in wild-type URAT1 (Figure 2A).

Plasma membrane expression levels of variants R434C and R434H were low whereas the intracellular localization was not strongly observed, possibly due to the stability of protein. For variant I75T the partial membrane expression was observed together with partial intracellular localization. For variants R347S and V388M strong plasma membrane expression, similar to wild-type levels was observed (Figure 2A).

Measurement of urate uptake in HEK293 cells demonstrates a significant reduction of urate transport function by I75T, R347S, R434C and R434H variants of URAT1 but not in V388M variant (Figure 2B).

Discussion

Idiopathic renal hypouricaemia is a disorder that has been characterized previously in patients from Far Eastern countries including Japan, Korea and China. The disease may be completely asymptomatic [23,25,26], but there are many reports, particularly from Japan, where patients present with acute kidney injury following strenuous physical activities [21,27,28,29,30,31,32,33,34]. The exact mechanism of acute kidney injury remains elusive, but it is believed that uric acid serves as an antioxidant, and in states of hypouricaemia this protective role is lost. Kaneko et al. demonstrated in a 15 year old girl with idiopathic renal hypouricaemia an oxidative imbalance soon after exercise with a predisposition to exercise-induced acute renal failure [35]. In contrast, some patients may present with more minor renal features including nephrolithiasis or hematuria [23,31,36].

In 2002, Enomoto et al. established that URAT1 transporter was responsible for tubular reabsorption of urate [3]. *SLC22A12* encodes the protein URAT1 and loss of function mutations are responsible for majority of patients with idiopathic renal hypouricaemia. The W258X variant of URAT1 is a typical mutation found in Japanese and Korean populations [23,31,37,38]. Allele frequency of W258X in the general population in Japan was found to be as high as 1.9% [39]. Heterozygous carriers of URAT1 mutations are usually asymptomatic but they may develop nephrolithiasis. Prevalence of hypouricaemia in Japan varies between 0.15% [40] and 0.23% from the analysis of serum urate levels in 1730 school children [41]. In Korea, the prevalence of hypouricaemia in healthy adults is 3.3% [42]. Therefore, school-age children who plan to performing competitive sporting activities are advised to have their serum uric acid level checked [43].

Loss of function mutations affecting URAT1 have not been previously reported in a Caucasian population. However, single case reports from European patients presenting with clinical and biochemical features of hereditary renal hypouricaemia exist [44,45,46,47]. Tzovaras et al. tested nine Greek subjects with primary renal hypouricaemia [48]. All had serum uric acid levels <2.5 mg/dl, associated with a $FE_{urate}>10\%$ and no other known cause of hypouricaemia. No definite pathogenic mutations were detected in this series and just one silent polymorphism (1246T>C) in exon 2 of the *SLC22A12* gene was noted. It is questionable why hereditary renal hypouricaemia is apparently so rare in Caucasian populations. Either the prevalence of URAT1 mutations is indeed low in this population, or a decreased awareness of this disease and its presentation outside of the Far East allows cases to go undetected. Even in Japan, there are patients who have clinical features of renal hypouricaemia but no *SLC22A12* mutations. Very recently, a genome-wide association study was performed in 6890 African Americans and 21708 European participants in order to try and identify risk alleles for elevated serum urate, associated with gout [49]. A novel URAT1 variant (G65W) was identified (rs12800450) and was associated with a reduction in serum urate of around 1.2 mg/dl per copy of the minor allele. Urate transport studies demonstrated a reduction in the urate transport for the G65W URAT1 variant [49]. This study validates heterozygous changes within URAT1 as a determinate of reduced serum urate levels. In this study the variant allele provided a protective affect against gout, but one may postulate that this may also be an at-risk allele for the hypouricaemia. Both biochemically and clinically, a single heterozygous change in URAT1 may be significant. Cheong et

Table 2. URAT1 variants and their *in silico* analysis for conservation and pathogenicity.

Missense change in URAT1	Evolutionary conservation	SNP ID (Average Hetrozygosity Index)	SIFT analysis	Snps3d analysis	PolyPhen-2 analysis
I75T	Not conserved between human, mouse and zebrafish	rs141570522 (0.001)	Predict Not Tolerated	Deleterious	Predicted to be benign
R347S	Conserved Human to Mouse	Novel variant	Predict Not Tolerated	Deleterious	Predicted to be probably damaging
V388M	Conserved between human, mouse and zebrafish	rs146388519 (0.000)	Predict Not Tolerated	Deleterious	Predicted to be benign
R434C	Conserved between human, mouse and zebrafish	rs145200251 (0.001)	Predict Not Tolerated	Deleterious	Predicted to be probably damaging
R434H	Conserved between human, mouse and zebrafish	rs147647315 (0.011)	Predict Not Tolerated	Deleterious	Predicted to be probably damaging

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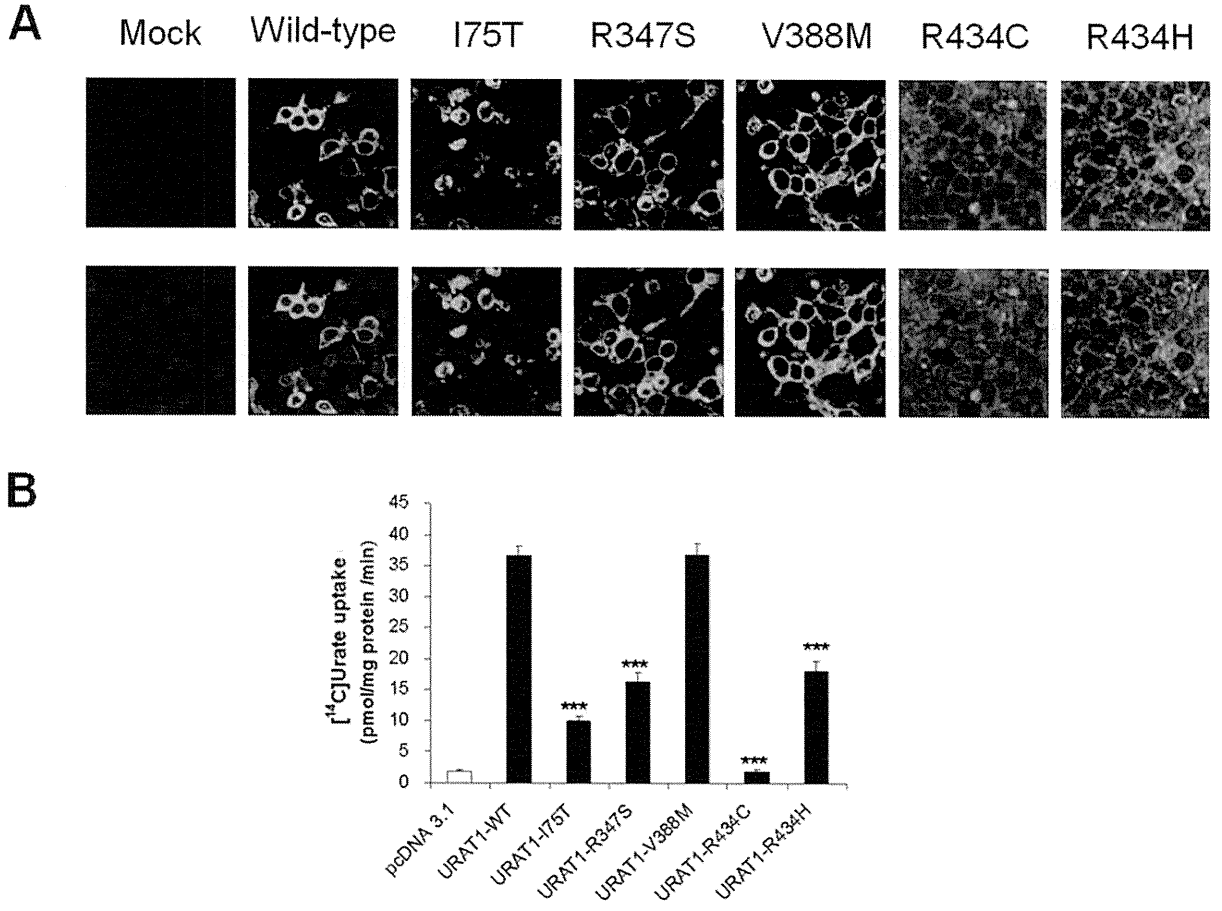

