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Reference intervals for 33 biochemical analytes in healthy Indian population: C-RIDL IFCC initiative

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Abstract

Background: In 2011, the IFCC Committee on Reference Intervals and Decision Limits (C-RIDL) initiated a worldwide multicenter study on references values facilitating the implementation of country-specific reference intervals (RIs). There has been no well-designed RI study in India. This study aims to derive RIs for 33 major biochemical analytes in carefully selected healthy Indians as defined in C-RIDL protocol.

Methods: A total of 512 healthy Indians were recruited. Sera collected from overnight fasting blood samples were measured collectively for the analytes. Multiple regression analysis (MRA) and nested analysis of variance (ANOVA) were used to identify the potential sources of variation (SV) of test results. RI were derived by both parametric and non-parametric methods for comparison. The need for secondary exclusion by latent abnormal values exclusion (LAVE) method was examined.

Results: MRA results indicated that both age and BMI were apparent SV for many analytes in both sexes. ANOVA revealed that partition of RIs by gender and age was required for 17 analytes (TC, HDL-C, TG, hsCRP, ALB, AST, ALT, ALP, GGT, TBil, Urea, CRE, UA, Fe, TTR, CK and IgM) and 5 (Glu, ALB, TC, ALP and Urea), respectively. RIs by parametric method were generally narrower than by non-parametric method, reflecting distorted peripheral distributions of test results. The LAVE method had no appreciable effect on RIs possibly due to inconsistency among abnormal values of related analytes.

Conclusions: This study has for the first time provided comprehensive RIs information in healthy Indians. The final RIs adopted were those derived by parametric method without LAVE procedure.

Keywords: Indian population; latent abnormal value exclusion (LAVE) method; multiple regression analysis; parametric method; reference value.

Abbreviations: IFCC, International Federation of Clinical Chemistry and Laboratory Medicine; C-RIDL, Committee on Reference Interval and Decision limits; RIs, reference intervals; CDLs, clinical decision limits; ANOVA, analysis of variance; MRA, multiple regression analysis; LAVE, latent abnormal values exclusion; SVs, sources of variation; SD, standard deviation; SDR, standard deviation ratio; ALT, alanine aminotransferase; ALB, albumin; ALP, alkaline phosphatase; AMY, amylase; AST, aspartate aminotransferase; Ca, calcium; Cl, chloride; C3, complement component 3; C4, complement component 4; CK, creatine kinase; CRE, creatinine; GGT, γ-glutamyltransferase; Glu, glucose; HDL-C, high-density lipoprotein cholesterol; hsCRP, high-sensitivity C-reactive protein; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IP, inorganic phosphate; Fe, iron; LDH, lactate dehydrogenase; LDL-C, low-density lipoprotein cholesterol; Mg, magnesium; K, potassium; Na, sodium; TBil, total bilirubin; TC, total cholesterol; TP, total protein; Tf, transferrin; TTR, transthyretin; TG, triglycerides; UA, uric acid.

Introduction

The term "reference value" was first introduced by Ralph Grasbeck and Nils-Erik Saris in 1969 [1]. Almost 17 years later on, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) published six guidelines covering different aspect of reference intervals (RIs) [2]. However, in spite of its immense clinical importance, most laboratories across many developing countries including India refers to reference values either from kit inserts provided by the manufacturers or from the scientific literature, which are based primarily

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on Western population. It is well known that population across the globe differs physiologically, genetically and ethnically which along with differences in lifestyle and diet have great impact on the various biochemical analytes. Thus, it is inappropriate to use RIs that do not represent the population. Establishing common RI by means of multicenter collaborative study is seen as a plausible solution for most of the issues involved in establishing RI [3]. In an attempt to determine the feasibility of determining common RIs for a large number of analytes on a global scale, the Committee on Reference Intervals and Decision Limits (C-RIDL) of the IFCC initiated a worldwide multicenter study on references values in 2011 facilitating the implementation of country-specific RI by providing a common protocol. Another important aspect of the multicenter study was to determine potential sources of variations (SVs) that could influence the test results.

Thus, as a part of the worldwide multicenter study and with limited literature on Indian population-specific RIs, the present study under the aegis of IFCC's C-RIDL was undertaken to determine the RI for a total of 33 routinely tested biochemical analytes in healthy Indian volunteers.

Materials and methods

Subjects

Under the aegis of IFCC's C-RIDL, a cross-sectional observational study for defining RIs was conducted at Biochemistry department, Hinduja Hospital, Mumbai, India. A common protocol recommended by C-RIDL [4] was used by all the participating countries in the IFCC global study. The study design was approved by hospital's Institutional Review Board (IRB) Committee, and throughout the study, adherence was made to the Declaration of Helsinki. A final sample size of minimum 500 healthy subjects was decided maintaining a gender ratio of 1:1 across all the different age groups. *A priori* approach was undertaken for selection of reference individuals who were mainly between 18 and 65 years of age and were mostly on no medications. Individuals above 65 years were also recruited, provided they comprise less than 20% of the study population. Further, the exclusion criteria were mainly those who were diabetic, had history of liver or kidney disease, donated blood as a donor in the past 3 months, HBV, HCV or HIV carrier and female volunteers who were pregnant or within 1 year after childbirth [4]. Primarily, employees working in Hinduja Hospital were recruited; however, their family members and those in healthcare business associated with the hospital were also recruited. Informed written consent was obtained from each volunteer prior to inclusion in the study. A health status questionnaire, which recorded information on the anthropometric measures, medical status, smoking, alcohol habits, physical activity and medications, if any, were obtained from each volunteer.

