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Original Article

Biochemistry Section

Reference Interval, Biological Variations and Distribution of Creatinine Level in Fasting Urine: A Cross-sectional Study

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ABSTRACT

Introduction: Serum creatinine, urine creatinine, and rate of urine formation are components of creatinine clearance and are under multiple influences. Of these, urine creatinine has the widest variability, especially in the tropical region. Reference intervals, biological variations, and distributions of urine creatinine in the reference individuals are related and important for clinical interpretations of kidney functions.

Aim: To establish the reference intervals, biological variations, and distributions of creatinine in urine formed during morning fasting state from reference individuals in a tropical location.

Materials and Methods: This observational cross-sectional study of the sample population was done from January 2017-November 2021 for the establishment of reference interval. Reference individuals, defined by inclusion and exclusion criteria, were from the population at near sea levels plains, in Thrissur district, Kerala, avoiding the mountainous Western Ghats. Morning fasting, third sample of urine was collected to ensure that the urine collected was formed in the morning (n=156). A second set of 44 urine samples, collected from 10 reference individuals consecutively for 4-5 days, was used for estimating the analytical Coefficient of Variation (CV_A), within-subject (CV_I), and between-subject (CV_G) components of biological variation. Outliers in the creatinine data were identified and removed by the Box-Whisker plot to exclude the subclinical influences that affected the urine creatinine, followed by Box-Cox transformation to bring the distribution to Gaussian and reference interval calculations of fasting urine creatinine.

Results: As the total sample number was >120, non parametric percentile method was used to calculate the reference interval of 17.14-325.89 mg/dL. As the female sample number was between 40 and 120, double-sided, 95% reference interval, based on normal distribution was calculated as 12.41-328.71 mg/dL. As the male sample was <50, method of robust calculation was used to define the reference interval of 21.91-379.12 mg/dL. The reference interval and biological variation data showed gender differences in the distribution, CV_I, and CV_G due to lower concentration of fasting urine creatinine in females resulting from lower muscle mass.

Conclusion: Reference intervals of creatinine in fasting urine formed in the morning were established. Gender differences were observed in the distribution of data, CV_I, and CV_G.

Keywords: Box whisker plot, Coefficient of variation, Distribution analysis, Gender differences, Uncertainty

INTRODUCTION

The quantitative creatinine assays in serum and urine are affected by bias, imprecision, interferences, biological variation, different types of errors, and difficulties in clinical interpretations [1-3]. Enzymatic assays of creatinine have lower calibration and analytical bias/imprecision (analytical CV_A) when compared to chemical picric acid-based methods [4-6]. Creatinine Standardisation Program has recommended standardising creatinine assays to isotope dilution mass spectrometry reference measurement procedure and High-Pressure Liquid Chromatography (HPLC) method [7,8]. In the present report, creatinine was assayed by the enzymatic method traceable to HPLC method with a correlation coefficient of 0.999, as in the procedure manual [9]. Reported Reference intervals of spot urinary creatinine by the same enzymatic method (OCD Vitros) of a sample population aged 30-79 years in men was 14.71-294.12 mg/dL (1.30-26 mmol/L) creatinine and in women it was 12.44-229.64 mg/dL (1.1-20.3 mmol) creatinine [10] with a CV_A of 1.9 [9].

The clinical interpretations of serum and urine creatinine, and rate of urine flow are on the Glomerular Filtration Rate (GFR) and these have interferences as follows: urine and serum creatinine are increased by muscle mass [2,11,12], muscle creatine is negatively affected in hepatic disease, and urine creatinine positively in muscle disease and injury [2,13], variations in GFR occur with age, gender, or ethnicity [2,14,15], creatinine secreted by renal tubules overestimate the true GFR [2,16], drugs interfering with tubular secretion, increased serum creatinine when GFR remains constant [17,18], serum creatinine and creatinine clearance are increased by a diet rich in cooked red meat [12,19,20]. These are lacunae in the interpretation of GFR using urine and serum creatinine concentrations and urine flow rate. There are also variations in the interpretations on GFR from estimated GFR from serum creatinine, creatinine clearance, and direct measurement of GFR with 'ideal' markers [3,21-23]. The third and the most serious lacuna is that there are no reports on fasting urine creatinine reference interval to the best of our knowledge, and this may be the first report. There is one report on the reference interval of spot urine creatinine [10].

