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Tubular phosphate transport: a comparison between different methods of urine sample collection in FGF23-dependent hypophosphatemic syndromes

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Abstract

Objectives: Tubular maximum phosphate reabsorption per glomerular filtration rate (TmP/GFR) is used to evaluate renal phosphate reabsorption and it is a useful tool for the differential diagnosis of hypophosphatemic syndromes. TmP/GFR is typically calculated from fasting plasma and second morning void urine samples, obtained 2 h after the first void (TmP/GFR 2 h). The purpose of this study was to evaluate if TmP/GFR calculated from 24 h urine collection (TmP/GFR 24 h) can be used as an alternative for TmP/GFR 2 h in patients with urine phosphate wasting.

Methods: We enrolled adult patients with X-linked hypophosphatemia (XLH) or tumor-induced osteomalacia (TIO). All patients underwent blood and urine sample collections, to calculate TmP/GFR 24 h and TmP/GFR 2 h.

Results: Twenty patients (17 XLH and 3 TIO), aged 24–78 years, were included. All patients had low TmP/GFR 2 h (0.35 mmol/L, IQR 0.24–0.47 mmol/L) and TmP/GFR 24 h (0.31 mmol/L, IQR 0.22–0.43 mmol/L). The concordance correlation coefficient between TmP/GFR 2 h and TmP/GFR 24 h was 0.86 (95 % CI: 0.69–0.93), with a systematic bias of 0.05 mmol/L (95 % limits of agreement: −0.10 to 0.20). Furthermore, in 70 % (i.e., 14 patients out of 20) and 80 % (i.e., 16 patients out of 20) of cases the difference between TmP/GFR 2 h and TmP/GFR 24 h was within ±30 % and ±35 %, respectively.

Conclusions: Despite TmP/GFR 2 and 24 h show a relatively suboptimal agreement, the difference between the two parameters appears to be small and not clinically significant in the setting of adult patients with FGF23-dependent urine phosphate wasting and secondary hypophosphatemia.

Keywords: tubular maximum phosphate reabsorption per glomerular filtration rate (TmP/GFR); phosphate metabolism; hypophosphatemic rickets; X-linked hypophosphatemia (XLH); tumor-induced osteomalacia (TIO)

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Introduction

Phosphate is an essential element in human physiology, as it is involved in several biological processes, including regulation of cellular membrane composition, intracellular metabolism and signal transduction, acid-base homeostasis, skeletal development, and metabolism. Therefore, the maintenance of an appropriate phosphate homeostasis is of crucial importance [1, 2]. Phosphate is mainly stored in calcified tissues (bone and teeth) and its serum concentration is primarily regulated by three hormones: (a) parathyroid hormone (PTH), which promotes phosphate resorption from bone and decreases its reabsorption in the proximal tubule; (b) 1,25-fibroblast growth

factor 23 (FGF23), which reduces tubular phosphate reabsorption, but also inhibits calcitriol synthesis [3].

Serum FGF23 may be assessed by two types of methods: (a) intact assays, which use two monoclonal antibodies against N-terminal and C-terminal portion of FGF23 respectively, thus measuring only biologically active molecules; (b) C-terminal assays, which use antibodies detecting only the C-terminal portion, that is considered to reflect the amount of FGF23 transcription or translation [4].

Together with calcium, phosphate is essential for bone mineralization. Thus, hypophosphatemia may be associated with the development of rickets and/or osteomalacia [5]. Hypophosphatemic rickets/osteomalacia can be classified in FGF23-dependent or FGF23-independent, with both groups including acquired or inherited forms. Among FGF23–dependent diseases, X-linked hypophosphatemia (XLH) and tumor-induced osteomalacia (TIO) represent the prototype of genetic and acquired forms, respectively [6]. In XLH, FGF23 excess is due to a loss-of-function mutation of phosphate regulating endopeptidase homolog X-linked (*PHEX*) gene [7], whereas TIO is a paraneoplastic syndrome in which FGF23 is hypersecreted by a phosphaturic mesenchymal tumor [8].

