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Derivation of sex and age-specific reference intervals for clinical chemistry analytes in healthy Ghanaian adults

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Abstract

Objectvies: This study is aimed at establishing reference intervals (RIs) of 40 chemistry and immunochemistry analytes for Ghanaian adults based on internationally harmonized protocol by IFCC Committee on Reference Intervals and Decision Limits (C-RIDL).

Methods: A total of 501 healthy volunteers aged ≥18 years were recruited from the northern and southern regions of Ghana. Blood samples were analyzed with Beckman-Coulter AU480 and Centaur-XP/Siemen auto-analyzers. Sources of variations of reference values (RVs) were evaluated by multiple regression analysis (MRA). The need for partitioning RVs by sex and age was guided by the SD ratio (SDR). The RI for each analyte was derived using parametric method with application of the latent abnormal values exclusion (LAVE) method.

Results: Using SDR≥0.4 as threshold, RVs were partitioned by sex for most enzymes, creatinine, uric acid (UA), bilirubin, immunoglobulin-M. MRA revealed age and body mass index (BMI) as major source of variations of many analytes. LAVE lowered the upper limits of RIs for alanine/aspartate aminotransferase, γ-glutamyl transaminase and lipids. Exclusion of individuals with BMI≥30 further lowered the RIs for lipids and CRP. After standardization based on value-assigned serum panel provided by C-RIDL, Ghanaian RIs were found higher for creatine kinase, amylase, and lower for albumin and urea compared to other collaborating countries.

Conclusions: The LAVE effect on many clinical chemistry RIs supports the need for the secondary exclusion for reliable derivation of RIs. The differences in Ghanaian RIs compared to other countries underscore the importance of country specific-RIs for improved clinical decision making.

Keywords: between-country differences; bias ratio; ethnicity; latent abnormal values exclusion method; multiple regression analysis; nonparametric method; parametric method; standard deviation ratio; standardization.

Abbreviations: Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMY, amylase; AG, anion gap; AST, aspartate aminotransferase; BD, Becton Dickinson; BMI, body mass index; BR, bias ratio; Ca, calcium; CK, creatine kinase; Cl, chloride; C3, complement component 3; C4, complement component 4; CDL, clinical decision limit; CI, confidence interval; Cre, creatinine; C-RIDL, Committee on Reference Intervals and Decision Limits; CRP, C-reactive protein; CV(b), CV of the regression slope b; DBil, direct bilirubin; eGFR, estimated glomerular filtration rate; F, female; GGT, gamma-glutamyl transferase; Glb, globulin; Glu, glucose; HbA_{1c}, hemoglobin A_{1c}; HDL-CHDL-C, high-density lipoprotein cholesterol; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IP, inorganic phosphate; K, potassium; LAVE, latent abnormal values exclusion; LDH, lactate dehydrogenase; LDL-C, low-density lipoprotein cholesterol; LL, lower limit; M, male; MF, male + female; Mg, magnesium; MRA, multiple regression analysis; Na, sodium; NP, non-parametric; P, parametric; RI, reference interval; r_p, standardized partial correlation coefficient; RV, reference values; SDR, standard deviation ratio; SV, sources of variation; TBil, total bilirubin; TG, triglycerides; TC, total cholesterol; TCO_2, total carbon dioxide; TP, total protein; UA, uric acid; UL, upper limit.

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Introduction

Clinical laboratory reference intervals (RI) are supposed to be established and verified in every country. However, few countries carry out their own RI studies due to challenges and cost involved in directly recruiting healthy individuals and deriving RIs based on well-defined procedures [1–3]. Consequently, in Ghana diagnostic laboratories rely on reference intervals provided by the manufacturers of *in vitro* diagnostic instruments.These RIs may have been derived from a population with different characteristics which might not be appropriate. Previous studies have reported regional differences in certain chemistry analytes [4, 5] either due to ethnicity, diet, and environmental conditions. This clearly points to the importance of establishing RIs for local populations. IFCC Committee on Reference Intervals and Decision Limits (C-RIDL) has elaborated a protocol that harmonizes the pre-analytical, analytical and post-analytical study processes, and promotes the common derivation of RIs through standardization and ensures ease of comparison of derived RIs across different regions, countries and ethnicities [6, 7].

Several studies conducted in African populations have reported variations in RIs for some chemistry analytes [8–10]. These observations emphasize the need for establishing RIs specific for the Ghanaian population, acknowledging that most diagnostic laboratories in Ghana adopt RIs from extant literature and manufacturers' inserts [11]. Hence, we conducted a nationwide study to establish standardized RIs for major chemistry analytes by recruiting well-defined healthy Ghanaian adults based on the C-RIDL protocol and value-assigned serum panel [12]. Additional objectives were (1) to evaluate sources of variation of reference values (RVs) specific to each analyte and (2) to explore unique features of Ghanaian/African RIs from an international perspective.

Materials and methods

Study population and recruitment

Healthy volunteers were recruited from Accra in the South and Tamale in the North, which represent two different ethnic groups. Recruitment was based on the C-RIDL protocol for harmonized implementation of multicenter studies [7]. A target sample size of 500 was set with equal sex and age (18–70 years) distributions. Through posters and media advertisement, volunteers were recruited from public institutions, health facilities, churches, etc. A questionnaire based on the protocol was administered to record volunteers' health status.

Inclusion/exclusion criteria

The exclusion criteria by the protocol were individuals with known diabetes mellitus under drug therapy, recent (≤14 days) recovery from acute illness, injury, or surgery requiring hospitalization, known carrier state of hepatitis viruses or HIV, pregnancy or within one year after childbirth. Additionally, we excluded individuals with body mass index (BMI)>35 kg/m^2, alcohol consumption≥70 g/day, smoking habit of >20 cigarettes/day, under regular medication. Upon completion of the questionnaire, the eligible volunteers from Accra were scheduled for blood collection at Medlab Ghana, while specimens taken in Tamale were transported to Medlab Ghana in Accra within 6 h each day. Participants' results were reported individually upon completion of measurements.

Laboratory analysis

Blood sampling and storage: Blood sample collection was done in two stages, the first from participants residing in the southern Ghana (Accra), and the second from the northern part (Tamale). The sample collection lasted for 6 months (June through December 2017). Blood was drawn under basal conditions as recommended in the protocol between 7:00 and 10:00AM after overnight fasting, and avoidance of strenuous muscular exertion (for three days) or night shift work prior to the sampling. Sitting posture of 20–30 min before venipuncture was requested to avoid postural changes in test results [13]. Thirty mL of blood was drawn into three evacuated serum separator tubes (BD-Vacutainer, West Africa), one sodium fluoride tube for glucose, and one vacutainer tube containing K_2-EDTA for HbA_{1c}. Anthropometric measurements which included weight, height, body mass index (BMI), waist circumference and blood pressure were taken on the day of sampling.

The blood was centrifuged at 1,200*g* for 10 min at room temperature and all the serum specimens were divided into five aliquots of 2 mL well-sealed freezing tubes (ThermoFisher, West Africa), and was immediately stored at −80 °C until the time of collective measurements. Also, some samples were preserved with dry ice at −80 °C and transported to the referral laboratory at SynLab, Germany for the analysis of tumour markers, thyroid test and immunological analytes. Sample analysis were performed from January 15th through February 13th, 2018. The purpose for preparing multiple serum aliquots was our plan of testing additional analytes by immunoassay, and for storage in anticipation of future testing of new markers.

Target analytes and measurements

The 34 analytes measured in serum were ALP, GGT, AST, ALT, LDH, CK, amylase (AMY), triglycerides (TG), total cholesterol (TC), HDL-C, LDL-C, Na, K, Cl, Ca, inorganic phosphate (IP), Mg, total CO_2 (TCO_2), anion gap (AG), total protein (TP), albumin (Alb), total bilirubin (TBil), direct bilirubin (DBil), urea, creatinine (Cre), uric acid (UA), glucose (Glu), $HbA_{1c,}$ CRP, complement component 3 and 4 (C3, C4), IgA, IgG and IgM. Six calculated parameters were globulin (Glb=TP − Alb), adjusted-Ca (aCa=Ca − 0.025 × [Alb − 40]), eGFR (CKD-EPI) [14], TC/HDL, HDL/LDL and non-HDL cholesterol (nonHDL=TC−HDL-C).