Most reports on urine creatinine are on 24 hour samples, spot urine creatinine-analyte ratio, and normalised analyte concentration for dilution and concentration of spot urine [21,24,25]. A report in 1988 from Scotland on spot urine creatinine has a within-subject diagram from which an approximate minimum-maximum was obtained as 56.56 mg/dL-169.68 mg/dL with a mean of 101.81 mg/dL in males, and it was 67.87-135.75 mg/dL with a mean of 90.50 mg/dL in females using the alkaline picrate method [24]. The biological variation for the whole sample (males+females) in the report was with a mean spot urine creatinine of 93.89 mg/dL, CV_A of 0.9, within-subject (CV_I) of 23.8 and between-subject (CV_G) 24.5 coefficient of variations [24]. A recent report in 2021 from Sweden using enzymatic method (Cobas 6000, Roche Diagnostics Scandinavia AB) for creatinine in a 24-hour urine sample gave values (mg/dL) of mean, median and (minimum-maximum) of 131, 126 (56-226) in males, and 82, 76 (40-174) in females [21]. This report also gave biological variation of spot urine creatinine (in gm/L) expressed as variance and 95% CI of mean as 0.09 (0.05, 0.16) in males, and

0.05 (-0.01, 0.10) in females [21]. Reference interval calculated from a 24-hour urine creatinine for men was 1070-2150 mg/day in men and 764-1200 mg/day in women [25].

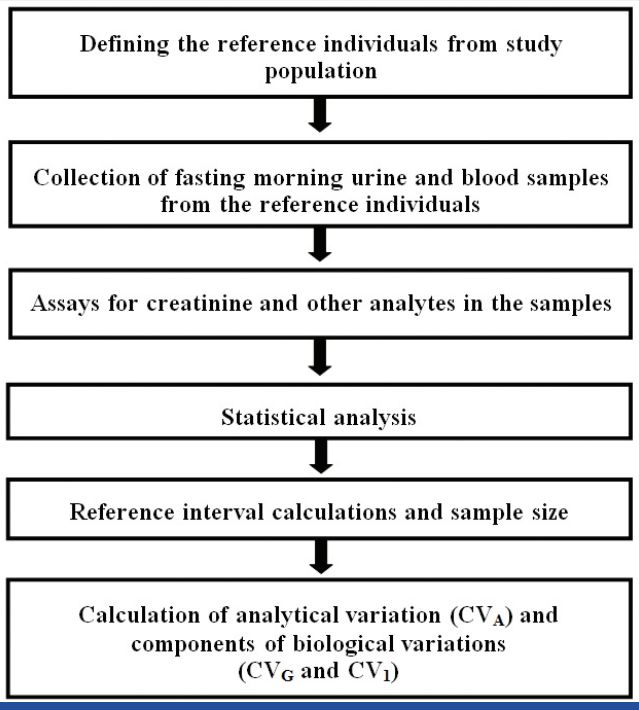
Urine creatinine is used to normalise the urinary concentration of analytes by dividing the test analyte in mmol/L by urine creatinine in mmol/L, for their dilution and concentration in urine [11]. Several studies [3,21-23] have shown that urine creatinine concentrations were highest in the more concentrated overnight samples and GFR is lowest at night. Urine creatinine concentrations were lowest at noon when the urine flow rate and GFR were highest [21]. These are lacunae in the sampling of urine for creatinine assay that necessitates the need for this study.

Authors hypothesised that better evaluation of the components of creatinine clearance can result in better interpretation of kidney functions in the clinical setting. The present report assayed creatinine concentrations in the morning fasting specimen of urine (avoiding the overnight, daytime, and postprandial samples) and collected from the defined reference individuals for calculations of reference interval.

Between-subject coefficient of variation (CV_G) and population reference intervals are useful for diagnosis of diseases [26,27]. Within-subject (CV_I) component of biological variation are less than CV_G and are better suited to predict the progress of the disease in a particular individual [27]. These estimates are currently available for many quantitative measurands [28] and are analysed for a better understanding of the reference interval.

MATERIALS AND METHODS

The study was designed as an observational cross-sectional study of the sample population for establishment of the reference interval of creatinine in fasting urine formed in the morning. Study population was from the tropical, near sea level plains in Thrissur district, Kerala, avoiding the mountainous Western Ghats. Urine and blood samples were collected directly (not indirectly from institutions) and at random from the study population from January 2017-November 2021. Study was approved by the Institutional Research and Ethics Committees (AIMSIEC/22/2016 dated 12/07/2016). The participants were approached directly by the investigators, and through the staff and students of this institution. Oral consent was obtained after describing about the research program and the method of sample collection. After including the participants by clinical examination, informed written consents were obtained, and then samples were collected, as given below [Table/Fig-1].



[Table/Fig-1]: Flow chart of the organisation of study.

Inclusion and Exclusion criteria: The reference individuals were selected from the study population by inclusion and exclusion criteria. The participants included in the study were above 18 years of age, apparently clinically healthy, without history of disease for three weeks, having regular diet and sleep for one week. The individuals excluded from the study were those with chronic diseases, alcoholism, smoking, Body Mass Index (BMI) ≥30, waist circumference ≥100 cm, diabetes mellitus, muscle diseases or muscle pain, and history of heart diseases. The final selection of participants was done by clinical biochemistry evaluation and exclusion of those with serum glucose, fasting ≥126 mg/dL and 2-hour postprandial ≥180 mg/dL, Alanine Aminotransferase (ALT) >60 U/L, serum creatinine ≥1.4 mg/dL, iron <50 µg/dL, ferritin <20 ng/mL, haemoglobin <11.5 g/dL and hsCRP >5 mg/L.