Phosphate homeostasis assessment is based on both serum phosphate levels and renal phosphate reabsorption. The tubular maximum phosphate reabsorption per glomerular filtration rate (TmP/GFR) was originally developed by Bijvoet et al. [9] and later modified by Barth et al. [10]. It consists in the maximum renal capacity to reabsorb phosphate per unit volume of glomerular filtration rate and represents the theoretical lower limit of serum phosphate below which all filtered phosphate is reabsorbed. TmP/GFR is considered the most reliable parameter for assessing renal phosphate wasting and its use is recommended for this purpose in the diagnosis and management of XLH [11] and TIO [12]. However, it is still unclear, at least in adults, which is the best urine sample to consider for its calculation. In the original paper by Bijvoet [9] an untimed urine morning sample was used, probably because it was developed for pediatric patients with hereditary rickets in which this type of collection is easier to be obtained. However, in a recent Consensus Statement on XLH management, it has been suggested the use of a second morning void sample obtained 2 h after the first void [11]. On the contrary, no fully established evidences exist regarding the possible contribution of 24 h urinary collection in the estimation of TmP/GFR. Furthermore, 24 h urinary collection remains essential in evaluating the excretion of other solutes, such as urinary calcium, oxalate, citrate, and uric acid, which is fundamental for estimating the risk of kidney stone formation [13, 14], a common disorder in patients hypophosphatemic diseases such as XLH [15].

Hence, calculating TmP/GFR on the 24 h urine collection, rather than the 2 h collection, would be advantageous for the patient as it involves collecting only one type of urine sample.

The aims of this study were to evaluate the agreement between TmP/GFR calculated from 24 and 2 h urine collections, and to identify any possible determinants which could influence the levels of these two parameters.

Materials and methods

Patients

We enrolled patients with FGF23-dependent hypophosphatemia evaluated in the outpatient clinic of the Internal Medicine and Endocrinology Units at the University Hospital of Padova, Italy. Inclusion criteria were: (1) age ≥18 years; (2) chronic hypophosphatemia, defined as fasting serum phosphate levels persistently below 0.8 mmol/L [6], before initiating any phosphate or calcitriol supplementation; (3) high serum FGF23 levels, defined as intact FGF23 above 95.4 pg/mL (97.5th percentile of healthy subjects) and/or C-terminal FGF23 above 0.8 pmol/L (median value of healthy subjects) with the assays described below; (4) diagnosis of XLH (defined by the presence of classic clinical features of adult XLH such as short stature or bowed legs and documented PHEX mutation) or diagnosis of TIO (defined by the presence of classic clinical features of adult TIO such as bone pain, fragility fractures and muscle weakness, and identification of a phosphaturic mesenchymal tumor using ^{68}Ga-DOTA-D Phe^{1}-Tyr^{3}-octreotide [^{68}Ga-DOTATOC] positron emission tomography). Exclusion criteria were: chronic kidney disease (CKD) stages 3–5 (estimated glomerular filtration rate [eGFR] <60 mL/min/1.73 m^2, according to the 2021 Chronic Kidney Disease Epidemiology Collaboration [CKD-EPI] Creatinine equation [16]), acute or chronic liver failure, intestinal malabsorption syndromes, presence of malignant neoplasm, previous or current use of anti-osteoporosis drugs and medications able to modulate PTH (i.e., cinacalcet) or FGF23 (i.e., burosumab), use of diuretics, chronic use of systemic corticosteroids, pregnancy or breastfeeding.

Data collection

Computerized medical records were reviewed and demographic, clinical and radiological data were collected for all patients. These included the following: age, sex, height, weight, body mass index (BMI), clinical features (bone deformities, previous orthopedic surgery, fragility fractures and enthesopathies on X-ray, dental abnormalities, renal lithiasis, gait impairment, musculoskeletal pain) and ongoing treatments (in particular, oral phosphate salts and calcitriol supplementation).

