Molecular Genetics and Metabolism 140 (2023) 107649

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Renal function, sex and age influence purines and pyrimidines in urine and could lead to diagnostic misinterpretation

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ARTICLE INFO

Keywords:
Glomerular filtration rate
Children
Chronic renal insufficiency
Purines
Pyrimidines
Urate
Lesch-Nyhan

ABSTRACT

Glomerular filtration rate (GFR) is commonly used in clinical practice for the diagnosis and follow-up of chronic kidney disease. Screening for inborn errors of metabolism (IEM) is based on analysis of biomarkers in urine, reported by their ratio to urinary creatinine (crn). Impaired renal function may complicate the interpretation of several biomarkers used for screening of IEM. Our goal was to investigate the influence of kidney function, in terms of measured GFR (mGFR) on purines and pyrimidines in urine, in addition to the relationship to sex, age, pH and ketosis. Children ($n = 96$) with chronic kidney disease (CKD), in different CKD stages, were included. Urine samples were obtained prior to the injection of iohexol. Serum samples at 7 time-points were used to calculate mGFR based on iohexol plasma clearance. The association with sex, age, ketosis and pH was examined in samples of the laboratory production from 2015 to 2021 ($n = 8192$). Age was a highly significant covariate for all markers. GFR correlated positively to several purines and pyrimidines; the ratios hypoxanthine/crn, xanthine/crn and urate/crn ($p = 2.0 \times 10^{-14}$, $< 3 \times 10^{-15}$ and 7.2×10^{-4}, respectively), and the ratios orotic acid/crn, uracil/crn, and carbamyl-β-alanine/crn ($p = 0.03$, 1.4×10^{-6} and 0.003, respectively). The values of urate/crn, xanthine/crn, uracil/crn, and carbamyl-β-alanine/crn were higher in females above 16 years of age. Ketosis and pH influenced some markers. In conclusion, decreased renal function interferes with the excretion of urinary purines and pyrimidines, and this could change decision limits substantially, e.g. result in false negative results in Lesch-Nyhan syndrome.

Synopsis: GFR influences purines and pyrimidines in urine.

Clinical Trial Registration: ClinicalTrials.gov, Identifier NCT01092260, https://clinicaltrials.gov/ct2/show/NCT01092260?term=tondel&rank=2

1. Introduction

Glomerular filtration rate (GFR) is used in daily practice for the diagnosis and follow-up of kidney disorders [1]. In addition to GFR, tubular reabsorption and secretion determine the kidneys' elimination rate of plasma molecules, e.g. diagnostic biomarkers [2]. Impaired renal function, affecting both GFR and tubular function and altering the biomarker concentrations in plasma and urine, may thus influence the interpretation of several non-renal biomarkers. A substantial part of the screening for inborn errors of metabolism (IEM) is based on analysis of different biomarkers in urine. In order to adjust for different degrees of water reabsorption, the markers are regularly reported by their ratio to the urinary creatinine (crn) concentration. With the use of iohexol clearance we have previously demonstrated that the levels of creatine and guanidinoacetate in plasma and their ratios to crn in urine were dependent on renal function as expressed by measured GFR (mGFR) to an extent that could lead to misinterpretation when diagnosing creatine deficiency syndromes in patients with chronic kidney disease (CKD) [3].

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https://doi.org/10.1016/j.ymgme.2023.107649
Received 5 December 2022; Received in revised form 28 June 2023; Accepted 1 July 2023
Available online 5 July 2023

Purines and pyrimidines in urine are important diagnostic markers for several IEMs, and are part of the general metabolic screening in patients with different symptoms in addition to kidney stones; e.g. unexplained intellectual disability, neurological symptoms, hearing loss, eye symptoms, immune deficiency, anemia and also if urea cycle disorder is a possibility based on the clinical picture [4]. In our laboratory, urine urate analysis is first tier in all IEM patients independent of clinical signs and symptoms [5]. To our knowledge, the effect of kidney function on the urine levels of purines and pyrimidines has so far not been published.

The goal of our study was to investigate how kidney function, in terms of measured GFR, acts on the levels of purines and pyrimidines in urine, and to which extent this could affect the reference- and decision limits in a cohort of children with CKD analogous to other IEM markers in urine (creatine and guanidinoacetate). In addition, we examined the relationship of sex, age, pH and ketosis with the levels of purines and pyrimidines in a reference population.