Sample collection and processing

Prior to blood collection, volunteers were requested to avoid excessive physical exertion/exercise for 3 days before the sampling and were also requested to avoid excessive eating/drinking the night before and fast overnight for 10–12 h. The sample collection started at 7:00 am till 10:00 am. To avoid variation due to postural influence and physical stress, the volunteers were requested to sit for 15–20 min prior to collection [5]. Approximately 8–10 mL of blood was collected in BD Vacutainers serum tubes, which after 45–60 min was centrifuged at 2000 *g* for 10 min. The serum was pooled, aliquoted (1.5 mL each) and immediately stored at −80 °C until analysis.

Target analytes and measurements

The frozen serum aliquots were measured collectively for 25 analytes by Beckman Coulter Unicel DxC 800 autoanalyzer: alanine aminotransferase (ALT); albumin (ALB); alkaline phosphatase (ALP); amylase (AMY); aspartate aminotransferase (AST); calcium (Ca); chloride (Cl); creatine kinase (CK); creatinine (CRE); γ-glutamyl-transferase (GGT); glucose (Glu); high-density lipoprotein cholesterol (HDL-C); inorganic phosphate (IP); iron (Fe); lactate dehydrogenase (LDH); low-density lipoprotein cholesterol (LDL-C); magnesium (Mg); potassium (K); sodium (Na); total bilirubin (TBil); total cholesterol (TC); total protein (TP); triglycerides (TG); Urea; uric acid (UA), and measured for eight analytes by Beckman Coulter Immage 800 autoanalyzer: complement component 3, and 4 (C3, C4); high-sensitivity C-reactive protein (hsCRP); immunoglobulin G, A, and M (IgG, IgA, IgM); transferrin (Tf); transthyretin (TTR) using the manufacturer's reagents, calibrators and controls as instructed. The Unicel DxC 800 autoanalyzer principle was based on ion selective electrode, conductivity electrode, glucose oxygen sensor, colorimetric, immuno-turbidimetric, enzyme immunoassay and rate end point, whereas the Immage 800 autoanalyzer was based on the principle of rate nephelometry and rate near infrared particle immunoassay (NIPIA).

Quality control (QC) and traceability

Internal QC was run for each analytes at both normal and pathological concentrations prior to sample testing. Internal QC monitoring was undertaken by preparing a mini-panel of sera from five healthy volunteers, which were measured with each subsequent batch of sample testing in order to monitor the between-day stability of the assay (Supplementary Table 1) [4, 6]. Further, to determine the traceability and to make the test results comparable among countries participating in the IFCC global study, a panel of 40 sera were provided by IFCC C-RIDL, which were measured in batches along with the volunteers' samples. The panel of sera were assigned values for 27/33 analytes (except ALB, Mg, Fe, AMY, LDH and ALP) by making it traceable to the reference measurement procedures [7]. The major axis linear regression between the assigned values and the observed values for panel of sera was used to assess the need for recalibration of the test results. When difference in the lower or upper limit of the RI before and after recalibration was more than 1/4 of standard deviation (SD) of the original RI, recalibration of test results was performed

by using regression coefficients (intercepts and slopes). This criterion corresponds to Frasers' allowable limit of bias [8–10].

Analysis of source of variation and partitioning

Multiple regression analysis (MRA) was performed to analyze potential SV such as age, BMI, drinking and physical exercise. The significance of each SV was evaluated as standard partial regression coefficient (rp), which corresponds to partial correlation coefficient and takes value between –1.0 and 1.0. We regarded $0.2 \leq |rp| < 0.4$ as slight to moderate association and $0.4 \leq |rp|$ as prominent association of test results with a given SV [6]. Nested analysis of variance (ANOVA) was performed to judge the need for partitioning RVs [4, 6, 11]. It separates the magnitude of variations attributable to each SV (age, gender, BMI, etc.) and expresses it in terms of SD. Derived were the variations of between-age (SDage), between-gender (SDsex), between-BMI (SD-BMI) components and net between-individual (SDindiv) component, which is adjusted for other SVs. We computed the SD ratio (SDR), which is the SD of a given SV over the SD-indiv. A ratio greater than 0.3 has been shown as a guide value for considering the need to partition RVs [6, 11]. The general-purpose statistical software, StatFlex for Windows version 6.0 (Artec Co. Ltd., Osaka, Japan), was used to carry out the MRA and nested ANOVA.

Derivation of RIs

For derivation of RIs, both non-parametric and parametric methods were used for comparative purposes. For the latter, modified Box-Cox power transformation formula [12] was used for Gaussian transformation of RVs, and values outside mean ± 2.57 SD were truncated once before computing the central 95% interval [12–14]. In the derivation of the final RIs, the effect of latent abnormal values exclusion (LAVE) method [6, 11] was examined. In applying the LAVE procedure, we allowed only one abnormal result (LAVE(+)Abn = 1) among 12 reference analytes: Glu, TG, LDL-C, HDL-C, UA, ALB, AST, ALT, LDH, GGT, CK and hsCRP. They were chosen to detect individuals with metabolic syndrome, inflammation, insufficient fasting and muscular exertion prior to the sampling. Ninety percent confidence intervals for the lower and upper reference limits (LL, UL) were calculated using the bootstrap method with iteration of 100 times, for both parametric and non-parametric methods. Making use of this procedure, the limits of the final RIs were set as the averages of repetitively computed LLs and ULs.

Results

Characteristics of study volunteers

A total of 512 volunteers were recruited primarily from Hinduja hospital employees (Table 1) with wide differences in their level of physical activities, right from managerial or hospital administrative personnel to physically demanding work profile such as that of laboratory technicians, nurse and hospital transporters. With respect to nutritional status, the BMI profile of volunteers was almost similar to that seen in general Indian population. Similar approach for volunteer recruitment was also adopted in the IFCC Asian study where more than 3500 volunteers from seven countries were recruited mainly from hospital personnel [15, 16].

Table 1: Clinical and anthropometric characteristics of healthy Indian volunteers recruited for the study.

