A reference interval study for common biochemical analytes in Eastern Turkey: a comparison of a reference population with laboratory data mining

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Abstract

Introduction: The aim of this study was to define the reference intervals (RIs) in a Turkish population living in Northeast Turkey (Erzurum) for 34 analytes using direct and indirect methods. In the present study, the regional RIs obtained were compared with other RI studies, primarily the nationwide study performed in Turkey.

Materials and methods: For the direct method, 435 blood samples were collected from a healthy group of females (N = 218) and males (N = 217) aged between 18 and 65 years. The sera were analysed in Ataturk University hospital laboratory using Roche reagents and analysers for 34 analytes. The data from 1,366,948 records were used to calculate the indirect RIs using a modified Bhattacharya method.

Results: Significant gender-related differences were observed for 17 analytes. There were also some apparent differences between RIs derived from indirect and direct methods particularly in some analytes (e.g. gamma-glutamyltransferase, creatine kinase, LDL-cholesterol and iron). The RIs derived with the direct method for some, but not all, of the analytes were generally comparable with the RIs reported in the nationwide study and other previous studies in Turkey.There were large differences between RIs derived by the direct method and the expected values shown in the kit insert (e.g. aspartate aminotransferase, total-cholesterol, HDL-cholesterol, and vitamin B12).

Conclusions: These data provide region-specific RIs for 34 analytes determined by the direct and indirect methods. The observed differences in RIs between previous studies could be related to nutritional status and environmental factors.

Key words: reference intervals; direct method; indirect method; Bhattacharya method; regional differences

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Introduction

Reference intervals (RIs) are very important for the interpretation of laboratory test results. RIs can be determined in some specified ways, and careful determination of RIs by a laboratory is extremely important (1). The International Federation of Clinical Chemistry (IFCC) has published several papers and recommended that each laboratory should have its own reference values and estimate the corresponding RIs according to defined procedures (2-4). The Clinical and Laboratory Standards Institute (CLSI) published in 2010 the C28-A3 guideline to describe the recommendations on RIs (5). However, the majority of clinical laboratories are not able to implement their RIs due to the difficulty in obtaining sufficient numbers of reference individuals from a representative population and subpopulations, and the high costs of numerous tests to be performed by individual laboratories. Thus, in practice, only a few clinical laboratories produce their own RIs, while the great majority use the RIs reported in literature or in the manufacturer's package inserts. The need to revisit the concept of RIs has recently been discussed and attempts have been made to overcome these difficulties through multicenter production of RIs (6) and/or using hospital and primary healthcare pa-

tients and applying different criteria for the detection of RIs (7).

Multicenter RI determination is a good alternative and a very important option for individual laboratory RI determination. As common standardization and traceability are crucial during production of reference values, each step of the pre-analytical, analytical and statistical application follows a well-defined protocol. In recent years, the IFCC Committee on Reference Intervals and Decision Limits (the C-RIDL) has been devoted to the determination of common or harmonized RIs. Recently, the C-RIDL published two papers including a protocol and comprehensive standard operating procedures (SOPs) (8) with indications on the utility of a panel of sera for the alignment of test results among laboratories in multicenter studies (9). The requirements for conducting the multicenter study, phase by phase, can be summarized as: a priori selection of reference subjects (i.e. inclusion-exclusion criteria, ethnicity and questionnaire), clear definition of pre-analytical phase (i.e. blood collection, sample proccesing, storage and transportation), clear definition of analytical phase (i.e. requirements for central laboratories and measurements, quality control, assay standardization and cross-comparison of values), statistical procedures for data analysis and reports of results (i.e. validation of data, analyses of sources of variation, partitioning criteria and derivation of RI) (8). Therefore, the global use of comprehensive SOPs and a common protocol is probably the most effective way to investigate globally applicable, common RIs (10). Such a nationwide multicenter RI study has been organized and performed in Turkey recently, using Abbott analysers for clinical chemistry analyses (11). Our laboratory was one of the participants in the nationwide multicenter RI study mentioned above (11), and we are currently using RIs derived from that study.

The city of Erzurum is located in Northeastern Anatolia and has the particular features of an average altitude of 1800 meters above sea level and long, extremely cold winters. Erzurum has a population of approximately 800,000 and the population is more homogenous than in other regions of Turkey. More than half of the population in the region live in rural areas, and the general economic status of the region is relatively low (12). Considering the importance of additional regional characteristics of the population in Eastern Anatolia and the nutritional, environmental, economic and socio-cultural factors, we organized and conducted a specific regional RI study in parallel to our participation in the nationwide multicenter study. The study aimed to (a) define region-specific direct and indirect RIs for 34 biochemical analytes and (b) explore possible regional differences in direct RIs derived from the nationwide multicenter study and other RI studies in Turkey. The RIs obtained through the direct method were compared with the RIs suggested by the manufacturer. The region-specific RIs were determined using both the direct and indirect methods. In the direct method, recommended by the IFCC, the RIs were determined in a healthy reference population from our region (Eastern Turkey), selected according to the IFCC recommendations. In the indirect method, the region-specific RIs were determined using a large amount of hospital patient data from our laboratory information system according to the modified Bhattacharya procedure (13). The modified Bhattacharya procedure is widely accepted as an alternative approach for the determination RIs of using the stored patient data.

Materials and methods

Subjects

For the direct RI determination, a total of 435 (217 males and 218 females) healthy individuals, selected according to the IFCC recommendations from our region (Eastern Turkey), were included in the study. The selected individuals were aged between 18 and 85 years. The main target age range was 20–65 years. A questionnaire comprising general health and lifestyle questions was completed in order to include proper subjects and exclude irrelevant subjects to avoid confounding and false results. Inclusion and exclusion criteria were set according to the IFCC/C-RIDL protocol (8). Exclusion criteria were: BMI ≥ 30, alcohol consumption ≥ 70 g/day, smoking > 20 cigarettes/day, chronic sys-

temic disease, having an acute disease within the last 14 days, currently known carrier state for HBV, HBC or HIV, pregnancy, and being in the postpartum first year. The volunteers gave written informed consent to participate in the study and they were informed of the results on request.

