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RESEARCH ARTICLE

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Comprehensive laboratory reference intervals for routine biochemical markers and pro-oxidant-antioxidant balance (PAB) in male adults

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Abstract

Background: Reference values of biochemical markers are influenced by various parameters including age, sex, region, and lifestyle. Hence, we aimed to determine age- and BMI-specific reference intervals (RIs) for important clinical biomarkers in a healthy adult male population from northeastern Iran. This is also the first study to investigate reference values for pro-oxidant-antioxidant balance (PAB).

Methods: Seven hundred and twenty (720) healthy men, aged 20-60 years, were recruited from Sarakhs in the northeast region of Iran. Reference values for lipid profiles (total cholesterol, triglyceride, HDL-C and LDL-C), fasting blood glucose, inflammatory factors (hs-CRP and PAB), minerals (zinc and copper), uric acid, and blood pressure were measured and statistically analyzed to establish accurate age- and BMI-specific RIs in alignment with CLSI Ep28-A3 guidelines.

Results: RIs for lipid profiles, inflammatory factors, minerals, and uric acid required no age partitioning with the exception of fasting blood glucose and blood pressure, which demonstrated significantly higher values in subjects aged 50 years and older. Among these biomarkers, only uric acid, blood pressure, and triglycerides demonstrated statistically significant increases in reference value concentrations with increasing BMI.

Conclusion: In this study, age- and BMI-specific RIs for several biochemical markers were determined in healthy adult Iranian men. Partitioning by age and BMI was only required for a few analytes with most demonstrating no statistically significant

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changes with these covariates. These data can be useful to monitor various diseases in male adults with varying BMI in this region and others.

KEYWORDS
biochemical markers, body mass index, inflammatory markers, pro-oxidant-antioxidant balance, reference values

1 | INTRODUCTION

Cardiovascular disease (CVD) is a major cause of morbidity and mortality globally and is responsible for one third of all deaths in individuals aged 35 and older in Western countries.[1-4] In comparison with Western countries, most Asian countries have higher mortality from stroke and ischemic heart disease.[5] Hypertension, high total cholesterol, smoking, glucose tolerance, and obesity are known to contribute to CVD burden.[6] Hence, monitoring some established risk factors for CVDs including high blood pressure, high levels of lipid profile markers (ie, total cholesterol, triglyceride (TG), low density of lipoprotein (LDL-C)) and glucose seems essential due to their prominent role in the development and progression of CVD.[7-9] Besides traditional markers of lipid metabolism, several risk factors, including markers relating to oxidative stress and inflammation, have been proposed to better assess the risk of cardiovascular events. Atherosclerosis is recognized as an inflammatory process that can elevate markers of systemic inflammation, such as serum high-sensitivity C-reactive protein (hs-CRP).[10-12] Furthermore, there is a strong positive association between serum hs-CRP and pro-oxidant-antioxidant balance (PAB).[13,14] An imbalance between the production of pro-oxidants and antioxidant defenses results in oxidative stress and increased formation of reactive oxygen species (ROS).[15] The level of oxidative stress markers is known to be elevated in CVD patients and may promote the pro-atherosclerotic inflammatory process, leading to enhancement of CRP concentrations.[16,17] Serum PAB may be considered as a cardiovascular risk predictor.[14,18] Serum trace elements such as zinc and copper may also be associated with inflammation and peroxidation.[19-21] Additionally, the findings of numerous studies support the relationship between serum uric acid and CVD progression.[22,23] The reference intervals (RIs) of serum values of these risk factors may be useful for the accurate assessment of CVD risk, and the clinical management of patients.[24]