No.	Characteristics	Males	Females
1	Total no of volunteers (n=512)	259	253
2	Gender distribution, years		
	18–29	63	63
	30–39	69	59
	40–49	60	63
	50–64	54	58
	≥65	13	10
3	Age, years	40.8 ± 13.2	40.8 ± 12.8
4	Body mass index (BMI), kg/m^2	24.6 ± 3.46	24.5 ± 4.4
5	Abdominal circumference, cm	89.3 ± 8.7	85.5 ± 10.5
6	Hypertension (HTN)	9 (3.4%)	4 (1.58%)
7	Alcohol consumption	66 (25.6%)	6 (2.3%)
8	Cigarette smoking	21 (8.1%)	0 (0%)
9	Vitamins and mineral supplement	20 (7.7%)	47 (18.6%)
	Vitamin D supplement	12 (4.6%)	16 (10.3%)
	Thyroid medication	4 (1.5%)	13 (5.1%)
	Anti-hypertensive medication	10 (3.8%)	0 (0%)

Analysis of source of variation (SVs)

MRA was performed analyte by analyte to identify the potential SVs (Table 2). The values shown are standard partial regression coefficients (rp) with positive sign highlighted by a background color of yellow (0.2–0.39) and orange (≥0.4) and negative sign highlighted by a light blue (0.2–0.39) or blue (≥0.4) background color. Those analytes with non-Gaussian skewed distribution of test results, such as TG, AST, ALT, GGT, TBil, hsCRP and CK, were logarithmically transformed before performing MRA.

The MRA revealed that age-related changes were observed for Glu in both sexes: TP, ALB and Ca in males; TC, LDL-C, TG, ALP, Urea, CRE, UA, LDH, IgA and IgM in females. BMI-related changes were observed for HDL-C, hsCRP, GGT, UA and C3 in both sexes; LDL-C, TG, AST and ALT in males; and Glu, Alb and AMY in females. However, regular exercise and alcohol consumption did not show any practically significant associations with the test values.

Table 2: Multiple regression analysis to identify potential source of variations.

Items	Males							Females						
	n	R	Age	BMI	SmkLvl	ExerLvl	DrkLvl	n	R	Age	BMI	ExerLvl	DrkLvl	
TP	254	0.35	−0.34	0.02	−0.06	0.05	0.07	253	0.17	−0.09	0.00	−0.06	−0.11	
ALB	255	0.48	−0.44	−0.16	−0.03	0.12	−0.01	253	0.34	−0.16	−0.24	−0.01	−0.11	
Glu	256	0.36	0.33	0.04	−0.05	−0.08	−0.04	252	0.47	0.35	0.26	−0.04	−0.08	
TC	255	0.24	0.19	0.08	0.06	0.05	0.08	251	0.36	0.33	0.09	0.02	0.06	
HDLC	256	0.29	0.01	−0.28	−0.07	0.06	0.00	253	0.29	0.17	−0.23	0.11	0.11	
LDLC	251	0.27	0.16	0.20	0.13	0.01	−0.02	248	0.35	0.28	0.15	0.01	0.05	
TG	249	0.37	0.18	0.32	−0.01	−0.02	0.07	251	0.43	0.34	0.19	−0.02	−0.11	
CRE	256	0.29	0.15	0.04	−0.18	0.07	0.22	252	0.33	0.21	0.11	0.12	0.12	
Urea	257	0.22	0.19	−0.05	0.07	0.05	0.00	253	0.44	0.40	0.06	−0.06	0.13	
UA	256	0.29	−0.01	0.28	−0.06	0.00	0.02	253	0.53	0.27	0.39	0.05	0.03	
AST	256	0.25	−0.08	0.21	−0.07	0.01	0.09	252	0.21	0.16	−0.11	0.09	−0.07	
ALT	240	0.39	−0.09	0.37	−0.03	−0.01	0.06	251	0.27	0.18	0.14	0.06	−0.11	
LDH	256	0.19	0.01	0.13	−0.13	0.00	−0.01	253	0.25	0.22	0.03	0.07	−0.07	
ALP	257	0.15	−0.11	0.02	0.03	−0.05	−0.10	252	0.30	0.26	0.12	−0.05	−0.07	
GGT	254	0.27	0.01	0.26	0.02	0.01	0.07	252	0.33	0.19	0.22	0.00	−0.12	
CK	254	0.15	−0.05	0.10	−0.06	0.09	0.00	252	0.15	0.08	0.10	0.00	0.04	
AMY	256	0.20	0.13	−0.12	−0.08	0.07	−0.01	250	0.31	0.11	−0.30	0.02	0.09	
TBil	256	0.17	0.00	−0.10	−0.02	0.12	0.07	253	0.10	0.09	−0.04	−0.02	0.05	
Na	256	0.12	−0.09	−0.05	−0.03	−0.07	0.04	251	0.24	0.18	−0.09	0.10	−0.10	
K	257	0.14	0.14	0.00	−0.02	−0.02	0.00	253	0.19	0.08	0.15	0.00	0.04	
Cl	256	0.17	0.06	0.05	−0.14	−0.04	0.09	251	0.08	0.03	0.00	0.06	0.03	
Ca	257	0.34	−0.33	−0.03	−0.01	0.06	0.02	253	0.16	0.06	−0.08	0.13	−0.01	
IP	257	0.32	−0.15	−0.16	0.17	−0.15	−0.08	252	0.14	0.14	−0.03	−0.03	0.03	
Mg	256	0.21	−0.05	−0.16	−0.14	0.04	0.01	253	0.16	0.10	−0.06	0.04	−0.11	
Fe	255	0.28	−0.16	−0.10	0.03	0.11	0.16	253	0.23	0.11	−0.10	0.08	0.17	
Tf	252	0.11	−0.08	−0.01	−0.07	−0.03	0.03	248	0.23	−0.17	−0.08	−0.07	−0.03	
hsCRP	256	0.41	0.14	0.38	0.07	−0.06	0.07	249	0.54	0.12	0.50	−0.05	−0.11	
IgG	257	0.11	−0.02	−0.02	−0.10	−0.03	0.08	253	0.12	0.03	0.02	−0.07	−0.09	
IgA	257	0.19	0.17	0.02	0.00	0.09	0.02	251	0.21	0.21	−0.04	−0.04	0.01	
IgM	257	0.17	−0.12	0.02	−0.02	0.12	0.00	252	0.36	−0.33	−0.10	−0.01	0.01	
C3	255	0.34	−0.09	0.30	−0.10	−0.03	−0.03	251	0.40	0.04	0.39	0.02	−0.04	
C4	257	0.17	0.05	0.14	0.01	−0.02	0.06	252	0.26	0.11	0.18	0.10	0.00	
TTR	257	0.21	−0.08	−0.14	−0.03	0.02	0.15	251	0.22	0.06	−0.13	0.16	0.08	