Methods

The study protocol was approved by the Ethics Committee of Ataturk University, School of Medicine (number 2012.4.1/11). In the direct method, the reference individuals were selected from a reference population using specific, well-defined criteria. For the direct method, preparation of the subjects for sampling and the procedures of sampling, and sample processing were conducted using the recently published IFCC/C-RIDL protocol (8). Blood specimens of 8 mL were collected into gel serum separator tubes, SST II (Becton, Dickinson and Company, Oxford, England). The subjects fasted prior to sample collection and the time of sampling was set at 7 to 10 am. Within 20 to 30 minutes of sampling, the samples were centrifuged at 1200 x g for 10 min at room temperature. Blood samples were centrifuged within 20–30 minutes of withdrawal from each volunteer. One aliquot of 1 ml was prepared and stored at -80 ± 2 ºC for up to six months until analysis. The frozen serum samples were transferred to a refrigerator (+4–6 ºC) for about 2–3 hours for thawing before analysis and then transferred to the analyser within 6 hours of thawing.

The following analytes were measured in each serum sample: alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), amylase (AMY), creatine kinase (CK), triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), calcium (Ca), inorganic phosphate (IP), magnesium (Mg), iron (Fe), total protein (TP), albumin (ALB), total bilirubin (TBIL), direct bilirubin (DBIL), glucose (GLU), urea (UN), creatinine (CRE), uric acid (UA), sodium (Na), potassium (K), chloride (Cl), free triiodothyronine (FT3), free thyroxine (FT4), thyroid stimulating hormone (TSH), vitamin B_{12} (VIT B_{12}), folate (FOL), ferritin (FER), parathyroid hormone (PTH) and insulin (INS). All analyses were performed using Cobas 8000 and E 170 (Roche Diagnostics, Mannheim, Germany) and following the manufacturer's instructions. The test list, assay methods, kit versions, calibrators and instrumentation profile are summarized in Table 1. The calibrators and controls were obtained from Roche (Roche Diagnostics, Mannheim, Germany).

For the indirect RI determination, the results of the laboratory analyses, stored for approximately one year, were used. During this period, approximately 5 million stored laboratory records of 1,063,605 individuals (aged 18–65 years old) were evaluated, and 1,366,948 refined records, of which 594,753 belonged to males and 772,195 belonged to females, were used for statistical analysis. All patient results have been stored in the laboratory information system, LIS, to constitute the original database. A specific Structured Query Language (SQL) was prepared for analyses and exclusions.

Assuming that persons with repeated measurements have a higher chance of being diseased, data from subjects who had repeated meaurements for any of the given 34 analytes within the 3-month observation period were excluded to ensure that the majority of the values for each analyte were health related. Together with this exclusion criterion, data for hospitalized patients and for ambulatory patients from the intensive care units were eliminated. Thus, the selected population can be named as outpatients.

The same analyser, Cobas 8000 and E 170 (Roche Diagnostics, Mannheim, Germany), was used for the direct and indirect studies. The time periods were the same (between January and December 2012) and the analytical issues were exactly the same in both studies.

Internal quality control was performed by daily assays of commercial lyophilized sera in two concentrations of each analyte. The desirable limits for between- and within-day analytical variations (CV_A) were set as ½ of within-subject biological variation (CV_I), as defined by Ricos *et al.* (14) and reported on the Westgard website (15). The within-

Table 1. Analytical and methodological characteristics of analytes with CV_A data

Analyte	Unit	Method	N	With-in day CV_A		Between-day CV_A		Analytical system / Kit Version / Calibrators / Calibrators' traceability
				C1	C2	C1	C2	
ALP	U/L	IFCC, p-Nitrophenyl Phosphate	20	1.20	1.31	2.34	2.57	Roche Cobas 8000 / 2 / CFAS / O.F. IFCC
GGT	U/L	Szasz, gamma- glutamylcar-boxynitroanilid	20	1.12	0.82	1.66	2.08	Roche Cobas 8000 / 2 / CFAS / O.F. Persijn / v.d.Slik
AST	U/L	IFCC, UV without P5P, 37 °C	20	2.01	1.93	2.49	2.24	Roche Cobas 8000 / 1 / CFAS / O.F. IFCC
ALT	U/L	IFCC, UV without P5P, 37 °C	20	1.68	1.50	2.69	2.45	Roche Cobas 8000 / 1 / CFAS / O.F. IFCC
LDH	U/L	IFCC, UV Lactate-pyruvate	20	1.04	0.86	1.48	1.46	Roche Cobas 8000 / 2 / CFAS / O.F. IFCC
AMY	U/L	IFCC, colorimetric PNP	20	0.86	0.93	1.40	1.81	Roche Cobas 8000 / 2 / CFAS / IFCC
CK	U/L	IFCC, UV NAC activated	20	1.21	1.03	1.46	1.24	Roche Cobas 8000 / 1 / CFAS / O.F. IFCC
TG	mmol/L	Enzymatic GPO-PAP colorimetric	20	1.80	1.53	2.12	1.92	Roche Cobas 8000 / 1 / CFAS / ID-MS
TC	mmol/L	Enzymatic CHOD-PAPcolorimetric	20	1.92	1.13	2.33	1.93	Roche Cobas 8000 / 2 / CFAS / Abell-Kendall
HDL-C	mmol/L	Colorimetric (oxidase)	20	1.21	1.12	2.46	1.57	Roche Cobas 8000 / 3 / CFAS Lipids / CDC Reference M.
LDL-C	mmol/L	Colorimetric (oxidase)	20	2.01	1.89	2.99	2.49	Roche Cobas 8000 / 1 / CFAS Lipids / Beta quantification
Ca	mmol/L	Ortho – crezolftalein complex	20	0.85	0.89	1.01	1.04	Roche Cobas 8000 / 2 / CFAS / SRM 956c
IP	mmol/L	UV Phosphomolybdate	20	1.05	1.23	2.05	2.53	Roche Cobas 8000 / 2 / CFAS / Primary reference material
Mg	mmol/L	Clorophosphonase III	20	2.12	1.23	2.61	1.56	Roche Cobas 8000 / 2 / CFAS / Atomic absorption
Fe	µmol/L	Ferro Zinc	20	0.96	0.92	1.32	1.71	Roche Cobas 8000 / 2 / CFAS / Primary reference material
TP	g/L	Biuret	20	0.72	0.65	2.03	1.41	Roche Cobas 8000 / 2 / CFAS / SRM 927b
ALB	g/L	Bromocresol Green	20	0.90	1.06	1.92	1.69	Roche Cobas 8000 / 2 / CFAS / ERM-DA470k / IFCC
TBIL	µmol/L	Jendrassik-Grof	20	1.70	1.00	1.60	1.26	Roche Cobas 8000 / 3 / CFAS / Doumas
DBIL	µmol/L	Diazo	20	0.80	0.60	2.10	1.29	Roche Cobas 8000 / 2 / CFAS / Doumas
GLU	mmol/L	Hexokinase	20	0.90	1.13	1.37	1.65	Roche Cobas 8000/3/CFAS/ ID-MS
UN	mmol/L	UV, urease	20	1.10	1.28	1.38	1.56	Roche Cobas 8000 / 1 / CFAS / SRM 909b
CRE	µmol/L	Kinetic	20	1.36	1.75	2.30	2.96	Roche Cobas 8000 / 2 / CFAS / ID-MS
UA	µmol/L	Uricase, colorimetric	20	1.43	1.68	2.38	2.56	Roche Cobas 8000 / 2 / CFAS / ID-MS