Biochemical analysis

Blood and urine samples were collected from all patients for biochemical analysis at the Laboratory Medicine Unit of the University of Padova, using methods whose quality performance are monitored according to ISO 15189 standard. Lithium-heparin plasma and 24 h urine samples have been collected for calcium and phosphate

Table 1: Formulae adopted for phosphate metabolism assessment.

a. uPi/Cr=uPi/uCr
b. TRP=1−(uPi/sPi) × (sCr/uCr)
c. TmP/GFR:
If TRP>0.86 then TmP/GFR=0.3 × sPi × TRP/(1−0.8 × TRP)
If TRP≤0.86 then TmP/GFR=sPi × TRP

sPi, serum phosphate concentration; sCr, serum creatinine concentration; uPi, urinary phosphate concentration; uCr, urinary creatinine concentration; uPi/Cr, urinary phosphate to creatinine ratio; TRP, tubular reabsorption of phosphate; TmP/GFR, tubular maximum phosphate reabsorption per glomerular filtration rate.

measurements using a colorimetric methods, and for creatinine enzymatic assay (with calibration traceable to the reference procedure), with the following reference range for plasma phosphate (0.87–1.45 mmol/L), calcium (2.10–2.55 mmol/L) and creatinine (45–84 µmol/L in females and 59–104 µmol/L in males). Albumin was measured by an immunoturbidimetric method, all tests carried out on Cobas 8000 (Roche Diagnostics, Mannheim, Germany). Serum 25-OH-vitamin D, 1,25-$(OH)_2$-vitamin D, PTH (third generation assay, reference range 6.5–36.8 ng/L) and intact FGF23 (plasma K_2-EDTA) were measured using an automated immunochemiluminescent methods (Liaison XL, DiaSorin, Saluggia, Italy), while serum C-terminal FGF23 with an ELISA kit (Pantec, Torino, Italy).

In order to ensure similar sampling conditions, all patients underwent overnight fasting and did not take oral phosphate supplements for at least 8 h prior to blood collection, that was obtained between 8.30 and 9.00 am. The 24 h urine sample was obtained discarding first morning void of the day prior to blood tests and collecting subsequent 24 h-urines, including the next morning's first void. After 2 h from the first morning void, the urine produced in this time lag were collected [11]. We decided to wait 2 h after the first void, in order to be sure that a second void might be produced from all patients. Patients were on a free diet in the days preceding the samplings.

The formulae shown in Table 1 were used to calculate urinary phosphate to creatinine ratio (uPi/Cr), tubular reabsorption of phosphate (TRP) and tubular maximum phosphate reabsorption per glomerular filtration rate (TmP/GFR), for both 24 h and 2 h urine collections [17].

Statistical analysis

Normally distributed continuous variables are expressed as mean ± standard deviations (SD). Non-normally distributed variables are expressed as the median and interquartile range (IQR). Pearson and Spearman correlation coefficients were used to assess the correlation between variables, depending on the data distribution. Lin's concordance correlation coefficient (CCC) [18], Bland-Altman method [19], and P30 and P35 accuracies (i.e., percentage of TmP/GFR 2 h values within ±30 and ±35 % of corresponding TmP/GFR 24 h values) were used to evaluate the agreement between the two variables. Multifactorial hypotheses were tested by multiple linear regression analysis. In multiple models, data were expressed as standardized regression coefficients (β) and p-value. The results were considered significant when the p-value was less than 0.05. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software.

Table 2: Patients' characteristics.