Chemistry analytes were measured in batches on AU480 autoanalyzer (Beckman-Coulter, US). While inflammatory markers were measured by immunoturbidometric assays using Centaur-XP auto-

analyzer (Siemens, Germany). HbA_{1c} was measured by Beckman Coulter AU 460 using the latex agglutination inhibition method. Detailed analytical methods and precisions are summarized in Supplementary Table 1.

Quality control

All measurements were carried out in Medlab Ghana and SynLab, Germany, all ISO15189 accredited Medical Laboratories, according to the laboratories' SOPs and Quality Manual to ensure reliable measurements. Additionally, a mini-panel of sera were prepared from healthy volunteers and measured each day in singleton to monitor the stability of the assay according to the protocol [7, 12].

Standardization based on value-assigned serum panel

Participating laboratories to the C-RIDL global study received a panel of 50 sera that had assigned values for 30 chemistry analytes [12]. The sera were measured to enable recalibration of reference values (RVs), where necessary. The test results also allowed for direct comparison of RVs among collaborating laboratories.

Ethical approval

The study protocol conforming to the Declaration of Helsinki was approved by the Ethical Review Committee of the College of Health Sciences, University of Ghana (CHS-Et/M.8-P4.14/2016–2017). Written consents were obtained from all volunteers. Participants' confidentiality was maintained at all times.

Statistical analyses

Sources of variation of RVs: Multiple regression analysis (MRA) was performed separately for each sex by setting RVs of each analyte as an objective variable. As explanatory variables, the following six factors were set: age, BMI, ethnicity (Akan=1; non-Akan=0), hours of standing/day (HrStand), habit of drinking alcohol (Yes=1; No=0), days/week of regular exercise (ExerLvl: 0–7). RVs of analytes that showed skewed distributions were transformed logarithmically (TBil, DBil, TG, ALT, AST, GGT, CK, CRP) or by square-root (LDH, ALP, AMY, IgA, IgM) to reduce the skewness. The association of each explanatory variable with the objective variable was expressed as standardized partial regression coefficient (r_p), which takes a value between −1.0 and 1.0. We regarded $|r_p| \geq 0.2$ as an appreciable effect size of correlation, which is between 'small' (0.1) and 'medium' (0.3) levels proposed by Cohen [15].

Partitioning criteria of RVs: The need for partitioning RVs by sex and age was guided by computing standard deviation ratio (SDR) [6]. The SDR represents a ratio of between-subgroup SD (variation of subgroup means from the grand mean) to between-individual SD (approximately 1/4 the RI width, representing between-individual SD). Two-level nested ANOVA was performed to compute between-sex SD and between-age SD after partitioning age at 30, 40, and 50 years. The SDR for between-sex SD (SDR_{sex}), and for between-age SD (SDR_{age}) were computed as a ratio to the residual SD (or between-individual SD). Since between-age variation changes by sex, we also computed SDR_{age} for each sex by one-way ANOVA. SDR≥0.40 was considered as practically significant between-subgroup difference to derive sex or age specific RIs [5].

However, SDR is sometimes insensitive to actual difference (bias) at lower or upper limit of the RI (LL, UL) after partitioning because SDR represents central tendency of between-group differences, not necessarily represents differences at LL or UL. Therefore, we set up a secondary criterion called bias ratio (BR) at LL or UL, BR_{LL} or BR_{UL}, as described elsewhere [16, 17].

$$BR_{LL} = \frac{|LL_M - LL_F|}{(UL_{MF} - LL_{MF})/3.92}, \qquad BR_{UL} = \frac{|UL_M - UL_F|}{(UL_{MF} - LL_{MF})/3.92}$$

where subscript M, F, and MF represent male, female, and male + female, respectively.

As a threshold of BR, 0.375 was used in previous studies [16, 17] following the specification of allowable analytical bias [16] at a minimum level. We adopted it in judging a bias at LL or UL before and after standardization or with/without LAVE procedure (see below). Whereas the threshold of 0.375 was found too sensitive when used in judging the need for partitioning by sex or age, hence we adopted 0.57 for the purpose (from relationship of bias (Δ) and SD: see Discussion). However, if actual between-group difference at LL or UL was too small from clinical perspective, partition was withheld.

Derivation of reference intervals: Parametric and non-parametric methods were used in deriving the RIs. For the parametric method, RVs were first transformed into Gaussian distribution using two parameter Box-Cox power transformation formula [6, 17], and the central 95% range was calculated as mean ± 1.96SD (after truncating values outside mean ± 2.81SD once). Then, the range was reverse transformed to get LL and UL in the original scale. The 90% confidence intervals (CIs) for LL and UL were estimated using the bootstrap method through iterative resampling of 50 times.

In calculating the RIs, to reduce the influence of inappropriate values attributable to latent diseases or noncompliance to the sampling requirements, the latent abnormal values exclusion (LAVE) method was used. It is an iterative RI optimization procedure [6, 17] with initial calculation of RIs done analyte by analyte independently. In the subsequent iterative calculation, individuals were excluded who had two or more results outside the RIs derived in the previous computation among mutually associated reference tests, which represent nutritional, inflammatory conditions or muscular damage: Alb, Glb, UA, Glu, AST, GGT, CK, LDH, TC, nonHDL, TG, and CRP.

Results

Demographic profiles of participants (Table 1)

The total sample size of reference individuals included in data analyses was 501, comprising 54% (n=270) males and 46% (n=231) females. The mean age and SD was 41.3 ± 13.5 years with almost flat distribution. The volunteers were from Accra (77%) and Tamale (23%). Regarding ethnicity, the participants were grouped into Akan and non-Akan based on their common language and diet. The Akan constituted

40.3% (n=202) and non-Akans 59.7% (n=299). Mean ± SD of BMI was 24.9 ± 3.5(M), 26.9 ± 4.0(F) kg/m^2, systolic/diastolic blood pressures were 121.2 ± 11.5/78.9 ± 7.2 mmHg. Almost all participants (99.4%) recruited were non-smokers, which is the cultural norm in Ghana. It is notable that individuals with any allergy, such as allergic rhinitis or atopic dermatitis, was as low as 1.9% in males and 0.4% in females. In relation to the testing of HbA_{1c}, we retrospectively ascertained the lack of disorders that may have affected its value, such as sever iron deficiency anemia, based on our previous report of hematology RI study [18]. While possible influence of sickle cell anemia in adults in calculating the RI was unlikely judged from its new-born incidence in Ghana of 2% [19].

Sources of variation analysis by MRA

The results of MRA performed for exploration of sources of variation (SVs) of each analyte are summarized in Table 2. By setting $|r_p|>0.2$ as the threshold, age and BMI were the key factors associated with many of the analytes. The notable age-related changes were mostly found in females for Na, urea, TCO_2, Cre, TG, UA, Mg, ALP, and TC in the descending order of strength, and only for TCO_2 and IgA in males. While, age-related decrease ($r_p<-0.2$) was observed in IgM, TP, and Glb in female, and Alb, TP, and Ca in males. Since there is no association of age in adjusted Ca (aCa), the decrease in Ca is obviously confounded by age-related decrease in Alb. These age-related changes are shown for BMI and RVs of eight representative analytes in Figure 1.

With respect to BMI-related changes, analytes tested in males showed generally higher associations with BMI compared to females. Positive association of RVs with BMI ($r_p>0.2$) were found in males for C3, TC/HDL, UA, nonHDL, ALT, GGT, LDL-C, TC, TG, Glu, HbA_{1c}, in the descending order of influence; while, in females for C3, CRP, ALT, TG, TC/HDL, UA, nonHDL, HbA1, LDL-C, and GGT; Conversely, negative association of RVs with BMI ($r_p<-0.2$) were noted for HDL/LDL in both sexes, and HDL-C in males, and AMY in

Table 1: Demography of the participants.

