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Determination of Biological Reference Interval for Serum Urea and Creatinine in Healthy Mid Adolescent Children in Rural South Indian Population.

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Introduction:
The reference interval given in laboratory reports of patient are used by clinicians for interpretation of clinical values, for supporting appropriate medical diagnosis and it helps in deciding on therapeutic interventions and other physiological assessments.

India is vast country with variable ethnicity and racial diversity. Variation is also observed in lifestyle, habits, socioeconomic status and environmental factors. Clinical laboratory standards and biological reference intervals established for Western, European and other Asian countries needs to be thoroughly evaluated and verified for our population. Most of the Indian diagnostic laboratories use reference interval available in the literatures, manufacturers package inserts, and standard textbooks or adopted from Western cohorts [1-5].

Standard guidelines suggest labs establish their own locally modified age-related reference intervals. The International federation of Clinical Chemistry (IFCC) have recommended list of factors to be considered while planning to determine the biological reference interval for any laboratory parameter [6] . The National Committee for Clinical Laboratory Standards (NCCLS) and Clinical and Laboratory standard Institute (CLSI) documents have described a standard detailed questionnaire and guidelines for proper selection of reference individuals [7, 8]. Several population based retrospective studies using Medical health check up data determining reference intervals of various biochemical parameters for Indians has been published in adult population between age group of 20-80 years [9, 10]. However no study to determine reference ranges for biochemical parameters in children, adolescents and geriatric age group has been attempted, hence we are taking up this study to determine the reference interval of serum urea and creatinine in healthy mid adolescent population in a south Indian town.

Materials and Methodology:
Type of study: Lab reference interval study.

Study Design: Cross sectional.
Duration of the study (in months): 2 months (August 2019 to September 2019)
Study Sampling and data collection: Multi-phase cluster random sampling [7]. Government and private school around our medical college were approached; all the students in the eligibility age group were explained regarding the study in their own understandable language. Written permissions were obtained from appropriate school authorities, written consents were taken from the parents of all study subjects as they were minors, accent was taken from study subjects as they were of adolescent age group.

Study Population:
Subjects' demographic data was taken and nutritional evaluation was done by measuring height and weight. Determination of body mass index of the participants was done using the formula wt in Kg/Ht in meters^2. A total 914 with 533 boys and 381 girls were screened for height and weight.
Inclusion criteria:
- All apparently healthy middle adolescents aged between 14-17 years [11] studying in various schools around our medical college hospital, willing to take part in the study were included.

Exclusion criteria:
1. Children or their parents who were not willing to take part in the study.
2. Children categorized as mal-nourished (< 10th percentile) using body mass index (BMI) using growth charts issued by Indian Academy of Pediatrics (IAP) 2015[11,12,13].
3. Children categorized as obese (>95th percentile) using body mass index (BMI) using growth charts issued by IAP 2015 [11,12,13].
4. Children suffering from any obvious illness or on medication for any chronic diseases.

All subjects satisfying the inclusion/exclusion criteria were included in the study. Out of 914 participants

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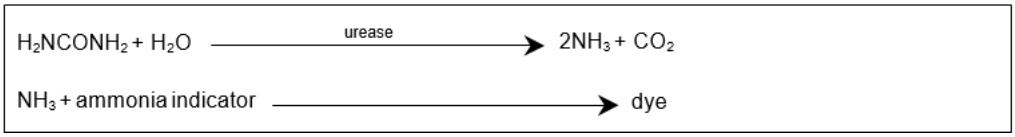
screened for BMI, 262 participants qualified according to our inclusion and exclusion criteria were recruited in the study, study group comprised of 125 mid adolescent boys and 137 mid adolescent girls.

Study material:

All subjects were requested to complete the questionnaire [7] for screening participants for previous acute or chronic illness, thyroid abnormality, diabetes mellitus, hypertension, tobacco, alcohol or any other drug consumption, menstrual/ hormonal abnormalities, surgery, pregnancy, lactation etc.3 ml random venous sample was collected from all participants in SST (serum separator tubes) yellow vacutainer of Becton, Dickinson and Company (BD) using all aseptic precautions as per our protocols documented in our sample collection manual. All required precautions were taken to minimize the complication and phlebotomists were trained properly for handling the same. No major complication was encountered during sample collection process. Samples were placed in cold storage box and transported to laboratory at a temperature between 4-8^{O}C. All samples were centrifuged as per our protocols at 3000 RPM for 10 minutes. Serum urea and creatinine was analyzed using Vitros 5.1 auto-analyzer Medical diagnostics new Jersey, United States, using urease and sarcosine, method respectively.