2. Materials and methods

2.1. Study population

2.1.1. GFR cohort

Children ($n = 96$) with CKD caused by different kidney disorders, in stable phase, were recruited from Oslo University Hospital and Haukeland University Hospital. The included children were in CKD stages 1–5 with: 28, 27, 23 and 18 patients in stages 1, 2, 3 and 4–5, respectively [6]. The median mGFR was 65.9 (range 6.3–153) mL/min/1.73 m^2 and median age 9.2 years (range 3 months to 17.5 years). Exclusion criteria were allergy to iohexol and X-ray contrast agents given less than five days prior to the study day. The study was approved by the Regional Ethics Committee of Western Norway, in accordance with the Helsinki Declaration and Good Clinical Practice. Written informed consent was obtained from patients and/or their parents.

2.2. Methods

In the GFR cohort serum samples (3 mL) and morning void urine samples were obtained prior to the injection of iohexol (Omnipaque®, 300 mg I/mL, GE Healthcare), for measurements of different biomarkers, including purines, pyrimidines, urate and creatinine. Omnipaque® was given intravenously, and iohexol clearance calculated as previously described [3,7]. Serum samples (0.5 mL) were collected from the contralateral arm of the iohexol injection at seven different time points ranging from 10 min to 300 min, to establish the gold standard GFR: 7-point mGFR based on plasma clearance [7,8]. Iohexol concentrations were analyzed by a HPLC system, as described elsewhere [7,9]. Quantification of purines (adenine, adenosine, deoxyadenosine, deoxyinosine, deoxyguanosine, inosine, guanosine, hypoxanthine and xanthine) and pyrimidines (carbamyl-β-alanine, uracil, orotic acid, pseudouridine, thymidine, thymine and uridine) in urine was performed by liquid chromatography-tandem mass spectrometry (LC-MSMS). Sample preparation included dilution to a creatinine concentration of 1 mmol/L using mobile phase A (described below) and filtering through syringe filter. LC-MSMS analysis was done using C18 reversed phase chromatography with gradient elution (mobile phase A: 50 mM acetic acid pH 4, mobile phase B: 50 mM ammoniumformate pH 5/methanol/acetonitrine, 2:1:1). Flowrate was 0,2 mL/min, injection volume 4 mL, and total runtime 20 min. The LC was coupled to a Sciex 4500 Q-Trap mass spectrometer running in negative ionization, MRM-mode, with MRM-transitions from deprotonized molecular ion (M^-) and formate-adducts ($M + formate^-$) for the purines and pyrimidines, respectively, to corresponding molecular fragments (fragments of M^-) and intact molecules (M^-). A 6 point calibration from 1 to 60 μmol/L of the purines and pyrimidines was included in every analysis sequence, and a mix of isotopically labelled internal standard (uracil, thymine, adenine, hypoxanthine, xanthine, orotic acid, adenosine) at a concentration of 31,4 mM each was added to all calibrators and samples to ensure high quality quantitative data. A reported analyte/creatinine value <0.01 μmol/mmol were concidered below the limit of quantification (LOQ). Urinary creatinine and urate were measured by enzymatic colorimetric methods (Roche Diagnostics®), IDMS traceable. Our laboratory participates in different external quality programs; European Research Network for evaluation and improvement of screening, diagnosis and treatment of Inherited Metabolic Disorders (ERNDIM) (purines and pyrimidines), LabQuality (creatinine, urate) and Equalis (iohexol clearance) with satisfactory results.

2.3. Statistical analysis

2.3.1. Reference population – adjustment for age dependence

As reference population we selected all first samples of individuals from the laboratory production in the period 2015.10.01 to 2021.11.30 ($n = 8192$) after all known cases with purine-, pyrimidine- or urea cycle disorders were excluded. As the urine levels of the purine- and pyrimidine/crn ratios (from now on called markers) vary significantly with age, we applied a slightly modified version of the LMS model to adjust for this covariate [10]. In short, a power transformation (Box-Cox) is used to obtain a normal distribution of the marker values, before constructing continuous curves for the age trend of the mean and standard deviation. This allows for the establishment of reference percentile charts, analogous to pediatric growth charts, as well as the expression of specific test results in terms of age-adjusted z-scores, i.e. the deviation from the age-specific mean, in multiples of the age-specific standard deviation. For example, a baby at 3.5 weeks with an urinary urate/crn ratio of 1.2 μmol/mmol might have the same z-score = 0 as a a 3 year old with an urinary urate/crn ratio of 0.6, as they would then be exactly at their age-specific mean, i.e. 0 standard deviations from the mean. *Z*-scores may also elegantly relate to reference or decision limits. A z-score of 1.96, for example, would mean that the patient's test result is just above the 97.5th percentile for that age.