		Male	Female	Sum
Age, years	n	270	231	501
	Mean ± SD	40.9 ± 13.5	41.8 ± 13.6	41.3 ± 13.5
Ethnicity 1	Akan	108 (40.0)	94 (40.7)	202 (40.3)
	Ewe	46 (17.0)	32 (13.9)	78 (15.6)
	Ga	38 (14.1)	29 (12.6)	67 (13.4)
	Other	78 (28.9)	76 (32.9)	154 (30.7)
Ethnicity 2	Akan	108 (40.0)	94 (40.7)	202 (40.3)
	Other	162 (60.0)	137 (59.3)	299 (59.7)
Waist circumference, cm	n	270	231	501
	Mean ± SD	34.59 ± 3.56	36.02 ± 4.56	35.25 ± 4.11
BMI, kg/m^2	n	270	231	501
	Mean ± SD	24.86 ± 3.54	26.93 ± 4.03	25.81 ± 3.91
SBP, mmHg	n	270	231	501
	Mean ± SD	122.6 ± 10.9	119.6 ± 12.0	121.2 ± 11.5
DBP, mmHg	n	270	231	501
	Mean ± SD	78.8 ± 7.0	79.0 ± 7.4	78.9 ± 7.2
Allergy	Yes	5 (1.9)	1 (0.4)	6 (1.2)
	No	265 (98.1)	230 (99.6)	495 (98.8)
Alcohol	Yes	42 (15.6)	24 (10.4)	66 (13.2)
	No	228 (84.4)	207 (89.6)	435 (86.8)
Regular exercise	Yes	121 (45.0)	85 (36.8)	206 (41.2)
	No	148 (55.0)	146 (63.2)	294 (58.8)
Hour stand per day	n	270	229	499
	Mean ± SD	3.5 ± 2.1	3.4 ± 2.0	3.5 ± 2.0
Hour sit per day	n	270	231	501
	Mean ± SD	4.94 ± 2.10	4.73 ± 1.92	4.84 ± 2.02
Hour sleep per day	n	270	231	501
	Mean ± SD	6.5 ± 1.1	6.5 ± 1.2	6.5 ± 1.2
Hour outdoor per day	n	269	230	499
	Mean ± SD	3.5 ± 2.7	2.9 ± 2.4	3.2 ± 2.5

Table 2: Multiple regression analysis for sources of variation of reference values.

Male	n	R	Age	Akan	BMI	Alcohol	ExerLvl	HrStand
TP	269	0.390	**0.35**	0.07	0.02	0.01	0.11	-0.06
Alb	269	0.461	**0.42**	0.00	0.05	0.05	0.11	-0.06
Glb	269	0.189	0.14	0.09	0.01	0.05	0.06	-0.04
TBil	268	0.201	0.07	0.10	0.02	0.13	0.07	0.07
DBil	267	0.207	0.08	0.04	0.08	0.07	0.06	0.11
UA	266	0.399	0.04	0.01	**0.38**	0.01	0.08	-0.01
Urea	269	0.208	0.04	0.07	0.06	0.09	0.15	-0.04
Cre	269	0.199	0.11	0.05	0.03	0.05	0.08	-0.11
Na	268	0.335	0.15	0.17	0.05	0.19	0.04	0.01
K	268	0.194	0.10	0.09	0.12	0.07	0.06	0.03
Cl	269	0.221	0.13	0.07	0.11	0.02	0.06	0.10
TCO_2	269	0.328	**0.27**	0.11	0.04	0.16	0.02	0.10
AG	269	0.411	0.20	**0.24**	0.03	0.19	0.11	-0.13
Ca	268	0.383	**0.34**	0.11	0.08	0.03	0.08	-0.04
aCa	268	0.176	0.03	0.14	0.03	0.09	0.02	-0.01
Mg	269	0.200	0.12	0.12	0.08	0.01	0.01	-0.08
IP	267	0.279	0.11	0.20	0.09	0.13	0.00	-0.02
AST	268	0.251	0.03	0.02	0.17	0.17	0.08	-0.03
ALT	267	0.416	0.16	0.08	**0.33**	0.19	0.05	-0.06
LDH	268	0.289	0.07	0.16	0.13	0.09	0.14	-0.04
ALP	268	0.109	0.04	0.08	0.01	0.05	0.03	-0.03
GGT	266	0.368	0.03	0.09	**0.32**	0.14	0.06	0.06
CK	269	0.208	0.11	0.01	0.13	0.11	0.02	-0.05
AMY	268	0.185	0.02	0.04	0.18	0.01	0.02	-0.04
Glu	265	0.288	0.04	0.01	**0.27**	0.06	0.04	-0.04
HbA_{1c}	269	0.333	0.13	0.10	**0.25**	0.01	0.06	-0.04
TC	269	0.315	0.07	0.09	**0.28**	0.03	0.03	-0.02
TG	269	0.433	0.17	0.03	**0.28**	0.02	0.04	-0.19
HDL-C	269	0.294	0.03	0.09	**-0.25**	0.00	0.02	0.10
LDL-C	269	0.329	0.04	0.06	**0.31**	0.03	0.02	-0.01
nonHDL	269	0.382	0.07	0.07	**0.35**	0.03	0.03	-0.04
TC/HDL	269	0.449	0.04	0.02	**0.42**	0.04	0.02	-0.09
HDL/LDL	269	0.411	0.04	0.00	**-0.39**	0.02	0.00	0.05
CRP	268	0.300	0.19	0.03	0.16	0.03	0.09	-0.06
IgG	268	0.168	0.06	0.04	0.08	0.11	0.01	-0.01
IgA	269	0.226	**0.22**	0.01	0.00	0.05	0.01	-0.02
IgM	267	0.170	0.04	0.07	0.11	0.06	0.08	-0.01
C3	269	0.515	0.01	0.00	**0.50**	0.06	0.11	-0.08
C4	267	0.328	0.16	0.09	**0.21**	0.12	0.08	-0.05

Females	n	R	Age	Akan	BMI	Alcohol	ExerLvl	HrStand
TP	229	0.318	**-0.21**	-0.04	-0.15	0.00	0.13	-0.03
Alb	229	0.273	-0.04	-0.07	-0.17	-0.01	**0.20**	0.01
Glb	229	0.255	**-0.22**	0.00	-0.08	0.00	0.03	-0.04
TBil	229	0.229	0.11	-0.18	0.01	0.09	0.07	-0.01
DBil	229	0.218	0.08	-0.18	-0.04	0.11	0.07	0.03
UA	229	0.420	**0.27**	0.06	**0.25**	0.00	-0.06	-0.04
Urea	229	0.408	**0.37**	-0.02	0.03	0.11	0.13	-0.05
Cre	229	0.330	**0.29**	0.03	0.06	0.08	-0.08	-0.04
Na	228	0.614	**0.57**	-0.10	0.01	0.03	-0.11	-0.06
K	228	0.229	0.17	0.11	-0.03	-0.05	0.08	-0.06
Cl	229	0.241	0.14	0.17	-0.03	0.01	-0.14	0.07
TCO_2	229	0.449	**0.32**	0.13	0.11	-0.11	0.16	-0.10
AG	229	0.345	0.06	**-0.30**	-0.05	0.08	-0.10	-0.03
Ca	229	0.170	0.09	-0.04	-0.03	0.07	0.13	-0.03
aCa	228	0.224	0.15	0.00	0.11	0.10	-0.01	-0.05
Mg	229	0.340	**0.26**	-0.18	-0.07	0.00	0.03	-0.10
IP	228	0.375	0.07	**-0.26**	-0.04	-0.04	-0.04	**-0.20**
AST	229	0.164	0.05	0.01	0.06	-0.07	-0.09	-0.04
ALT	228	0.312	-0.10	0.03	**0.29**	-0.04	0.10	0.03
LDH	229	0.250	0.08	-0.18	0.11	0.07	0.01	-0.07
ALP	228	0.300	**0.23**	-0.09	0.09	-0.05	-0.02	-0.01
GGT	229	0.269	0.08	0.00	**0.21**	0.08	-0.01	0.10
CK	227	0.237	0.07	0.05	0.17	-0.04	0.10	0.03
AMY	227	0.342	-0.03	0.11	**-0.32**	-0.02	-0.02	-0.04
Glu	229	0.297	0.19	0.10	0.14	-0.07	0.03	0.04
HbA_{1c}	229	0.314	0.14	-0.05	**0.23**	-0.05	0.01	0.06
TC	229	0.354	**0.23**	0.12	0.18	0.01	0.07	0.01
TG	229	0.453	**0.27**	-0.06	**0.28**	-0.04	-0.05	0.04
HDL-C	227	0.138	0.11	0.05	-0.07	0.02	0.04	-0.04
LDL-C	228	0.333	0.18	0.11	**0.21**	0.03	0.07	-0.02
nonHDL	228	0.368	**0.20**	0.10	**0.24**	0.02	0.06	-0.01
TC/HDL	229	0.316	0.11	0.04	**0.26**	0.00	0.04	0.05
HDL/LDL	229	0.279	-0.10	-0.06	**-0.22**	-0.02	-0.05	-0.03
CRP	227	0.399	0.12	0.03	**0.34**	0.07	-0.04	-0.08
IgG	229	0.229	-0.11	0.09	-0.14	0.01	0.01	-0.08
IgA	229	0.251	0.18	0.18	-0.08	-0.05	-0.02	-0.01
IgM	227	0.247	**-0.23**	0.00	-0.02	-0.01	0.05	-0.09
C3	228	0.366	0.04	0.00	**0.35**	0.00	-0.03	0.02
C4	228	0.314	0.15	0.10	**0.20**	-0.05	0.08	0.01