Figure 2. Functional analysis of changes in URAT1 expressed in HEK293 cells. A. HEK293 cells were transiently transfected with Flag-tagged URAT1 cDNA constructs (wild-type and variants I75T, R347S, V388M, R434C and R434H). Plasma membrane expression was detected using an anti-FLAG monoclonal antibody, secondarily detected using an Alexa Fluor® 488 (green) antibody. Nuclei are counter stained using DAPI (blue). B. Uric acid uptake by HEK293 cells transiently transfected with wild-type URAT1 or its mutants was measured using [^{14}C]Urate at 2 min, at 37°C and pH7.4. Significant reductions in urate transport activity was seen in some of the disease-associated variants. Data are mean ± S.E.M with n = 4. ***, P<0.001 when compared with wild type.
doi:10.1371/journal.pone.0028641.g002

al. reported a W258X homozygous mutation in a 7 year old child, whose mother and brother were also heterozygous for W258X and had mild hypouricaemia and abnormally high FE_{urate}, whilst his father who was also heterozygous for W258X, had a normal serum uric acid level of 4.6 mg/dL [23]. In the same report, an 11-year-old girl with asymptomatic microscopic hematuria and low serum urate was a compound heterozygote for W258X/R477H. Her mother had a heterozygous W258X mutation associated with renal hypouricaemia (serum uric acid 2.3 mg/dl and FE_{urate} 16.3%) [23]. In addition, a 15 year old girl with haematuria, proteinuria and renal hypouricaemia (serum uric acid 1.3 mg/dl and FE_{urate} 14.5%) was noted to have a single heterozygous W258X mutation, whilst a 36-year-old female, with recurrent episodes of uric acid stones in the right ureter had a serum uric acid of 1.8 mg/dL and an elevated FE_{urate} of 28.1%. She was heterozygote for a R90H variant in URAT1 [23]. Vázquez-Mellado et al. reported patients heterozygous for C850G in URAT1 with primary gout and low serum uric acid concentrations [50] whilst Ichida, et al. reported 5 individuals with a W258X heterozygous change, one of whom had a history of acute kidney injury and renal stones [23,31,36].

Recently, the GLUT9 glucose transporter, encoded by *SLC2A9* gene has been shown to have an important functional role in transporting uric acid from the renal tubular cells through the basolateral membrane into interstitium [22]. Two additional reports have confirmed this finding [51,52]. Matsuo et al. detected two heterozygous mutations in GLUT9 in two hypouricaemic subjects whom were negative for URAT1 mutations (R380W in exon 10 and R198C in exon 6) and confirmed their reduced transport activity in *Xenopus* oocyte expression system [51] whilst Dinour et al. recently described two families with recessively inherited hypouricaemia who were negative for URAT1 mutations [52]. Among hypouricaemic subjects in both families, three subjects had nephrolithiasis and three subjects had a history of exercise induced acute kidney injury. With genome wide homozygosity screen and linkage analyses they established *SLC2A9* as a causative gene for renal hypouricaemia in these families. In these families, homozygous carriers of *SLC2A9* mutations had very low serum levels of uric acid and extremely high values of FE_{urate} (at around 150%).

In this report we present combined data from Macedonian and English patients who are heterozygous carriers of *SLC22A12* sequence variants. Although the presentations of the patients were varied, a systematic search for hypouricaemia identified patients with possible hereditary renal hypouricaemia and who are suitable for mutational analysis of *SLC22A12* and *SLC2A9* genes to try and

identify abnormalities in the encoded urate transporters URAT1 and GLUT9, respectively. We identified seven patients harboring five *SLC22A12* variants. Interestingly, three Macedonian patients carried mutations at amino acid position 434 of URAT1.

Some of these variants in *SLC22A12* were discovered following significant clinical episodes including nephrolithiasis in patients SK-1, NC-1 and NC-2. There were however, no episodes of exercise induced acute kidney injury that we are aware of.

The sequence variant R434H was identified in one of our patients with hypouricaemia. This variant has a reported heterozygosity index of 0.011 (Table 2), although this variant was not detected in our normal control subjects. Additional studies are required to demonstrate whether this variant is a common cause of hypouricaemia.

Of note, in our cohort of patients, we found no novel sequence variants in the GLUT9 transporter (*SLC2A9*), leaving a number of patients with unexplained hypouricaemia. However, given the complexity of proximal tubule urate handling, other urate transporter protein variants may account for hypouricaemia in the remaining patients.

In patient SK-1 nephrolithiasis was likely to be due to multiple risk factors including renal wasting of urate, episodes of cyclic vomiting leading to concentrated urine and hypocitraturia. In patient SK-4, who also had a molecular genetic diagnosis of Alport syndrome, attacks of gross hematuria were confusing due to presence of colicky pain and eumorphic red blood cells (100%), which are not usual features of Alport syndrome. In this patient, we found the mutation p.R347S. Cheong et al. reported a similar case to this, where a 14 year old girl who presented with acute post-streptococcal glomerulonephritis [23]. Although her nephritis had a favorable course, the microhematuria persisted more than one year. On reevaluation this girl was found to have low serum uric acid (1.3 mg/dl) with increased FE_{urate} (14.5%) in favor of idiopathic hypouricaemia. Mutational analysis in this patient revealed heterozygous W258X mutation in *SLC22A12*.

Patient SK-2 has hypertension and moderate proteinuria due to reflux nephropathy, presumably as a coincidental finding to the functionally significant p.R434H variant. As hypertension in the context of renal disease is often treated with ACE inhibitors or angiotensin receptor antagonists, some caution is advisable. Both losartan [8] and irbesartan [53] have an inhibitory action on URAT1. Thus treatment with these agents has potential to have a marked uricosuric effect in patients with homozygous URAT1 mutations.

Patient SK-3 had a complex phenotype of distal renal tubular acidosis and renal hypouricaemia, associated with the p.R434C mutation within URAT1. It is well known that hypouricaemia may be associated with distal renal tubular acidosis at diagnosis [17,18] as a part of transitory proximal tubular dysfunction. In additional, pharmacological agents may also disrupt proximal tubular handing of urate. In our case, hypouricaemia persisted for more than 2 years despite a normalization of other proximal tubular functions. Definitively, mutational analysis of *SLC22A12* with a functionally significant change (p.R434C) explained the persistent hypouricaemia in this patient.

Patient NC-1 was a recurrent calcium stone former, with a past medical history of type 1 diabetes. Here, serum urate levels were only borderline low and the FE_{urate} was also only transiently raised at 16%. The missense mutation p.V388M was associated with negative functional data, with no significant change in urate transport in HEK293 cell experiments. Inclusion of this case is helpful as the negative functional data and the transient hypouricaemia are consistent with this variant being benign. The dataset derived from the V388M variant allow a comparison to be made between it and the more functionally significant variants, acting as another negative control. Given the hypouricaemia was transient in this case, we do not assume that this sequence variant is causative. From our sequence variants of URAT1, mutations with an impact upon uric acid handling (when modeled in HEK293 cells) are associated with a persistent hypouricaemia. Patient NC-2 was also a recurrent stone former, with persistently low serum uric acid. The heterozygous missense mutation, p.I75T was confirmed to be functionally significant in HEK293 urate uptake studies.

In previously reported Japanese and Korean cases of idiopathic renal hypouricaemia secondary to *SLC22A12* mutations, the majority of patients have been shown to have either homozygous mutations or compound heterozygote mutations [23]. In our Caucasian population, disease associations have been made with single heterozygous changes in URAT1, suggesting that such a change is sufficient and that a dominant pattern of inheritance may be present.

In conclusion, we have identified Macedonian and British patients with hypouricaemia, who presented with symptoms including renal stone disease and haematuria. We have identified missense mutations in *SLC22A12* encoding URAT1. This data highlights the importance of renal urate transporters in determining serum urate concentrations and the of clinical phenotypes that should lead the clinician to suspect an inherited form of renal hypouricaemia.

Materials and Methods

Ethics Statement

For Macedonia patients, the Institutional Review Board and the Ethics Committee at the Medical Faculty and The Ministry of Education and Science, Skopje, Republic of Macedonia approved the study. Informed written consent was obtained by participants' parents or legal guardians. For UK patients, full ethical approval was obtained by the Newcastle and North Tyneside Research Ethics Committee and informed written consent were obtained from participants.

Recruitment of patients

Macedonian patients: Children attending in-patient or out-patient nephrology service at the University Children's Hospital, Skopje were screened for hypouricaemia. Serum uric acid level was determined in children with contraction or expansion of the extracellular volume, acute kidney injury, chronic kidney disease, nephrolithiasis, tubular disorders, those receiving diuretics or nephrotoxic drugs, liver diseases, diabetes mellitus and thyroid diseases. Children less than two years of age were excluded from this study since they have physiologically lower levels of uric acid due to immaturity of the liver and renal tubular function. Where serum uric acid levels were <2 mg/dl and Fractional Excretion of urate (FE_{urate}) was $>10\%$ (normal range 2–8%) and these biochemical findings persisted despite clinical improvement and normalization of other biochemical indices, or if no explanation was found for lower serum levels of uric acid, than idiopathic renal hypouricaemia was considered to be a likely diagnosis [23]. Patients with suspected renal hypouricaemia underwent additional studies, including ultrasound imaging of the urinary tract (if previously not performed) and molecular analysis of *SLC22A12* and *SLC2A9*.