Sample size calculation: The sample sizes and reference interval calculations were done after removal of outliers, analysis of data distribution, and data transformations according to criteria laid down by International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), and Clinical and Laboratory Standards Institution (CLSI) guidelines by using the software, MedCalc [26]. The methods for reference interval calculation were selected according to the sample sizes of partitioned and unpartitioned groups [29]. The formula for the parametric minimum sample size calculation of the 95% reference limits (2.5^{th} to 97.5^{th} percentile) depends on the sample number required for the lower 2.5^{th} percentile as recommended by IFCC [26], which is equal to 1/p=1/0.025=40 sample numbers per partition, where 'p' is expressed as a fraction (0.025) or percentile (2.5^{th}). The sample size calculation for 95% non parametric reference interval depends on the 90% Confidence Interval (CI) of upper and lower limits of the reference intervals [26], for which the minimum sample number is 120. As the male sample number (n=45) in the present study was much less than the female sample number, both parametric and robust method of calculation of reference intervals were done.

Sample Collection and Sample Preparation for Assays

Fasting urine and blood samples were collected after overnight fasting, before 8.30 am, more than two hours after waking up from sleep. First morning urine sample was voided immediately after waking up, to exclude overnight concentrated urine in the bladder [3]. The second sample was voided after about 30 or more minutes to exclude urine in the collecting system of the kidney and residual urine in bladder. The third sample of fasting urine formed in the morning for about one hour or more was collected before 8.30 am, for creatinine assay. The participant could take about 150 mL of water if there was a feeling of thirst or dryness of the mouth. Two sets of samples were collected [27]. First set was collected from January 2017-November 2021 (n=156). Second set of 44 urine samples collected from September 2021-November, 2021 from 10 reference individuals consecutively for 4-5 days (As one female participant did not give sample on the 5^{th} day, female samples were reduced from 25-24. Total sample number=males 20 + females 24=44) was used for estimating CV_A, and components of biological variation, CV_I and CV_G.

The urine sample was prepared for assay by centrifuging at 3000 rpm for 5 minutes to sediment particles, cells, bacteria and casts. Supernatant was separated, aliquoted for assays immediately for creatinine, and for storage at -20°C [30].

The fasting serum sample was prepared from 6-10 mL of blood collected in polypropylene tubes, without clot activators, were centrifuged immediately at 3000 rpm for 5 minutes to sediment cells before clotting. The supernatant was transferred to glass tubes for clotting, followed by clot separation and second centrifugation. If clotting was observed after the first centrifugation, then the plasma was allowed to clot in the same tube and then centrifuged again. This procedure reduced haemolysis and increased the yield of serum

which was preferred over plasma for storage. Serum samples were assayed immediately and stored at -20°C in aliquots [30]. The blood sample was collected in EDTA tubes for haemoglobin assay which was done manually by Drabkin's method [31].

Assays, Traceability and Quality Management

Chemistry assays were done with Vitros 5,1 FS autoanalyser and multilayered dry chemistry slide reagents for serum glucose, creatinine, ALT, highsensitivity C-Reactive Protein (hsCRP), iron, and urine creatinine {Ortho Clinical Diagnostics (OCD), USA}. Creatinine was assayed in Vitros 5,1FS using the VITROS CREA multilayered slides [9]. Conversion of conventional units to International System (SI) units of urine creatinine was done using a MW of 113.12 g/mol: (mg/dL×0.0884)=mmol/L). Immunochemistry assay for serum ferritin was done in Beckman Coulter Access 2 or DXi 600 immunochemistry autoanalysers by the sandwich immunoassay method [32].

Traceability of creatinine calibrations were to the certified National Institute of Standard and Technology Reference material, SRM 914a. Vitros 5,1FS creatinine assay was traceable to High Performance Liquid Chromatography (HPLC) comparative method indirectly through Vitros 950 system with a correlation coefficient of 0.999 [9].

Quality control assays were done continuously twice a day for glucose, creatinine, ALT, hsCRP, iron, and once a month for external quality assurance program (EQAS, Biorad, USA). Quality control assays for ferritin (Biorad, USA) were done before each assay. Quality control data were analysed by Westgard rules for acceptance or rejection of analyte data [33,34]. If there was a rejection, measures were taken to set right errors in machine functioning, reagents, storage or calibrations. EQAS gave Z scores (SDI) <1.0 in the months of sample assays.