R=multiple correlation coefficient. Akan=dummy variable indicating ethnicity (Akan=1; nonAkan=0) Alcohol=habit of drinking alcohol (Yes=1; No=0); ExerLvl=days/week of regular exercise (0–7); HrStand=hours of standing/day. The width of bar in each cell represents partial correlation coefficient (r_p) with negative value shown by red bar. $|r_p| \geqq 0.2$ was marked by bold font.

females. These findings are shown graphically for 12 representative analytes in Figure 2.

None of the analytes, including GGT or ALT, showed any significant association with drinking, obviously attributable to infrequent drinking habits of our volunteers: 13.2% (male: 15.6% female: 10.4%). The level of regular exercise or hours of standing per day was not significantly associated with any analyte. We did not include smoking habit in MRA because only three subjects were smokers. Using the dummy variable of Akan or not to examine the effects of ethnicity, only AG and IP showed weak associations in both sexes: i.e., their RVs in Akan were lower than non-Akan.

Standard deviation ratio (SDR) as a guide for partitioning RVs

The magnitude of between-sex differences is shown in Table 3 expressed as SDR_{sex} that was derived by two-level nested ANOVA with age as covariate. $SDR_{sex} \geq 0.4$ was observed for

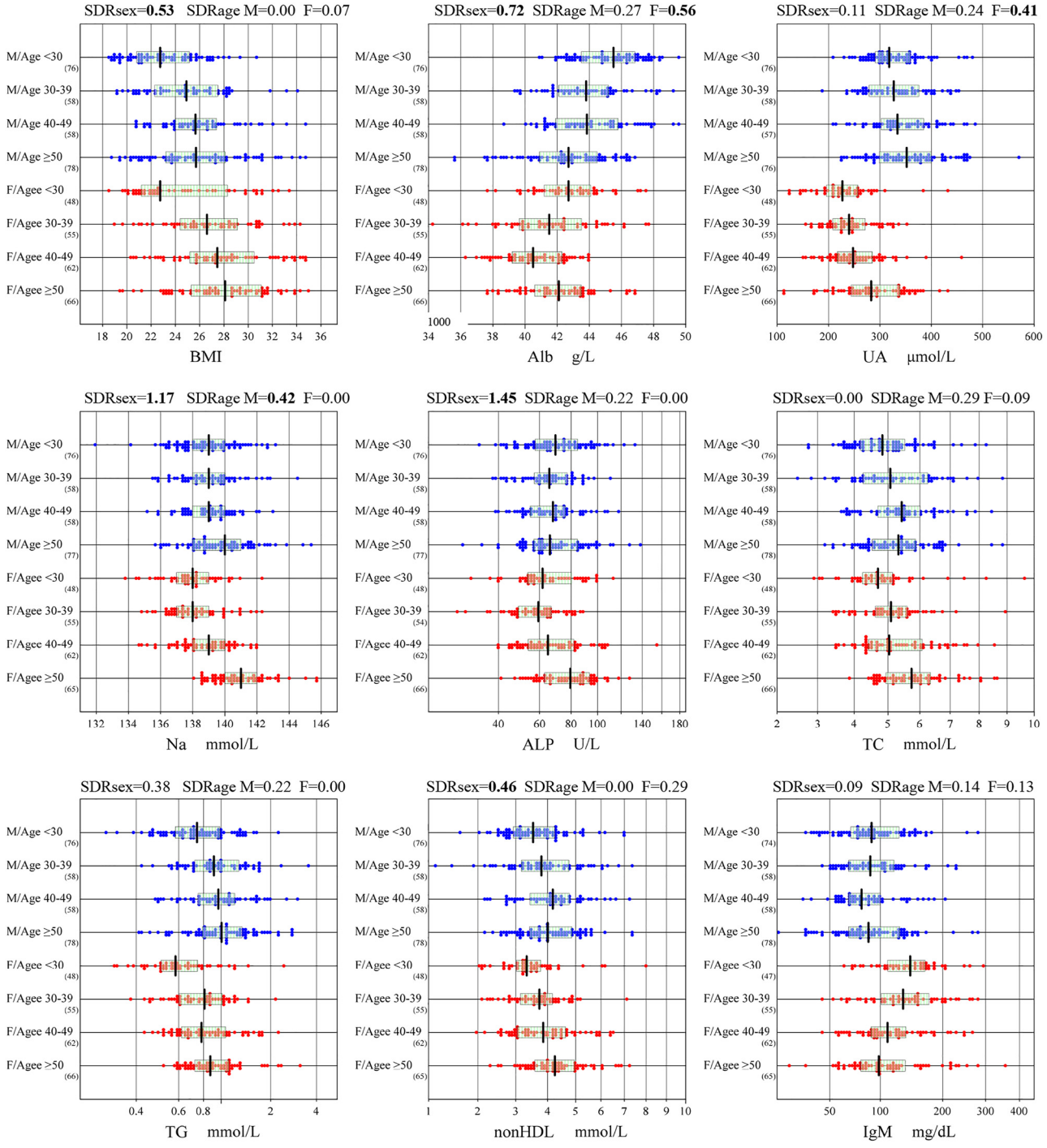

Figure 1: Sex and age-related changes of representative chemistry analytes.
RVs were partitioned by sex (male: M, female: F) and age-subgroups (~29, 30–39, 40–49, 50~). The box in the center of each scattergram indicates the mid 50% range of RVs, and its central vertical bar represents the median. The data size of each subgroup is shown at the right bottom of the age group labels. Because no secondary exclusion was done for RVs, the range of the scattergrams may not match to the RI, which is to be derived with inclusion of single mean ± 2.81 SD truncation step under the transformed scale.