United Kingdom Patients: Adult kidney stone formers attending for lithotripsy at the Newcastle upon Tyne NHS Foundation Trust Hospital, UK were recruited (following informed consent) and screened for serum and urinary biochemical abnormalities.

Twelve patients with hypouricaemia (<2.6 mg/dl) underwent molecular analysis of *SLC22A12* and *SLC2A9*.

Molecular analysis

Mutational analysis of *SLC22A12* and *SLC2A9* genes was performed using exon PCR and direct sequencing. (Oligonucleotide primer sequences are listed in Table S1). For control patient DNA screening, 92 samples were obtained from blood donor (healthy control) panels.

In silico analysis of mutations

Online in silico analyses were performed when sequence variants were identified. These included SIFT (http://sift.jcvi.org/), PolyPhen (http://genetics.bwh.harvard.edu/cgi-bin/ggi/ggi.cgi) and SNPS3D (www.snps3d.org/).

Functional expression studies

Full-length cDNA of human URAT1 was obtained as described previously [3] and tagged with 3× FLAG at the N-terminus. The QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce point mutations into URAT1 cDNA in the expression vector according to the instructions. Complementary oligonucleotides used for mutagenesis are described in Table S2. All the final cDNA sequences were confirmed by DNA sequencing.

For the evaluation of transport function of *SLC22A12* mutations, we used a mammalian cell expression system as described previously [54]. Briefly, HEK293 cells [55] were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C and 5% CO_2. Transient transfection with Lipofectamine 2000 (Invitrogen) was performed according to the manufacturer's instructions. After transfection, the cells were grown 36–48 h before the experiments.

Cellular uptake of [^{14}C]UA were measured in hURAT1 (or its mutants)-transfected HEK293 cells [55] grown on poly-D-lysine-coated 24-well plates. The cells were incubated in chloride-free Hanks' balanced salt solution (HBSS) containing the following in mM: 125 Na gluconate, 4.8 K gluconate, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.3 Ca gluconate, 5.6 glucose and 25 HEPES, pH 7.4) for 10 min. The uptake study was started by adding HBSS containing [^{14}C]UA or to the plate. After 2 min, the cells were washed three times in ice-cold HBSS, then lysed in 0.1 M NaOH for 20 min followed by measurement of radioactivity by scintillation counting. The experiments were performed in quadruplicate per experiment and repeated twice. All the data are given as the mean ± S.D. The Student's *t*-test was used to determine significant differences. A value of $P<0.05$ was considered to be significant.

Immunocytochemical analyses were performed as previously described [56]. URAT1 (or its mutants)-transfected HEK293 cells were fixed with methanol and incubated with the anti-FLAG antibody (1:50) (Sigma) followed by Alexa Fluor® 488-labelled goat anti-rabbit immunoglobulin (Invitrogen; diluted 1:200). The staining was observed under a confocal laser scanning microscope (Fluoview FV1000, Olympus). Alexa 488 fluorescence was excited by Argon laser light of wavelength 488 nm.

Supporting Information

Table S1 Oligonucleotide primers for exon PCR of SLC22A12 and SLC2A9.
(DOC)

Table S2 Oligonucleotide primer pairs used for site-directed mutagenesis of SLC22A12.
(DOC)

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Author Contributions

Conceived and designed the experiments: VT NA JAS. Performed the experiments: AMH KK HIC VJL ZG PJ NA JAS. Analyzed the data: VT AMH KK HIC NA JAS. Contributed reagents/materials/analysis tools: VT JAS NA. Wrote the paper: VT JAS.

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ORIGINAL ARTICLE

Performance of the Japanese GFR equation in potential kidney donors

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Abstract
Background Japanese GFR equation was developed from mainly chronic kidney disease (CKD) subjects. Only a small number of healthy subjects were included in the development and validation of the GFR equation. We assessed the performance of the equation in potential kidney donors.
Methods A total of 113 potential kidney donors was included. The data of CKD subjects that were previously reported were also included for comparison. GFR (mGFR) was measured by inulin clearance. The estimated GFR (eGFR) was calculated by the Japanese GFR equation. Bias of the equation (eGFR − mGFR) and urinary creatinine excretion were evaluated.
Results There was no significant difference between eGFR and mGFR in 340 CKD subjects (54.2 ± 31.6 and 55.7 ± 33.2 ml/min/1.73 m^2, respectively). Contrarily, the eGFR was significantly lower than mGFR in 113 potential kidney donors (78.9 ± 16.2 and 93.6 ± 19.2 ml/min/1.73 m^2, respectively). The biases in potential kidney donors with eGFR 30–59 and 60–89 ml/min/1.73 m^2 were significantly greater than those in CKD subjects (−19.2 ± 12.2

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and -18.3 ± 16.4 ml/min/1.73 m^2 in potential kidney donors and -3.8 ± 15.6 and -3.4 ± 17.6 ml/min/1.73 m^2 in CKD subjects, respectively). Creatinine excretion per body weight of potential kidney donors was significantly higher than that of CKD subjects, suggesting higher creatinine generation in potential kidney donors.

Conclusion The Japanese GFR equation underestimated GFR in potential kidney donors. Higher creatinine generation compared with CKD subjects may contribute to the underestimation of GFR by the Japanese GFR equation in potential kidney donors.

Keywords Inulin clearance · GFR equation · Potential kidney donor · Creatinine excretion

Introduction

The glomerular filtration rate (GFR) is accepted as the best indicator of overall kidney function. It is ideal to measure the clearance of exogenous GFR markers such as inulin, but the method is time-consuming and not widely available. Use of GFR-estimating equations has been recommended in clinical practice [1]. The Modification of Diet in Renal Disease (MDRD) Study equation [2] is most commonly used worldwide. However, it has been reported that the limitations of the equation are imprecision and systematic underestimation of measured GFR at higher values [3–5]. The equation was developed mostly in chronic kidney disease (CKD) subjects. Therefore, the performance of the equation is good in subjects with GFR under 60 ml/min/1.73 m^2, but has a negative bias in subjects with normal GFR [3]. The MDRD equation systematically underestimates GFR in healthy persons [4]. The Japanese GFR equation was also developed in mainly CKD subjects [6]. Detailed study about the performance of the equation in healthy subjects has been required. In the present study, performance of the equation was analyzed in 113 potential kidney donors.

Methods

Potential kidney donors and CKD subjects

A total of 113 potential kidney donors were included. Ten of them were from a previous study. Performance of the Japanese GFR equation was validated using 350 subjects consisting of 10 kidney donors and 340 CKD subjects previously [6]. The ten kidney donors were included as the potential kidney donors in the present study. The other 103 of the 113 potential kidney donors were obtained from 2008 to 2010 in four hospitals. The 340 CKD subjects of the previous study were used for comparison between the CKD subjects and potential kidney donors.

Inulin renal clearance and creatinine excretion

Inulin renal clearance was measured from three sets of 30-min urine collections during 2 h of fasting and hydrated condition by the continuous infusion method. The details were reported previously [6]. Urinary creatinine excretion was measured simultaneously from the three sets of 30-min urine collections (total 90 min). Creatinine excretion per day was calculated from the creatinine excretion during 90 min. Because urinary creatinine was not available in four participants, they were not included in the study for creatinine excretion.

Serum creatinine measurement

Serum creatinine of 350 subjects from the previous study was measured by enzymatic method in a single laboratory (central laboratory) [6]. Creatinine value of the laboratory was IDMS-traceable [6]. Serum creatinine of 56 of 103 potential kidney donors was measured by same method in the central laboratory. Serum creatinine of 47 of 103 potential kidney donors was measured in one participating laboratory by enzymatic method. The creatinine value of the participating laboratory was calibrated to the value of the central laboratory using a regression line ($Y = 0.9X + 0.031$, Y central laboratory, X participating laboratory) that was made from 30 samples that were measured in both laboratories.

GFR equation

Estimated GFR was calculated using the Japanese GFR equation [6].

$$\text{eGFR (ml/min/1.73 m}^2) = 194 \times \text{Cr}^{-1.094} \times \text{age}^{-0.287} \ (\times 0.739 \text{ if female})$$

Statistical analysis

Data were expressed as means ± standard deviation (SD). Bias of the equation was expressed as the difference between eGFR and mGFR (eGFR − mGFR). The biases in potential kidney donors and CKD subjects were compared by Student's t test. Stat view version 4.02 (SAS, USA) was used for statistical analysis.