Table 1. Analytical and methodological characteristics of analytes with CV_A data (continued)

Analyte	Unit	Method	N	With-in day CV_A		Between-day CV_A		Analytical system / Kit Version / Calibrators / Calibrators' traceability
				C1	C2	C1	C2	
K	mmol/L	Ion Selective Electrode Direct	20	0.90	1.20	1.97	2.16	Roche Cobas 8000 / 2 / CFAS / P.C. gravimetrically prepared
Cl	mmol/L	Ion Selective Electrode Direct	20	1.00	1.30	1.48	1.63	Roche Cobas 8000 / 2 / CFAS / P.C. gravimetrically prepared
FT3	pmol/L	ECLIA	20	3.34	3.25	3.73	3.91	Roche E170 / 3 / FT3 Calset / FT3 assay (REF 11731386)
FT4	pmol/L	ECLIA	20	2.02	2.15	2.44	2.51	Roche E170 / 2 / FT4 Calset / Equilibrium dialysis
TSH	mU/L	ECLIA	20	2.45	2.66	4.26	4.72	Roche E170 / 1 / TSH Calset / WHO Reference Std.80 / 558
VIT B12	pmol/L	ECLIA	20	3.25	3.47	4.82	4.64	RocheE170 / 1 / VITB12 Calset / VITB12 assay (REF1182075)
FOL	nmol/L	ECLIA	20	3.42	3.64	4.59	4.77	Roche E170 / 3 / FOL Calset / FOLassay (REF03253678)
FER	µg/L	ECLIA	20	3.24	3.83	5.54	6.61	Roche E170 / 1 / FER Calset / 1st (IS) NIBSC
PTH	pmol/L	ECLIA	20	2.53	2.82	4.05	4.53	Roche E170 / 1 / PTH Calset / RIA
INS	pmol/L	ECLIA	20	4.22	4.61	6.62	5.89	Roche E17 / 1 / INS Calset / Reference STD. 66 / 304

CV_A – Analytical variation, C1 – Control 1, C2 – Control 2, CFAS – Calibrator for automated systems, CFAS Lipids – Calibrator for automated systems of lipids, IFCC – International Federation of Clinical Chemistry and Laboratory Medicine, P5P – Pyridoxal-5-phosphate, GPO-PAP – Glycerol phosphate oxidase / peroxidase aminophenazone, CHOD-PAP – Cholesterol oxidase / peroxidase aminophenazone, UV – Ultraviolet, PNP – para-nitrophenol, NAC – N-acetylcysteine, CDC – Centers for Disease Control and Prevention, ECLIA – Electro-chemiluminescence immunoassay, SRM – Standard reference material, ERM – European reference material, ID-MS – Isotope Dilution-Mass Spectrometry, O.F. – Original formulation, P.C. – Primary calibrators, RIA – Radioimmunoassay, (IS) NIBSC – (International Standard) National Institute for Biological Standards and Control, STD – Standard.

and between-day CV_A for all analytes, listed in Table 1**,** did not exceed the desirable limits reported (14). External quality control was performed using EQAS (Bio-Rad Laboratories, Milano, Italy). The results of the external quality control were acceptable when compared with the same peer group.

Statistical analysis

Data obtained by the direct method were transferred to SPSS version 20.0 (SPSS Inc., Chicago, USA) and MedCalc version 14.12.0 (MedCalc Software, Mariakerke, Belgium) to calculate RIs. Reference limits define the central 95% of the reference population. Nonparametric statistic was used for the estimation of the direct RIs. Nonparametric methods typically encompass the central 95th percentile of reference values and use the 2.5th and 97.5th percentile as the lower and upper reference limit, respectively. Dixon's range test, recommended by the IFCC for statistical analysis in reference interval studies, was used to detect and eliminate extreme values as outliers. Dixon's outlier range statistic typically identifies the single most extreme value at the upper or lower limit as an outlier (16). The simplest criterion of rejection (r criteria) is $D/R > 0.3$, where D is the absolute difference between the most extreme value and the next nearest value divided by the range of all values (R) including the extreme value (s). Confidence intervals of 90% (90% CI) of reference limits were estimated following IFCC recommendations (4).

The magnitude of the standard deviations (SD) of test results between-gender (SD-gender) variations were computed by three-level nested ANOVA. A standard deviation ratio (SDR) greater than 0.3 was regarded as a guide to consider partitioning reference values by the factor (17).

A modified Bhattacharya procedure (13) was used to estimate the indirect RIs from hospital patient data. In the unmodified Bhattacharya method, the distribution of the main population is assumed to be Gaussian (18). Since most laboratory data are not Gaussian, Baadenhuijsen *et al.* described a modification (13). The data of the total unselected hospital population obviously cannot be transformed to a normal distribution because the deviation from the normal distribution is caused in part by results that are pathological. Only the analytical data near the mean were used (at least 40% of the results). It was assumed that calculation on this subpopulation was not influenced by pathological test results (13). The 90% CI of the lower and upper limits of the RIs were calculated using the bootstrap method, through random resampling (200 times) of the same dataset.

For each analyte, the values of both groups of RIs, direct and indirect, were calculated for males and females, separately. The difference was taken into consideration and noted if the difference between the lower limits or upper limits was more than 10% (> 10%) of the compared RI.