Laboratory testing requires health-associated benchmarks for disease interpretation, known as RIs. RIs can be defined as the range of values (commonly the 2.5th and 97.5th percentiles) for a parameter observed in a healthy reference population.[24] The International Federation of Clinical Chemistry (IFCC) has published several papers recommending that each laboratory determines specific reference values for its population or validates a preexisting RI.[25] Unfortunately, most clinical laboratories cannot establish their own RIs due to the difficulties associated with recruiting an adequate number of healthy subjects and also the high cost of sample analysis.[26] Nevertheless, in 2008, a guideline for determining RIs was published by the Clinical and Laboratory Standards Institute (CLSI)[27] and many countries have used this guideline to define their RIs for various clinical parameters.[28-32]

Previously, several studies have presented reference intervals for various routine laboratory tests in Europe,[33,34] Asia,[29-31,35] and Canada.[32] This is the first study to investigate reference values for several biochemical laboratory tests in the population from north-eastern Iran. In this study, we also aimed to determine age- and BMI-specific reference intervals for important markers of CVD.

2 | MATERIALS AND METHODS

2.1 | Study area and population

In 2010, a total of 720 men aged 20-60 years old were recruited from employees of the Shahid Hasheminejad Gas Processing Company (SGPC), Sarakhs, Iran. Patients with poorly controlled diabetes, severe hypertension, endocrine abnormalities, overt signs/symptoms of CVD, liver and kidney diseases, cancer and chemotherapy, major surgery, hepatitis and any chronic and/or acute health conditions which were professionally diagnosed were excluded from the study. This led to changes in sample size for each index (Table 1). Furthermore, participants had not undertaken any weight control diet and/or special exercise program. The Human Research Ethics Committee of Mashhad University of Medical Sciences (MUMS) approved the study protocol. Informed, written consent was obtained from all participants.[13]

2.2 | Biochemical analysis and quality control

All subjects were asked to fast for 12-14 hours before blood collection. Blood samples were collected in Vacutainer® tubes. Samples were then centrifuged at 5,000 g for 15 minutes at 4°C and aliquots of serum were kept frozen at −80°C for future analysis. All biochemical markers were measured less than 24 hours after sampling in October 2010, except PAB measurement which was assessed in 2016-2017.

Sera were analyzed by enzymatic methods for uric acid, TG, total cholesterol (TC), using Pars Azmun kits, and low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein

TABLE 1 Reference intervals of biochemical parameters in male Iranian population

		Male				
Index	Age	n	Lower limit	Upper limit	Lower Confidence Interval	Upper Confidence Interval
LDL-C (mg/dL)	20-60	672	77.9	197	(75.1, 79.5)	(191, 208)
HDL-C (mg/dL)	20-60	685	27.2	59.0	(26.6, 27.8)	(56.0, 61.2)
TC (mg/dL)	20-60	683	130	256	(127, 132)	(254, 264)
TG (mg/dL)	20-60	697	51.0	315	(46.0, 55.6)	(291, 338)
Uric acid (mg/dL)	20-60	684	2.80	7.59	(2.61, 2.96)	(7.39, 7.70)
SBP (mm Hg)	20-50	514	90	140	(90, 90)	(135, 150)
	50-60	180	90	170	(90, 100)	(160, 200)
DBP (mm Hg)	20-50	496	60	90	(60, 60)	(90, 90)
	50-60	169	70	100	(70, 70)	(100, 105)
Hs-CRP (mg/L)	20-60	668	0.51	7.82	(0.48, 0.54)	(7.24, 8.35)
PAB (% H_2O_2)	20-60	698	18	65	(16.88, 19.40)	(62.95, 68.05)
Zinc (μg/dL)	20-60	668	90	218	(86, 92)	(207-, 235.55)
Copper (μg/dL)	20-60	504	85	233	(81, 86)	(227, 251)
FBG (mg/dL)	20-40	464	78	109	(78, 79)	(108, 112)
	40-60	169	86	243	(84, 87)	(235, 251)