In more detail, this was achieved by a multistep process. The age axis was divided into bins of $n = 120$ samples and age was Box-Cox transformed ($\alpha = [B\text{-}C; \lambda]$ age) to approximate a rectangular distribution (this to ensure even weight across the age span in the polynomial regression later on). The marker in question (X) was Box-Cox-transformed to remove skewness $\Xi = [B\text{-}C;\lambda]$ X. Within each bin the marker values were sorted in ascending order and given a rank k. Then a Q-Q plot was constructed, with abscissa = Ξ and ordinate = the z-score corresponding to the percentile = $(k - 3/8)/(n + ¼)$ in the standard normal distribution [11]. In the further calculations, the central 95% part of the Q-Q plot was used. The Box-Cox parameter λ did not seem to show any significant dependence on age. Thus as optimal λ was chosen the value that gave maximal mean of all bin specific correlation coefficients between Ξ and z, rendering the central 95% part of the Q-Q plot fairly rectilinear. This implies that Ξ follows approximately a normal distribution, from which estimates of the mean μ and the standard deviation s were calculated by regression analysis. In the situations where the limit of quantification (LOQ) exceeded the 2.5%-ile, the values below LOQ were still retained for the ranking and calculation of percentiles, but not used in the regression calculation of μ and s. The estimated values of μ and s were subjected to low power polynomial regression (order ≤ 3) in segments along the covariate scale (transformed age = α) that were seamlessly connected at their intersections. From the continuous functions μ(α) and s(α) an experimental z-score: $z_{exp} = (\Xi - \mu(\alpha))/s(\alpha))$ was calculated for each marker value. *Z*-scores outside the Tukey fences $z = \pm 3.37$, corresponding to a Tukey factor = 2 were considered as outliers and excluded. The whole estimation process was repeated until no outliers were detected. Age-adjusted percentiles were constructed from the expression $\mu(\alpha) + z_p \cdot s(\alpha)$, where z_p is the theoretical z-score in the standard normal distribution. Reverse transformation established reference percentile charts in original units. Dependence between z_{exp}

and urine dipstick ketosis results and pH was depicted by scatter plots.

2.3.2. GFR cohort

The markers' dependence on renal function in the case group (GFR cohort) was best described by a rectilinear relation between z_{exp} and lg (mGFR), which was fitted by a non-parametric rectilinear Theil-Sen regression. A couple of extreme outliers as judged by visual inspection were removed from the regression, and subsequent correlation testing was done using Pearson's correlation coefficient and the HC4 estimator of standard errors to account for heteroscedasticity [12]. A statistically significant effect size, as Cohen's d or a z-score difference was considered to be biologically relevant when being >0.25 (Gowans' criterion) [13]. Several markers had a substantial number of values below LOQ. The difference in the relative portion of non-measurable values between the GFR cohort and a comparable reference group (REF-960) was tested by χ2-test. The latter group was a subset of the reference population constructed by matching by age each individual in the GFR cohort with 10 individuals in the total reference population. Median relative matched age difference between the two groups was exactly 0%, interquartile and total ranges were (−0.1 to +0.1) and (−1.1 to +2.2) % respectively. This procedure served as an alternative method to indicate the degree of GFR influence, since the GFR cohort doubtlessly comprises a much higher relative amount of individuals with low renal function than REF-960. Microsoft Excel (version 2016) and R (version 4.1.3) were used for statistical analysis. To compensate for multiple testing the significance level was set at a value $P_i = i \cdot 0.05/m$, determined by the Benjamini-Hochberg procedure, where i is the rank of the actual *p*-value and m is the number of comparisons.

2.3.3. Adjustment for covariates

The general reader may apply the theory developed here to adjust for the dependence on the covariates, although being rather complex and highly marker specific. The patient median of the local laboratory production should be calculated in a narrow age interval with an appreciable amount of data. An eventual substantial systematic deviation of the median from what is reported through the percentile curves provided here, may then be corrected for in terms of z-score, i.e. by subtracting the z-score of the local patient median from the z_{exp}. As will be shown below, age carries the major part of the covariate variation. Hence, as a first approximation the adjustment for reduced renal function (low GFR) is performed by adding to z_{exp} the actual z-score deviation from 0 obtained from the graphs exhibiting the linear relationship between the z_{exp} and the logarithm of GFR. Using the scatter plots between z_{exp} and dipstick readings the deviations for urine dipstick ketosis and pH have finally to be added to z_{exp} (after age and GFR adjustment) to see if the total displacement then causes z_{exp} to cross the diagnostic decision limit.