The values for each SVs are standard partial regression coefficients (rp). rp values with positive sign are highlighted by a background color of light yellow (rp: 0.2–0.39) and orange (≥0.4). Similarly, rp with negative sign are highlighted by a light blue (rp: 0.2–0.39) and darker blue background color (≥0.4). R, multiple regression coefficient; BMI, body mass index; SmkLvl, smoking; ExerLvl, physical exercise; DrkLvl, alcohol consumption.

Partitioning of reference values

To determine whether there is need to establish different RIs with respect to gender and age, nested ANOVA was carried out (Table 3). Actual differences in LL and UL of RIs before and after partition were rather small when SDR is near 0.3. Therefore, we adopted a criterion of SDR ≥ 0.4 for both gender and age partitioning. However, some analytes (TC, TG, Urea, ALP and IgM) with SDRsex < 0.4 were partitioned by sex when SDRage for either males or females was around 0.4 with noticeable difference in the UL between age groups.

Therefore, gender partitioning was required for 17 analytes, TC, HDL-C, TG, hsCRP, ALB, AST, ALT, ALP, GGT, TBil, Urea, CRE, UA, Fe, TTR, CK and IgM, whereas age partitioning required five analytes, Glu, ALB, TC, ALP and Urea. Sex- and age-related changes of RVs are shown as one-dimensional scattergram for eight representative analytes in Figure 1, and for all analytes in Supplementary Figure 1. Sex- and BMI-related changes are also shown

Table 3: NESTED ANOVA for partitioning of reference values.

No	Analytes	Nested ANOVA		One-way ANOVA	
		SDRsex	SDRage	SDRage (male)	SDRage (female)
1	TP	0.00	0.23	0.22	0.23
2	ALB	**0.61**	0.34	**0.42**	0.20
3	Glu	0.00	**0.46**	**0.44**	**0.47**
4	TC	0.00	0.35	**0.41**	0.28
5	HDLC	**0.47**	0.04	0.00	0.12
6	LDLC	0.00	0.23	0.26	0.19
7	TG	**0.39**	0.30	0.26	0.36
8	CRE	**1.32**	0.08	0.00	0.16
9	Urea	0.26	0.34	0.11	**0.51**
10	UA	**1.11**	0.18	0.06	0.29
11	AST	**0.47**	0.03	0.00	0.18
12	ALT	**0.70**	0.15	0.00	0.33
13	LDH	0.00	0.20	0.00	0.29
14	ALP	0.00	0.26	0.00	**0.42**
15	GGT	**0.42**	0.00	0.00	0.21
16	CK	**0.46**	0.17	0.23	0.00
17	AMY	0.10	0.00	0.00	0.10
18	TBil	**0.55**	0.00	0.00	0.00
19	Na	0.10	0.04	0.00	0.19
20	K	0.15	0.07	0.06	0.09
21	Cl	0.26	0.00	0.00	0.00
22	Ca	0.26	0.22	0.28	0.06
23	IP	0.12	0.19	0.14	0.24
24	Mg	0.05	0.10	0.00	0.18
25	Fe	**0.57**	0.10	0.00	0.17
26	Tf	0.34	0.15	0.15	0.15
27	hsCRP	**0.40**	0.14	0.00	0.19
28	IgG	0.17	0.00	0.00	0.00
29	IgA	0.03	0.09	0.00	0.15
30	IgM	**0.31**	**0.41**	0.39	**0.43**
31	C3	0.27	0.18	0.27	0.00
32	C4	0.09	0.09	0.13	0.00
33	TTR	**0.82**	0.00	0.00	0.00

SDR, standard deviation ratio. SDR ratio ≥0.4 was used as a criterion for partition of reference values (highlighted in yellow). Those highlighted in green also showed noticeable difference in UL and were partitioned accordingly.

for four representative analytes in Figure 1 and for total of 12 selected analytes with known association with BMI revealed by the MRA in Supplementary Figure 2.

Derivation of RIs

The RIs were derived using both parametric and non-parametric methods after applying secondary exclusion based on the LAVE method. RIs derived by parametric method and their 90% confidence limits were in general narrower than those observed by non-parametric methods as shown for representative analytes in Figure 2, and for all in Supplementary Figure 4. Therefore, we adopted RIs by parametric method for all analytes, except for hsCRP that failed to give Gaussian shape by the power transformation. Interestingly, the figures also show that, for majority of analytes, there was little or no difference in the RIs by applying the LAVE method (see Discussion for possible reasons). These findings are clearly shown in Supplementary Table 2, which presents the complete RI data derived by parametric and non-parametric method with 90% CI, and with/without LAVE method. The final list of RIs adopted in consideration of SDRsex and SDRage is shown in Table 4.