Abbreviations: DBP, diastolic blood pressure; FBG, fasting blood glucose; HDL-C, high-density lipid cholesterol; hs-CRP, high-sensitivity C-reactive protein; LDL-C, low-density lipid cholesterol; PAB, pro-oxidant-antioxidant balance; SBP, systolic blood pressure, TC, Total cholesterol; TG, triglyceride.

cholesterol (HDL-C) using Pishtaz Teb kits on a BT-3000 auto analyzer (Biotechnical). FBG was measured using the standard kit (made by Pars Azmoon Inc.). Serum hs-CRP levels (mg/L) were determined by polyethylene glycol (PEG-)-enhanced immunoturbidimetry using an Alcyon analyzer (Abbott). PAB was measured using an enzyme-linked immunosorbent assay (ELISA), as previously described.[36,37] The values of PAB are expressed in arbitrary Hamidi-Koliakos (HK) units, which represent the percentage of hydrogen peroxide in the standard solution.

The quality of assays was monitored using serum quality control materials at two different concentrations. Intra- and inter-assay coefficient of variation (CV) were both 2.0% for TC, 4.0% for TG, 5% for HDL-C and LDL-C, 2% for FBG, 17.0% for hs-CRP, and 2.0% for uric acid. For the determination of the precision of the modified PAB method, the intra- and inter-assay CV were determined. The intra-assay CV for PAB samples analyzed (n = 28) in triplicate was between 1.4% and 3.5%, with a mean of 2.1%. The inter-assay CV for PAB samples (n = 20), analyzed over 3 days, was between 4.1% and 8.5%, with a mean of 6.1%. Flame atomic absorption spectrometry (Perkin Elmer model 3030, USA, 1980) was used to measure serum concentrations of copper and zinc. To reduce the effect of signal interference of the serum matrix, the reverse osmosis/ deionized (RO/ DI) water was used to dilute the samples (1 in 5). A standard sample made with (RO/ DI) water was used to compare the samples. Values of 654 mL and 637 mL, the standard zinc (BDH product no. 14150 3C), and copper (BDH product No. 14139 2N), respectively, diluted by 100 mL of (RO/ DI) water in a volumetric flask. 0.5 mL of samples, standard and control were diluted with 2.0 mL RO/DI water and mixed well. The wells were aspirated and analyzed by atomic absorption spectroscopy. Serum copper and zinc concentrations were determined using a standard curve. The spectrophotometer setting for copper and zinc measurements were as previously described.[38] Also, the intra-assay and inter-assay CV were 1.5 ± 0.2% and 2.6 ± 0.4% for Zn and 1.3 ± 0.12% and 2.11 ± 0.32% for Cu, respectively. The limit of detection was less than 0.1 mg/L.

Height was measured to the nearest 0.1 cm, and weight was measured to the nearest 0.1 kg, in fasting state for all individuals. BMI (body mass index) was calculated as weight (Kg)/height (m^2). Blood pressure (mm Hg) was measured in duplicate using the DONG BANG ACUPRIME device with each subject being requested to sit for 15 minutes before each measurement. Systolic (SBP) and diastolic (DBP) blood pressures were recorded, and the average was used in statistical analysis.

2.3 | Statistical methods

Before analysis, we excluded individuals who were confirmed to be on treatment, or with a diagnosis of diabetes mellitus,

TABLE 2 Reference intervals for biochemical parameters based on BMI partition

Variables	BMI < 25						BMI ≥ 25					
	Age	n	Lower limit	Upper limit	Lower Confidence Interval	Upper Confidence Interval	Age	n	Lower limit	Upper limit	Lower Confidence Interval	Upper Confidence Interval
TG (mg/dL)	20-60	192	41.5	248	(32.0, 45.0)	(232, 266)	20-60	493	60	344	(55.6, 60.0)	(329, 363)
Uric acid (mg/dL)	20-60	189	2.61	7.18	(2.23, 2.86)	(6.97, 7.70)	20-60	472	3.45	8.17	(3.34, 3.45)	(7.83, 8.38)
SBP (mm Hg)	20-60	179	90	131	(90, 90)	(129, 138)	20-50	370	90	140	(90, 100)	(135, 150)
							50-60	138	100	180	(90, 100)	(160, 190)
DBP (mm Hg)	20-60	137	60	90	(60, 60)	(88, 92)	20-50	310	60	80	(60, 60)	(80, 80)
							50-60	106	80	100	(80, 80)	(99, 106)