3. Results

3.1. GFR cohort

Details about the GFR cohort ($n = 96$) are presented in Table 1. Table 2 shows to which extent GFR affects the biomarkers. Several disease markers (hypoxanthine, xanthine, urate, orotic acid, uracil, carbamyl-β-alanine and pseudouridine) in the GFR group exhibit all or the majority of values above LOQ. For these markers the regression analysis gives an adequate description of the GFR dependence. GFR has a highly significant correlation with three purines; the ratios hypoxanthine/crn, xanthine/crn and urate/crn in urine. These three purines show clearly decreased levels with lower GFR ($p = 2.0 \times 10^{-14}$, $< 3 \times 10^{-15}$ and 7.2×10^{-4}, respectively) (Fig. 1, Tables 2 & 3). In addition, the same relationship is observed regarding three pyrimidines: the ratios orotic acid/crn ($p = 0.03$), uracil/crn ($p = 1.4 \times 10^{-6}$), and carbamyl-β-alanine/crn ($p = 0.003$) (Fig. 2 and Tables 2 & 3). The effect size in terms of Δz-score (difference from the reference population) at specific GFR levels ranging from 90 to 15 mL/min/1.73 m^2 is listed in Table 3. Among those markers with considerable number of values below as well as above the LOQ (but with non-significant slope in the regression analysis), adenine, deoxyguanosine, deoxyinosine and guanosine showed a statistically significant lower fraction of non-measurable values in the GFR cohort than the REF-960 group as demonstrated with a 2 × 2 table χ2-test. This might indicate that a reduced GFR is associated with some increase in these markers. The relationship for uridine goes in the opposite direction. However, as the fraction of non-measurable values could be sensitive to imprecision in the determination of LOQ these results should interpreted with some caution and ought to be controlled by a measuring method with a higher analytic sensitivity.

For the remaining markers, adenosine, inosine, thymine and pseudouridine, both the regression analysis and 2 × 2 table χ2-test failed to reveal any significant relation to GFR.

Table 1
Characteristics of the GFR cohort.

Variable	Value [median (range) or number]
Total number (F/M)	96 (41/55)
Age, years	9.2 (0.3–17.5)
Body weight, kg	28.3 (6.6–84.6)
Height, cm	134 (59–177)
Measured GFR (7-point iohexol clearance), mL/min/1.73 m^2	65.9 (6.3–153)

F = female, M = male.

3.2. Reference population

The Box-Cox λ's needed to make the markers in the reference population approximately normally distributed, together with the association with sex, pH and ketosis are listed in Table 4. Two purines (urate and xanthine) and three pyrimidines (carbamyl-β-alanine, pseudouridine and uracil) exhibited statistically significant and biologically relevant higher values in females after age 16 years, Δz-score (mean z-score females – mean z-score males) > 0.25, presumably coinciding with the cessation of puberty. In the reference population we observed elevated xanthine/crn, urate/crn, and orotic acid/crn when pH was high (> 7) (Table 4 and Suppl. 2I, 2 J, 2 K). Higher levels of inosine/crn, hypoxanthine/crn and carbamyl-β-alanine/crn were observed in samples with ketosis (Table 4 and Suppl. 3D, 3H, 3P), together with a modest elevation in deoxyguanosine/crn, deoxyinosine/crn, xanthine/crn, and uracil/crn (Table 4 and Suppl. 3E, 3F, 3I, 3 N). Of these markers, only xanthine showed a significant effect of pH; high pH leading to elevated level. For urate/crn and orotic acid/crn, on the contrary, there were lower levels in marked ketosis (dipstick ketones 4+) (Table 4 and Suppl. 3 J, 3 K). Here levels increased with increasing pH. However pH and ketosis showed only a weak interdependence, mean pH = 6,24, 6.14, 6.10, 5.98 and 5.73 for dipstick ketones 0, +1, +2, +3 and + 4 respectively. All this taken together supports the assumption that the markers' dependencies of pH and ketosis are largely independent of each other. The age-dependent reference percentiles for all the purines and pyrimidines are presented in Supplementary material (Suppl.1A-Q).

4. Discussion

This study demonstrates that age has a profound effect on the reference limits of urinary purines and pyrimidines ratios to creatinine. For some markers, sex also is an important denominator in adults with higher values in females. Low renal function interferes substantially with the filtration, reabsorption and excretion of several of these markers, resulting in significant alterations in the urinary levels of many diagnostic biomarkers. Furthermore, the presence of ketones and a deviant pH also may influence the urine levels.