General	
Age, years	
Mean ± SD	46 ± 14
Range	24–78
Gender	
Female, n (%)	11 (55)
Male, n (%)	9 (45)
Height, cm	
Mean ± SD	155.4 ± 9.2
Range	144–173
Weight, kg	
Mean ± SD	60.6 ± 10.0
Range	45–77
BMI, kg/m^2, mean ± SD	24.9 ± 2.3
Diagnosis	
XLH, n (%)	17 (85)
TIO, n (%)	3 (15)
Clinical features	
Fragility fractures, n (%)	8 (40)
Bone deformities, n (%)	15 (75)
Previous orthopedic surgery, n (%)	14 (70)
Enthesopathy on X-ray, n (%)	11 (55)
Dental abnormalities, n (%)	10 (50)
Renal lithiasis, n (%)	10 (50)
Gait impairment, n (%)	6 (30)
Musculoskeletal pain, n (%)	15 (75)
Ongoing treatment	
Oral phosphate salts, mg/day, median (IQR)	3,060 (2,452–3,836)
Oral calcitriol, µg/day, median (IQR)	0.75 (0.50–0.94)

Data are expressed as mean ± SD, median and IQR, or absolute number and percentage, as appropriate. SD, standard deviation; IQR, interquartile range; BMI, body mass index; XLH, X-linked hypophosphatemia; TIO, tumor-induced osteomalacia.

Results

Patient characteristics

A total of 20 patients were included in the study, 17 with XLH and 3 with TIO (see Table 2). Eleven patients (55 %) were female, and mean age was 46 ± 14 years (range: 24–78 years), with patients affected by XLH being younger than patients with TIO (43 ± 10 vs. 62 ± 19 years, p=0.02). Patients with XLH were also significantly shorter than patients with TIO (152.6 ± 6.9 vs. 171.0 ± 1.7 cm, p<0.001). All patients with TIO had symptomatic fragility fractures, compared to 29 % (i.e., 5/17) of patients with XLH. No patients with TIO had bone deformities, dental abnormalities, renal lithiasis or gait impairment, which were instead present in the majority of XLH subjects (Table 2). All patients were treated with oral phosphate salts (overall median dose: 3,060 mg/day, median dose for patients with XLH: 2,500 mg/day, median dose for

patients with TIO: 4,000 mg/day), while 18 patients were also treated with oral calcitriol (overall median dose: 0.75 µg/day, median dose for patients with XLH: 0.75 µg/day, median dose for patients with TIO: 0.50 µg/day). Patients with prior serum 25-OH-vitamin D levels lower than 50 nmol/L were also treated with oral cholecalciferol supplements.

Renal phosphate handling measures

Laboratory test results are summarized in Table 3. Median plasma phosphate levels were 0.63 mmol/L (IQR 0.51–0.71 mmol/L), and all patients had high serum levels of both intact (median 165.1 pg/mL, IQR 106.1–455.0 pg/mL) and C-terminal FGF23 (median 4.35 pmol/L, IQR 1.58–8.60 pmol/L). As expected, all patients had low TmP/GFR 2 h (median 0.35 mmol/L, IQR 0.24–0.47 mmol/L) and TmP/GFR 24 h (0.31 mmol/L, IQR 0.22–0.43 mmol/L) towards age- and gender-specific reference range [20].

The Bland-Altman analysis revealed that TmP/GFR 2 h urine sample had a positive bias of 0.05 mmol/L (95 % limits of agreement: −0.10 to 0.20) vs. TmP/GFR 24 h (Figure 1A). Overall, in 70 % (i.e., 14/20) and 80 % (i.e., 16/20) of cases the difference between TmP/GFR 2 h and TmP/GFR 24 h was within ±30 and ±35 %, respectively. A systematic negative bias of −0.79 mmol/mmol (95 % limits of agreement: −2.87 to 1.29) was found for uPi/Cr 2 h vs. uPi/Cr 24 h (Figure 1B). The CCC between TmP/GFR 2 h and TmP/GFR 24 h was 0.86 (95 % CI 0.69–0.93) (Figure 1C), whereas the CCC between uPi/Cr 2 h and uPi/Cr 24 h was 0.81 (95 % CI 0.62–0.91) (Figure 1D).

Table 3: Laboratory tests.