Results

Clinical characteristics of the study population are shown in Table 1. There was no significant difference between

Table 1 Characteristics of the subjects

	Potential kidney donors	CKD subjects
Male (%)	43 (38%)	201 (59%)
Age (years)	56 ± 11	54 ± 18
BSA (m^2)	1.62 ± 0.19	1.63 ± 0.19
BMI (kg/m^2)	23 ± 3	23 ± 4
Serum creatinine (mg/dl)	0.70 ± 0.14	1.59 ± 1.39
mGFR (ml/min/1.73 m^2)	94 ± 19	56 ± 33
eGFR (ml/min/1.73 m^2)	79 ± 16	54 ± 32
Cr excretion/BW (mg/day/kg)	19.5 ± 5.6	18.6 ± 6.3

BSA body surface area, *BMI* body mass index

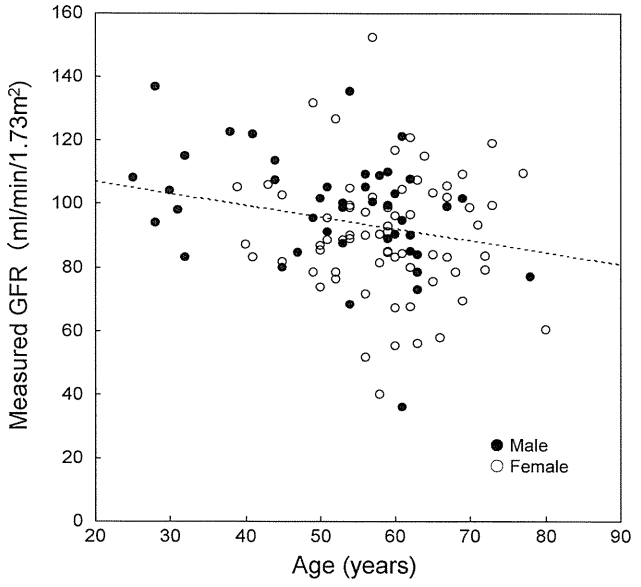

Fig. 1 Relationship between age and measured GFR in potential kidney donors. *Dotted line* is a regression line of the measured GFR against age obtained from 113 subjects

eGFR and mGFR in 340 CKD subjects (54.2 ± 31.6 and 55.7 ± 33.2 ml/min/1.73 m^2, respectively). Contrarily, the eGFR was significantly lower than the mGFR in 113 potential kidney donors (78.9 ± 16.2 and 93.6 ± 19.2 ml/min/1.73 m^2, respectively, $p < 0.001$). Figure 1 shows the relationship between age and mGFR in potential kidney donors. The regression line showed that GFR was about 100 ml/min/1.73 m^2 in young subjects and slightly decreased with aging. Figure 2 shows the relationship between mGFR and eGFR in potential kidney donors. GFR was underestimated in most subjects. The bias of the GFR estimation is shown in Table 2. The bias in total subjects of potential kidney donors were −14.6 ± 17.2 (−15.8 ± 17.6 in male and −13.9 ± 7.1 in female) ml/min/1.73 m^2, which was significantly higher than the value of −1.6 ± 17.5 ml/min/1.73 m^2 in the CKD subjects ($p < 0.001$). The biases in potential kidney donors with eGFR 30–59 and 60–89 ml/min/1.73 m^2 were −19.2 ± 12.2 and −18.3 ± 16.4 ml/min/1.73 m^2, respectively. The differences between eGFR and mGFR were significantly greater than the values of CKD subjects with the same eGFR range.

Creatinine excretion per body weight (mg/day/kg) was plotted against age in potential kidney donors and CKD subjects (Fig. 3). Dotted lines are the regression lines obtained from CKD subjects. Mean creatinine excretion per body weight in potential kidney donors and CKD subjects were 22.3 ± 4.4 and 20.2 ± 6.7 mg/day/kg in male and 17.8 ± 5.5 and 16.4 ± 4.9 mg/day/kg in female, respectively. Creatinine excretion per body weight of potential kidney donors seems to be higher than those of CKD subjects. The regression lines obtained from CKD subjects indicated that creatinine excretion per body weight decreased as aging. The age distribution was different between the potential kidney donors and CKD subjects. Therefore, we compared the creatinine excretions using subjects aged 50–69 or under 50 years old. Creatinine excretions of male subjects of potential kidney donors aged 50–69 years old were significantly higher than those of CKD subjects with the same age range [22.3 ± 5.7 ($N = 44$) and 18.7 ± 4.7 mg/day/kg ($N = 84$), respectively]. Creatinine excretions of female subjects of potential kidney donors aged 50–69 years were also significantly higher than those of CKD subjects [17.7 ± 6.1 ($N = 53$) and 15.6 ± 4.1 mg/day/kg ($N = 48$), respectively]. Creatinine excretions of male potential kidney donors aged 25–49 years old ($N = 14$) and male CKD subjects with the same age range ($N = 58$) were 22.5 ± 2.6 and 21.7 ± 5.3 mg/day/kg, respectively. Creatinine excretions of female potential kidney donors aged 39–49 years old ($N = 8$) and female CKD subjects with the same age range ($N = 23$) were 17.7 ± 2.7 and 17.6 ± 4.6 mg/day/kg, respectively. Creatinine excretion per body weight of potential kidney donors under 50 years old was slightly higher than those of CKD subjects, although the difference was not significant. The number of subjects was small to evaluate the difference of the creatinine excretion in the age range.

Discussion

Mean value of measured GFR was about 100 ml/min/1.73 m^2 in young potential kidney donors and slightly decreased with aging (Fig. 1). Measured GFR over 100 ml/min/1.73 m^2 was not rare in potential kidney donors aged over 60 years old. Estimated GFR was significantly lower than measured GFR in the potential kidney donors (78.9 ± 16.2 and 93.6 ± 19.2 ml/min/1.73 m^2, respectively). The bias in the potential kidney donors was significantly greater than that of CKD subjects. We showed

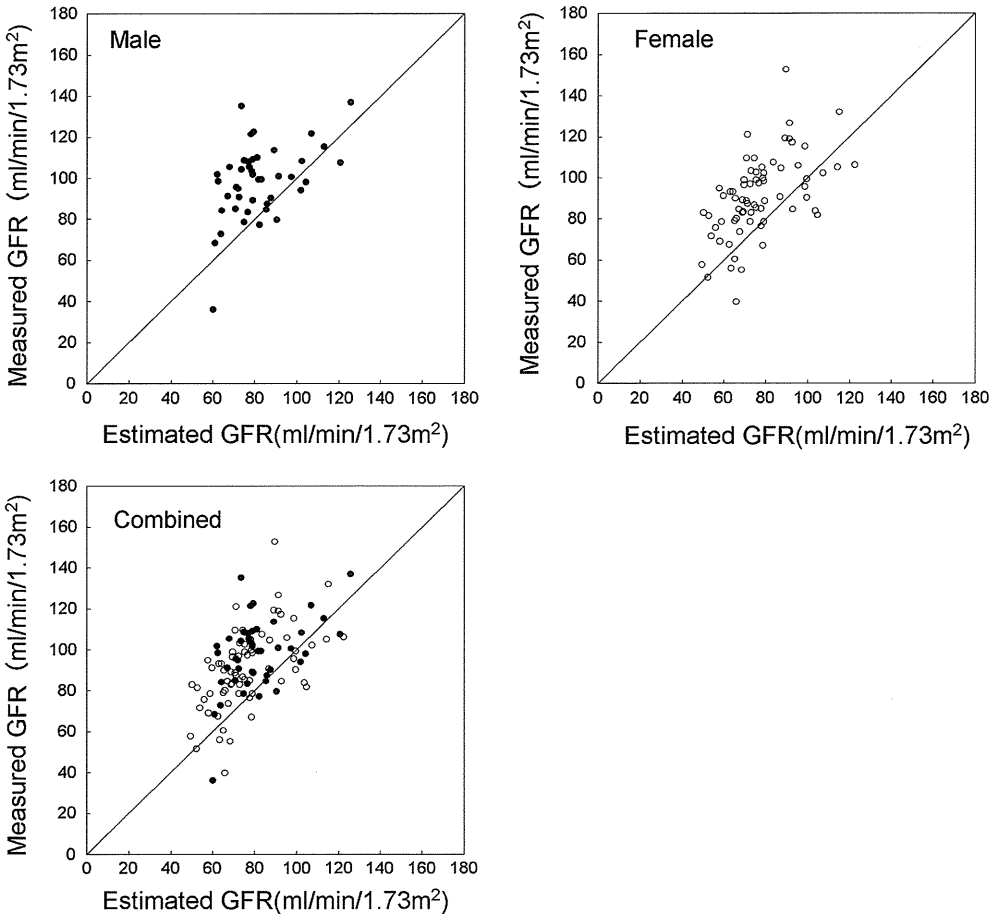

Fig. 2 Relationship between the estimated GFR and measured GFR in potential kidney donors. *Solid line* represents the line of identity