Quality control monitoring and traceability

All the five sera in the mini-panel analyzed with every batch (approximately 100 samples/batch) of sample testing had an acceptable between-day CV of all <10% (Supplementary Table 1), which were within the desirable limits [17]. For traceability, the panel of 40 sera was measured, correlated with the assigned values [7] and presented as correlation matrix graph (Supplementary Figure 3). RVs for 10 analytes (Glu, TC, LDL-C, CRE, AST, ALT, GGT, TBil, C3 and C4) exhibited bias in either LL or UL judged by the criteria described elsewhere [8–10], and thus, they were recalibrated using the major-axis regression line.

Discussion

With limited literature on Indian reference values for most of biochemical analytes, and as a part of the IFCC worldwide study, the present study was aimed to determine the RIs for 33 analytes. Due to a difficulty of the parametric method in attaining Gaussian transformation by use of the conventional Box-Cox formula, the CLSI C28-A3 guidelines recommended the use of the non-parametric method, which simply sorts RVs and sets 2.5 and 97.5 percentiles as RI limits. In the present study, we used modified Box-Cox formula for the parametric method instead. It invariably succeeded in attaining Gaussian transformation for accurate prediction of central 95% range. Features of the parametric method are (1) a consistently narrower 90% confidence interval of the RI limits and (2) generally narrower range of the RIs, compared to the non-parametric method. The latter phenomenon suggest a significant number of abnormal results present in the tail of distribution: Glu and TG partly due to insufficient fasting; Glu, TG,

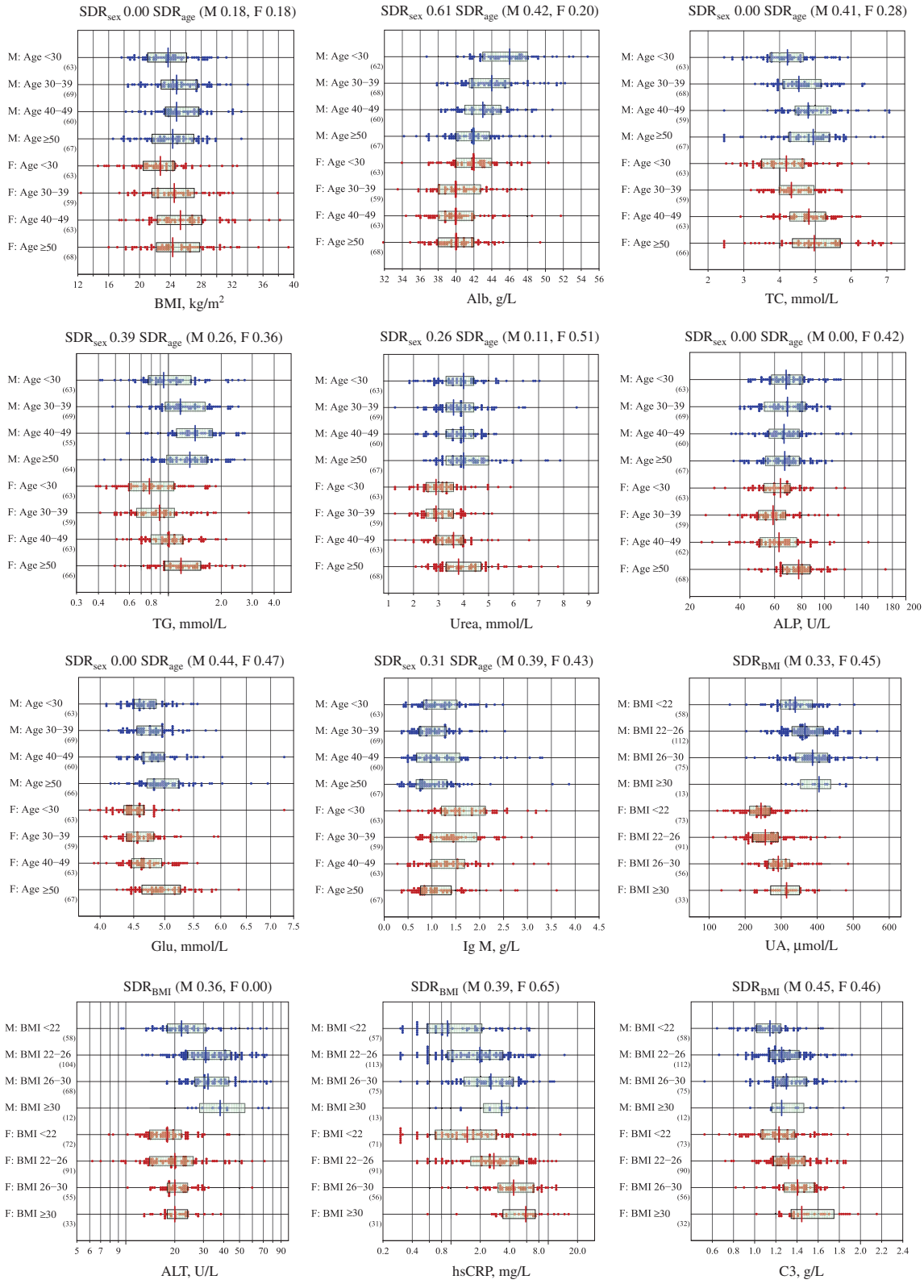

Figure 1: Distribution of RVs partitioned by sex and age or BMI for selected analytes.
For the first eight analytes, RVs of each analyte were first partitioned by sex and then partitioned by age into four groups at the boundary of 30, 40, 50 and above 50 years. For the last four analytes, RVs partitioned by sex and by BMI using boundary values of 22, 26 and 30 kg/m². The box and center vertical line in each scattergram represent the central 50% range and the median of RVs. Please note that all the RVs were plotted without any exclusion, and thus the distribution range by visual inspection may not match the RI of respective analyte.