Table 2
Association of GFR with the different biomarkers.

Marker	2 × 2 table test: Percentage below limit of quantification					Theil-Sen regression: z-score vs. lg(GFR) in the GFR cohort							Conclusion
	REF-960*	GFR cohort	$\chi^{2\dagger}$	*p*-value	Effect of decreased GFR††	n	Outliers	Intercept	Slope	Pearson's corr. Coeff.	*p*-value (HC4)	Effect of decreased GFR††	Effect of decreased GFR††
Purines													
U-Adenine/crn	78%	45%	49	2.6×10^{-11}	↑	51	2	1.8	−0.25	−0.095	0.76	n/s**	(↑)
U-Adenosine/crn	88%	84%	0.9	0.37	n/s	15	1	0.46	0.38	0.010	0.98	n/s	No effect
U-Inosine/crn	8.2%	4.2%	1.5	0.29	n/s	92		−0.85	0.81	0.16	0.26	n/s	No effect
U-Deoxyadenosine/crn	100%	100%	n/a			0							n/a
U-Deoxyguanosine/crn	87%	61%	41	6.0×10^{-10}	↑	37		1.3	0.22	0.39	0.67	n/s	(↑)
U-Deoxyinosine/crn	93%	82%	12	0.002	↑	16	1	1.3	0.38	0.025	>0.99	n/s	(↑)
U-Guanosine/crn	96%	89%	7.8	0.010	↑	11		3.8	−0.99	−0.19	0.68	n/s**	(↑)
U-Hypoxanthine/crn	0.0%	0.0%	n/a			96		−7.3	3.5	0.69	2.0×10^{-14}	↓	↓
U-Xanthine/crn	0.0%	0.0%	n/a			96		−11	5.5	0.81	$< 3.0 \times 10^{-15}$	↓	↓
U-Urate/crn	0.0%	0.0%	n/a			96		−3.0	1.5	0.39	7.2×10^{-4}	↓	↓
Pyrimidines													
U-Orotic acid/crn	9%	16%	3.7	0.08	(↓) n/s	82		−1.7	1.3	0.25	0.03	↓	↓
U-Thymidine/crn	98%	100%	1.0										n/a
U-Thymine/crn	36%	31%	0.6	0.45	n/s	65	1	0.25	0.14	0.11	0.60	n/s	No effect
U-Uracil/crn	0.0%	9.4%	n/a			87		−4.4	2.1	0.49	1.4×10^{-6}	↓	↓
U-Uridine/crn	46%	63%	9.3	0.005	↓	36		−0.14	0.35	−0.006	> 0.99	n/s	(↓)
U-Carbamyl-β-alanine/crn	1.0%	0.0%	n/a			96		−2.4	1.3	0.41	0.003	↓	↓
Quality marker													
U-Pseudouridine/crn	0.0%	0.0%	n/a			96		−0.09	−0.06	−0.040	0.851	n/s	No effect

p-values are adjusted for multiple testing by the Benjamini-Hochberg procedure with $\alpha = 0.05$. Parentheses enclosing the arrows symbolize that only one of the tests (2 × 2 table and regression) reached statistical significance. n/a = not applicable. n/s = not significant. * REF-960 is a subgroup of the full reference group matched to the GFR cohort with respect to age and sex. ** Indicates a regression slope that goes in the same direction as the 2 × 2 table test. † Yates corrected. †† The arrow indicates in which direction the marker level changes when GFR decreases. HC4 = heteroscedasticity-consistent estimator of standard errors.

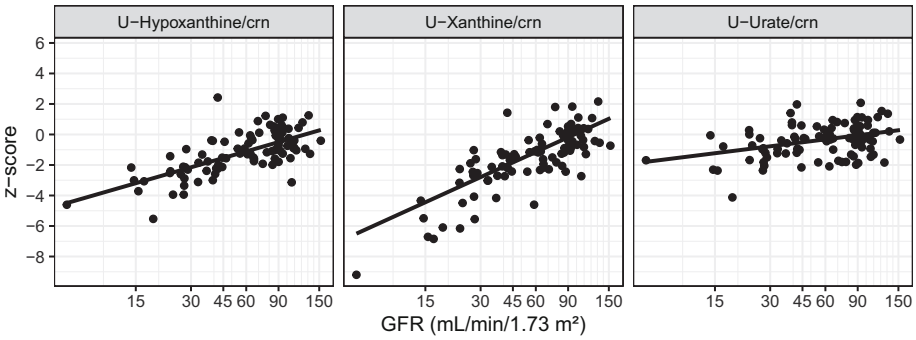

Fig. 1. Theil-Sen regression on lg(mGFR) (black lines) shows a biologically relevant association between GFR and three purines in urine (regression slopes in parentheses): hypoxanthine/crn (3.46), xanthine/crn (5.47) and urate/crn (1.50).