Variable	Results
Serum phosphate, mmol/L	0.63 (0.51–0.71)
TmP/GFR 2 h, mmol/L	0.35 (0.24–0.47)
TmP/GFR 24 h, mmol/L	0.31 (0.22–0.43)
uPi/Cr 2 h, mmol/mmol	3.75 (2.65–5.18)
uPi/Cr 24 h, mmol/mmol	4.41 (3.55–5.66)
Serum creatinine, µmol/L	69.0 (53.9–82.2)
Creatinine clearance, mL/min	117 (93–129)
Serum albumin, g/L	44.0 (41.5–47.0)
Serum calcium, mmol/L	2.39 (2.31–2.43)
Urinary calcium, mmol/24 h	3.73 (2.75–4.95)
PTH, ng/L	28.0 (22.8–41.4)
25-OH-vitamin D, nmol/L	90.5 (65.3–103.0)
1,25-$(OH)_2$-vitamin D, pmol/L	93.0 (64.0–142.3)
Intact FGF23, pg/mL	165.1 (106.1–455.0)
C-terminal FGF23, pmol/L	4.35 (1.58–8.60)

Data are expressed as median and interquartile range (IQR). TmP/GFR, tubular maximum phosphate reabsorption per glomerular filtration rate; uPi/Cr, urinary phosphate to creatinine ratio; PTH, parathyroid hormone; FGF23, fibroblast growth factor 23.

To further insight into the reasons of the relatively sub-optimal agreement between TmP/GFR 2 h and TmP/GFR 24 h, a sensitivity analysis was performed by excluding the 3 patients having the highest absolute differences (0.18 mmol/L for two patients and −0.18 mmol/L for one patient) between TmP/GFR 2 h and TmP/GFR 24 h values (see Figure 1A). These three patients were females in 2 cases and male in 1 case, were affected by XLH, aged 34–54 years, and they did not significantly differ from the other patients in terms of clinical, anthropometric characteristics, and laboratory tests.

The exclusion of these three patients showed that the systematic bias remained almost unchanged but the 95 % limits of agreement substantially improved for both TmP/GFR (mean 0.05 mmol/L, 95 % limits of agreement: −0.03 to 0.13, Figure 2A) and uPi/Cr (mean −0.80 mmol/mmol, 95 % limits of agreement: −2.19 to 0.55, Figure 2B) and this was also true for the CCCs (TmP/GFR 2 h vs. TmP/GFR 24 h, CCC: 0.94, 95 % CI 0.85–0.97, Figure 2C; uPi/Cr 2 h vs. uPi/Cr 24 h, CCC: 0.89, 95 % CI 0.75–0.95, Figure 2D).

TmP/GFR determinants

To identify the determinants of TmP/GFR 2 h and TmP/GFR 24 h, a correlation analysis was performed. TmP/GFR 24 h showed significant correlations with intact FGF23 (r=−0.590, p=0.006) and C-terminal FGF23 (r=−0.623, p=0.003), as well as TmP/GFR 2 h (with intact FGF23, r=−0.620, p=0.004; C-terminal FGF23, r=−0.687, p=0.001). Neither TmP/GFR 24 h nor TmP/GFR 2 h were significantly correlated with serum PTH (r=−0.105, p=0.661, and r=−0.319, p=0.170, respectively). A multiple linear regression model was built up to test whether intact FGF23 and PTH resulted to be significant and independent correlates of TmP/GFR. Of note, intact FGF23 significantly predicted both TmP/GFR 2 h (β=−0.430, p=0.049) and TmP/GFR 24 h (β=−0.482, p=0.031), while PTH did not significantly predict neither TmP/GFR 2 h (β=−0.402, p=0.065) nor TmP/GFR 24 h (β=−0.318, p=0.139).

Furthermore, no significant correlation was observed between the dose of oral calcitriol taken by the patients and serum phosphate (r=−0.362, p=0.117), TmP/GFR 2 h (r=−0.407, p=0.075), and TmP/GFR 24 h (r=−0.240, p=0.307). Similarly, no significant correlation was found between the dose of oral phosphate taken by the patients and serum phosphate (r=−0.048, p=0.841), TmP/GFR 2 h (r=−0.420, p=0.066), and TmP/GFR 24 h (r=−0.403, p=0.078).