STATISTICAL ANALYSIS

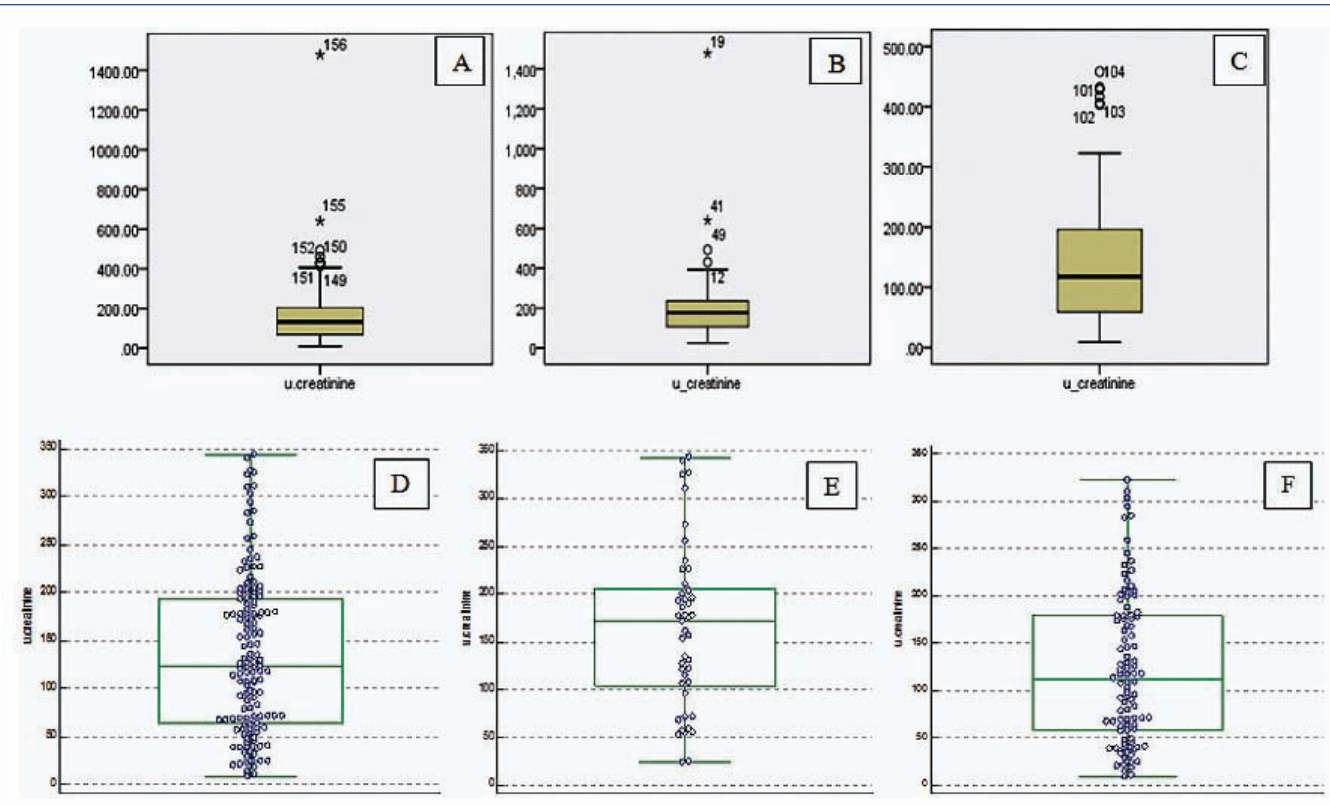
Data analysis for distribution characteristics and for statistical removal of outliers. Outliers were identified by the Box-Whisker plot with Statistical Package for the Social Sciences (SPSS). Outliers were urine creatinine values outside the upper and lower extreme of the Box-Whisker plot. The upper extreme was identified by addition of (1.5×interquartile range) with the upper quartile (Q3). Similarly, the lower extreme was identified by subtracting (1.5×interquartile range) from the lower quartile (Q1). The individual data distribution was visualised graphically by the Box-Whisker plot with MedCalc and by histogram. If the distribution was Gaussian, as seen by Shapiro-Wilk and Kolmogorov-Smirnova (K-S) tests, the data may be used for parametric statistical methods. After removal of outliers, if the data did not have Gaussian distribution (p-value ≤0.05), then data was transformed by log10 or Box-Cox transformations [35,36]. Sample size and reference intervals were calculated [26].

CV_A, CV_G and CV_I

Analytical and biological variations were calculated [27] from a second set of 44 urine samples as given above, from 5 males and 5 females. The coefficient of variation (CV) was calculated by dividing standard deviation (SD) by mean and multiplying by 100. Each urine sample was assayed repeatedly five times to calculate CV_A. Each participant gave urine samples for 4 (male) or 5 (female) days. CV_I was calculated from all assays done on all days for that participant. CV_G was calculated by taking the average of 5 repeated assays for each urine sample of all participants in a day, followed by calculating the CV_G from the mean and SD of all the average values. There were 20 average values for males and 24 for females. CV_G was also calculated from the reference interval sample (n=145).