Note: RIs were also calculated for individuals with a BMI of < 25 and ≥ 25 to assess the influence of BMI on CVD-related risk indicators based on CLSI guideline.
Abbreviation: BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure; TG, triglyceride.

hypertension, hyperlipidemia, osteoporosis, autoimmune diseases, malignancies, hematologic disorders, cardiovascular disease, hepatitis, and participants with hs-CRP ≥ 10 mg/L. RIs were calculated for the total population, after excluding those who were ineligible, as previously described by the Canadian Laboratory Initiative on Paediatric Reference Intervals (CALIPER)[39] and recommended by the CLSI Ep28-A3 guidelines.[40] Specifically, outliers were excluded using the Tukey statistical method twice. To obtain RIs, defined as the 2.5th and 97.5th percentiles, for partitions with ≥120 sample size, we used a nonparametric rank method and for partitions with <120 and ≥40 sample size, we used a robust statistical algorithm. The lower and upper reference limits were considered and corresponding 90% confidence intervals were calculated. RIs were also calculated for individuals with a BMI of <25 and ≥25, when statistically significant differences were observed. Statistical significance of partitions were determined by using the Harris and Boyd statistical method.[41]

3 | RESULTS

Calculated RIs for twelve biochemical factors including lipid profile, inflammatory factors, and minerals are provided in Table 1. Additionally, the RIs for parameters requiring partitioning by BMI (<25 and 25≥) are indicated in Table 2. The age-specific scatter plots showcasing the distribution of reference values across the adult age (20-60 years) are shown in Figures 1-4.

3.1 | RIs for lipid parameters and FBG

For all parameters assessed (Table 1), only FBG required age partitioning at 40 years. In general, reference values for lipid parameters remained relatively stable throughout the age range (Figure 1) with some demonstrating slightly higher concentrations at higher BMI values (ie, TG). Lower and upper reference limits for HDL-C were 27.2 and 59.0 mg/dL, respectively (Figure 1A). For LDL-C, lower and upper reference limits were 77.9 and 197mg/dL, respectively (Figure 1B). There was also a significant change in the upper reference limit for TG increasing from approximately 250 to 350 mg/dL in subjects with low and high BMI, respectively (Figure 1D). For TC, no age or BMI-specific differences were observed (Figure 1C). According to Figure 1E, FBG required two age partitions, with significantly higher concentration in subjects aged 40 years and older. Lower and upper reference limits for FBG were 78 and 109 mg/dL in 20-40 years old and 86 and 243 mg/dL for those 40 years and older.

3.2 | RIs for inflammatory parameters

There was a rising change in the concentration of uric acid with increasing BMI. The upper reference limit for subjects with lower BMI was 7.18 mg/dL, while it rose to 8.17 mg/dL in the higher BMI group