Discussion

In the present study, we observed that TmP/GFR – regardless of the urine sample used to calculate it – was markedly

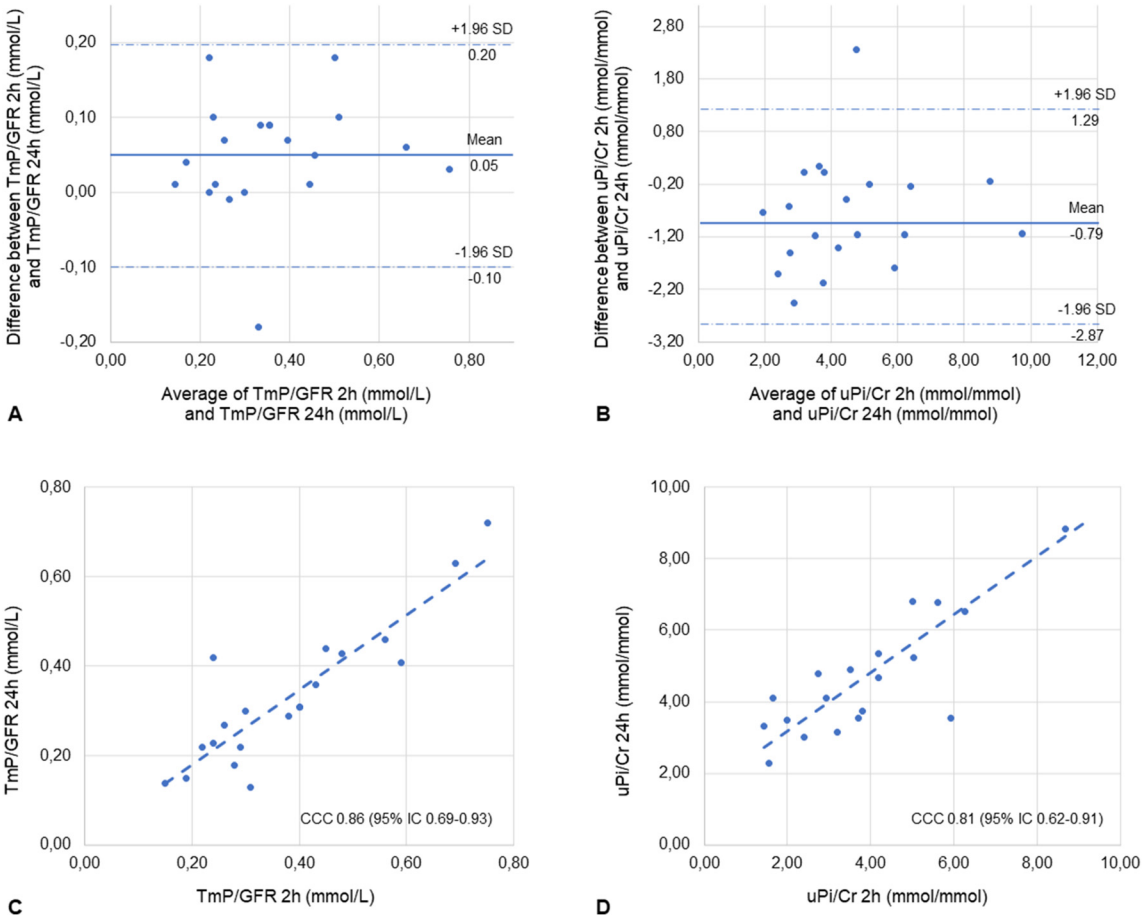

Figure 1: Bland-Altman analysis (A) and Lin's concordance correlation coefficient (C) for TmP/GFR 2 h and TmP/GFR 24 h. Bland-Altman analysis (B) and Lin's concordance correlation coefficient (D) for uPi/Cr 2 h and uPi/Cr 24 h.