RESULTS

Fasting urine creatinine data distribution before and after removal of outliers: Fasting urine creatinine from 156 samples was analysed by Box-Whisker plots (SPSS) for the distribution characteristics and outliers. The total sample (male+female) had six outliers and was positively skewed [Table/Fig-2a]. The male and female urine samples had four and six outliers, respectively, and they were also positively skewed [Table/Fig-2b,c]. The sample number was reduced to 145 with 45 males and 100 females, after removal of 11 outliers in 2 steps by Box-Whisker-Plots from the total sample, 5 outliers in 2 steps from male and 6 outliers in 1 step from the female samples. The sample distributions after removal of outliers were nearer to Gaussian distribution [Table/Fig-2d-f].



[Table/Fig-2]: Distribution of creatinine (mg/dL) in the morning fasting sample of urine by Box-Whisker plots.
Before removal of outliers: A, B, C by SPSS; After removal of outliers: D, E, F by MedCalc; Where, A and D (Males + Females); B and E Males; C and F Females

Before removal of outliers, fasting urine creatinine showed high CV_G in all groups, higher in males (101.70) and lower (74.80) in females [Table/Fig-3]. The SD had decreased markedly after the removal of outliers, resulting in lower CV_G in males (52.39) and higher in females (64.90). After removal of outliers, the distribution was not Gaussian in the total and female samples (p-value <0.001) by Shapiro-Wilk test, but was almost Gaussian (p-value=0.097) in males [Table/Fig-3].

Distribution characteristics fasting urine creatinine	Before removal of outliers			After removal of outliers		
	Male+Female (n=156)	Male (n=50)	Female (n=106)	Male+Female (n=145)	Male (n=45)	Female (n=100)
Age	18-92	18-92	18-69	18-92	18-92	18-69
Mean±SD	164.07±154.88	216.49±220.17	139.35±104.23	135.28±83.48	164.20±86.03	122.27±79.35
CV_G	94.40%	101.70%	74.80%	61.71%	52.39%	64.90%
Minimum-Maximum	9.00-1479.00	24.20-1479.00	9.00-458.00	9.00-344.20	24.20-344.20	9.00-322.50
95% CI of mean	139.58-188.57	153.92-279.06	119.27-159.42	121.58-148.99	138.36-190.05	106.52-138.01
Shapiro-Wilk test, (p-value)	<0.001	<0.001	<0.001	<0.001	0.097	<0.001
Kolmogorov-Smirnov test, (p-value)	<0.001	<0.001	0.005	0.001	0.200	0.005

[Table/Fig-3]: Distribution of urine creatinine (mg/dL) before and after removal of outliers from the morning fasting sample of urine.
Outliers were removed by Box-Whisker plot (SPSS) from (Male+ Female) data in 2 steps, male data in 2 steps, and female data in 1 step

Calculation of reference intervals: Data obtained after the removal of outliers was used for reference interval calculation. Box-Cox transformation of data was undertaken for parametric calculation with an optimum lambda of 0.46 for the total sample (MedCalc). The non parametric percentile method of reference interval calculation was preferred for the total sample, as the sample number was >120. The calculated reference interval was from 17.14-325.89 mg/dL.

When the sample number was 40-120 (n=100 in females), 95%, double-sided reference interval calculation method based on normal distribution after Box-Cox transformation was the preferred method of reference interval calculation in the female sample. Box-Cox method of transformation was done with an optimum lambda of 0.59 for males and 0.40 for females for parametric reference interval calculation. The 95% double-sided reference interval calculated from the normal distribution of urine creatinine data was 12.41-328.71 mg/dL in the female sample [Table/Fig-4].

Measurements	Total (n=145)	Male (n=45)	Female (n=100)
Mean	119.98	154.26	105.55
Minimum-Maximum	9.00-344.20	24.20-344.20	9.00-322.50
Coefficient of Skewness	-0.1160 (p=0.5560)	-0.09049 (p=0.7879)	-0.1097 (p=0.6394)
Coefficient of Kurtosis	-0.8062 (p=0.0014)	-0.5608 (p=0.3873)	-0.8715 (p=0.0033)
Shapiro-Wilk test (p-value)	0.0267	0.3141	0.0968
95% Reference interval, Double-sided			
A. Method based on Normal distribution			
Lower limit (90% CI), mg/dL	14.36 (8.84-21.36)	25.82 (10.61-46.14)	12.41 (7.16-19.48)
Upper limit (90% CI), mg/dL	343.97 (310.17-379.66)	361.09 (309.97-415.38)	328.71 (286.67-374.24)
B. Non parametric percentile method (CLSI C28-A3)			
Lower limit (90% CI), mg/dL	17.14 (9.00-24.20)	-	-
Upper limit (90% CI), mg/dL	325.89 (302.60-344.20)	-	-
C. Robust method (CLSI C28-A3)			
Lower limit (90% CI*), mg/dL	-	21.91 (9.21-44.58)	11.84 (7.19-18.63)
Upper limit (90% CI*), mg/dL	-	379.12 (326.28-423.84)	337.75 (299.27-375.65)

[Table/Fig-4]: Calculation of reference interval of fasting morning urine creatinine by MedCalc and Box-Cox transformations.
Creatinine data after the removal of outliers were used. *Bootstrap Confidence Interval (CI) (10000 iterations; random number seed: 978)

The robust method of reference interval calculation was preferred when the sample number was small, <40. But as the sample number of males (n=45) was much less than that of females the robust method was preferred: 21.91-379.12 mg/dL [Table/Fig-4].