Table 3
Estimated mean z-scores at specified GFRs.

Marker	Mean z-score at specified GFR			
	90	60	30	15
Purines				
U-hypoxanthine/crn	−0.50	−1.11	−2.15	−3.19
U-xanthine/crn	−0.19	−1.15	−2.80	−4.44
U-urate/crn	−0.06	−0.32	−0.78	−1.23
Pyrimidines				
U-orotic acid/crn	0.79	0.56	0.18	−0.21
U-uracil/crn	−0.26	−0.63	−1.26	−1.89
U-carbamyl-β-alanine/crn	0.10	−0.13	−0.51	−0.89

Estimated mean z-scores at specified GFRs: 90, 60, 30 and 15 mL/min/1.73 m^2.

The defect in hypoxanthine:guanine phosphoribosyltransferase (HPRT) enzyme typically leads to elevated hypoxanthine, xanthine and urate in urine, and elevated urate in plasma. This is an X-linked recessive deficiency in the purine salvage pathway. Symptoms in the severe spectrum with complete enzyme deficiency include intellectual disability, involuntary movements, severe neurological symptoms and self-injury/behavioral problems, commonly named Lesch-Nyhan syndrome, (OMIM 300322) [14]. A partial HPRT deficiency, Kelley-Seegmiller syndrome (OMIM 300323), may generally have milder degrees of intensity. In addition, some of these patients have been described with acute kidney failure due to hypercalciuria and nephrocalcinosis, however, the main cause of renal failure in these patients is uric acid nephrolithiasis [15].

Our study demonstrated reduced levels of hypoxanthine/crn, xanthine/crn and urate/crn in the GFR cohort when the renal function decreases. These three markers are used in the routine diagnostics for several IEM, including Lesch-Nyhan syndrome and Phosphoribosyl pyrophosphate synthetase superactivity [14,16]. Since these diseases can affect the kidneys, the levels might fall below the reference limits, which could lead to false negative screening for these diseases. Thumfart et al. reported a patient with Lesch-Nyhan syndrome and renal failure, and xanthine excretion was not elevated but in the upper normal range, however, urate and hypoxathine were increased [17]. Urate/crn ratio is commonly used for 1.tier screening for IEM, as a part of general metabolic screening in urine, and both low and high urate/crn ratios are of clinical importance [4]. GFR was positively correlated with urate/crn ratio, however not as pronounced as for hypoxanthine/crn and xanthine/crn. The kidney handling of urate is complex and is a balance between production, renal elimination and intestinal elimination [18]. Tsai et al. have previously published a case with Lesch-Nyhan syndrome and normal uric acid in serum. The uric acid/creatinine ratio was used for the diagnosis instead. The authors discuss the possibility of borderline hyperuricemia due to increased renal clearance. Urinary uric acid/creatinine ratio is used as a screening test for inherited disorders of purine metabolism [19]. Another study of a patient with Lesch-Nyhan syndrome and end-stage renal disease demonstrated normal 24-h urinary uric acid excretion and abnormal uric acid/creatinine ratio [20].

Increased orotic acid and/or uracil are associated with several IEM as phosphoribosyl pyrophosphate synthetase 1 deficiency (OMIM 311850), Miller syndrome (OMIM 126064) and are important markers in the diagnostics of urea cycle disorders, e.g. ornithine transcarbamylase deficiency (OMIM 300461) [21–23]. A non-targeted metabolomics study from 2018 found a decrease in relative concentration of several urinary markers in decreasing estimated GFR, including uracil [24]. This is in accordance with our results from the GFR cohort, showing lower absolute uracil levels by the use of LC-MS technique. There are scarcely any published studies on the association between GFR and orotic acid or carbamyl-β-alanine.