Principle of urea estimation by urease method: The VITROS BUN/UREA Slide is a multilayered, analytical element coated on a polyester support. A drop of patient sample is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. Water and non-proteinaceous components then travel to the underlying reagent layer, where the urease reaction generates ammonia.

Reaction Sequence:

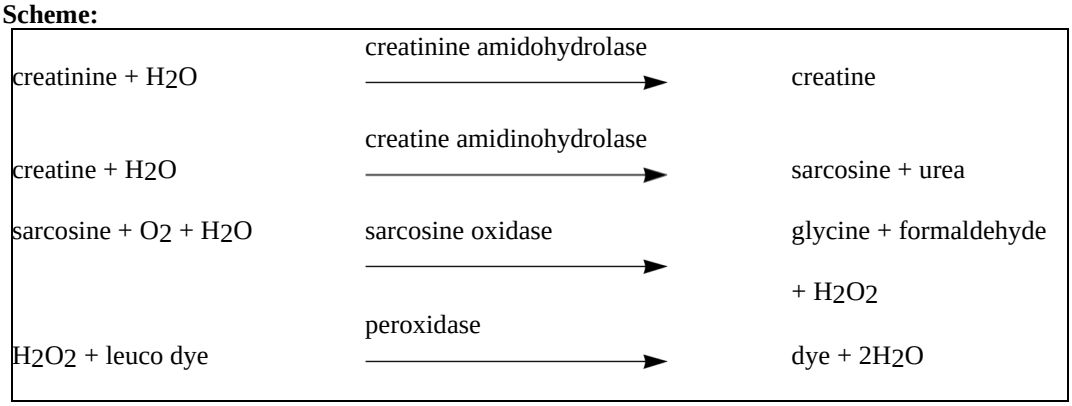


The semi-permeable membrane allows only ammonia to pass through to the color-forming layer, where it reacts with the indicator to form a dye. The reflection density of the dye is measured and is proportional to the concentration of urea in the sample [14].

The VITROS CREA Slide is a multilayered, analytical element coated on a polyester support.

A drop of patient sample is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. Creatinine diffuses to the reagent layer, where it is hydrolyzed to creatine in the rate-determining step. The creatine is converted to sarcosine and urea by creatine amidinohydrolase. The sarcosine, in the presence of sarcosine oxidase, is oxidized to glycine, formaldehyde, and hydrogen peroxide. The final reaction involves the peroxidase-catalyzed oxidation of a leuco dye to produce a colored product.

Reaction Scheme:



Following addition of the sample, the slide is incubated. During the initial reaction phase, endogenous creatine in the sample is oxidized. The resulting change in reflection density is measured at 2 time points. The difference in reflection density is proportional to the concentration of creatinine present in the sample [14].

Quality assurance: Our laboratory is an NABL accredited laboratory, quality assurance are performed as per laboratory protocols. All quality assurance program run are from Bio-Rad laboratories, Diagnostics group, California, United States.

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Laboratory ran 2 levels per day of Bio-Rad lyphocheck assayed chemistry control(C-310-5) with lot number 26450 with monthly Bio-Rad External Quality Assurance scheme (EQAS) of clinical chemistry monthly EQAS program (BC-50) with lot number 211700 cycle 18 sample No 2 & 3 for the month of August and September 2019.

Statistical analysis:

Sample size was calculated as per NCCLS [7] guidelines as 120 subjects in each group with 90% confidence interval. For reference value estimation, Dixon [15] test were used which suggest "ratio of D/R, where *D* is the absolute difference between an extreme observation (large or small) and the next largest (or smallest) observation, and *R* is the range of all observations, including extremes". As suggested by Reed et al [16] the value 1/3 as a cut-off value; i.e., if the observed value of *D* were equal to or greater than one-third of the range *R*, the extreme observation would be deleted. The reference limits determined from selected subjects sample are estimates of the corresponding percentiles in the population of person's studied.2.5- 97.5 percentile were included as reference interval [7].