below the age- and gender-specific reference range in all patients with FGF23-dependent hypophosphatemia, confirming the disruption of renal phosphate handling by which they are affected. TmP/GFR shows higher values when calculated for 2 h urine sample than 24 h sample. Since for calculating both parameters we used the same values of serum phosphate and creatinine, this difference is necessarily due to the extent of urinary phosphate excretion as confirmed by the calculation uPi/Cr, which instead is lower on 2 h sample. Our hypothesis is that phosphate excretion is lower during the first hours of the morning, reflecting circadian changes in serum and urinary phosphate concentrations as previously described [21]; in addition, these circadian changes may be accentuated in patients taking oral phosphate supplements, as we expect them to increase serum phosphate levels and, consequently, urinary phosphate levels only during the daytime, with a subsequent decrease during overnight fasting.

Under physiological conditions renal phosphate handling depends mainly on PTH and FGF23, whose action is to reduce the expression of the same sodium-dependent phosphate cotransporters, NaPi-2a and NaPi-2c, on the luminal membrane of the proximal renal tubule, thus inhibiting phosphate tubular reabsorption. In FGF23--dependent hyperphosphaturic hypophosphatemia instead, according to our regression model, TmP/GFR seems to depend more on FGF23, suggesting that when present in excess it may predominate over PTH.

Despite TmP/GFR 2 and 24 h show a relatively suboptimal agreement, the difference between the two parameters appears to be small and not clinically significant in the setting of FGF23-dependent hypophosphatemia. In fact, by calculating the TmP/GFR 2 h from the corresponding TmP/GFR 24 h value and adding the positive bias calculated with the Bland-Altman analysis, the TmP/GFR values still turn out to be significantly reduced compared to the reference range, thus minimizing the risk of failing to identify a patient with a hypophosphatemic hyperphosphaturia.

Patients with XLH and TIO are often affected by conditions such as primary or tertiary hyperparathyroidism,