The age-dependent reference limits for all the purines and pyrimidines in our study (reference group) deviate from the reference values used in our own laboratory today or what is available in standard textbook of metabolic disorders and publications. We here divulge examples of age-continuous percentile charts instead of values in wide

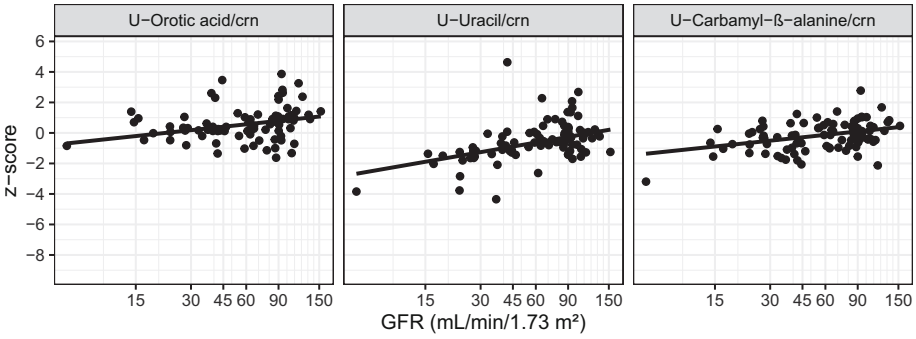

Fig. 2. Theil-Sen regression on lg(mGFR) (black lines) reveals a biologically relevant relationship between GFR and three pyrimidines in urine (regression slopes in parentheses): orotic acid/crn (1.28), uracil/crn (2.09), and carbamyl-β-alanine/crn (1.26).

Table 4
Association between sex, pH, ketosis and the biomarkers together with the optimal Box-Cox transformation parameters in the reference population.

Marker	Below limit of quantification[†]	Sex difference after 16 years			Box-Cox transformation parameter λ			Relation to pH	Relation to ketosis
Purines		***p*-value**	Cohen's *d*	Conclusion	All	Female	Male		
U-Adenine/crn	68%	0.13	−0.19	No	0.84			Elevated when pH > 7	No effect
U-Adenosine/crn	87%	0.10	−0.44	No	0.90			Slight inverse dependence	No effect
U-Inosine/crn	6.3%	0.79	0.03	No	1.19			Variable	Increase
U-Deoxyadenosine/crn	99.6%	0.16		n/a	n/a			n/a	n/a
U-Deoxyguanosine/crn	79%	0.79	−0.23	No	0.56			No effect	Moderate increase
U-Deoxyinosine/crn	83%	0.80	−0.10	No	1.87			No effect	Moderate increase
U-Guanosine/crn	94%	0.14	0.10	No	1.21			No effect	No effect
U-Hypoxanthine/crn	0.2%	$<2 \times 10^{-13}$	0.09	No	−0.08			No effect	Great increase
U-Xanthine/crn	0.0%	$<9 \times 10^{-14}$	−1.02	F > M	−0.09	−0.097	−0.11	Increasing	Increasing, but low at ketones 4+
U-Urate/crn	0.0%	7.1×10^{-7}	−0.59	F > M	−0.24	−0.26	−0.26	Increasing	Decrease at ketones ≥4+
Pyrimidines									
U-Orotic acid/crn	7.5%	0.03	−0.14	No	0.75			Increasing	Decrease at ketones ≥3+
U-Thymidine/crn	97%	0.93	0.06	No	0.74			n/a	n/a
U-Thymine/crn	35%	0.81	0.04	No	0.89			No effect	No effect
U-Uracil/crn	0.8%	5.7×10^{-7}	−0.28	F > M	0.37	0.35	0.36	No effect	Increase at ketones ≥3+
U-Uridine/crn	40%	0.90	−0.01	No	1.13			No effect	No effect
U-Carbamyl-β-alanine/crn	0.9%	1.2×10^{-6}	−0.28	F > M	−0.01	0.13	0.18	Low at pH = 5	Great increase
Quality marker									
U-Pseudouridine/crn	0.0%	$<6 \times 10^{-14}$	−0.54	F > M	−0.02	0.042	0.06	Moderate increase	Variable

p-values are adjusted for multiple testing by the Benjamini-Hochberg procedure with α = 0.05. n/a = not applicable. n/s = not significant. [†] Proportion of samples below the limit of quantification in the reference population. F = female, M = male.

discrete age-intervals commonly used in laboratories, which emphasize the importance of age-continuous percentiles to avoid false negative and false positive results. Other laboratories may utilize methods that have analytic accuracy (trueness and precision) different from ours. Still it is be expected that the covariate dependence, especially what concerns age, will be of the same functional form. It is important to consider this when a marker value is evaluated.