Fasting urine creatinine showed gender differences in CV_I and CV_G: As expected, the minimum-maximum of CV_A was overlapping in the 5 male and 5 female samples on different days [Table/Fig-5].

But CV_I and CV_G showed gender differences. CV_I in the male sample extended from 17.77-34.86. In the female sample, it extended from 2.17-60.25, indicating a wider range of within-subject variation in females. The CV_G or between-subject variation was also higher in the female sample [Table/Fig-5]. CV_G in the reference interval sample after removal of outliers (n=145) was also higher in females [Table/Fig-3].

Gender differences in biological variations and distributions resulted from lower fasting urine creatinine in the female sample: Histogram of the distribution of fasting urine creatinine after removal of outliers in the male sample showed near Gaussian distribution, while the female sample was positively skewed [Table/Fig-6], thus illustrating gender differences, also seen in the Shapiro-Wilk statistic of distribution [Table/Fig-3]. The histogram [Table/Fig-6] and the mean [Table/Fig-3] demonstrated lower concentrations of creatinine in the female sample. The male sample, distribution showed 24.4% of the samples below 100 mg/dL creatinine, while the female sample showed 46% of the samples below 100 mg/dL creatinine [Table/Fig-6a,b]. The major mode in the male sample was between 150 and 200, while in the female sample it was between 0-50. The lower concentrations of fasting urine creatinine nearer to the lower limit of urine creatinine in the female sample resulted in the non-gaussian distribution, resulting in higher CV_G. In the biological variation sample set, 25% of the male and 37.5% of the female samples were below 100 mg/dL creatinine [Table/Fig-6c,d].