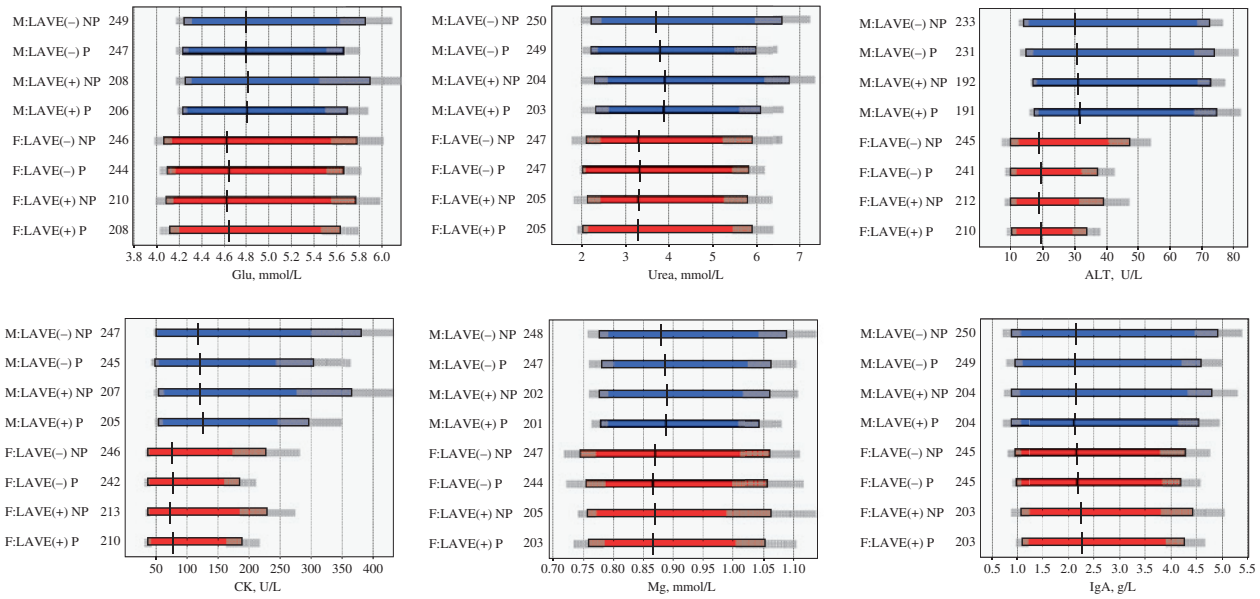

Figure 2: Comparison of RIs and their 90% CI derived in four ways.
The RIs were derived in four ways: by parametric (P) or non-parametric (NP) method with or without application of latent abnormal values exclusion method (LAVE) method. Each horizontal bar represents the RI, and the vertical line in the center corresponds to the midpoint. The shades on both ends of the bar represent 90% CI for the limits of the RI predicted by the bootstrap method. The results for six representative analytes for males (M) and females (F) are shown in this figure. The same figures for all analytes are available in Supplementary Figure 4.

ALT, GGT, C3 and C4 due to inclusion of individuals with prevalent metabolic syndrome with their close relationship with BMI; TP, IgG, IgA, IgM, C3 and C4 due to concurrent inflammation; CK, AST and LDH due to muscular exertion prior to the sampling, etc. Those outlying points in high proportion are expected to have undesirable effect on the RIs by the non-parametric method but not much on those by parametric method, which puts more weight to the center of the distribution and includes a tertiary exclusion step by truncating data outside mean ± 2.57 SD once [13]. In Figure 2, this fact is well reflected in a small reduction of data size by the parametric method regardless of use or nonuse of the LAVE method. This feature of the parametric method underscores its advantage over non-parametric method for RI derivation.

Furthermore, in order to reduce influences of latent diseases or sampling under inappropriate conditions, the LAVE method was used [6, 12]. The advantage of the LAVE method is that it helps to narrow the RI for analytes that have some association with the reference analytes, whereas the RIs for those analytes that do not associate with the reference analytes are unaffected by the procedure. However, in the present study, unlike in Turkish, Saudis and Chinese studies [8–10], Indians showed only minor change in the RI after applying the LAVE procedure. This finding possibly indicates that metabolic syndrome, and related inflammatory conditions are relatively milder and the association of abnormal test results among related analytes are less clear. In fact, if we applied LAVE method in a stricter way allowing no abnormal results in other analytes, its effect in narrowing the range of the RI was noted, although the number of RVs left for calculation was greatly reduced. Effectiveness of the LAVE method depends on association among reference tests used in identifying inappropriate individuals. Therefore, in order to understand the reason for the failure of the LAVE method, a comparison of correlation matrices of volunteers' values for six analytes (TG, UA, AST, ALT, GGT and CRP) across six countries (i.e. IND, CHN, JPN, SAU, TUR and RUS) were made. We found that the levels of associations among six analytes in Indians are as a whole weaker than those in other countries (Supplementary Table 3). Therefore, we assumed that, although the prevalence of metabolic syndrome in India is as high as other countries [18], its severity is generally less with weaker associations among nutritional markers, resulting in lack of noticeable effect of the LAVE method.

There have been very few published papers on Indian RIs for lipid-, renal- and liver-related analytes [19–25]. Therefore, an attempt to compare the present study's RIs with those from previous Indian, IFCC and the C-RIDL studies was undertaken (Table 4). The present study's RI for TC, TG and LDL-C were consistent with the various RI studies conducted across India covering the culturally and

Table 4: Final summary of the Indian reference intervals derived for all the 33 analytes in comparison with the previous IFCC C-RIDL reference interval studies on different population.