The levels of some purines and pyrimidines are influenced by sex. As far as we know, this has not previously been published in the literature. Xanthine/crn, urate/crn, uracil/crn, and carbamyl-β-alanine/crn ratio were higher in adult females than adult males in the reference group. Part of this may be attributed to creatinine, which has a higher value in male urine due to higher muscle mass. Flynn et al. recently investigated the sex-specific genetic effects across different biomarkers, and found sex differences, with higher levels in females, in the heritability of 17 of 33 biomarkers (mainly biomarkers in blood), including urate, however urate in urine was not included [25]. Costanzo and collegues reviewed recently the literature for evaluation of differences between male and female metabolome in blood and urine. Both sex and age are important to take into account in metabolomics studies, due to differences in genome, transcriptome and proteome [26].

Samples with pH above 7 showed elevated levels of xanthine/crn, urate/crn and orotic acid/crn (reference group). Urate is produced from hypoxanthine and xanthine by the enzyme xanthine oxidase. Low urinary pH is well known to predispose for uric acid nephrolithiasis, and an increase in pH would decrease uric acid crystallization [27]. A report from Ankem et al. discuss the importance of correct pH level in patients with Lesch-Nyhan syndrome and uric acid stones, and at a urinary pH level of 7, >1600 mg/dL of uric acid can be suspended in the urine as soluble salt. The same effect of altering urinary pH is not described in xanthine calculi [28].

Several studies have shown that ketones can inhibit the tubular secretion of uric acid and depress the clearance of this metabolite [29]. This is in accordance with our finding of lower urate/crn ratio with dipstick ketones 4+. It is also previously demonstrated that ketones can inhibit biosynthesis of pyrimidines in fetal rat brain, affecting some of the enzymes catalyzing the formation of orotic acid [30]. In accordance with this, we also found lower levels of orotic acid/crn when ketones were high (dipstick 4+). On the other hand, our study demonstrated increased levels of several purines and pyrimidines in patients with ketosis (reference group), of uncertain cause. The reason for ketosis in most of our patients is probably due to overnight fasting. Ketogenic diet is a well-known treatment in refractory epilepsy. Masino et al. discussed the possibility of increase in adenosine (a purine), due to ketolysis, either through ketogenic diet or starvation [31].

There are some limitations in this study. One is the number of patients in the GFR cohort ($n = 96$). However, to our knowledge there are no other published studies on CKD and purines and pyrimidines. Only one patient was younger than 1 year, but nine patients were < 2 years. It is also a disadvantage that we do not have samples from patients with purine- and pyrimidine disorders combined with kidney failure, and a simultaneous GFR measurement.

The strengths of our study are the large number of individuals in the reference population and the multi-point iohexol clearance to give an accurate mGFR in the GFR cohort, instead of the commonly used estimated GFR formulas based on creatinine and cystatin C [7,32].

In conclusion, our study demonstrates that decreased renal function interferes with the excretion of urinary purines and pyrimidines, and this could potentially change decision limits and lead to erroneous interpretation with false negative or false positive results. Age has a substantial effect on the reference limits, and sex and the presence of ketones and a deviant pH may also influence the levels of several of these biomarkers.

Author contributions

Cathrin Lytomt Salvador; Conceptualization, Project administration, Investigation, Resources, Writing – original draft, Writing – review & editing. Per Trygge Kjelland Flemmen; Formal analysis, Investigation, Writing – review & editing. Camilla Tøndel; Project administration, Resources, Writing – review & editing. Yngve Thomas Bliksrud; Resources, Writing – review & editing. Ellen Fun Fong Tsui; Formal analysis, Writing – review & editing. Atle Brun; Formal analysis, Writing – review & editing. Anna Bjerre; Resources, Writing – review & editing. Lars Mørkrid; Conceptualization, Project administration, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing. All authors read and approved the final manuscript for submission.

Ethics statement

The study was approved by the Regional Ethics Committee of Norway and written informed consent was obtained prior to inclusion in the study. The procedures followed were in accordance with the Helsinki Declaration and Good Clinical Practice.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Acknowledgements

We wish to express our gratitude to the pediatric study nurses Mai Britt Lynum (Oslo University Hospital), Hildur Grindheim and Renathe Håpoldøy (Haukeland University Hospital) for their technical assistance with the sample collection from the children and to the laboratory engineer Kjersti Carstensen for the iohexol analyses (Haukeland University Hospital). The study was supported by grants from the Health Trust of Western Norway, The Norwegian Society of Nephrology, Haukeland University Hospital and Oslo University Hospital. One of the authors (YTB) is member of the European Reference Network for Hereditary Metabolic Disorders (MetabERN). Berit Woldseth (Oslo University Hospital) generously read and corrected the final version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgme.2023.107649.

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