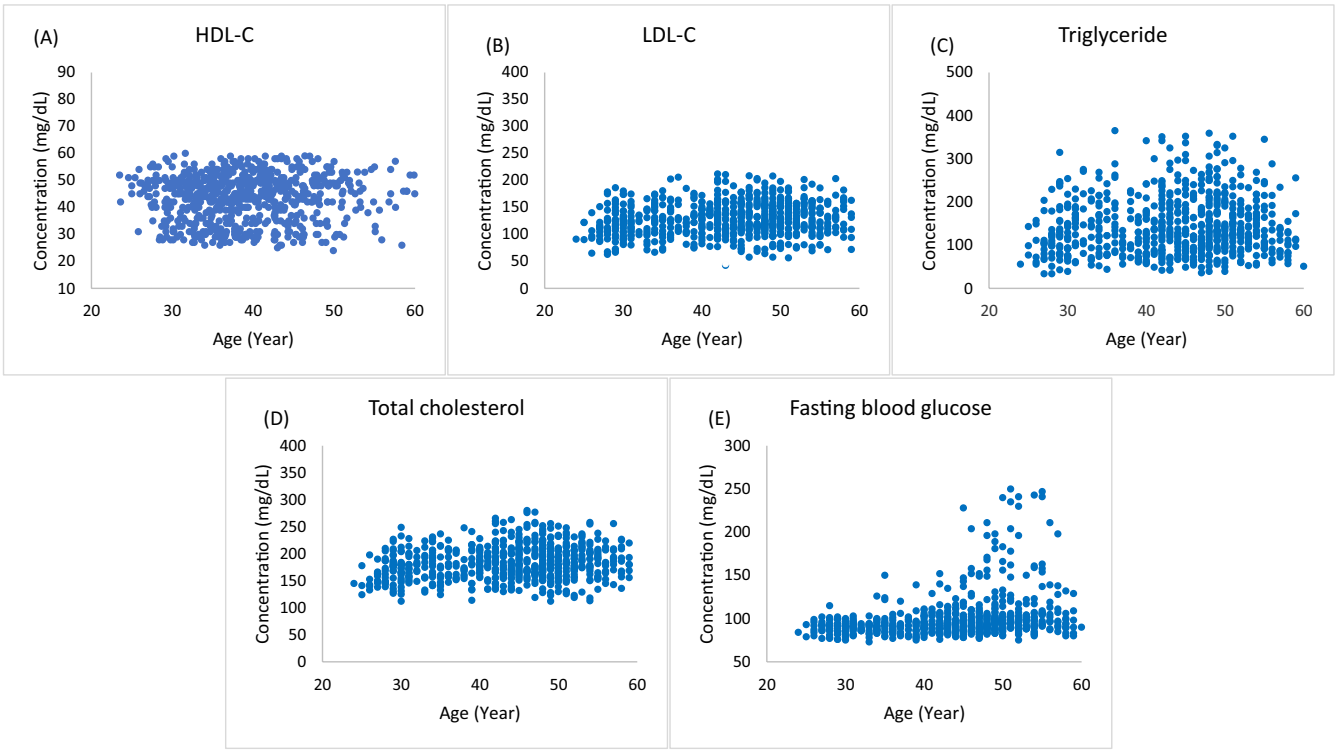

FIGURE 1 Scatter plot distributions for serum HDL-C (A), LDL-C (B), triglyceride (C), total cholesterol (D), and glucose (E) in total population

(Table 2). As shown in Table 1 and 2, no age or BMI partitions were required for hs-CRP and PAB. Lower and upper reference limits were 0.51 and 7.82 mg/L for hs-CRP and were 18 and 65% H_2O_2 for PAB (Figure 2B,C).

3.3 | RIs for minerals and blood pressure

RIs for minerals are presented in Table 1 as well as Figures 3 and 4. No age or BMI partitions were required for zinc and copper. In contrast, age- and BMI-specific partitions were required for systolic and diastolic blood pressure (Table 2). Lower and upper reference limits for systolic blood pressure were 90 and 140 mm Hg in subjects aged 20-50 years. While, for subjects aged 50-60 years, lower and upper reference limits were 90 and 170 mm Hg, respectively (Figure 4A). Lower and upper reference limits for diastolic blood pressure were 60 and 90 mm Hg in subjects aged 20-50 years and 70 and 100 mm Hg in subjects aged 50-60 years (Figure 4B). Systolic and diastolic blood pressure also increased significantly in the higher BMI group (Table 2).