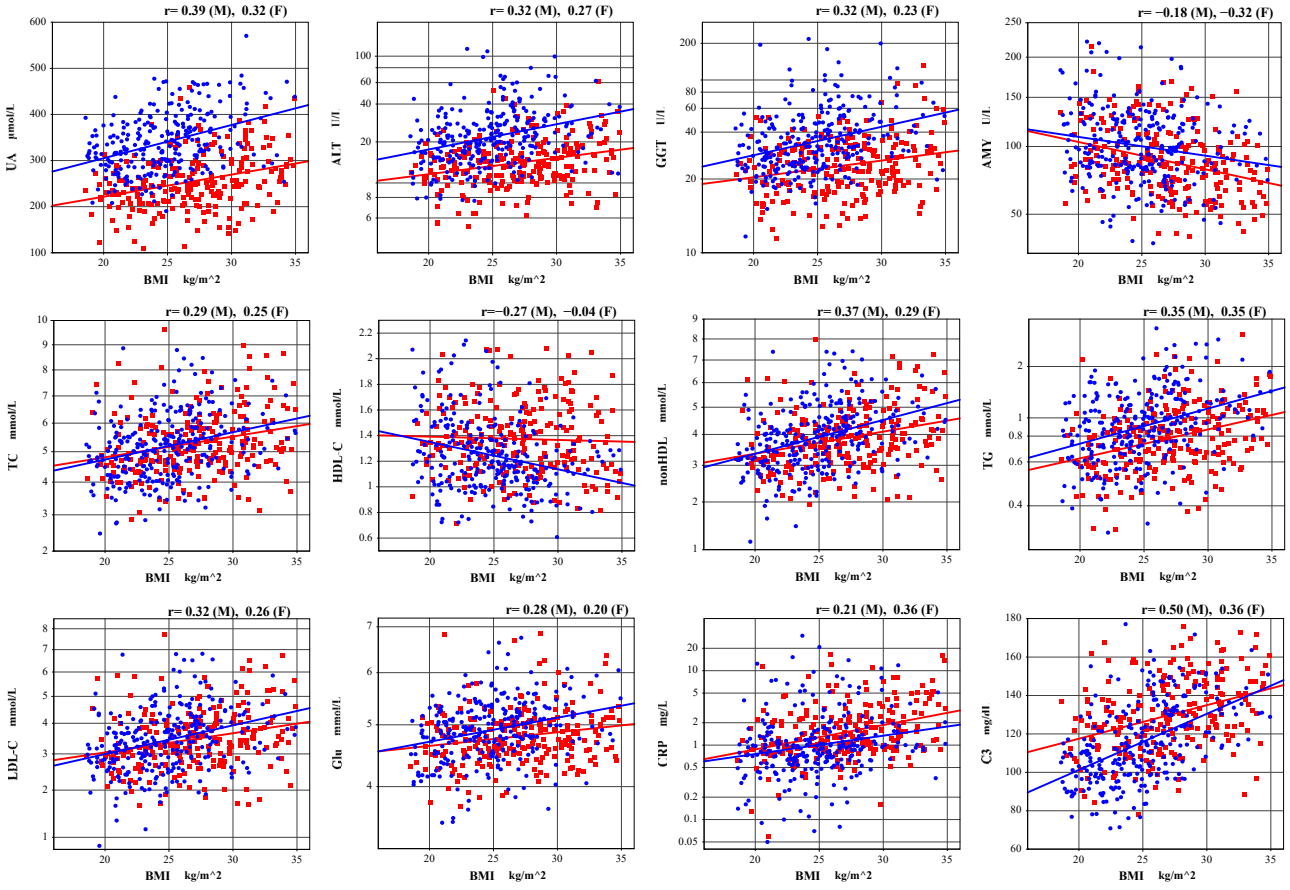

Figure 2: Association of BMI with RVs of nutritional/inflammatory markers.
Associations of BMI (on x-axis) with RVs of 12 analytes (on y-axis) are shown separately for males (blue dots) and females (red dots) by 2D-scattergram. The least-square regression lines are drawn and Spearman's correlation coefficients are calculated for both sexes.

Cre, UA, ALT, Alb, AST, CK, DBil, TBil, Cl, GGT, IgM, C3, and Ca, in the descending order of magnitude. The direction of gender difference in RVs was all higher in males except for Cl, and IgM. For age related change, SDR_{age} specific for each gender was derived as SDR_{ageM} or SDR_{ageF} by one-way ANOVA. For males, only SDR_{ageM} for Alb and eGFR were significant, while for females, SDR_{ageF} were significant for Na, eGFR, Ca, TG, and ALP. From Supplementary Figure 1, the direction of changes is increase with age for all analytes except for Ca and Alb.

Derivation of RIs

RIs were calculated in four ways by parametric (P) and nonparametric (NP) methods with/without application of the LAVE method. The comparisons of RIs for all analytes are shown in Supplementary Figure 3. It was obvious that UL by NP method was biased to the higher side and its 90% CI was generally wider especially for analytes with skewed distribution such as ALT, CK, GGT, and CRP. Judged from BR of >0.375 with/without LAVE shown in Supplementary Table 2, the LAVE procedure had a notable effect in lowering ULs for AST, ALT, GGT, TC, TG, nonHDL, LDL-C, TC/HDL, CRP in both sexes, UA and Cre in females, and CK and IgM in males.

The effect of excluding individuals with high BMI (≥30 kg/m^2) was examined for the analytes with high r_p by MRA as shown in Supplementary Table 3. Based on BR of >0.375 as a significant effect of BMI restriction, we adopted the BMI-restricted RIs for UA, TC, LDL-C, and CRP for both sexes and, for nonHDL and TG for females only.

Table 3: SDRs representing between-sex, between-age differences.

		2-level ANOVA		One-way ANOVA	
Analyte	N	SDRsex	SDRage	SDRageM	SDRageF
TP	500	0.12	0.38	0.37	0.38
Alb	500	**0.62**	**0.43**	**0.48**	0.36
Glb	500	0.19	0.22	0.11	0.30
TBil	499	**0.58**	0.00	0.00	0.00
DBil	498	**0.59**	0.00	0.00	0.00
UA	497	**0.95**	0.28	0.16	0.39
Urea	500	0.25	0.25	0.00	0.36
Cre	500	**1.15**	0.22	0.06	0.34
eGFR	498	0.14	**0.60**	**0.48**	**0.69**
Na	498	0.00	**0.54**	0.19	**0.82**
K	498	0.00	0.16	0.00	0.26
Cl	500	**0.56**	0.19	0.21	0.16
TCO_2	500	0.33	0.34	0.31	0.37
AG	500	0.00	0.20	0.27	0.00
Ca	499	**0.43**	**0.41**	0.36	**0.45**
aCa	498	0.00	0.18	0.00	0.28
Mg	500	0.00	0.19	0.09	0.27
IP	497	0.24	0.00	0.00	0.00
AST	499	**0.61**	0.11	0.13	0.09
ALT	497	**0.67**	0.21	0.26	0.05

		2-level ANOVA		One-way ANOVA	
Analyte	N	SDRsex	SDRage	SDRageM	SDRageF
LDH	499	0.00	0.00	0.00	0.07
ALP	498	0.00	0.28	0.00	**0.42**
GGT	496	**0.54**	0.11	0.12	0.11
CK	498	**0.59**	0.19	0.14	0.25
AMY	497	0.29	0.18	0.00	0.30
Glu	496	0.14	0.22	0.20	0.24
HbA1c	500	0.00	0.28	0.24	0.32
TC	500	0.00	0.27	0.14	0.36
TG	500	0.22	0.36	0.30	**0.43**
HDL-C	498	0.31	0.00	0.00	0.00
LDL-C	499	0.00	0.21	0.13	0.29
NonHDL	499	0.00	0.25	0.18	0.33
TC/HDL	500	0.22	0.19	0.18	0.21
HDL/LDL	500	0.15	0.17	0.17	0.17
CRP	497	0.27	0.23	0.24	0.20
IgG	499	0.00	0.08	0.04	0.11
IgA	500	0.00	0.21	0.26	0.16
IgM	496	**0.49**	0.16	0.00	0.24
C3	499	**0.49**	0.13	0.15	0.10
C4	497	0.38	0.24	0.25	0.23

The value of SDR ≧0.4 was marked by bold letter, and SDR ≧0.5 was highlighted by a gray shade. SDRage was computed for males (M) and females (F), seprately as SDRageM and SDRageF.

In Figure 3, we illustrated the effect of LAVE procedure and/or BMI restriction for four representative analytes: ALT, GGT, LDL-C and CRP. Effect of LAVE in lowering UL of the RI was observed more or less in all four analytes, while additional lowering of UL by BMI restriction was noted only in LDL-C and CRP. In other words, for RVs of ALT and GGT, the LAVE procedure had nearly the same effect as the BMI restriction.