Table 2. Age distribution of participants in the direct RI study

Group	N	Age, years
Males and females	435	38 (29–48)
Males	217	38 (29–47)
18–29 years	56	27 (24–29)
30–39 years	56	33 (31–37)
40–49 years	55	44 (42–46)
50–64 years	40	54 (51–57)
65–85 years	10	71 (67–76)
Females	218	39 (29–48)
18–29 years	57	27 (23–29)
30–39 years	56	35 (31–38)
40–49 years	55	45 (42–46)
50–64 years	40	53 (51–55)
65–85 years	10	72 (68–78)

Age is expressed as median (interquartile range).

Results

The median (interquartile range, IQR) age of males, females and the total group were 38 (29–47), 39 (29–48) and 38 (29–48) years, respectively. The age distribution for the direct population is presented in Table 2.

Table 3 shows the RIs of 34 analytes determined by the direct method in males (N = 217) and females (N = 218) participants and 90% CIs of reference limits. Significant gender-related differences (SDR > 0.30) were observed for 17 analytes; ALP, GGT, AST, ALT, CK, TG, TC, HDL-C, LDL-C, Fe, TBIL, UN, CRE, UA, FER, VIT B_{12} and PTH. However, RIs for all analytes were stated as separate RIs for males and females (Table 3). The upper limits of RIs determined by the direct method in males were higher (> 10%) than in females for GGT, ALT, AST, AMY, CK, TG, LDL-C, TBIL, DBIL, CRE, UA, FER and INS, and lower (> 10%) than in females for HDL-C, FOL and PTH (Table 3). The lower limits of RIs in males were higher (> 10%) than females for GGT, ALT, AMY, CK, Fe, UN, CRE, UA, FER, TBIL and FT3, and the values in females were higher (> 10%) than in males for HDL-C, TSH, IP and INS (Table 3).

The calculated indirect RIs for each analyte and 90% CIs of reference limits are shown for males and females in Table 4. The upper limits of RIs for males were higher (> 10%) than in females for CK, Fe, TBIL, CRE, UA and FER. The lower limits of RIs for males were higher (> 10%) than in females for ALP, GGT, CK, TG, Fe, TBIL, DBIL, UN, CRE, UA, VIT B_{12} and FER (Table 4). The lower limits of RIs for males were lower (> 10%) than in females for HDL-C, TC, FT3 and TSH. The upper limits of RIs for males were lower (> 10%) than in females for FT3 and PTH (Table 4).

As seen in Tables 3 and 4, there were some differences (> 10% lower or higher) in the upper limits of the observed RIs for GGT, ALT, LDH, CK, HDL-C, LDL-C, Fe, Alb, FT3, FT4 and FER determined by di-

Table 3. Reference intervals estimated with direct method using non-parametric calculation

Analyte	Unit	Males				Females			
		N	RI			N	RI		
			LL–CI	LL–UL	UL–CI		LL–CI	LL–UL	UL–CI
ALP	U/L	196	31–42	40–127	107–131	206	31–42	40–116	107–130
GGT	U/L	210	8–9	9–58 (*, #)	54–60	210	6–8	7–32	30–34
AST	U/L	210	8–11	9–28 (*)	24–30	214	8–11	9–24	22–38
ALT	U/L	217	9–12	10–55 (*, #)	51–60	218	5–9	6–30	26–42
LDH	U/L	217	88–117	104–212	193–220	218	22–112	105–202	185–212
AMY	U/L	217	20–36	34–131 (*, #)	118–139	218	9–37	23–116	107–125
CK	U/L	217	37–54	42–228 (*, #)	223–232	218	28–39	31–142	135–152
TG	mmol/L	210	0.51–0.66	0.59–3.12 (*)	3.03–3.16	213	0.38–0.72	0.63–2.71	2.52–3.08
TC	mmol/L	217	2.27–3.14	3.02–6.68	6.42–6.81	218	0.70–3.26	2.90–6.53	6.04–6.74
HDL-C	mmol/L	217	0.49–0.65	0.54–1.30	1.24–1.63	218	0.50–0.80	0.72–2.02	1.83–2.09
LDL-C	mmol/L	217	1.28–1.71	1.49–4.98 (*)	4.77–5.18	218	0.53–1.74	1.45–4.48	4.27–4.79
Ca	mmol/L	217	2.0–2.2	2.1–2.5	2.4–2.6	218	2.0–2.2	2.1–2.5	2.4–2.8
IP	mmol/L	217	0.75–0.85	0.76–1.51	1.45–1.54	218	0.73–0.98	0.85–1.39	1.34–1.55
Mg	mmol/L	217	0.69–0.72	0.70–0.90	0.86–0.99	218	0.65–0.70	0.68–0.89	0.87–0.93
Fe	µmol/L	217	8.1–8.9	8.5–30.1 (#)	29.5–30.4	218	3.6–6.7	5.2–27.8	25.2–30.4
TP	g/L	217	58–64	62–79	77–80	218	24–63	62–78	77–79
ALB	g/L	217	39–42	42–50	49–51	218	35–40	38–50	48–51
TBIL	µmol/L	212	3.7–3.9	3.8–23.9 (*, #)	22.4–24.1	212	3.0–3.2	3.1–20.7	19.8–22.1
DBIL	µmol/L	213	1.0–1.3	1.2–5.3 (*)	5.1–5.4	214	0.9–1.2	1.1–4.6	4.5–4.8
GLU	mmol/L	217	3.5–3.8	3.7–6.1	6.0–6.2	218	2.9–4.1	3.9–6.3	5.6–6.6
UN	mmol/L	217	3.0–3.5	3.1–7.2 (#)	6.5–7.5	218	1.9–2.7	2.3–6.9	6.1–8.1
CRE	µmol/L	217	56–66	59–107 (*, #)	98–154	218	39–48	45–83	76–90
UA	µmol/L	217	190–227	202–489 (*, #)	458–546	218	120–167	148–369	331–427
Na	mmol/L	217	125–137	134–147	146–149	218	132–135	134–146	144–147
K	mmol/L	217	3.5–3.9	3.7–5.1	4.9–5.4	218	3.7–4.0	3.8–5.1	5.0–5.3
Cl	mmol/L	217	92–99	96–108	107–109	218	96–99	98–108	107–115
FT3	pmol/L	217	3.3–3.9	3.7–6.0 (#)	5.9–7.0	218	2.7–3.6	3.1–6.2	5.9–6.8
FT4	pmol/L	217	10.4–13.3	11.9–20.4	19.4–29.7	218	2.5–12.3	11.3–20.9	19.5–21.9
TSH	mU/L	212	0.28–0.40	0.36–4.78	4.22–5.12	211	0.40–0.52	0.49–4.92	4.75–4.95
VIT B_{12}	pmol/L	202	59–79	70–368	321–388	200	56–90	71–395	372–413
FOL	nmol/L	215	7–10	9–28	26–33	216	7–11	9–33	29–38
FER	µg/L	217	42–49	46–399 (*, #)	355–436	218	13–17	15–209	143–418
PTH	pmol/L	217	1.6–2.0	1.8–8.1	6.9–9.2	218	1.6–2.0	1.9–11.9	10.1–23.7
INS	pmol/L	212	20–25	21–190 (*)	150–210	208	22–28	26–161	131–176