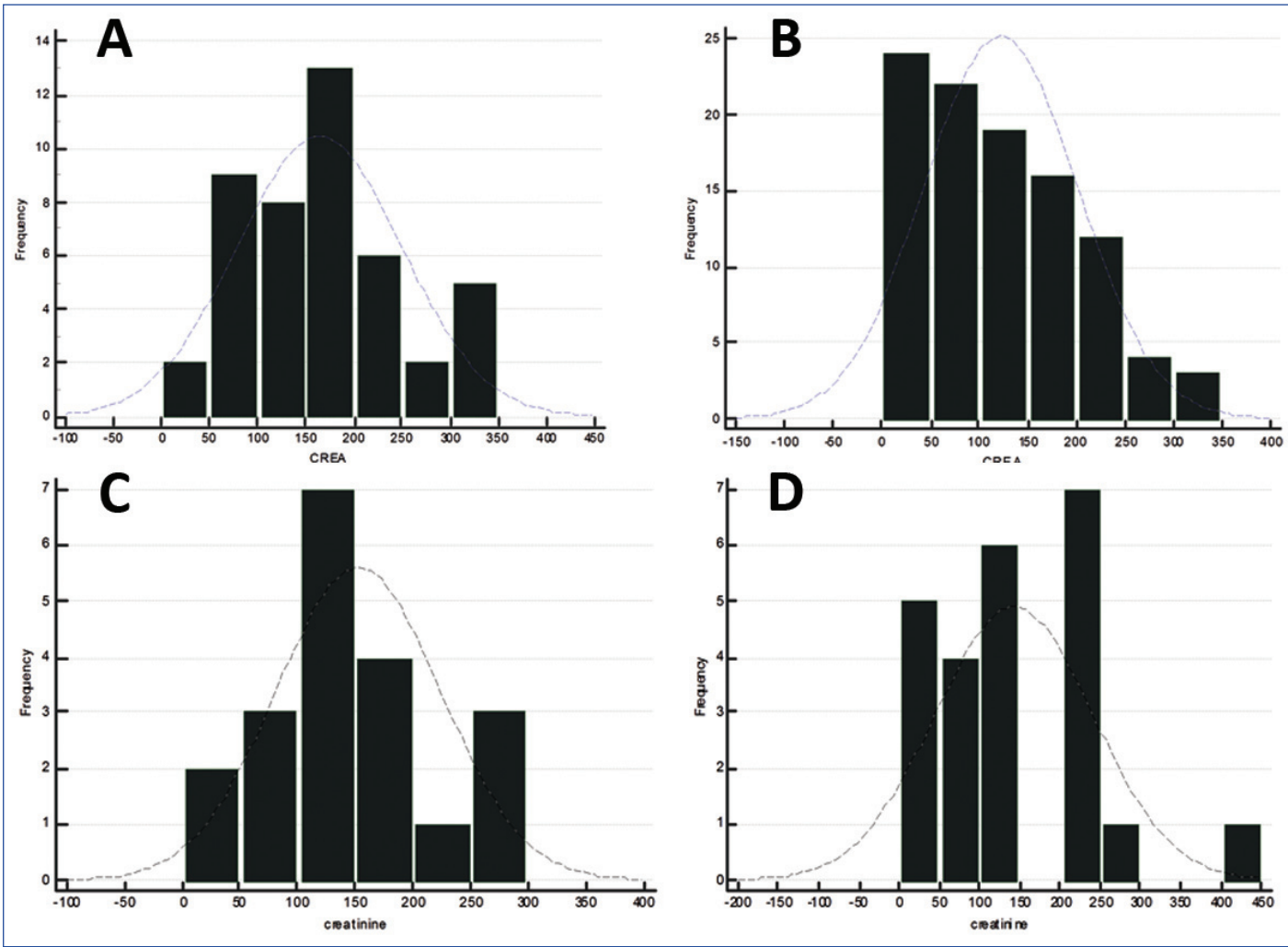
DISCUSSION

The two earlier reports on spot urine creatinine from temperate zones, such as Canada on reference limits [10], and Scotland on minimum-maximum [24] showed upper limits much lower than that were given in our present report [Table/Fig-4]. The present study was from a tropical zone, with higher environmental temperature round the year making the urine sample more concentrated than that from the temperate zones [Table/Fig-7]. In these reports, including the current study, the upper limits of these values were lower in the female sample, and may be attributed to the lower urine creatinine in the female samples, due to lower muscle mass [2,11,12].

Most reports on urine creatinine are on the 24-hour sample, spot urine creatinine-analyte ratio, normalised analyte concentration for dilution and concentration of spot urine, GFR and creatinine clearance [3,21,24,25]. The 24-hour urine creatinine reference interval report from Japan [25], a temperate zone, also showed lower values in the female sample (1070-2150 mg/day in men and 764-1200 mg/day in women). The 24-hour urine creatinine reference interval [25] cannot be compared with the fasting urine reference

Participants	1st day	2nd day	3rd day	4th day	5th day	Average of 4 or 5 CV_A	Average of 20 or 24 CV_A	CV_I	CV_G (n=20 or 24)
Males									
1	1.62	0.97	2.24	2.85	-	1.92	1.66 (Min-Max: 0.45-2.91)	30.38	46.70
2	1.36	1.50	1.24	0.45	-	1.14		28.92	
3	1.07	0.73	1.61	2.68	-	1.52		17.77	
4	1.52	2.15	0.78	2.58	-	1.76		34.86	
5	2.67	1.49	2.91	0.71	-	1.95		28.21	
Females									
6	0.61	0.87	1.07	0.47	0.39	0.682	1.49 (Min-Max: 0.39-3.18)	2.17	68.52
7	1.10	0.94	0.81	2.38	1.55	1.36		20.81	
8	2.48	2.29	1.70	1.54	1.78	1.96		60.25	
9	1.47	1.50	0.86	3.18	3.17	2.04		37.52	
10	1.19	1.35	1.96	1.20	-	1.43		35.22	

[Table/Fig-5]: Estimation of CV_A, CV_I, and CV_G of creatinine (mg/dL) from fasting morning urine sample in males and females.
Each urine sample was assayed 5 times repeatedly to calculate the 44 CV_A or within-run imprecision. Average CV_A (analytical imprecision) was calculated for each participant and for all male and female participants. CV_I (within-subject variation) of each participant was calculated from all the assays done for all days for that participant. There were 20 assays for each male and 25/20 assays for each female participant. CV_G (between-subject variation) was calculated by taking the average of 5 repeated assays for each urine sample in a day, followed by calculating the CV_G from the mean and SD of all the average values from all samples of all male and female participants. There were 20 average values for males and 24 for females



[Table/Fig-6]: Gender differences in the histogram of fasting urine creatinine (by MedCalc) in the reference interval sample (n=145; A and B) and in the biological variation sample (n=44; C and D). (A and C) males; (B and D) females.

Author's name and year	Place of study of publication of study	Number of subjects	Climatic zone (Tropical/Temperate)	Time for sample collection	Reported reference Interval/Minimum -maximum values
Adeli K et al., (2015) [10]	Canada	Male=1300 Female=1340	Temperate	Spot urine, daytime	95% Reference Interval by enzymatic method, healthy individuals. Male=14.71-294.12 mg/dL Female=12.44-229.64 mg/dL
Gowans EMS and Fraser CG, (1988) [24]	Scotland	Male=7 Female=8	Temperate	Spot urine, daytime	mean (min-max), by picric acid-rate method. Male=101.81 (56.56-169.68) mg/dL, Female=90.50 (67.87-135.75) mg/dL.