Table 2 Bias of estimated GFR

Bias: difference between eGFR and mGFR (ml/min/1.73 m^2)

*, **Significance of the difference between CKD subjects and potential kidney donors ($p < 0.05$ and $p < 0.001$, respectively)

eGFR (ml/min/1.73 m^2)	CKD subjects		Potential kidney donors	
	N	Bias	*N*	Bias
30–59	118	−3.8 ± 15.6	9	−19.2 ± 12.2*
60–89	86	−3.4 ± 17.6	79	−18.3 ± 16.4**
90–119	43	−1.1 ± 23.3	22	−2.7 ± 15.2
>120	9	35.4 ± 34.8	3	6.5 ± 15.1
Total	340	−1.6 ± 17.5	113	−14.6 ± 17.2**

that the Japanese GFR equation underestimated GFR in potential kidney donors. Kakuta et al. [7] reported similar results in potential kidney donors. They measured GFR by inulin clearance and showed underestimation of GFR by the Japanese GFR equation in potential kidney donors. They reported that measured GFR and estimated GFR were 96.1 and 72.6 ml/min/1.73 m^2, respectively. They also pointed out the possibility of systemic bias of creatinine measurement between their laboratory and the central laboratory for developing the GFR equation. When the creatinine value in their laboratory is systematically higher than the value of the central laboratory, the GFR calculated by the equation will be underestimated. In the present study, creatinine was measured by the central laboratory or calibrated to the laboratory. We minimized the possibility of the bias of creatinine measurement.

The serum creatinine level is affected by kidney function, skeletal muscle mass and protein intake [8]. In the steady state, creatinine generation from muscle can be calculated by urinary excretion of creatinine. We estimated creatinine generation by urinary excretion of creatinine. Subjects with a higher average muscle mass and creatinine generation rate have a higher GFR with the same serum creatinine level. Creatinine excretion per body weight in

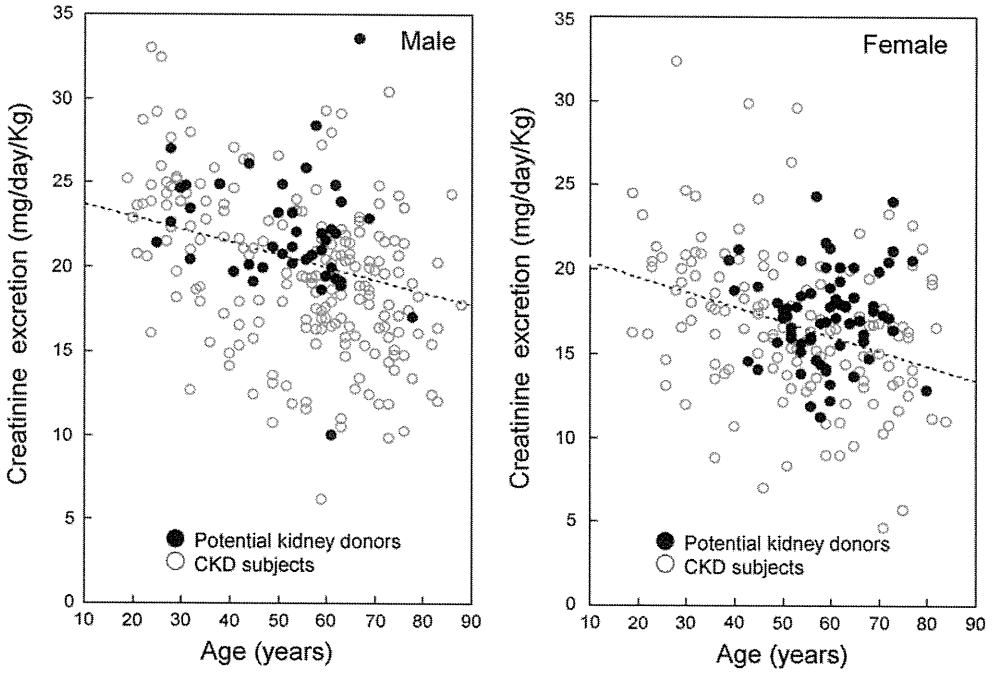

Fig. 3 Relationship between age and creatinine excretion per body weight in potential kidney donors and CKD subjects. *Dotted lines* are regression lines of creatinine excretion per body weight against age obtained from CKD subjects

potential kidney donors aged 50–69 years old was significantly higher than that of CKD subjects with the same age range, suggesting a higher generation of creatinine in potential kidney donors. Higher muscle mass or higher protein intake or both in potential kidney donors compared with CKD subjects may be the possible explanation for the higher generation of creatinine. These differences in creatinine generation could be reflected as differences in serum creatinine levels between CKD subjects and potential kidney donors. The Japanese GFR equation was derived from serum creatinine values of mainly CKD subjects. Higher serum creatinine levels because of higher generation of creatinine leads to underestimation of GFR by the equation. Matsuo et al. [6] mentioned that the eGFR according to the Japanese GFR equation may not be applicable to the healthy population, because the equation was derived mostly from patients with CKD. We confirmed the limitation of the equation that GFR is systematically underestimated in healthy subjects, although potential kidney donors are a highly selected population of healthy subjects in the general population.

Low GFR and proteinuria are used for the diagnosis of CKD in the health check program. The GFR estimation equation is useful to detect low GFR (<60 ml/min/1.73 m^2) subjects in the general population. Accuracy of the GFR equation expressed as percentage of subjects whose eGFRs are within ±30% of mGFR is about 75% [6]. About 25% of the CKD subjects are overestimated or underestimated over 30% of the mGFR. The GFR equation is easy to use and useful for screening of CKD, but the accuracy is not enough. This is one of the limitations of the equation. Underestimation of GFR in healthy subjects is another limitation of the equation. Some healthy subjects are included in subjects without proteinuria, but with eGFR around 60 ml/min/1.73 m^2. We have to know the limitation of the GFR equation based on serum creatinine and should select the more accurate methods such as inulin clearance if necessary. We recommend that the kidney donor's GFR should be measured by inulin renal clearance.

In the future, the accuracy of the estimated GFR will be increased using multiple GFR markers. Serum cystatin C was recently proposed as an alternative marker of GFR. Better performance of cystatin C compared with creatinine has been suggested [9]. The serum concentration of cystatin C was less influenced by muscle mass compared with serum creatinine, suggesting higher performance of estimated GFR based on cystatin C or the combination of serum creatinine and cystatin C. Further study is required.

Conclusion

The Japanese GFR equation underestimated the GFR in potential kidney donors. Creatinine excretions per body weight in potential kidney donors were significantly higher compared with CKD subjects. Higher generation of creatinine possibly due to higher muscle mass or higher protein intake may contribute to the underestimation of the GFR in potential kidney donors.

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Conflict of interest All the authors have declared no competing interest.

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ORIGINAL ARTICLE

Validation of the equations for estimating daily sodium excretion from spot urine in patients with chronic kidney disease

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Abstract
Background Measuring sodium excretion in a 24-h urine collection is the most reliable method of estimating salt intake, but it is not applicable to all patients. As an alternative, equations for estimating Na excretion from Japanese by a spot urine sample were created, but they have not been validated in patients with chronic kidney disease (CKD), which are frequently associated with nocturia and medication.
Methods We enrolled 136 patients with CKD and collected both 24-h urine and the first morning urine. Na excretion was estimated from the first morning urine by Kawasaki's equation, which was originally used for the second morning urine, and Tanaka's equation, which is applied for spot urine samples taken at any time from 9 am to 7 pm. We evaluated the two equations for bias, RMSE and accuracy within 30 and 50% of the measured Na excretion.
Results Bias, RMSE and accuracy within 30% of the estimated Na excretion were 48 ± 69 and 2 ± 69 mmol/day, 84 and 69 mmol/day, and 35 and 49% using Kawasaki's equation and Tanaka's equation, respectively. Na excretion in the first morning urine was accurately estimated by Tanaka's equation, but it was overestimated by Kawasaki's equation. Nocturia and medication such as diuretics and ACE inhibitor or angiotensin receptor blocker did not affect the accuracy with which Na excretion was estimated by Tanaka's equation substantially.
Conclusion Tanaka's equation for estimating Na excretion from the first morning urine in patients with CKD is accurate enough for use in clinical practice.

Keywords Sodium · Creatinine · CKD · Estimated Na excretion

A part of this study was presented at ERA-EDTA 2011 at Plague.