UL – upper limit. LL – lower limit, CI – confidence interval, (*) – UL of males (> 10%) higher than females, (#) – LL of males higher (> 10%) than females.

Table 4. Reference intervals estimated with indirect method using modified Bhattacharya algorithm

Analyte	Unit	Males				Females			
		N	RI			N	RI		
			LL–CI	LL–UL	UL–CI		LL–CI	LL–UL	UL–CI
ALP	U/L	19,116	33–37	35–125 (#)	122–128	40,787	30–32	31–118	115–122
GGT	U/L	16,871	4–6	5–38 (#)	36–40	22,112	3–5	4–36	35–37
AST	U/L	27,941	9–11	10–29	28–31	37,488	9–11	10–28	27–29
ALT	U/L	28,260	5–6	5–28	27–30	37,187	4–6	5–26	25–27
LDH	U/L	27,217	136–144	140–261	255–266	39,041	136–149	138–260	254–265
AMY	U/L	8555	19–21	20–123	120–126	12,276	18–20	19–122	119–125
CK	U/L	16,224	21–23	22–173 (*, #)	171–175	15,752	16–18	17–146	144–148
TG	mmol/L	24,973	0.27–0.32	0.29–2.85 (#)	2.81–2.88	28,206	0.07–0.16	0.10–2.86	2.83–2.89
TC	mmol/L	13,834	2.44–2.57	2.49–7.23	7.13–7.31	15,695	2.69–2.80	2.75–6.97	6.87–7.10
HDL-C	mmol/L	16,674	0.49–0.54	0.52–1.57	1.53–1.61	19,303	0.59–0.69	0.64–1.74	1.67–1.81
LDL-C	mmol/L	4370	0.98–1.09	1.04–4.09	4.04–4.14	4992	1.00–1.05	1.03–4.22	4.17–4.27
Ca	mmol/L	16,304	2.0–2.1	2.1–2.7	2.6–2.8	23,204	2.0–2.2	2.1–2.6	2.5–2.7
IP	mmol/L	24,927	0.61–0.68	0.65–1.52	1.45–1.58	36,873	0.61–0.74	0.68–1.52	1.45–1.58
Mg	mmol/L	1830	0.62–0.70	0.66–0.95	0.90–0.99	15,394	0.62–0.70	0.66–0.95	0.90–0.99
Fe	µmol/L	1296	2.6–2.9	2.8–27.0 (*, #)	26.5–27.4	2774	1.8–2.1	1.9–23.4	22.9–24.0
TP	g/L	22,668	60–64	62–84	81–87	29,002	60–66	63–84	81–87
ALB	g/L	25,930	32–34	33–59	57–60	33,973	33–38	35–55	53–57
TBIL	µmol/L	22,247	3.7–4.1	3.9–23.9 (*, #)	21.9–25.6	29,121	2.5–2.7	2.6–20.7	18.9–22.1
DBIL	µmol/L	20,380	1.1–1.3	1.2–5.5 (#)	5.4–5.6	26,334	0.8–1.0	0.9–5.2	4.2–4.3
GLU	mmol/L	22,375	3.5–3.7	3.6–6.1	5.9–6.2	29,140	3.6–3.8	3.7–5.9	5.8–6.0
UN	mmol/L	18,847	2.7–2.9	2.8–7.6 (#)	7.1–8.2	22,543	1.6–1.8	1.7–7.5	7.1–7.9
CRE	µmol/L	22,476	46–51	49–104 (*, #)	101–106	27,329	27–44	35–81	79–84
UA	µmol/L	13,492	137–161	149–494 (*, #)	482–506	19,801	83–95	89 -4 46	428–464
Na	mmol/L	42,197	133–137	135–146	144–147	51,325	133–137	135–146	144–147
K	mmol/L	44,011	3.4–3.7	3.6–5.1	5.0–5.2	52,738	3.4–3.6	3.5–5.1	5.0–5.2
Cl	mmol/L	3104	97–99	98–111	110–113	3659	96–100	98–112	110–114
FT3	pmol/L	20,030	1.5–1.8	1.7–5.4	5.3–5.5	21,367	1.5–2.3	2.1–6.2	4.7–6.5
FT4	pmol/L	20,431	9.3–10.2	9.8–25.2	24.9–25.4	21,535	8.9–9.7	9.3–25.2	24.9–25.4
TSH	mU/L	19,027	0.30–0.36	0.32–4.43	4.39–4.46	19,636	0.37–0.45	0.42–4.31	3.04–4.4
VIT B_{12}	pmol/L	8846	72–76	75–336 (#)	334–338	9213	55–58	57–314	311–317
FOL	nmol/L	6952	4–6	5–29	28–30	7261	4–6	5–29	28–30
FER	µg/L	4978	33–56	46–356 (*, #)	342–385	5710	14–25	17–193	181–206
PTH	pmol/L	1629	1.4–1.8	1.6–7.8	7.7–7.9	3649	1.3–1.7	1.6–9.0	8.8–9.1
INS	pmol/L	6741	8–12	10–179	160–194	7775	8–11	10–177	167–188

UL – upper limit. LL – lower limit, CI – confidence interval, (*) – UL of males (> 10%) higher than females, (#) – LL of males higher (> 10%) than females.

rect and indirect methods for males and GGT, AST, ALT, LDH, HDL-C, Fe, DBIL, UA, FT4, TSH, VIT B_{12}, FOL and PTH for females. There were some differences (> 10% lower or higher) in the lower limits of the observed RIs for AST, ALP, GGT, ALT, LDH, AMY, CK, TG, TC, LDL-C, IP, Fe, ALB, CRE, UA, FT3, FT4, TSH, FOL, PTH and INS for males and ALP, GGT, AST, ALT, LDH, AMY, CK, TG, HDL-C, LDL-C, IP, Fe, DBIL, TBIL, UN, CRE, UA, FT3, FT4, TSH, VIT B_{12}, FOL, FER, PTH and INS for females.