Results and observation:

Government and private school students studying in 9^{th}, 10^{th}, 11^{th} and 12^{th} standard of 14, 15, 16 and 17 years were included in the study. A total of 124 boys and 137 girls were included in the study. Mean age among the boys and girls was 15.30±0.4 & 15.41± 0.7 respectively.

Table 1: Age distribution of participants:

Age	Boys	Girls	Total
14 yrs	44	41	85
15 yrs	35	27	62
16 yrs	7	40	47
17 yrs	38	29	67
Total	**124**	**137**	**261**

Table 2: BMI of boys included in study

BMI	14 years (%)	15 years (%)	16 years (%)	17 years (%)
17.3 - 18	4 (9%)	1(2.7%)	11(4.2 %)	1(2.7 %)
18-22.99	34 (77.2%)	26(72.2 %)	45(7.1 %)	25(67.5 %)
23 – 25	6 (13.6%)	9(25 %)	22(8.5 %)	11(29.7 %)

Table 3: BMI of girls included in study

BMI	14 year Girls (%)	15 year Girls (%)	16 year Girls (%)	17 year Girls (%)
17.3 - 18	7(17.0%)	5(17.8%)	11(27.5%)	6(20.68%)
18-22.99	31(75.60%	19(70.37%)	28(70%)	21(72.41%)
23 – 25	3(7.3%)	3(11.11%)	1(2.5%)	2(6.89%)

All participants recruited in the study were with BMI of 17.3 to 25 (adult equivalent of 95^{th} percentile).

Table 4: Data of internal quality control (IQC)

Parameters	Month	IQC Level	Mean	Coefficient of Variance (CV)%
Urea (mg/dl)	August 2019	Level 1	31.82	3.3
		Level 2	88.49	1.7
	September 2019	Level 1	31.67	2.5
		Level 2	88.70	1.6
Creatinine (mg/dl)	August 2019	Level 1	1.79	2.7
		Level 2	5.41	1.9
	September 2019	Level 1	1.809	1.9
		Level 2	5.4	1.5

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Table 5: Data of External quality assurance scheme (EQAS)

Parameters	EQAS	Month / sample No	Our result	EQAS provider Mean	Z score
Urea (mg/dl)	Biorad	August/2	15.1	14.9	0.24
		September/3	57.8	57.1	0.42
Creatinine mg/dl)	Biorad	August/2	3.8	3.62	1.5
		September/3	3.2	3.11	0.86

Table 6: Reference intervals estimated with frequency and percentile calculation

Variable	**Boys**		**Girls**	
	Urea (mg/dl)	Creatinine (mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)
Number	124	124	137	137
Mean	19.36	0.62	18.84	0.52
Median	18.45	0.60	17.9	0.5
Standard deviation	5.63	0.14	5.2	0.08
Mean - 3SD	2.45	0.19	3.15	0.26
Mean + 3SD	36.28	1.05	34.53	0.79
2.5 percentile	10.40	0.4	11.08	0.4
97.5 percentile	37.27	0.98	32.99	0.7
Variance	31.79	0.21	27.3	0.008
minimum	9.5	0.4	9.8	0.3
Maximum	41.6	1.4	36.7	0.8
Range	9.5 – 41.6	0.4 – 1.4	9.8 – 36.7	0.3 – 0.8
Biological ref int	10.4 – 31.79	0.4 – 0.98	11.08 – 32.99	0.4 – 0.7

Table 7: comparison of values between girls and boys

	Boys		Girls		P Value
	Mean	SD	Mean	SD	
Urea (mg/dl)	19.36	5.63	18.84	0.52	0.00*
Creatinine (mg/dl)	0.62	0.14	0.52	0.08	0.00*

Table 8: Comparison of biological reference interval as per study and reference interval used by the lab currently:

		Biological Reference Interval (BRI) as per study	BRI currently used by the laboratory
Boys	Urea (mg/dl)	10.4 – 31.79	19 - 43
	Creatinine (mg/dl)	0.4 – 0.98	0.5 – 1.0
Girls	Urea (mg/dl)	11.08 – 32.99	19 - 43
	Creatinine (mg/dl)	0.4 – 0.7	0.5 – 1.0

Presently laboratory uses the reference interval from either manufactures specified values (14) or from values specified in Tietz text of clinical chemistry (17), the reference intervals are seen to be similar for males and females of mid adolescent age group. The values obtained for Indian population are slightly different when compared to presently used values.