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Introduction

High salt intake is associated with higher risk for stroke and cardiovascular disease [1, 2]. High salt intake causes high blood pressure [3], while reducing salt intake results in a decrease in blood pressure [4]. Reducing salt intake is a key action for preventing cardiovascular diseases and all-cause mortality. It was estimated that reducing dietary salt by just 1 g per day could result in reducing the annual number of coronary heart disease cases by 20,000–37,000, stroke cases by 13,000–20,000, and deaths by any cause by 17,000–28,000 in the USA, where the average consumptions of salt were 10.4 and 7.3 g in men and women, respectively [5].

Despite the alarming risk associated with salt intake, there is poor awareness in the general population of high

salt intake, and even in patients with hypertension or CKD. To increase the awareness of the risk of high salt intake, and to educate the general population about the benefits of reducing their salt intake, the first step is to get individuals to find out their daily salt intakes by measuring their sodium (Na) excretion in urine.

Twenty-four hour urine collection is regarded as the most reliable method of measuring the Na excretion, but it is cumbersome and cannot be used for everyone. An alternative method is to use an equation to estimate Na intake from a spot urine sample. This method is less reliable than 24-h urine collection, but it is a practical way to let individuals roughly gauge their salt intakes [6]. There are 3 equations for estimating Na intake in Japanese [7]. Kamata's equation estimates the 24-h Na excretion from night-time urine data and the lean body mass estimated by bioelectrical impedance [8]. Kawasaki's equation estimates the 24-h Na excretion by measuring the Na/creatinine (Cr) ratio in the second morning urine sample [9]. This equation has been validated in population studies [10–12] and a hypertensive population [13], and its accuracy was confirmed in these studies. Tanaka's equation estimates the 24-h Na excretion by measuring the Na/Cr ratio from a spot urine sample [14]. Tanaka's equation has also been used to estimate the sodium intake in the general population [15]. These equations were generated based on data from healthy volunteers.

Tanaka's equation is recommended for use in the estimation of salt intake by the Japanese Society of Hypertension [6]. Its accuracy has not, however, been validated in patients with chronic kidney disease, who frequently have nocturia. In this study, we validated the accuracy, bias and RMSE of two equations (Kawasaki's and Tanaka's) for estimating 24-h Na intake in patients with chronic kidney disease (CKD).

Participants and methods

The protocol of the study was approved by the ethical committee of the Nagoya University Hospital. The study was conducted in accordance with the guidelines of Nagoya University Hospital. We enrolled patients with CKD who regularly visited the Department of Nephrology at Nagoya University Hospital from March 2009 to September 2010. All of them provided written informed consent to participate in this study. All study participants were given a 2.5-L bag for urine collection and instructed to collect a 24-h urine sample by discarding the first voided urine upon rising in the morning and then collecting all voided urine up to and including the first void of the following morning. We collected the 24-h and the first morning urine samples. Four milliliters of the first morning urine sample were collected, and the rest of it was combined with the 24-h urine sample by patients according to the urine collection instructions provided by the nurses.

Sodium and creatinine was measured by an auto-analyzer (BM2250, JEOL Ltd., Tokyo, Japan). Creatinine was measured by an enzymatic method.

The equations used in the study were as follows:

Kawasaki's equation [9]:

$$\text{24-h urinary Na excretion (mmol/day)} = 16.3 \times \{(\text{UNa/UCr}) \times \text{estimated 24-h UCr}\}^{0.5}$$

$$\text{Male: estimated 24-h UCr(mg/day)} = 15.12 \times \text{weight} + 7.39 \times \text{height} - 12.63 \times \text{age} - 79.90.$$

$$\text{Female: estimated 24-h UCr(mg/day)} = 8.58 \times \text{weight} + 5.09 \times \text{height} - 4.72 \times \text{age} - 74.95.$$

Tanaka's equation [14]:

$$\text{24-h urinary Na excretion (mmol/day)} = 21.98 \times \text{UNa/UCr} \times \{-2.04 \times \text{age} + 14.89 \times \text{weight (kg)} + 16.14 \times \text{height (cm)} - 2244.45\}^{0.392}.$$

We compared these two equations in terms of RMSE, bias, accuracy, and correlation coefficient. The RMSE in the Na excretion estimated by the equation was calculated as the square root of [sum of squared errors of the estimate/(N)]. The bias of each equation was expressed as the mean difference between Na estimated using each equation and the measured Na excretion. The accuracy was expressed as the percentages of subjects with estimated Na excretions that were within 30 and 50% of the measured 24-h Na excretion.

Table 1 Patient characteristics

	Male	Female
Number	98	38
Age (years old)	67.5 ± 12.0	69.1 ± 10.3
Height (cm)	166.5 ± 6.3	152.6 ± 5.9
Weight (kg)	69.6 ± 12.6	55.5 ± 11.4
BMI (kg/m^2)	25.0 ± 3.8	23.7 ± 4.0
Urinary Na excretion (mmol/day)	166.7 ± 82.5	133 ± 75.4
Urinary Cr excretion (mg/day)	1365.9 ± 463.6	854.1 ± 276.7
eGFR (mL/min/1.73 m^2)	41.0 ± 17.4	39.9 ± 19.8
CKD stage	*n* (%)	*n* (%)
Stage 1	1 (1.0)	2 (5.3)
Stage 2	9 (9.2)	3 (7.9)
Stage 3	61 (62.2)	21 (55.3)
Stage 4	24 (24.5)	7 (18.4)
Stage 5	3 (3.1)	5 (13.2)
Hypertension	70 (71.4)	29 (76.3)
Diabetes	37 (37.8)	4 (10.5)

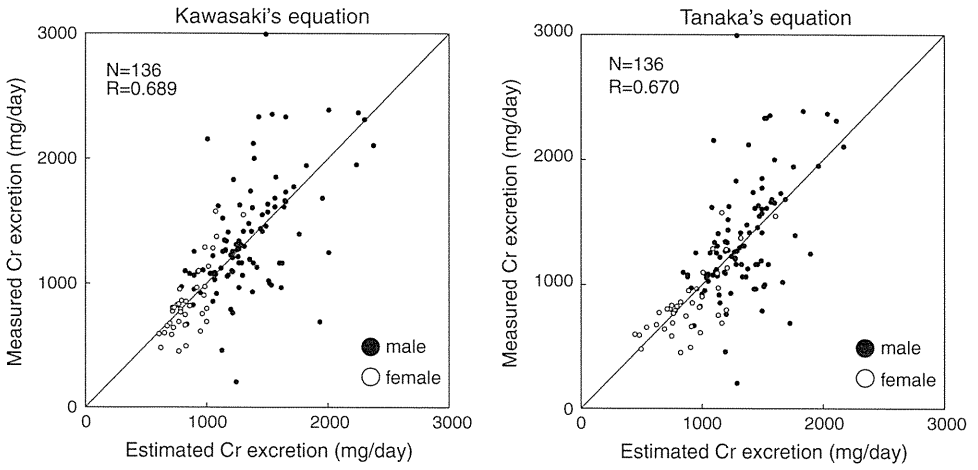

Fig. 1 Correlation between estimated Cr excretion and measured 24-h Cr excretion. Correlations between the measured 24-h urinary excretion of Cr and the estimated Cr excretion in 24-h calculated by Kawasaki's equation (*left*) and by Tanaka's equation (*right*) are shown

Statistical analysis

Data were expressed as mean ± SD. Bias was obtained by calculating (estimated Na excretion—measured Na excretion). Differences in the accuracy of their estimated Na excretion values between the equations were evaluated by chi-square tests. The differences in the bias (absolute value) of the estimated Na excretions were evaluated between the equations with Student's *t* test. A difference with $P < 0.05$ was considered to be statistically significant. Statview (version 4.02, SAS Institute, Cary, NC, USA) was used for statistical analysis.

Results

We screened 197 patients with CKD who regularly visited Nagoya University Hospital. Sixty-one were excluded from this study because they could not collect a urine sample. One hundred thirty-six patients were enrolled and collected 24-h urine and first morning urine samples. The characteristics of the patients are shown in Table 1. The urinary creatinine excretions of males and females in 24 h were 1366 ± 463 and 854 ± 276 mg/day, respectively. The median (Q1, Q2) sodium excretion of the patients was 145 mmol/day (99, 194). The average (± SD) sodium intake was 157.4 ± 60.7 mmol/day. Nocturia was observed in 33 patients (24.2%). Diuretics and ACE inhibitor/angiotensin receptor blocker were used in 26 patients (19.1%) and 92 patients (67.6%), respectively.

Cr excretion

The Cr excretion estimated by each equation was significantly correlated with the measured 24-h Cr excretion ($r = 0.670$ and 0.689 with Tanaka's equation and Kawasaki's equation, respectively, Fig. 1). The estimated Cr excretion did not significantly differ between the two equations ($r = 0.953$, Fig. 2).