Table 5 includes several categories of data for the 34 analytes: (a) direct RIs obtained from reference individuals according to the IFCC recommendations of our region, (b) the RIs of three regional studies [(R1 (19), R2 (20), and R3 (21)] in Turkey, and the RIs of the recently conducted multicenter RI study for Turkey (11) and (c) the values expected by the manufacturer for these analytes.

The RIs observed in the present study by the direct method for some, but not all analytes, were more or less comparable with the values reported in the MC, R1, R2 and R3 studies (Table 5). The values of RIs for some analytes in the present study and the multicenter study were comparable. However, the upper limits of RIs determined in the multicenter study for ALT, CRE and LDL-C. The lower limits of RIs determined in the present study for males were lower (> 10%) than those of the multicenter study for GGT, AST, LDH, CK, HDL-C, Mg and DBIL. The upper limits of RIs determined in the present study for females were higher (>10%) than those of the multicenter study for GGT, ALT, LDL-C, IP, CRE, TBIL, TG and GLU. The lower limits of RIs determined in present study for males were higher (> 10 %) than those of the the present study for females were lower (> 10%) than those of the multicenter study for CRE, TC, HDL-C, AST, ALT, LDH, AMY, Mg and DBIL (Table 5).

The RIs in the lower and/or upper limits observed in the present study were also different (> 10 % lower or higher) from the values reported in R1 study for ALP, GGT, AST, ALT, LDH, AMY, CK, TG, HDL-C, IP, TBIL, DBIL, GLU, UN, CRE, UA, TSH, VIT B_{12}, FOL and FER, and the values reported in R2 study for ALP, TG, HDL-C, LDL-C, CRE, FT3, FT4 and TSH, and the values reported in R3 study for ALP, AST, ALT, TG, HDL-C, UN, UA, CRE, K, FT3, FT4, TSH, VIT B_{12}, and FOL (Table 5). As seen in Table 5, there are some differences (> 10% lower or higher) in the lower and/or upper limits of the observed RIs for ALP, GGT, AST, ALT, LDH, AMY, CK, TG, TC, HDL-C, LDL-C, Fe, Mg, TP, ALB, TBIL, DBIL, GLU, FT3, TSH, FER, VIT B_{12}, PTH, FOL and INS from the values expected by the manufacturer for these analytes.

Discussion

The present data provided direct and indirect RIs for 34 biochemical analytes for our laboratory in Erzurum in Eastern Anatolia. The results of this study showed some differences between the RIs derived by the direct method and the indirect method. The RIs derived with the direct method for some, but not all, of the analytes were comparable with the RIs reported in the nation-wide study and other previous studies in Turkey.

The data of the present study showed clearly that there are some differences in the lower and/or upper limits of the RIs derived from direct and indirect methods in some analytes, such as GGT, ALT, TG, TC, HDL-C, LDL-C, ALB, Fe, UA, FT3, FT4, and VIT B_{12}. ALB and UA have a wider RI in the indirect method when compared to the direct method. Results similar to those for ALB have been observed for UA, where the lower limit is lower in the indirect method. Another analyte, with higher upper limits in the direct method is VIT B_{12}. Others, such as GGT, ALT and CK have a narrower RI in the indirect method when compared to the direct method. The differences between the results can be attributed in part to different statistical methods (Bhattacharya and IFCC methods), and in part to differences between populations (hospital and healthy populations). With some limitations, the Bhattacharya method can be of help in the determination of reference values, as asymmetricaly distributed test results can lead to low reference values when calculated with the Bhattacharya methods (22). The C28-A3 guideline recommends using the direct method to establish and verify RIs (5) whenever possible. However, some authors favour the indirect method as the results are clinically relevant and much simpler for an individual laboratory to implement than the time-consuming

Table 5. Comparison of the present study data with previous RI studies

ANALYTE (unit)	Gender	PS	MC	R1	R2	R3	MEV
		LL–UL	LL–UL	LL–UL	LL–UL	LL–UL	LL–UL
ALP (U/L)	M	40–127 (*, #)	42–120	64–176	36–129	44–134	40–130
	F	40–116 (*, #)	36–110	51–141	31–120	44–134	35–105
GGT (U/L)	M	9–58 (*, #)	11–58	6.5–34.1			8–61
	F	7–32 (*, #)	7–27	6.0–26.4			5–36
AST (U/L)	M	9–28 (*, #)	13–36	10–45		12–32	5–40
	F	9–24 (*, #)	11–28	9–32		10–28	5–32
ALT (U/L)	M	10–55 (*, #)	7–38	8–45		6–44	5–41
	F	6–30 (*, #)	7–23	6–26		3–40	5–33
LDH (U/L)	M	104–212 (*, #)	126–231	190–364			135–225
	F	105–202 (*, #)	120–231	190–364			135–214
AMY (U/L)	M	34–131 (*, #)	31–121	21–90			28–100
	F	23–116 (*, #)	32–117	21–90			
CK (U/L)	M	42–228 (*, #)	47–252	67–266			7–190
	F	31–142 (*, #)	32–135	43–220			7–170
TG (mmol/L)	M	0.59–3.12 (*, #)	0.52–3.47	0.39–3.37	0.45–2.44	0.57–3.54	< 1.69‡
	F	0.63–2.71 (*, #)	0.46–2.39	0.27–2.47	0.41–1.95	0.44–2.25	
TC (mmol/L)	M	3.02–6.68 (*, #)	3.15–6.17	2.64–6.63	2.87–6.29	3.13–6.97	< 5.18‡
	F	2.90–6.53 (*, #)	3.25–5.92	2.64–6.63	2.87–6.29	3.13–6.97	
HDL–C (mmol/L)	M	0.54–1.30 (*, #)	0.77–1.57	0.77–1.39	0.73–1.74	0.8–1.71	> 1.5‡
	F	0.72–2.02 (*, #)	0.92–1.97	0.80–2.15	0.91–2.15	0.91–2.02	
LDL–C (mmol/L)	M	1.49–4.98 (*, #)	1.52–4.25		1.06–4.56	1.55–4.77	< 2.5‡
	F	1.45–4.48 (*, #)	1.27–3.87		1.06–4.56	1.55–4.77	
Ca (mmol/L)	M	2.1–2.5	2.17–2.5	2.20–2.60		2.28–2.64	2.15–2.55
	F	2.1–2.5	2.12–2.47	2.18–2.63		2.16–2.57	
IP (mmol/L)	M	0.76–1.51	0.76–1.4	0.74–1.52			0.8–1.45
	F	0.85–1.39 (*, #)	0.83 – 1.4	0.74–1.52			
Mg (mmol/L)	M	0.70–0.90 (*, #)	0.82–1.1	0.66–1.03			0.7–1.07
	F	0.68–0.89 (*, #)	0.82–1.1	0.66–1.03			
Fe (µmol/L)	M	8.5–30.1 (*, #)		7.3–31.1			5.83–34.5
	F	5.2–27.8 (*, #)		4.9–30.1			
TP (g/L)	M	62–79 (*)	67–82	61–80			66–87
	F	62–78 (*)	66–82	61–80			
ALB (g/L)	M	42–50 (#)	41–50	37–51			35–52
	F	38–50	40–49	37–51			
TBIL (µmol/L)	M	3.8–23.9 (*, #)	3.6–23.9	5.1–25.7			1.71–20.5
	F	3.1–20.7 (*, #)	3.4–17.1	3.4–18.8			