In Table 4, a list of RIs adopted for all analytes are presented. For the selection of RIs with/without partition by sex and age, we principally used SDR_{sex} and SDR_{age}, but we also considered the bias at the RI limits (B_{LL} and BR_{UL}), which are presented in Supplementary Table 2. For partitioning RVs by sex based on SDR_{sex}≧0.4, the analytes selected were as noted above. However, actual between-sex difference at RI limits for Cl and Ca was less than three reporting units, and thus their RIs were determined without partition by sex. Whereas RVs for eGFR, IP, LDH, ALP, AMY, nonHDL, TG, TC/HDL, CRP and C4 recorded SDR_{sex}<0.4, yet, their actual between-sex differences at LL or UL expressed as BR_{LL} or BR_{UL} were high (>0.57), and thus their RIs were determined for each sex. For partitioning by age, SDR_{age}≧0.4 was noted, in females for Na, Ca, ALP, and TG, in males for Alb, in both sexes for eGFR (Supplementary Table 3). However, for Alb, Na, and Ca, actual differences at RI limits after partition at 45 years of age were small (3 g/L, 2 mmol/L, 0.06 mmol/L, respectively), and thus no partitioning was done (Supplementary Table 4). For TG in females, despite SDR_{age}>0.4, BR_{UL} after partition by age was small (<0.57). Hence, the RIs was not partitioned by age.

Standardization of RIs

Comparisons of our test results for 50 panel sera with assigned values are shown in Supplementary Figure 2. The need for recalibrating the derived RI (LL~UL) was assessed by computing adjusted RI (LLa~ULa) by using major axis regression line coefficients [6] shown in the Figure, and by calculating BR_{LL} and BR_{UL}.

By regarding BR>0.375 as exceeding allowable analytical bias, recalibration of RIs was performed for Alb, Ca, IP, AST, ALT, LDH, ALP, and HDL-C, but not for Na and

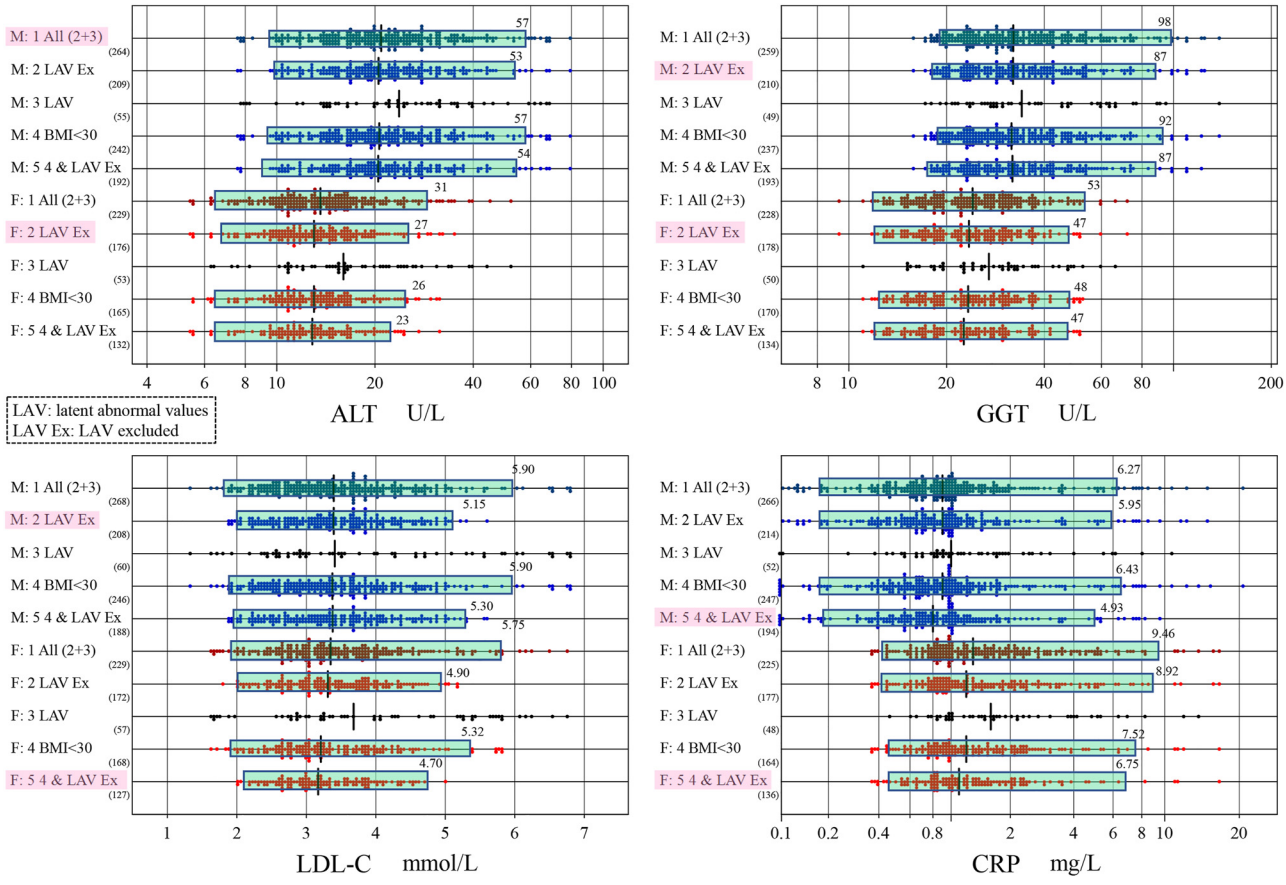

Figure 3: Effect of LAVE and BMI restriction on RIs of nutritional markers.
RIs of four representative nutritional markers were calculated for each sex in four ways: (1) using all RVs [1: All (2 + 3)], (2) using RVs that remained after latent abnormal values exclusion (LAVE) procedure [2: LAV Ex], (4) using RVs from individuals with BMI<30 kg/m^2 [4: BMI<30], (5) using RVs that remained after BMI restriction plus LAVE [5: 4 & LAV Ex]. The RI determined for each category of RVs is shown as rectangular region drawn over the scattergram. The scattergram (3) in the third row of each sex represents the subset of RVs that were excluded by the LAVE procedure [3: LAV]. Hence the scattergram (1) is broken down into scattergrams (2) and (3). In applying LAVE, the following mutually associated reference tests were used for identifying individuals with latent abnormal values: Alb, Glb, UA, Glu, AST, GGT, CK, LDH, TC, nonHDL, TG, and CRP. It is of note that, there seems to exist excessive number of points outside the RI overlaid, but, in the derivation of the RI, values outside mean ± 2.81 SD were truncated once.

Cl because of high imprecision of slope b [CV(b)>11%] of the regression line as described elsewhere [6].

Discussion

In this study to establish RIs suitable for Ghana, sources of variation analyses of RVs were performed using MRA and ANOVAs. Sex, age, and BMI were major SVs requiring consideration in deriving RIs, while ethnicity was not relevant in any analyte except AG and IP, which were marginally lower among Akan who constituted 40% of the study subjects, but below the level requiring separate RIs. Both smoking and drinking did not have any impact as they were infrequent among the volunteers.

In judging the need for partitioning RVs, we primarily relied on SDR by using 0.4 as the cut-off threshold, which was empirically derived based on traditional practice of setting sex-specific RIs in most clinical laboratories [4]. As a secondary criterion for partitioning, we relied on bias ratio (BR) or between-group difference at RI limits (e.g., UL_1–UL_2) divided by SD_{RI}. To make threshold of BR aligned to SDR with common denominator (SD_{RI}), we multiplied SDR=0.4 by $\sqrt{2}$ to obtain its threshold of $0.4 \times \sqrt{2}$ |=0.57: i.e., absolute difference (bias) of two values (x_1 and x_2), or $|x_1 - x_2|$, is $\sqrt{2}$ times larger than SD of the two values. It is of

Table 4: List of RIs after recalibration.