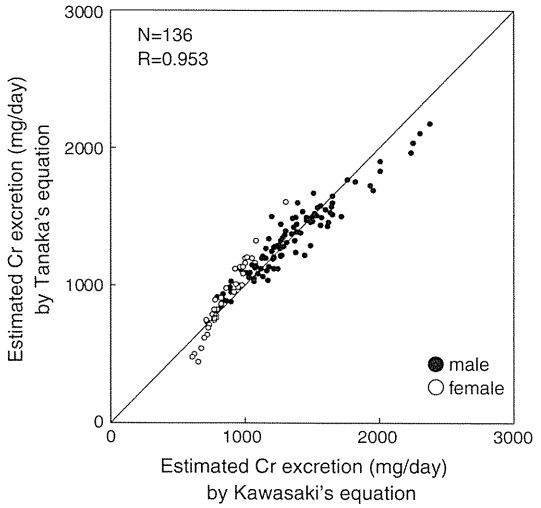

Fig. 2 Comparison of the Cr excretions estimated by the two equations. The correlation between the estimated Cr excretions in 24-h obtained by Kawasaki's equation and by Tanaka's equation is shown

Na excretion

The Na/Cr ratio from the first morning urine was highly correlated with the 24-h Na/Cr ratio in the CKD population ($r = 0.599$, Fig. 3). The estimated Na excretion obtained using each equation was compared with the measured 24-h Na excretion ($r = 0.533$ and 0.564, Tanaka's equation and Kawasaki's equation, respectively; see Fig. 4). The estimated Na excretion calculated by Kawasaki's equation was higher than the measured Na excretion and also higher than the Na excretion estimated by Tanaka's equation (Figs. 4, 5). The performance of each formula was

evaluated; see Table 2. Bias was significantly less for Tanaka's equation than for Kawasaki's equation ($P < 0.001$). Tanaka's equation estimated the Na excretion with significantly better accuracy to within 30 and 50% than Kawasaki's equation ($P < 0.05$ and 0.001, respectively). Kawasaki's equation generally overestimated the Na excretion and resulted in 48 mmol/day of bias and 84 mmol/day of RMSE compared to the measured Na excretion. Tanaka's equation tended to overestimate when the Na excretion was 200 mmol/day or higher, while it tended to underestimate when the Na excretion was less than 200 mmol/day.

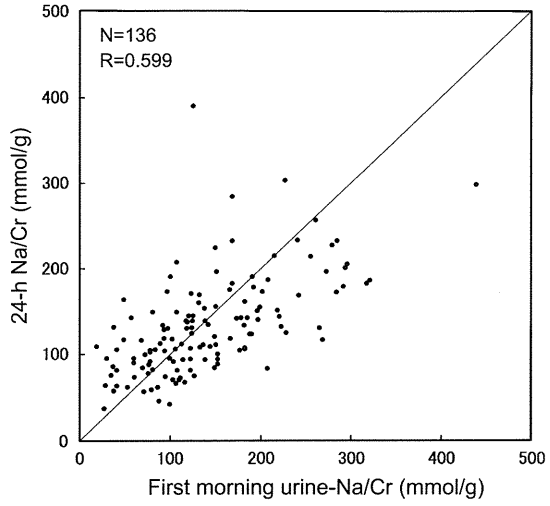

Fig. 3 Correlation between the measured 24-h Na/Cr ratio and the measured Na/Cr ratio in the first morning urine. Na and creatinine were measured in 24-h urine and in the first morning urine. The Na/Cr ratio was calculated and the correlation between the 24-h Na/Cr ratio and the Na/Cr ratio of the first morning urine is shown

Effect of nocturia on the estimation of Na excretion

The bias associated with the use of Tanaka's equation did not differ significantly between patients with and without nocturia (Fig. 6a). In contrast, the bias associated with the use of Kawasaki's equation was significantly less in patients without nocturia than in patients with nocturia ($P < 0.01$) (Fig. 6a). When we compared the biases for patients with or without nocturia, the bias was significantly less when Tanaka's equation was used than when Kawasaki's equation was applied in patients without nocturia ($P < 0.01$) and with nocturia ($P < 0.0001$).

Effects of medication on the estimation of Na excretion

Treatment with diuretics did not affect the accuracy of the estimate of Na excretion in either equation (Fig. 6b). For patients treated without diuretics, Tanaka's equation was more accurate than Kawasaki's equation ($P < 0.0001$). For patients treated with diuretics, the bias did not differ between the two equations.

Treatment with ACE inhibitor or ARB did not affect the accuracy of the estimated Na excretion in either equation (Fig. 6c). Bias was significantly less using Tanaka's equation than for Kawasaki's equation for patients treated with RAS inhibitor ($P < 0.01$) and without RAS inhibitor ($P < 0.0001$).

Discussion

The purpose of this study was to evaluate the accuracy of the equation for estimating Na excretion from the first morning urine in a Japanese CKD population. The hypothesis that 24-h urinary sodium excretion can be estimated from spot urine was satisfied on the basis of a good correlation between 24-h Na/Cr ratio and the Na/Cr

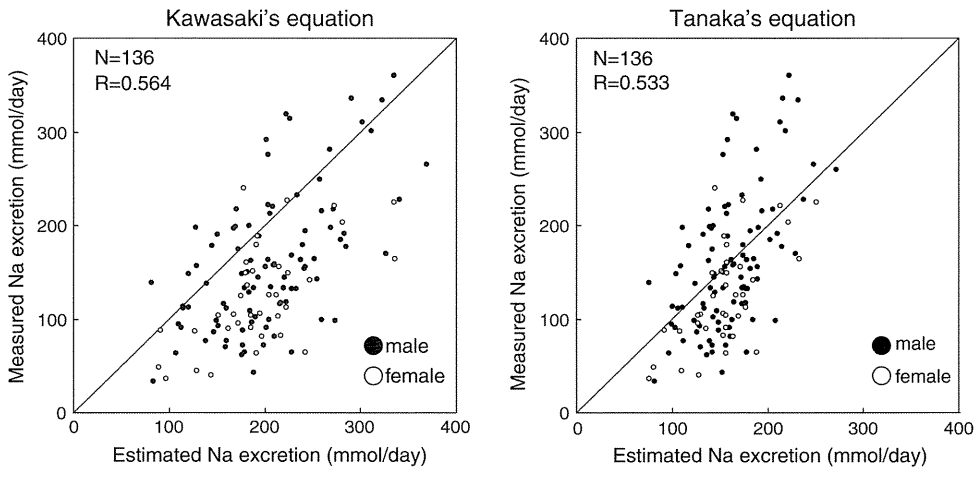

Fig. 4 Correlation between the measured Na excretion and the estimated Na excretion in 24-h. Correlations between the measured 24-h Na excretion and the estimated Na excretion calculated by Kawasaki's equation (*left*) and by Tanaka's equation (*right*) are shown

ratio of spot urine. The correlation of the Na/Cr ratios in 24-h urine and the first morning urine was found to be good in the present study ($r = 0.599$, $P < 0.001$, Fig. 3), suggesting that first morning urine is suitable for estimating the 24-h Na excretion. Estimated Na excretion calculated by either equation showed a good correlation with the measured 24-h Na excretion in CKD patients. Kawasaki's equation, however, generally overestimated the Na excretion and resulted in 48 mmol/day of bias and 84 mmol/day of RMSE compared to the measured Na excretion, leading to reduced accuracy when estimating sodium intake. In contrast, Tanaka's equation showed good performance, with 2 mmol/day of bias and 69 of RMSE compared to the measured Na excretion, which suggests that Tanaka's equation performs significantly better than Kawasaki's equation for first morning urine. We suggest using Tanaka's equation to estimate Na excretion in the first morning urine of CKD patients.

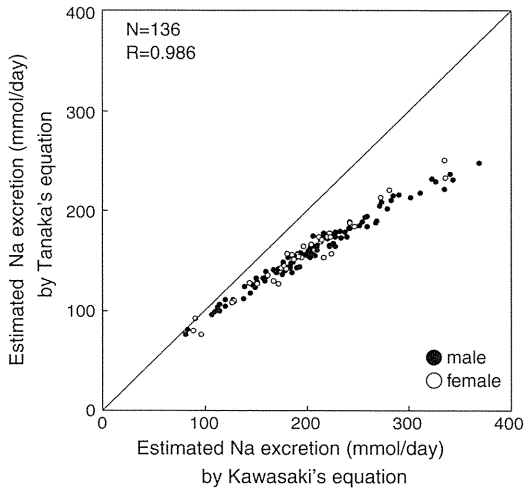

Fig. 5 Comparison of estimated Cr excretions. The correlation between the estimated Cr excretions calculated by Kawasaki's equation and Tanaka's equation is shown

The average creatinine excretion was 1223 ± 476 mg/day, which implied that urine collection was performed well in this study. Cr excretion was estimated differently in the two equations. Tanaka's equation calculated it based on age, weight and height in both sexes. In contrast, Kawasaki's equation was calculated differently for each sex. However, the accuracy of the estimated Cr excretion was similarly good in both methods, in that the correlation between the estimated Cr excretion and the measured Cr excretion was good in both equations ($r = 0.670$ and 0.689, respectively).