Table 5. Comparison of the present study data with previous RI studies (continued)

ANALYTE (unit)	Gender	PS	MC	R1	R2	R3	MEV
		LL–UL	LL–UL	LL–UL	LL–UL	LL–UL	LL–UL
DBIL (μmol/L)	M	1.20–5.30 (*, #)	1.71–8.50	1.71–10.26			1.54–5.13
	F	1.10–4.60 (*, #)	1.71–6.84	0.68–7.01			
GLU (mmol/L)	M	3.7–6.1 (#)	3.9–5.9	3.55–5.60		4.00–5.83	< 5.5‡
	F	3.9–6.3 (*, #)	3.8–5.6	3.55–5.60		4.00–5.83	
UN (mmol/L)	M	3.10–7.20 (*, #)	2.87–7.41	2.66–6.83	2.83–8.15	2.86–6.78	3.16–7.34
	F	2.30–6.90 (*, #)	2.21–6.78	2.00–6.17	2.14–6.78	2.14–6.07	2.5–6.7
CRE (μmol/L)	M	59–107 (*, #)	58–89	62–133	80–142	71–101	62–106
	F	45–83 (*, #)	51–69	44–106	62–115	56–92	44–80
UA (μmol/L)	M	202–489 (*, #)	213–476	160–350		220–480	202–417
	F	148–369 (*, #)	148–363	60–240		130–380	143–339
Na (mmol/L)	M	134–147	137–144	133–151		139–147	136–145
	F	134–146	137–144	133–151		139–147	
K (mmol/L)	M	3.7–5.1 (*)	3.7–5.0	3.4–5.0		3.7-5.7	3.5–5.5
	F	3.8–5.1 (#)	3.7–5.0	3.4–5.0		3.6-5.07	
Cl (mmol/L)	M	96–108	98–107	97–108		103-111	98–107
	F	98–108	100–107	97–108		103-111	
FT3 (pmol/L)	M	3.7–6.0 (*, #)			2.00-6.77	4.57-8.02	3.1–6.8
	F	3.1–6.2 (*, #)			2.00-6.77	4.57-8.02	
FT4 (pmol/L)	M	11.9–20.4 (*, #)			10.29-24.45	13.2-25.0	12.00–21.93
	F	11.3–20.9 (*, #)			10.29-24.45	13.2-25.0	
TSH (mU/L)	M	0.36–4.78 (*, #)		0.51–3.51	0.30-4.17	0.60-6.25	0.27–4.2
	F	0.49–4.92 (*, #)		0.51–3.51	0.30-4.17	0.60-6.25	
VIT B_{12} (pmol/L)	M	70–368 (*, #)		158–1139		142-953	14 1–489
	F	71–395 (*, #)		235–1473		142-953	
FOL (nmol/L)	M	9–28 (*, #)		6.7–39.4		12.7-45.3	7.02–39.64
	F	9–33 (*, #)		8.2–49.8		12.7-45.3	
FER (μg/L)	M	46–399 (*, #)		10–190			30–400
	F	15–209 (*, #)		3.2–56			13–150
PTH (pmol/L)	M	1.8–8.1 (*, #)					1.58–6.84
	F	1.9–11.9 (*, #)					
INS (pmol/L)	M	21–190 (#)					17.8–173
	F	26–161 (#)					

LL – lower limit, UL – upper limit, PS – present study, R1 – RIs investigated in the region of Bursa and presented by Ilcol and Aslan (19), R2 – RIs investigated in the region of Denizli by Enli *et al.* (20), R3 – RIs investigated in the region of Izmir by Koseoglu *et al.* (21), MC – global, multicenter RIs for Turkey (11), MEV – manufacturer expected values, M – male, F – female, (‡) – clinical decision limits for TG, TC, HDL-C, LDL-C and GLU, (*) – UL of PS different (> 10% lower or higher) from MC, R1, R2, R3 or MEV, (#) – LL of PS different (>10% lower or higher) from MC, R1, R2, R3 or MEV.

direct a priori method (23,24). Furthermore, a recent study has suggested that an indirect method can be as accurate as a direct method when the right indirect method is used (25).

The exclusion criterion of more than one access per test to the laboratory during the data collection period has been applied in previous studies (23,24) and the rationale for that has been already explained. Approximately 20% of patient records were eliminated after applying this rule. The next exclusion rule (elimination of the data for hospitalized patients and for ambulatory patients from the intensive care unit) was applied to obtain a population that could be labeled as outpatients so that the majority of the values for each analyte could be more health related. By applying this exclusion cascade, 37% of patient records were eliminated and the 5 million stored laboratory records were reduced to 1,366,948, refined records. This limitation was balanced by the large amount of available data, which allowed the production of gender-specific RIs. Thus, the reference limits for many analytes were calculated on sample sizes of several thousands of test results.