Sallsten G and Barregard L (2021) [21]	Sweden	Male=29 Female=31	Temperate	Spot urine	mean, median (min-max) by enzymatic method M=131, 126 (56-226) mg/dL Female=82, 76 (40-174) mg/dL
Present study, (2022)	Kerala, India	Male=45 Female=100	Tropical	Fasting morning third urine sample	95% Reference Interval by enzymatic method, strict inclusion-exclusion criteria Male=21.91-379.12 mg/dL Female=12.41-328.71 mg/dL

[Table/Fig-7]: Comparison of 95% reference interval of fasting creatinine in the morning third urine sample in the present study with the reported spot urine creatinine reference interval and minimum-maximum values [10,21,24].

interval, as there is a stronger influence of muscle mass [2,11,12], there is an averaging of the concentrated overnight urine sample and the more diluted daytime sample [21,37-39], and there is the influence of diet [12,19,20] on the 24-hour urine creatinine and not on fasting urine creatinine.

In a recent report with spot urine sample (n=60) from Sweden [21] on CV_G using variance and (95% CI min-max), {Males, 12 and (4-19); Females, 9 and (3-15)} showed higher values in the male sample, and was seen in this (n=156) report also. But the earlier reported values were very much lower, the percent difference between the upper and lower CI limits were much higher than that in this report. Authors may attribute these observations to the higher physical activity and muscle mass of young adult males, and the higher tropical zone temperatures. The lower 95% CI range in our study was possible only in the fasting urine sample, as the influences are less. As CV_G is dependent on sample number, the higher sample number in our study gives more credibility to our results.

But the CV_I is less dependent on large sample number and is commonly used for delta checking for previous values and for assessing quality of laboratory reports [40], and for long or short-term checking of previous values as an intuitive subject-based reference level checking for diagnosis and progress of a disease state, done frequently in clinics. This method is made more systematic and error-free by estimating CV_I and CV_A [26,27]. The CV_I was much lower than CV_G giving more validity to the data. The CV_A was quite low in the present study, indicating that there was almost no influence of the analytical method on urine creatinine values.

The gender difference of the histogram was due to lower concentration of urine creatinine in the female sample. The lower concentration of urine creatinine in the female sample was closer to the lower limit of creatinine in urine resulting in a positively skewed appearance [Table/Fig-6]. As the male sample had the higher creatinine, the mode and mean were farther away from the lower limit of creatinine resulting in a near Gaussian distribution.

The sample size calculation for establishment of reference interval was quite unlike in other studies were the minimum sample size for partitioned and non partitioned groups may be estimated before the study [41,42]. For reference interval calculation, higher the sample number better will be the estimation of reference interval. But in this report due to strict exclusion criteria and sampling, it was difficult to obtain high sample numbers. But the sample number was much higher than in that in the earlier reports [21,24,25]. The second, important aspect was that in reference interval calculation, the method used for calculation was selected by the sample number in the partitioned and non partitioned groups. The principles of sample size calculation for the non parametric methods are complex, and have been made easier by MedCalc software [27].

In this report, the clinical exclusion criteria did not remove most of the outliers. Therefore, we resorted to statistical exclusion of outliers using the Box-Whisker plot [Table/Fig-2]. Several studies [21,37-39] have shown diurnal variation in urine creatinine concentrations, GFR and creatinine excretion rates. The lower GFR at night is partly counteracted by the higher tubular secretion of creatinine [38,39]. There is a positive relationship between urine flow and creatinine excretion rate [43,44]. In the present study, postprandial, overnight, and daytime urine samples were excluded to decrease the influence on urine creatinine as shown by several studies [3,21-23]. A mild increase in ALT from 35-60 U/L from fatty liver may not have influence on urine creatinine. But a moderate to large increase of ALT and hsCRP >5 mg/L resulting from steatohepatitis and cirrhosis liver has influences on muscle and urine creatinine [3,12,13] and was exclusion criteria for this study. Participants deficient in haemoglobin, iron, and ferritin were excluded, as they were found to have widespread influences on a number of measurands in our earlier studies [45,46]. In this report, participants with BMI ≥30 were excluded, as obesity was reported to have influences on kidney functions [47,48].

Limitation(s)

The outliers or α-errors were identified and removed by statistical and clinical methods. But there are still β-errors or internal errors which are within the reference interval limits and cannot be easily identified in health and in disease states. Efforts are being made in our laboratory to identify these β-errors. Much more clinical work has to be done to use the fasting urine creatinine reference interval for diagnosing disease states.

CONCLUSION(S)

Reference intervals of fasting urine creatinine concentrations in the urine formed in the morning were calculated. The urine sample formed in the morning fasting state was preferred, as there was a decrease in the interferences and to get consistent results. The reference intervals in the present study from a tropical warmer location had higher upper limits than the reported reference intervals, and were interpreted to result from the more concentrated urine at higher temperatures. The gender differences seen in the distribution and biological variations were due to lower concentrations of urine creatinine in the female sample resulting from the lower muscle mass. The future studies may be on identification of β-errors of fasting urine creatinine in health and disease states, as this study identifies only α-errors.

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