Items	Units	Age, years	Present study – Indian			IFCC – Asian study [15, 16]			China's study [10]			Turkey's study [8]			Saudi Arabia's study [9]		
			M+F	M	F	M+F	M	F	M+F	M	F	M+F	M	F	M+F	M	F
TP	g/L	18–65	68–86	–	–	–	–	–	65–79	–	–	66–82	–	–	62–77	–	–
ALB	g/L	18–65	–	–	36–47	41–51	41–51	40–50	41–52	–	–	41–49	–	–	39–50	–	–
		<45	–	39–52	–	–	–	–	–	–	–	–	–	–	–	–	–
		≥45	–	37–49	–	–	–	–	–	–	–	–	–	–	–	–	–
Glu	mmol/L	<45	4.1–5.5	–	–	–	–	–	4.0–5.9 (age 20–64)	–	–	3.96–5.88 (age 20–64)	–	–	4.0–5.9 (age 20–64)	–	–
		≥45	4.3–6.0	–	–												
TC	mmol/L	18–65	–	–	2.9–6.6	3.5–6.7	3.5–6.7	3.5–6.8	–	3.19–6.16	–	3.22–6.45	3.20–6.42	3.20–6.38	3.5–6.36	–	–
		<45	–	3.1–6.2	–	–	–	–	–	–	3.12–5.68 (age 20–49)	–	–	–	–	–	–
		≥45	–	2.5–6.7	–	–	–	–	–	–	3.65–6.87 (age 50–64)	–	–	–	–	–	–
HDL-C	mmol/L	18–65	–	0.7–1.5	0.8–1.8	0.94–2.43	0.89–2.11	1.07–2.55	–	0.80–1.95	0.91–2.18	–	0.85–1.52	0.95–1.56	–	0.74–1.76	0.98–2.19
LDL-C	mmol/L	18–65	1.7–4.4	–	–	1.65–4.55	1.83–4.85	1.62–4.42	–	1.33–3.83	1.28–3.41 (age 20–49) 1.75–4.43 (age 50–64)	1.47–3.92	1.60–4.01	1.32–3.92 (age 20-49) 1.78–4.91 (age 50–64)	1.80–4.34	–	–
TG	mmol/L	18–65	–	0.6–2.7	0.5–2.1	0.4–2.2	0.5–2.8	0.4–1.7	–	0.53–3.43	0.46–2.28	–	0.53–3.39	0.46–2.52	–	0.50–3.58	0.39–1.60
CRE	µmol/L	18–65	–	58–95	35–74	–	61–97	42–71	–	57–102	42–73	–	59–92	50–71	–	66–111	50–74
Urea	mmol/L	18–65	–	2.2–6.0	–	2.7–7.1	2.9–7.3	2.6–6.8	–	3.1–7.6	2.4–6.4 (age 20–49) 3.0–7.7 (age 50–64)	–	2.95–7.20	2.21–6.12 (age 20–49) 2.85–7.96 (age 50–64)	–	2.8–7.3	2.1–6.4
		<45	–	–	1.9–5.1	–	–	–	–	–	–	–	–	–	–	–	–
		≥45	–	–	2.4–6.7	–	–	–	–	–	–	–	–	–	–	–	–
UA	µmol/L	18–65	–	248–509	159–404	–	233–471	153–344	–	247–540	178–406	–	226–458	166–345	–	223–444	148–321
AST	U/L	18–65	–	20–53	17–39	14–32	16–35	14–29	–	16–39	14–34	–	13–30	11–25	–	11–28	10–24
ALT	U/L	18–65	–	15–74	10–37	11–44	14–54	11–31	–	10–59	6–34	–	9–57	7–28	–	7–39	5–18
LDH	U/L	18–65	104–206	–	–	138–235	142–240	136–233	–	120–224	112–202 (age 20–49) 133–233 (age 50–64)	126–220	–	–	10–238	–	–
ALP	U/L	18–65	–	41–111	–	34–90	39–96	32–84	–	50–124	39–96 (age 20–49) 52–127 (age 50–64)	38–112	43–116	34–97 (age 20–49) 47–133 (age 50–64)	39–114	–	–
		<45	–	–	35–100	–	–	–	–	–		–	–		–	–	–
		≥45	–	–	43–117	–	–	–	–	–		–	–		–	–	–
GGT	U/L	18–65	–	14–62	11–40	14–54	15–68	15–43	–	10–62	8–31	–	11–69	7–33	–	11–65	7–21
CK	U/L	18–65	–	48–304	36–184	43–226	58–261	40–152	–	65–277	44–180	–	48–227	34–131	–	54–266	27–138
AMY	U/L	18–65	36–135	–	–	47–136	45–131	51–148	29–92	–	–	34–119	–	–	31–117	–	–
TBil	µmol/L	18–65	–	6.2–23.7	4.0–17.3	–	–	–	–	7.1–28.8	6.0–22.0	–	3.8–24.1	2.7–15.9	–	3.6–22.4	2.2–15.5
Na	mmol/L	18–65	135–146	–	–	139–146	–	–	136–144	–	–	137–144	–	–	135–144	–	–

Table 4 (continued)