In a previous study, a validation of Tanaka's equation in 336 subjects showed that the estimated Na was 24 mmol/L lower than the measured Na (178.5 mmol/L), and that the correlation coefficient between the estimated 24-h Na excretion and the measured 24-h Na excretion was $r = 0.32$ [14]. In the present study, the correlation coefficient was much higher than that ($r = 0.67$). The previous validation of Tanaka's equation was performed on the basis of a casual urine specimen that was collected in a medical check-up with no restriction on collection time (8:00 am to 7:00 pm) [14]. In our study, urine collection was limited to the first morning urine, which may improve the accuracy of the estimation. In addition, in the previous validation, the ages of the patients were 20–69 years old, while the mean age in the present study was 68 ± 11 years old. This difference of age may affect the accuracy of the estimation.

Table 2 Performances of the equations for estimating Na excretion

	N	Bias (mmol/day)	RMSE (mmol/day)	Accuracy	
				±30%	±50%
Kawasaki's equation					
<100 mmol/day	5	19 ± 48	47	20	40
100–148	16	18 ± 46	48	56	69
150–199	45	50 ± 49	69	36	56
200–249	44	48 ± 90	101	23	43
≤250	26	69 ± 70	97	42	65
Total	136	48 ± 69	84	35	54
Tanaka's equation					
<100 mmol/day	8	10 ± 36	35	38	63
100–148	44	7 ± 48	48	48	77
150–199	65	4 ± 81***	81	49	74*
200–249	17	−25 ± 81	83	53*	94**
≤250	2	18 ± 11	20	100	100
Total	136	2 ± 69***	69	49*	77***

* $P < 0.05$, ** 0.01, *** 0.001 versus Kawasaki's equation

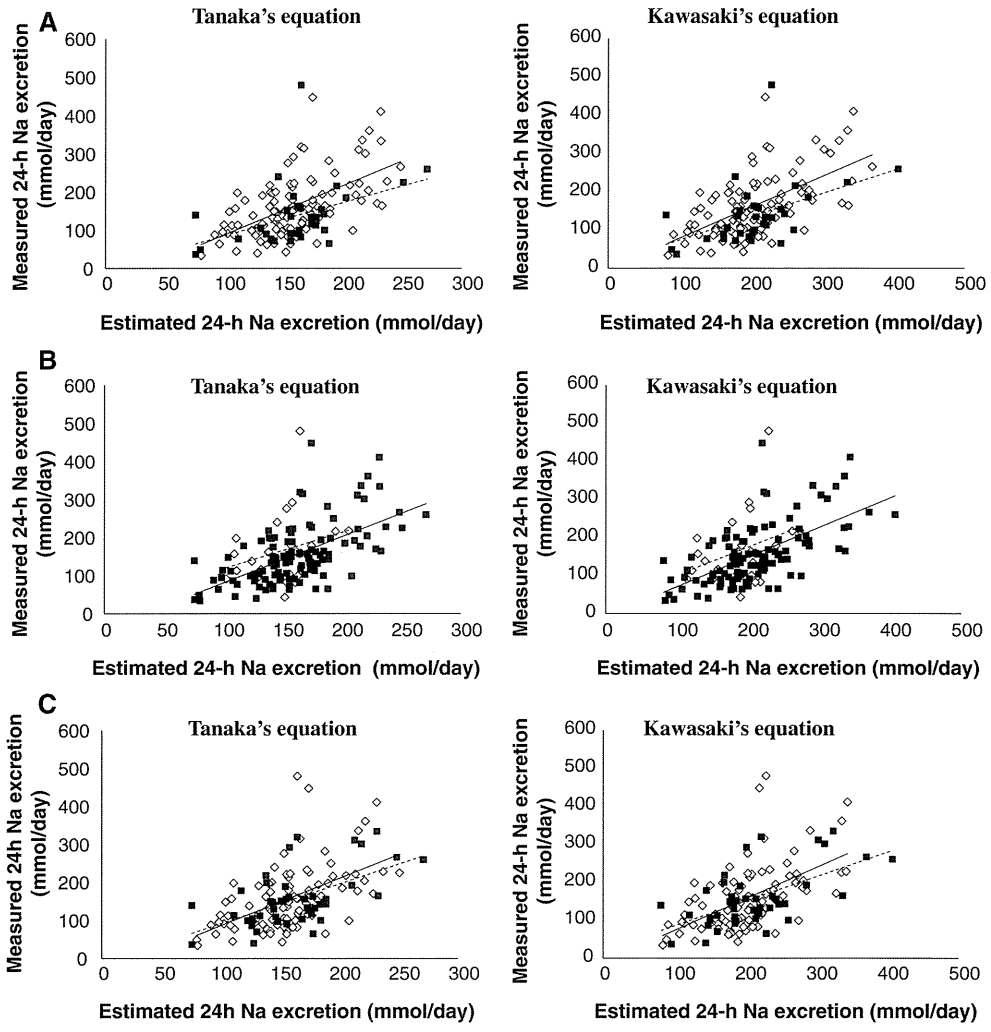

Fig. 6 **a** Effect of nocturia on the estimation of Na excretion. *White squares* show patients without nocturia ($n = 103$). *Black squares* show patients with nocturia ($n = 33$). The correlation between the estimated Cr excretions obtained by Kawasaki's equation and Tanaka's equation is shown for patients with nocturia (*dotted line*) and without nocturia (*continuous line*). **b** Effect of diuretics on the estimation of Na excretion. *White squares* show patients treated with diuretics ($n = 26$). *Black squares* show patients treated without diuretics ($n = 110$). The correlation between the estimated Cr excretions calculated by Kawasaki's equation and Tanaka's equation is shown for patients with diuretics (*dotted line*) and without diuretics (*continuous line*). **c** Effect of RAS inhibitor on the estimation of Na excretion. *White squares* show patients treated with RAS inhibitor (ACE inhibitor or angiotensin receptor blocker) ($n = 92$). *Black squares* show patients treated without ACE inhibitor and/or angiotensin receptor blocker ($n = 44$). The correlation between the estimated Na excretions obtained by Kawasaki's equation and Tanaka's equation is shown for patients with RAS inhibitor (*continuous line*) and without RAS inhibitor (*dotted line*)

We used the first voiding of urine to evaluate the equations mainly because the patients are accustomed to collect the first morning urine and also because this avoided the effects of medications, including diuretics, in the morning. In fact, it is difficult to collect second morning urine samples from patients with CKD because most of them are old and frequently have polynocturia, so they take pre- and postprandial medications in the morning. The patients with CKD have nocturnal polyuria due to natriuresis during the night. It was suggested that the night-time Na excretion was higher than the daytime one in a CKD population [16]. The mean ratio of Na/Cr in the first morning urine samples from CKD patients was higher (147 ± 89 mmol/g) than the Na/Cr ratio in 24-h Na/Cr samples (134 ± 58 mmol/g) in the present study. Nocturia did not affect the accuracy of the estimation of Na excretion by Tanaka's equation. Tanaka's equation consistently provided more accurate values of estimated Na excretion irrespective of nocturia compared with Kawasaki's equation. Kawasaki's equation was less accurate when estimating Na excretion from the first voiding of urine because that equation was derived based on data for the second morning urine, which may be more dilute than the first urine.

Diuretics and RAS inhibitor facilitate Na excretion and improve nocturnal hypertension in the CKD population [17, 18]. Medication by diuretics or RAS inhibitor, which induces daytime Na excretion and creates a negative Na balance, did not affect the accuracy of the estimate calculated by Tanaka's equation, but it did affect the estimation performed by Kawasaki's equation.

The limitations of this study were as follows. First, the number of participants was small. Second, the influence of posture on the estimate was not evaluated. When using Kawasaki's equation, it is suggested that the urine should be collected in a sitting or standing position to maintain accuracy [19]. All of the patients who participated in this study were outpatients, and they probably collected the first morning urine just after they woke up.

The average salt intake of the present study was 9.2 g/day, which was 1.5-fold higher than the recommended values for salt intake in the guidelines of the Japanese Society of Hypertension [20] and the Japanese Society of Nephrology [21]. We need strong and tenacious endeavor to reduce the levels of sodium intake in the CKD population.

In conclusion, Tanaka's equation for estimating Na excretion was accurate for a CKD population when the first morning urine was used. The accuracy of this equation means that it can be applied to estimate Na excretion in clinical practice, and the value of the estimated salt intake from this equation can be used to persuade patients with CKD to reduced their salt intake.

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