3.4 | Discussion

Although variables such as age, sex, and region can affect reference values of given markers, there have been few studies in Iran investigating differences from definite guidelines generally used in laboratories. In this study, we determined RIs for 12 biochemical markers relating to various diseases mainly CVD in a healthy male population of Iran.

Our data demonstrated no age-specific differences for lipid parameters (TC, TG, LDL-C, and HDL-C). It is important to note that the upper limits of TG, TC, and LDL-C were higher than defined guidelines (IFCC and NECP) which may relate to exclusion criteria and/or population differences. In a study completed in a Danish population[42] with varying BMI, reference values for TC, TG, and LDL-C showed a significant increase with age. In addition, reported upper limits of RIs for TC were elevated when compared to our data, while data for other lipid markers were more comparable. Another Iranian study[43] reported similar results (reported in median) for lipid profiles in which they excluded individuals with BMI ≥ 25. In contrast to our method, the LDL-C concentration was calculated using Friedewald formula and there were four age ranges calculated for lipid profiles.[43] In this study, the influence of BMI on lipid parameters was also assessed. Only TG demonstrated a considerable rise in concentration with increasing BMI. No significant differences were determined in other lipid parameters.

FBG was one of few parameters that demonstrated significant age-specific differences, requiring two age partitions (Table 1). In the present study, the established FBG upper limit in men aged over 50 was not similar to other reported reference values.[44,45] This rising level of FBG can be influenced by age due to increasing insulin resistance.[46] Indeed, it has been suggested that the capacity of insulin resistance is more frequent in Asian populations compared with people of other ethnicities, despite similarities in BMI.[47,48]

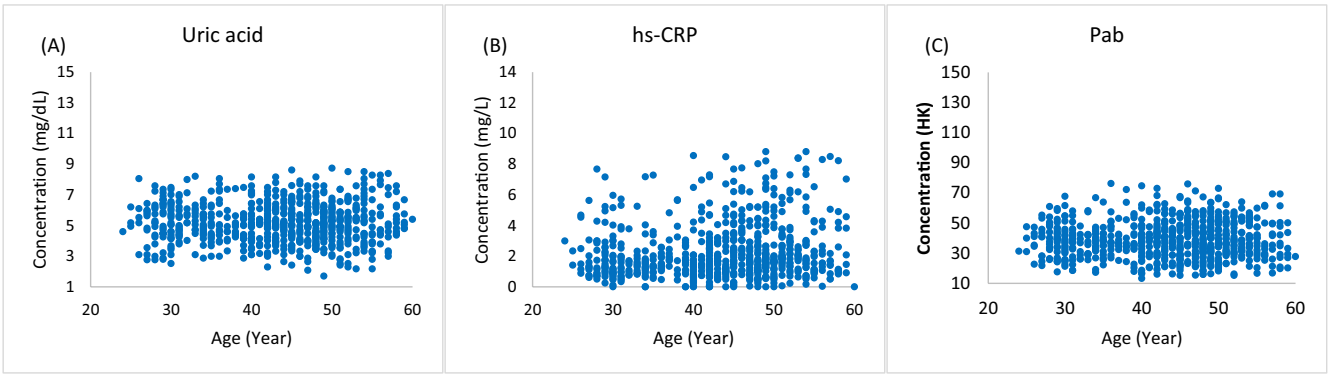

FIGURE 2 Scatter plot distributions for serum uric acid (A), hs-CRP (B), and pro-oxidant-antioxidant balance (C) in total population