							Reference Interval by parametric method						
Item	unit	Sex	BMI	Age	LAVE	n	90%CI of LL		LL	Me	UL	90%CI of UL	
TP	g/L	MF	All	18-70	(-)	499	64.5	65.7	**65**	**74**	**84**	82.8	84.3
Alb	g/L	MF	All	18-70	(-)	501	37.2	38.3	**38**	**44**	**49**	49.0	49.8
Glb	g/L	MF	All	18-70	(-)	499	23.0	24.4	**24**	**31**	**40**	38.9	40.4
TBil	µmol/L	M	All	18-70	(-)	267	6.3	7.6	**7**	**14**	**36**	32.6	38.6
		F	All	18-70	(-)	229	4.9	5.9	**5**	**10**	**24**	21.0	26.6
DBil	µmol/L	M	All	18-70	(-)	267	1.2	1.5	**1.4**	**2.8**	**7.0**	6.3	7.7
		F	All	18-70	(-)	229	0.8	1.1	**1.0**	**1.9**	**4.8**	4.2	5.4
UA	µmol/L	M	<30	18-70	(-)	247	221	237	**229**	**331**	**467**	452	482
		F	<30	18-70	(+)	132	131	164	**147**	**239**	**347**	330	365
Urea	mmol/L	MF	All	18-70	(-)	500	2.08	2.27	**2.2**	**3.5**	**5.7**	5.49	5.97
Cre	µmol/L	M	All	18-70	(-)	269	55	61	**58**	**80**	**109**	104	113
		F	All	18-70	(+)	174	38	43	**40**	**59**	**82**	78	85
eGFR	ml/min /1.73m^2	MF	All	18-44	(-)	302	85	97	**91**	**133**	**200**	193	207
		MF	All	45-70	(-)	196	70	76	**73**	**109**	**167**	158	176
Na	mmol/L	MF	All	18-70	(-)	497	135.3	135.9	**136**	**139**	**143**	142.6	143.7
K	mmol/L	MF	All	18-70	(-)	498	3.39	3.52	**3.5**	**4.2**	**5.0**	4.88	5.07
Cl	mmol/L	MF	All	18-70	(-)	500	98.1	99.0	**99**	**103**	**108**	107.3	108.3
TCO_2	mmol/L	MF	All	18-70	(-)	501	17.6	18.4	**18.0**	**22.8**	**27.3**	26.9	27.7
AG	mmol/L	MF	All	18-70	(-)	501	8.1	8.8	**8.5**	**12.9**	**21.1**	20.4	21.8
Ca	mmol/L	MF	All	18-70	(-)	500	2.16	2.19	**2.17**	**2.34**	**2.51**	2.50	2.53
aCa	mmol/L	MF	All	18-70	(-)	498	2.14	2.17	**2.16**	**2.27**	**2.41**	2.40	2.43
Mg	mmol/L	MF	All	18-70	(-)	501	0.71	0.73	**0.72**	**0.83**	**0.97**	0.96	0.98
IP	mmol/L	M	All	18-70	(-)	266	0.70	0.82	**0.76**	**1.03**	**1.44**	1.37	1.51
		F	All	18-70	(-)	228	0.83	0.95	**0.89**	**1.11**	**1.63**	1.51	1.75
AST	U/L	M	All	18-70	(+)	210	18.4	21.0	**20**	**27**	**43**	38.8	46.6
		F	All	18-70	(+)	176	17.2	18.1	**18**	**22**	**32**	29.8	33.4
ALT	U/L	M	All	18-70	(-)	264	8.5	10.0	**9**	**21**	**57**	51.0	61.8
		F	All	18-70	(+)	176	6.2	7.4	**7**	**13**	**27**	24.9	30.1
ALP	U/L	M	All	18-70	(-)	268	46	53	**49**	**80**	**130**	122	138
		F	All	18-44	(-)	136	38	50	**44**	**74**	**119**	110	129
		F	All	45-70	(-)	85	46	59	**52**	**88**	**139**	124	155
GGT	U/L	M	All	18-70	(+)	209	14.6	19.2	**17**	**33**	**87**	71.3	103.3
		F	All	18-70	(+)	176	11.3	12.9	**13**	**23**	**47**	43.6	52.4
CK	U/L	M	All	18-70	(+)	211	76	111	**94**	**207**	**518**	433	604
		F	All	18-70	(-)	227	41	64	**52**	**139**	**300**	257	343
LDH	U/L	M	All	18-70	(-)	269	150	163	**157**	**218**	**294**	285	304
		F	All	18-70	(-)	230	159	172	**166**	**221**	**308**	298	319
AMY	U/L	M	All	18-70	(-)	268	42	55	**48**	**101**	**181**	166	196
		F	All	18-70	(-)	228	40	53	**46**	**85**	**162**	149	175
Glu	0.09	MF	All	18-70	(-)	494	3.84	4.06	**3.95**	**4.85**	**5.94**	5.78	6.09
HbA1c	%	MF	All	18-70	(-)	498	4.11	4.40	**4.26**	**5.37**	**6.26**	6.14	6.39
TC	mmol/L	MF	**<30**	18-70	(+)	331	3.25	3.69	**3.47**	**5.02**	**7.10**	6.85	7.36
HDL-C	mmol/L	MF	All	18-70	(-)	498	0.87	0.97	**0.92**	**1.41**	**2.18**	2.12	2.25
nonHDL	mmol/L	M	All	18-70	(+)	216	1.93	2.36	**2.15**	**3.85**	**6.12**	5.70	6.54
		F	**<30**	18-70	(+)	132	2.18	2.55	**2.36**	**3.55**	**5.45**	4.99	5.91
TG	mmol/L	M	All	18-70	(+)	214	0.39	0.46	**0.42**	**0.88**	**2.08**	1.84	2.33
		F	**<30**	18-70	(+)	130	0.35	0.44	**0.40**	**0.70**	**1.57**	1.22	1.93
LDL-C	mmol/L	M	**<30**	18-70	(+)	208	1.87	2.09	**1.97**	**3.40**	**5.15**	4.97	5.29
		F	**<30**	18-70	(+)	127	2.06	2.24	**2.14**	**3.21**	**4.70**	4.48	4.98
TC/HDL		M	All	18-70	(+)	208	2.38	2.72	**2.55**	**4.15**	**6.58**	6.21	6.96
		F	All	18-70	(+)	173	2.47	2.79	**2.63**	**3.68**	**5.85**	5.40	6.31
HDL/LDL		MF	All	18-70	(-)	497	0.18	0.21	**0.20**	**0.38**	**0.74**	0.70	0.78
CRP	mg/L	M	**<30**	18-70	(+)	194	0.13	0.21	**0.17**	**0.83**	**4.93**	3.76	6.07
		F	**<30**	18-70	(+)	136	0.42	0.49	**0.44**	**1.16**	**6.75**	4.75	7.86
IgG	mg/dL	MF	All	18-70	(-)	498	1133	1193	**1163**	**1589**	**2170**	2123	2217
IgA	mg/dL	MF	All	18-70	(-)	500	106	122	**114**	**225**	**417**	387	446
IgM	mg/dL	M	All	18-70	(+)	207	34	44	**39**	**86**	**181**	158	204
		F	All	18-70	(-)	228	46	57	**51**	**119**	**269**	249	289
C3	mg/dL	M	All	18-70	(-)	270	79	85	**82**	**113**	**164**	158	169
		F	All	18-70	(-)	230	90	98	**94**	**129**	**170**	166	175
C4	mg/dL	M	All	18-70	(-)	268	13	16	**15**	**28**	**46**	44	48
		F	All	18-70	(-)	230	15	19	**17**	**33**	**53**	49	57

M=male; F=female; MF=M+F; LL=lower limit; Me=mid-point; UL=upper limit; CI=confidence interval. LAVE=latent abnormal values exclusion. (–) LAVE denoted with color light green shows that the LAVE method was not applied. (+) LAVE with a green color shows LAVE method was used. Yellow color code depicts the reference interval derived for all age and Bmi. Violet color code represents both male and female (M + F) reference interval. light blue color shows male reference interval. pink shows female reference interval.

note that, in judging the bias in LL or UL caused by LAVE or BMI restriction, we adopted the distinct threshold of 0.375 that corresponds to the limit of allowable analytical bias.

Our findings of high between-sex differences (SDR_{sex}) observed in 12 analytes shown in Table 3 were all consistent with most previous studies [2, 3, 20, 21]. A notable exception was HDL-C, whose SDR_{sex} and BRs were not large enough to require sex-partition. This lack of gender-difference in Ghanaian HDL-C may be attributable to its decrease by prevalent obesity observed in females, with an opposing increase in males due to higher physical activity.