Our laboratory recently participated in the nationwide multicenter study of RI determination for the Turkish population by Ozarda *et al.* (11). Data from the present study extended the data obtained from the nationwide study in respect of the number of samples and parameters examined, by providing region-specific RIs for 34 biochemical analytes for males and females. The RIs observed for some analytes with the direct method were in good accordance with the reported RIs in the multicenter study in Turkey (11). However, noticeable differences were observed in the lower limits and/or upper limits of ALT, LDH, TG, TC, HDL-C, LDL-C, Mg, TBIL, DBIL, GLU, CRE and K between the current and the multicenter study (11). The differences between our regional data and the data of the multicenter study could firstly be attributed to differences of analyzers and reagents. Abbott kits and analyzers were used in the multicenter study, whereas Roche kits and analyzers were used in the current study. In the multicenter study, the RIs were calculated using parametric and non-parametric methods. Since the RIs were derived by the non-parametric method in the present study, these were compared with those calculated by the non-parametric method in the multicenter study. However, the only difference for the calculation of the RIs between the two studies was the elimination of extreme values as outliers; the latent value exclusion method (17) in the multicenter study (11) and the Dixon range test (16) in the present study.

Comparisons were made with previous RI studies: Ilcol and Aslan, Enli *et al.* and Koseoglu *et al.* (19-21). These studies have been abbreviated R1, R2 and R3, respectively (Table 5). Differences were determined in some of the lower limits and/or upper limits between the current direct RIs and those of the R1, R2 and R3 studies. These studies were performed in different cities of Turkey using different analysers. R1, R2 and R3 were conducted in Bursa, Denizli and Izmir, respectively. The analysers were Technicon DAX-72 (Bayer Diagnostics, New York, USA) and ACS 180 (Bayer Diagnostics, New York, USA) for R1, ILAB 900 (Instrumentation Laboratory Company, Lexington, USA) and DPC Siemens Immulite One (Siemens Healthcare Diagnostics, Los Angeles, USA) for R2 and Architect (Abbott Diagnostics, Wiesbaden, Germany) and Advia Centaur (Siemens Diagnostics, New York, USA) for R3. Taken together, the instrumentation, the methodology and the laboratory environment of the present study were different from these studies and all these differences may also be related to the different characteristics (e.g. nutritional, environmental, economic and socio-cultural factors) of the population used in the present study.

For reproducibility and standardization, the pre-analytical aspects must be accurately defined and described for the implementation of a multicenter RI study (10). Haemolysis is the most common pre-analytical interference and still one of the biggest challenges to the laboratory specialists (26). There is clear evidence for clinically significant differences caused by visually undetectable to moderate hemolysis for LDH, AST, K and TBIL, whereas biases for some other parameters were found to be statistically significant, but remained within the conventional Clinical Laboratory Improvement Amendments (CLIA) limits ALB, ALP, AMY, CL, HDL-C, CK, GLU, Mg, TP, TG, unsaturated iron binding

capacity (UIBC) and UA (26). In addition, it is known that the storage temperature and the length of storage time have some effects on the samples (27). However, as a participant of the multicenter study, we carefully followed the SOPs and common protocol for all preanalytical aspects to standardization of the multicenter study and believe that these preanalytical effects are negligible for our study. Furthermore, the freezer has continuous temperature recording. Based on this recording, the serum samples were stored at -80 ± 2 °C for up to a maximum six months until analysis to minimize the effects of the storage temperature and the length of storage time.

In the present study, upper limits were found to be unexpectedly higher for some analytes, such as GGT, ALT and AST. However, the participants were excluded based on self-reported alcohol/tobacco consumption. There is a possibility that under-reporting of alcohol/tobacco consumption may have affected the data (e.g the upper limit of GGT). In addition, the enzyme analytes in this study are non-functional plasma enzymes and their lower limits have no diagnostic meaning, but upper limits are critical from a diagnostic perspective. Thus, the differences in the lower limits of the enzymes between studies can be ignored. However, there is an exceptional condition that low values of ALP may indicate hypophosphatasia which is caused by deactivating mutations within the gene that encodes the tissue-nonspecific ALP characterized by deficiency of serum ALP with defective bone and teeth mineralization (28). A similar approach could also be applied to the lower limits of TG, TC, HDL-C, DBIL, UN, and CRE as these are also not critical for diagnosis. The upper reference limits derived for GLU and lipid parameters in serum are not meant to be used for clinical decision-making, and it is therefore more appropriate to apply clinical decision limits for these parameters in order to identify risk for certain diseases.

It is known that Turks have a high prevalence of coronary heart disease, associated with some known risk factors. Turks have distinctively low concentrations of HDL-C, associated with elevated hepatic lipase activity and fasting triglyceridemia (29). Genetic and environmental factors are also important in modulating HDL-C concentrations in Turks (30). In the present study, the upper limits of HDL-C in males and females were 1.3 mmol/L and 2.0 mmol/L, respectively. The upper limits of TG and TC in males and females were 3.12 mmol/L and 2.71 mmol/L, and 6.68 mmol/L and 6.53 mmol/L, respectively. These values are comparable to those reported for males and females living in other regions in Turkey (R1, R2, R3 and MC). Our data support previous studies on the risk factors and cardiovascular disease in Turkey.

The findings of this study have shown that there are some differences in lower limits and/or upper limits between the direct method and the manufacturer's expected values. From the total of 34 analytes examined in the previous study, the lower limits and/or upper limits of 25 analytes were determined as different (>10 lower or higher) using the direct method compared to the manufacturer's expected values. The difficulties experienced by manufacturers when conducting appropriate RI studies must be taken into consideration and especially the challenge of accounting for potential differences in various patient populations.

In summary, the data from the present study provide RIs for 34 biochemical analytes specific to our region (Erzurum). The observed RIs for the majority of analytes are comparable to the RIs reported recently by the nationwide multicenter RI study (11). The observed differences in lower limits and upper limits of some analytes may reflect regional characteristics of the population in Eastern Anatolia, and nutritional and environmental factors. The national study concluded that: "With the lack of regional differences and the well-standardized status of test results, the RIs derived from this nationwide study can be used for the entire Turkish population." (11). Although we agree with this conclusion in general, our data allow us to conclude that the region-specific RIs will provide detailed information to better understand the health status of the locally served population.

Potential conflict of interest

None declared.

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