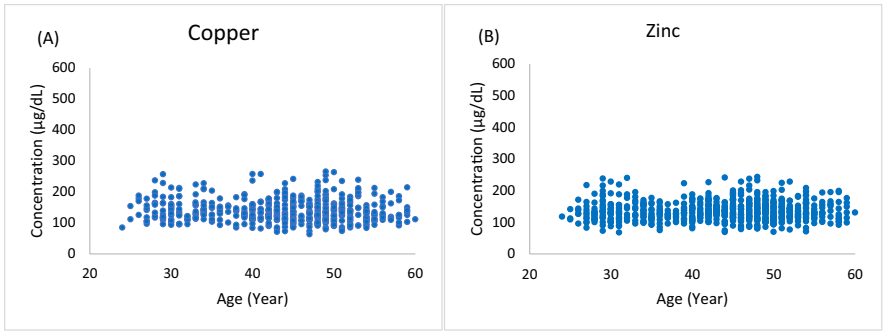

FIGURE 3 Scatter plot distributions for serum copper (A), zinc (B)

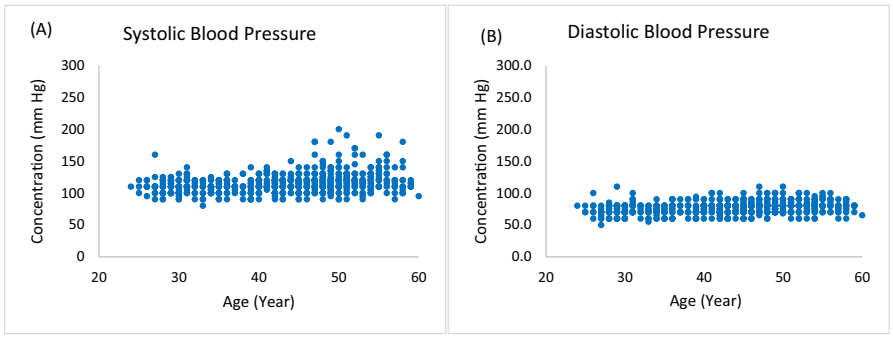

FIGURE 4 Scatter plot distributions for systolic blood pressure (A), diastolic blood pressure (B)

The reference values of uric acid in this study agreed with other reported results in a similar age- and sex-specific populations.[23,32,49-51] Although some of these studies presented a mean value of approximately 6 mg/dL for healthy males,[52,53] an Indian study provides a RI for uric acid of 3.5-8.7 mg/dL for males aged 35-86.[50] Also, a Canadian study established a RI for uric acid as 3.7-7.7 mg/dL for males aged 13-79, similar to our results.[32] It has been found that uric acid can impair nitric oxide synthesis, resulting in vascular endothelial dysfunction and pro-inflammatory status.[54,55] Enhanced uric acid levels are also associated with increased systemic inflammation and oxidative damage related to atherosclerosis.[56] Furthermore, some parameters as body weight, blood pressure, alcohol intake and diabetes can affect the level of uric acid.[57,58] As expected, both lower and upper limits of uric acid RIs increased in men with higher BMI (Table 2).

In our study, the distribution of hs-CRP concentration ranged from 0.51 to 7.82 (mg/L) (Table 1). In a study in an adult Nigerian population,[59] the RI for hs-CRP was reported as 0.62-11.64 mg/L for healthy individuals with a mean of BMI 18 kg/m^2, using the synthron CRP ultrasensitive enzyme-linked immunosorbent assay method. Also, in another study, the established RI for CRP for healthy Thai adults was 0.2-6.4 mg/L.[60] Anthropometric indices of study population were not indicated in this publication, and CRP reference values were measured by hs-CRP reagent kit using Integra-700 (Roche Diagnostics). Such differences in established RIs may be related to study methods, the technique of measurement and also population sample size.[61] Additionally, some factors as diet, physical activity, and stress can affect the health status of subjects.[60] Furthermore, according to the Helsinki Ageing Study, there is an association between aging and increased concentration

of hs-CRP which may be associated with enhanced production of interleukin-6 due to aging.[62] BMI and waist circumference have also been shown to positively correlate with a high CRP likely due to increased interleukin-6 release in the adipose tissue of overweight individuals.[63,64] In this study, no age and BMI partitions were observed for hs-CRP.