With respect to age-related changes, r_p for age (by MRA) and SDR_{age} (by ANOVA), the RVs of Alb and eGFR showed appreciable lowering with age in both sexes. While age-related increase was found for Na, Ca, TG, and ALP in females, however actual differences in RIs partitioned at 45 years were rather small for Alb, Na, and Ca (less than three reporting units) and for TG (BR_{UL}<0.57), therefore, only ALP of females and eGFR was partitioned at 45 years of age. This finding of ALP in females is consistent with other reports [3, 20].

BMI was a prominent SV for many analytes in our study judged from r_p for BMI and 2D-scattergram shown in Figure 2. BMI-related increase of RVs with r_p>0.2 has been noted in many nutritional and inflammatory markers in both sexes and indeed is consistent with what was reported in the interim report of the global multicentre study and other studies [12, 22, 23]. Also, BMI was inversely associated with HDL-C and AMY in both sexes; a finding consistent with several reports [22]. Although low HDL-C levels are well known to be associated with cardiovascular disorders, low serum AMY is also associated with obesity and risk of diabetes mellitus [24]. Hence, in this study, we excluded individuals with BMI≥30 kg/m^2 which is regarded as excessively obese and constitutes 16.6% of the volunteers (male, 8.1%; female, 26.4%). The exclusion procedure resulted in significant lowering of ULs (BR_{UL}) for UA, TC, LDL-C, and CRP in both sexes, and for TG and nonHDL in females (Supplementary Table 3) while, in other nutritional markers, the LAVE procedure effectively reduced the influence of high BMI on RIs.

In comparison to the starndardized RIs reported from nine countries that collaborated in the C-RIDL global study, we identified features that were unique to the Ghanaian RIs as partly shown in Figure 4. The most conspicuous ones are a higher-side shift of RIs for TBil, LDH, CK and AMY. Even though high levels of these analytes among African population have been reported [8–10, 25, 26], the level of CK, TBil and AMY were even higher among Ghanaian population. The higher AMY might be attributable to high carbohydrate consumption in Ghana besides genetical factors [25, 27]. Remarkably, the RIs of TC and LDL-C were also shifted to higher levels in contrast to other countries except for Russia, and similarly for HDL-C except for the RIs of Japan. Hence, these features must be considered in interpreting lipid profiles in the management of atherosclerotic diseases. These between-country variations may be attributed to differences in lifestyle and/or genetic factor among the study population [3].

The LAVE procedure effectively narrowed RIs especially for hepatic and lipid markers as reported elsewhere [2, 17, 21], which emphasizes the importance of secondary exclusions in deriving RIs for chemistry analytes that frequently are observed to be abnormal among healthy individuals.

Limitations

Our volunteers largely represented the urban population, whose socioeconomic status, nutrition and lifestyles are likely to be different from the rural population. Hence some of derived RIs may differ in the rural setting. Another limitation was that we relied on self-reporting of health status and might have inevitably included individuals with latent, metabolic or inflammatory disorders. However, the application of the LAVE method and BMI restriction, if appropriate, seem to have coped with those conditions or in compliance to the sampling requirements.

Conclusions

This study demonstrated that sex, age, and BMI were critical factors which required objective assessments in deriving country specific RIs. The application of LAVE and BMI restriction methods were found to be essential in reducing the influence of abnormal values frequently observed in chemistry test results among healthy subjects. The availability of value-assigned serum panel allowed us to ensure the RIs were standardized and furthermore able to identify Ghanaian features of RIs that were unique in comparison to those of other collaborating countries.

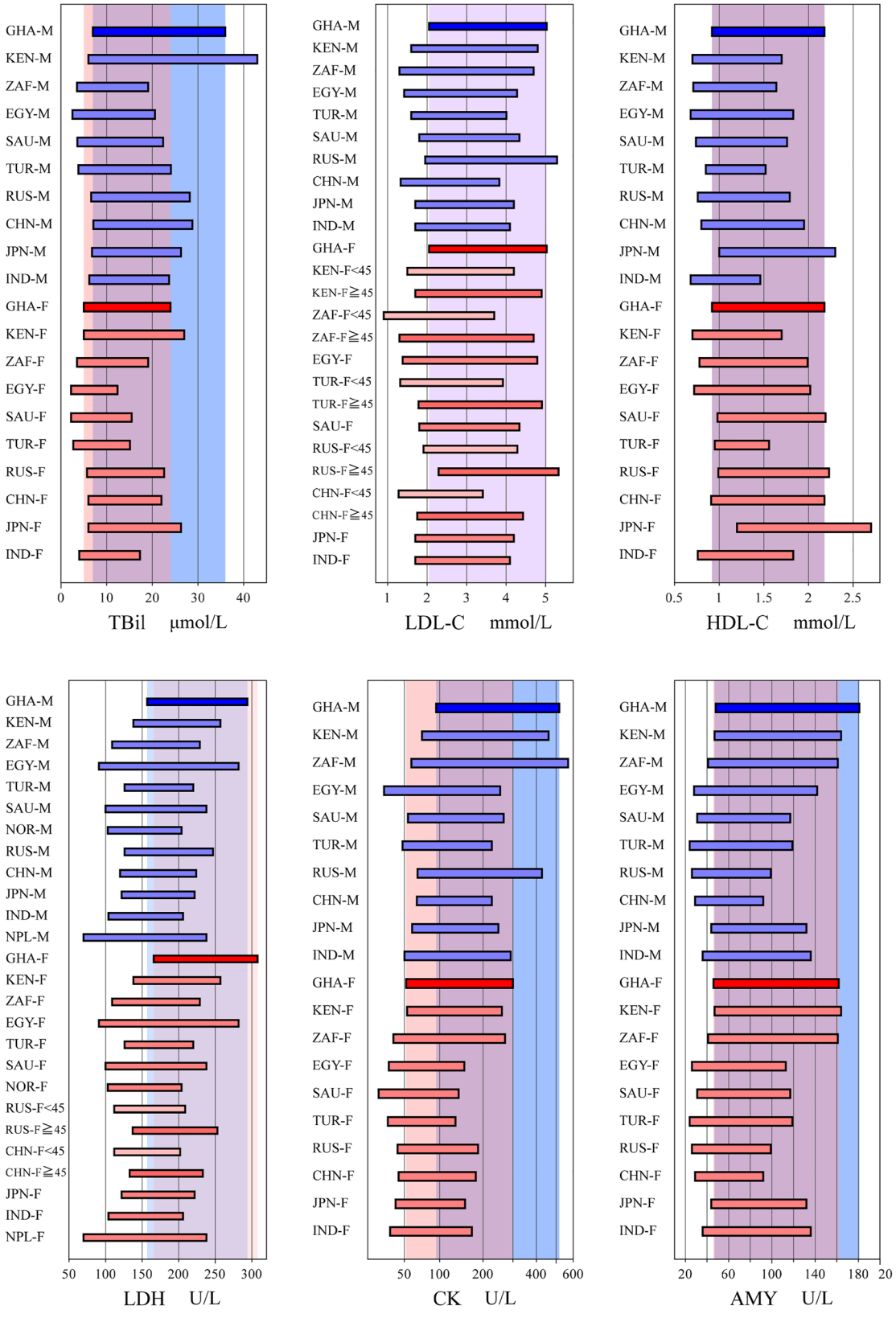

Figure 4: RIs of 10 countries in collaboration were compared for six analytes with Ghanaian specific features.
RIs of 10 countries that collaborated in the global study were compared by bar-chart for six representative analytes that exhibited features specific for Ghanaian and/or Africans. The blue, pink, and purple background shades denote the RIs of Ghana derived for male (M), female (F), and M + F, respectively. Please note that all the RIs were made comparable through standardization based on the value-assigned serum panel measured in common. ZAF denotes RIs determined selectively from African in South Africa [28]. TUR=Turkey [21]; JPN=Japan [29]; CHN=China [3]; SAU=Saudi [2], RUS=Russia [30]; IND=India [31]; KEN=Kenya [26]; EGY=Egypt [32].

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Informed consent: Written consents were obtained from all volunteers. Participants' confidentiality was maintained at all times.

Ethical approval: The study protocol conforming to the Declaration of Helsinki was approved by the Ethical Review Committee of the College of Health Sciences, University of Ghana (CHS-Et/M.8-P4.14/2016–2017).

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