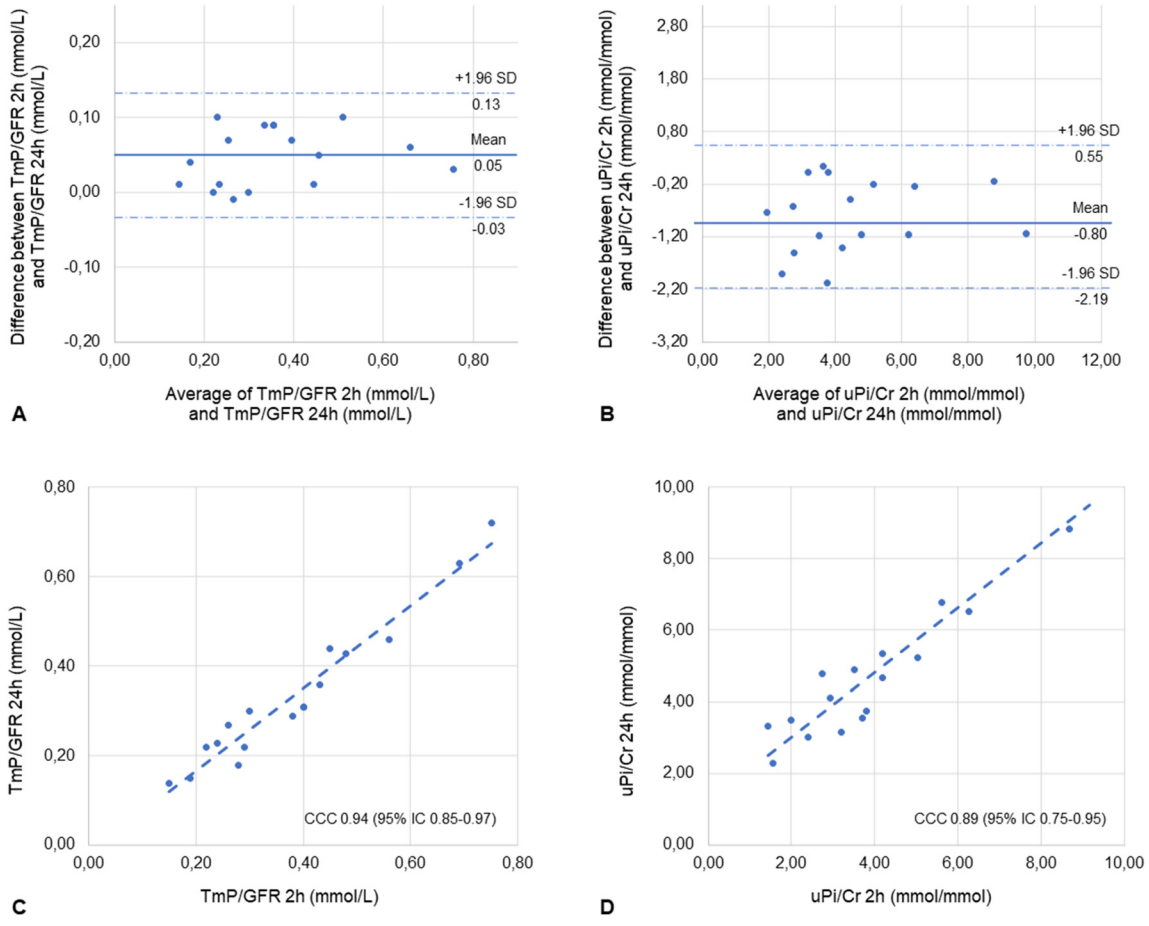

Figure 2: Bland-Altman analysis (A) and Lin's concordance correlation coefficient (C) for TmP/GFR 2 h and TmP/GFR 24 h, after excluding the 3 patients with the highest absolute differences. Bland-Altman analysis (B) and Lin's concordance correlation coefficient (D) for uPi/Cr 2 h and uPi/Cr 24 h, after excluding the 3 patients with the highest absolute differences.

hypercalciuria, nephrolithiasis, or nephrocalcinosis [15, 22, 23]. Thus, a 24 h urinary collection may be necessary to assess the extent of urinary calcium and other solutes (e.g., urinary citrate, oxalate, uric acid), as their excretion cannot be adequately measured with 2 h or spot morning samples [14]. Considering the data presented in this study, the 24 h urine sample could be an alternative to the 2 h morning sample for calculation of TmP/GFR. This could enhance patient compliance, as they would only need to collect one type of urine sample.

There are several limitations of this study. Firstly, the sample size is relatively small even considering the prevalence of the diseases that were included. Then, participants were not prescribed a study diet and were not in a fasting state during urine sample collections, particularly the 24 h sample. However, neither calcitriol therapy nor phosphate supplements were determinants of both TmP/GFR 2 and 24 h. Furthermore, since we included only patients with FGF23-dependent hyperphosphaturic hypophosphatemia and excluded those with eGFR<60 mL/min/1.73 m^2, we cannot predict if in other settings TmP/GFR 2 and 24 h have a different level of agreement or if they may depend also on other factors. In particular, in patients with CKD, there are both a reduced capacity for urinary phosphate excretion and an increase in PTH, which, when present in high concentrations, can amplify the TmP/GFR lowering effect of FGF23 excess [24].

In conclusion, in adult patients affected by XLH or TIO, TmP/GFR 2 and 24 h are markedly reduced towards reference range and their difference is within ±30 % in most cases. Further research is needed to evaluate TmP/GFR 24 and 2 h concordance in other clinical settings.

Research ethics: This study was conducted in accordance with the principles of the Declaration of Helsinki, and was performed according to ethics committee approval (391n/AO/23).

Informed consent: Informed consent was obtained from all individuals included in this study.

Author contributions: The authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Competing interests: All other authors state no conflict of interest.
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Data availability: The raw data can be obtained on request from the corresponding author.

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