It has been reported that the level of oxidative stress parameters are elevated in CVD patients.[15] Oxidative stress may promote the pro-atherosclerotic inflammatory process which leads to enhancement of CRP concentrations.[16,17] Although no study has investigated PAB reference values to date, some results were presented from a healthy Iranian population [14,65] and compared with CVD patients. Both studies demonstrated that PAB level increased in patients with coronary artery disease (CAD) (66.4 ± 2.84 and 131.1 (HK units) in healthy individuals vs 77.37 ± 33.51 and 147.6 (HK units) in patients).[51,66] Our study thus provides novel information of PAB reference distributions in a large healthy cohort.

In the present study, the upper limit of reference values for serum zinc and copper in men (Table 1) were higher than RIs used in our clinical laboratory (65-110 μg/dL for zinc and 70-140 μg/dL for copper).[38] Some publications are in agreement with these values[67-70]; however, other reports differed.[71-74] Such discrepancies can be explained by the effects of various factors including sex, age of population, dietary intake, region, and standards of living and working environment.[72,75] For example, the mean value of zinc in people living in Islamabad/Rawalpindi was reported to be 2629.19 μg/dL (126.99 μmol/L). High zinc concentrations were considered to relate to their geographical area and diet/drinking water.[76] Also, the mean value for zinc in an Austrian population with mean age of 25 years was reported to be 497.30 μg/dL due to the rich zinc ores in their region.[77] Regarding data obtained from healthy Iranian males, people living in Tehran [73] demonstrated a higher level of zinc than the northeast population.[72] Our findings confirm the importance of RI determination for main minerals in a particular population living in a specific region.

For systolic and diastolic blood pressure, we observed a rise in upper and lower limits with aging. According to the Framingham Heart Study,[78] a continuous increase is expected for SBP from 30 years and older. However, DBP showed a varying pattern with age, as it increased until the fifth decade and then slowly decreased from 60 years to 84 years of age.[78] Regarding the age range of our study (20-60 years), our results agreed with these reports. Based on American College of Cardiology/American Heart Association (ACC/AHA) Guideline, upper limits for both SBP and DBP from 20 to 50 years were relatively close to other reports.[79,80] These rose over the suggested normal range for the second age partition; 50 to 60 years. Hypertension was defined as mean brachial SBP ≥ 140 mm Hg, DBP ≥ 90 mm Hg.[81,82] Moreover, it is known high blood pressure can be affected by overweight and obesity [83] which can also be influenced by age due to changes in lifestyle and less physical activity.[84]

In conclusion, age- and BMI-specific reference intervals for 12 biochemical markers were determined in a large cohort of healthy adult Iranian men. Partitioning by age and BMI was only required for a few analytes with most demonstrating no significant changes with these covariates. These data support the development of population-derived RIs and will be useful to monitor various diseases in this region and others.

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CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

AUTHOR CONTRIBUTIONS

We declare that we contributed significantly toward the research study, that is, data analysis was performed by Atieh Kamel Khodabandeh and Maryam Tayefi; and Conception and interpretation of results was performed by Hamideh Ghazizadeh, Marzieh Emamian, Mary Kathryn Bohn, and Maryam Mohammadi-Bajgiran; Reza Zare-Feyzabadi, Naghmeh Jaberi, and Maryam Saberi-Karimian designed the presented idea. Drafting the article was performed by Roshanak Ghaffarian Zirak, Payam Sharifan, and Ameneh Timar and developed by Samaneh Silakhori. Revising the paper critically for important intellectual content was carried out by Mohammad Reza Oladi and finally the article was approved by Habibollah Esmaily. The project supervised by Gordon A. Ferns, Khosrow Adeli, and Majid Ghayour-Mobarhan. Also, all authors read and approved the final manuscript.

ETHICAL APPROVAL

This study was approved by the Ethics Committee of Mashhad University of Medical Sciences (Mums), Mashhad, Iran.

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