Discussion:

Biological reference intervals given in laboratory reports alongside patient values are used commonly by clinicians for interpretations of the values obtained by the patient [18]. The reliability of these values is highly dependent over healthy population from which reference values are obtained [19, 20]. As suggested by Eugene and James the characteristics of reference population considered as sample for biological reference interval should be same as that of patient population [21]. Indian laboratories either use manufactures' specified reference interval or as specified in standard text books both can be the reference values from the west. Indian population is a diverse group containing people of different race and ethnicity.

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Our country is presently facing public health challenges with plethora of communicable and non-communicable illness. In this phase clinical laboratories play a major role in helping clinicians to identify and treat the disease in early stages.

Not many studied are done for determining reference intervals of the parameters in Indian population, few studies available in literature are a product of data mining done on health check-up patients. Such retrospective studies might not correctly represent healthy population as many participants would have been referred for some clinical purposes as trend to attend clinics without any specific clinical condition has not yet picked up among Indian population, and more so in rural areas. However, to the best of our knowledge no such reference interval studies have been conducted over adolescent children. Hence there is a need to conduct more of prospective studies among the population to verify the reference interval and contribute to the national data. Such region specific representations of the population might help set specific health goals.

To address all these issues, we took up this study as per the recommendation and protocols specified by The National Committee for Clinical Laboratory Standards (NCCLS) and Clinical and Laboratory standard Institute (CLSI) of estimating the region specific biological reference interval for urea and creatinine in adolescent boys and girls.

The values observed in study population were found comparable with presently used laboratory reference interval. Presently used reference interval is not gender specific and available age specific urea levels are for 10 -20 years individuals. Our study shows statistically significant difference when the values of males when compared with females, hence our study have contributed the gender specific and age specific data in mid adolescent age group. However, comparison of this data cannot be done with values available from different regions of India in different studies published previously as other studies have focused mainly over adult age group as our study is first of its kind among this age group. Frequent validation of reference interval is recommended by IFCC, hence we are of the opinion that the age specific, gender specific and region specific prospective studies shall help establish biological reference interval for all the Indian population thus helping clinicians to take better clinical decisions.

The limitations of the studies were due to financial constrains all the clinical chemistry parameter were not analyzed in the study, which if done would have broadly widened the scope of the study.

Conclusion:

Biological reference interval determined by this study is comparable with the values used by the laboratory. However gender specific reference intervals are always desirable in any age group. Our study attempts to fill the gap of availability of the blood values of healthy individuals of Indian origin, it also contributes to the gender specific reference values in adolescent population. However we are of the opinion that this is a small attempt towards this direction and feel the need for many such studies to contribute to our national data.

Summary

The study was undertaken to determine the biological reference interval of the mid adolescent boys and girls of Indian origin in the age group of 14 -17 years. As per the recommendation and protocols specified by The National Committee for Clinical Laboratory Standards (NCCLS) and Clinical and Laboratory standard Institute (CLSI) this study was conducted. After appropriate permission from school authorities and consent from parents and accent from participants, 3 ml blood was collected in SST vacutainer. After the separation of serum, serum was used for estimation of urea and creatinine in vitros FS 5.1 auto analyzer Jamovi software(22,23) was used to calculate the mean, standard deviation and frequency distribution, 2.5 percentile and 97.5 percentile was obtained and biological reference range was established for urea as 10.4 – 31.79 and 11.08 – 32.99 for boys and girls respectively and for creatinine as 0.4 – 0.98 and 0.4 – 0.7 for boys and girls respectively. Our study has contributed age specific gender specific biological reference interval for urea and creatinine for adolescent population residing in a south Indian town.

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