Items	Units	Age, years	Present study – Indian			IFCC – Asian study [15, 16]			China's study [10]			Turkey's study [8]			Saudi Arabia's study [9]		
			M+F	M	F	M+F	M	F	M+F	M	F	M+F	M	F	M+F	M	F
K	mmol/L	18–65	3.8–5.0	–	–	3.7–4.7	–	–	3.7–4.7	–	–	3.7–4.9	–	–	3.7–4.9	–	–
Cl	mmol/L	18–65	102–113	–	–	101–108	–	–	101–109	–	–	99–107	–	–	101–111	–	–
Ca	mmol/L	18–65	2.10–2.44	–	–	2.19–2.47	2.21–2.49	2.18–2.45	2.16–2.48	–	–	2.15–2.47	–	–	2.11–2.56	–	–
IP	mmol/L	18–65	0.80–1.43	–	–	–	–	–	0.84–1.48	–	–	0.80–1.40	–	–	0.81–1.44	–	–
Mg	mmol/L	18–65	0.77–1.07	–	–	–	–	–	0.78–1.00	–	–	0.77–1.06	–	–	0.71–0.96	–	–
Fe	µmol/L	18–65	–	7–33	4–26	–	–	–	–	10–34	5–30	–	–	–	–	7.9–29.6	3.7–26.0
Tf	g/L	18–65	2.2–4.0	–	–	1.9–3.5	1.9–3.1	2.0–3.6	–	1.83–3.02	1.95–3.52	–	–	–	–	2.0–3.2	2.1–3.9
hsCRP	mg/L	18–65	–	0.33–7.34	0.35–11.90	0.01–2.81	0.04–3.74	0.01–2.57	–	–	–	–	–	–	–	–	–
IgG	g/L	18–65	9.1–20.4	–	–	9.8–18.8	8.8–18.0	9.5–19.1	–	–	–	–	–	–	–	–	–
IgA	g/L	18–65	0.94–4.35	–	–	1.06–4.33	0.93–4.19	1.02–4.26	–	–	–	–	–	–	–	–	–
IgM	g/L	18–65	–	0.40–2.54	0.51–3.10	0.37–2.38	0.33–1.78	0.49–2.65	–	–	–	–	–	–	–	–	–
C3	g/L	18–65	0.85–1.82	–	–	0.73–1.4	0.75–1.42	0.71–1.35	–	–	–	–	–	–	–	–	–
C4	g/L	18–65	0.16–0.55	–	–	0.12–0.34	0.12–0.34	0.11–0.34	–	–	–	–	–	–	–	–	–
TTR	mg/L	18–65	–	161–360	133–278	193–391	230–401	186–331	–	210–390	180–320	–	–	–	–	–	–

ethnically different Indian population as well as those observed in the Asians, Chinese, Turkish and Saudis [8–10, 15, 16, 19–23], whereas RI for HDL-C were found to be lowest among the Asian, Turkey and Saudi Arabia study. A similar trend was seen when compared with the clinical decision limits (CDLs) recommended by the NCEP. The RIs derived are aimed at reflecting the actual health scenario of a given population as against CDLs, which are defined by consensus among clinicians and meant for prevention and early intervention of diseases [26]. In any case, derivation of the RI from healthy individuals is important for management of non-CDL-dependent diseases for, e.g. UL of LDL-C for cholestasis or hypothyroidism, and its LL for hyperthyroidism and malnutrition. However, for those analytes with their CDLs specified in clinical guidelines, CDLs should be mainly used in the clinical management of the relevant clinical conditions.

For liver- and renal-related analytes, our RIs were found to be almost comparable to those in the literature except for AST, ALT (males), for which our UL was the highest. However the UL for Urea was the lowest among the Asians, Turkish, Saudis as well as in the Nordic countries [8–10, 15, 16, 27]. It is notable that the UL for ALT (37 U/L) in females was lowest across Indian studies (50–75 U/L) [24, 25]; however, it was comparable to the Chinese and higher than that seen in Turkish and Saudis [8–10]. The varying levels of liver enzymes across the different populations could possibly be due to vitamin B_6 deficiency or due to different analytical platform used for estimation [27–29]. The UL for UA was higher than most population except to those seen in Chinese population [10]. Also, the UL for CRE was comparable across the Turkish, Chinese, Saudis and in the Asian study [8–10, 15, 16].

The RIs for inflammatory markers like hsCRP, IgG, C3 and C4 were highest as compared to those seen in the Asian study. This finding has been consistent with the IFCC Asian study where a strong regionality was seen in inflammatory markers, including IgG, C3 and hsCRP, suggesting the regions closer to the equator tend to have a higher serum levels of inflammatory markers, most likely due to greater exposure to infectious agents in regions that are close to the earth's equator than towards the poles [15, 16]. RIs for the remaining analytes (Na, K, Cl, Mg, Ca, IP, Fe, Tf, TTR, CK, AMY and LDH) were almost comparable with the literature except for CK, AMY and TTR where the UL were different than those seen in the Asians, Turkish, Chinese as well as the Saudis [8–10, 15, 16].

The effect of SVs on reference values was determined by MRA, which revealed that age and BMI were the most common SVs as compared to alcohol consumption and regular exercise, which did not significantly influence the

test results, a finding consistent in Turkish and Saudis study [8, 9]. Age-related changes were observed for Glu in both sexes, whereas HDL-C, hsCRP, GGT, UA and C3 were influenced by BMI. The positive correlation of age with Glu and lipids (females) levels has been apparent for a long time and with increase in the incidence of metabolic syndrome in Indians, such observation in the present study is not surprising. The positive correlation of BMI with liver enzymes (AST, ALT and GGT) especially in males, with inflammation (hsCRP, C3) and with serum uric acid, has been observed in the literature [30–33]. As the BMI increases, the adipocytes promote inflammation, predispose towards insulin resistance and even enhance the secretion of uric acid. High BMIs also have a negative impact on the HDL-C metabolism, thereby adversely affecting the HDL-C levels in the circulation [34]. Thus, increasing BMI certainly has the potential to influence the test results of analytes.

The limitation of this study could be the sample size, which is still small. With India being ethnically and culturally so diverse, it is very difficult to select a reference group that represents the entire population. However, since Mumbai being India's financial capital and a major metropolitan city, people migrate from all parts of the country, and therefore the present study population represents people from all caste and socioeconomic status. Nonetheless, it would be worthwhile to further validate the study findings on a large cohort of healthy Indian volunteers.

In conclusion, the present IFCC C-RIDL study undertaken to derive RIs in India has for the first time comprehensively provided information on RIs, SV and partition of reference values for the routinely tested 33 biochemical analytes in well-defined Indian healthy volunteers.

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