Background: This study aimed to determine for the first time the age- and gender-specific reference intervals for biomarkers of bone, metabolism, nutrition, and obesity in a nationally representative sample of the Iranian children and adolescents.

Methods: We assessed the data of blood samples obtained from healthy Iranian children and adolescents, aged 7 to 19 years. The reference intervals of glucose, lipid profile, liver enzymes, zinc, copper, chromium, magnesium, and 25-hydroxy vitamin D (25(OH)D) were determined according to the Clinical & Laboratory Standards Institute C28-A3 guidelines. The reference intervals were partitioned using the Harris–Boyd method according to age and gender.

Results: The study population consisted of 4800 school students (50% boys, mean age of 13.8 years). Twelve chemistry analyses were partitioned by age and gender, displaying the range of results between the 2.5th to 97.5th percentiles. Significant differences existed only between boys and girls at 18 to 19 years of age for low density lipoprotein-cholesterol. 25(OH)D had the only reference interval that was similar to all age groups and both sexes.

Conclusions: This study presented the first national database of reference intervals for a number of biochemical markers in Iranian children and adolescents. It is the first report of its kind from the Middle East and North Africa. The findings underscore the importance of providing reference intervals in different ethnicities and in various regions.

Key words: adolescents; biomarkers; chemistry; pediatrics; reference intervals

Introduction

The practice of pediatric laboratory medicine is associated with a number of unique problems related to the influence of nutrition, growth, and disease during childhood and adolescence. Age-related changes from birth to adulthood complicate the process of health diagnosis in growing children. The assessment of pediatric diseases depends on the measurement of a variety of disease biomarkers in clinical laboratories, which serve to guide some important clinical decisions. Medical systems rely on the availability of suitable and consistent reference intervals to accurately interpret the results of laboratory tests collected by medical examinations. Many available reference intervals of laboratory tests were determined years ago and by using old technologies, therefore they may be no longer reliable. Currently, critical gaps exist in the literature, particularly in pediatric laboratory reference intervals, which make the accurate interpretation of laboratory tests impossible. In turn, this would increase the
A number of international and national institutes have begun initiatives to address these challenges. Moreover, some researchers have conducted literature reviews to evaluate whether existing studies provide useful interpretive guidance based on patient selection criteria, analytic methods, and sample sizes. Currently, published values are estimated to be incomplete because of using insufficient data. Indeed, many reference values have been derived from samples collected from hospitalized children, thus they may not reflect the levels appropriate for healthy populations. In some cases, age-appropriate and gender-specific reference intervals are lacking. Such reference interval values might be required for group-specific analysis based on age or gender, particularly for the pediatric patients. The Canadian laboratory initiative on pediatric reference intervals (CALIPER) is one of the most important initiatives focusing on these gaps, and is working to create a comprehensive database for many pediatric biomarkers in a multi-ethnic population.

The current study aims to determine the age- and gender-specific reference intervals for biomarkers of bone, metabolism, nutrition, and obesity in a nationally representative sample of the Iranian pediatric population. These values that serve as the first data from the Middle East and North Africa (MENA) are compared with some data obtained from Western populations.

Methods

The population of this cross-sectional study consisted of Iranian children and adolescents recruited in the third survey of a national surveillance program, entitled the childhood and adolescence surveillance and prevention of adult non-communicable diseases (CASPIAN) study. It was conducted in urban and rural areas of 27 provinces in 2009-2010. We once reported the methodology of this study, herein we described it briefly.

Schools were stratified and randomly selected, and then by multistage random cluster sampling, 5570 healthy students aged 7-19 years were selected from these schools. Study protocols, complying with the principles of the Declaration of Helsinki, were reviewed and approved by the Ethics Committee and other relevant national regulatory organizations. Following complete explanation of the study objectives and protocols, oral assent and written consents were obtained from students and their parents, respectively. Students were invited to the nearest health center to their school, and expert nurses obtained 12-hour fasting venous blood samples from them. All students were Iranian and had white skin tone; all blood samples were collected in the spring.

Healthy students were selected based on absence of underlying disease, e.g., diabetes, hypertension, cardiovascular disease, anemia or renal disorders. Those having any chronic diseases, experiencing an acute illness within the previous month, or currently using medication were not included in the study. Weight and height were measured under standard protocols. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m²). Weight disorders were defined by the sex- and age-specific Z-score curves of the World Health Organization (WHO). The data of students with obesity [BMI >+2 standard deviation (SD)] and severe thinness (BMI <-3SD) were excluded from the current study. Severe thinness was used instead of thinness since the prevalence of thinness is likely to be overestimated by the WHO standard. HIL index measurements were also checked for all serum samples. This index estimates the amount of hemolysis (H), icterus (I), and lipemia (L) in the specimens to ensure the validity of the test results.

In addition to the blood-sample data, questionnaires were completed for the students and their parents. The questionnaire included questions about the relationship with peers, body image, and psychosocial environment of schools, dietary habits, lifestyle habits, physical activity pattern, unintentional injuries, violent behaviors, active, passive smoking, and psychosocial relations with families.

Blood samples were analyzed to assess serum levels of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), fasting blood sugar, and liver enzymes (alanine aminotransaminase and aspartate aminotransaminase). All biochemical analyses were performed in the central provincial laboratory that met the standards of the National Reference Laboratory, a WHO-collaborating center in Tehran by using standard kit (Pars Azmoon, Iran). Serum samples were collected in plastic vacutainer tubes with or without gel. Moreover, serum subsamples (stored at -70°C) were used to determine the serum concentrations of zinc, copper, chromium, magnesium, and 25-hydroxy vitamin D [25(OH)D]. Frozen samples were subjected to only one freeze-thaw cycle prior to testing. We applied the methodology of the CALIPER study to determine the reference intervals.

Statistical analysis

The data were analyzed in accordance with Clinical & Laboratory Standards Institute (CLSI) C28-A3 guidelines. Statistical analysis was similar to that of CALIPER study; it was conducted using SPSS ver. 16 and Matlab ver. 7 software. The only difference with the CALIPER protocol was that an adjusted boxplot was used to determine the reference intervals.
was used instead of Tukey's boxplot for the detection of outliers. Although the Tukey's boxplot may be applicable for symmetric data, more skewed data result in a greater number of valid observations being detected as outliers. This is due to the fact that the Tukey's method is based on robust measures as lower and upper quartiles and the interquartile range, it does not consider the skewness of the data. In this method, the interval of the boxplot is adjusted by a robust measure of skewness for a skewed distribution. After analyzing the data and their partitioning based on the age and/or gender using the Harris and Boyd method, a nonparametric rank method was used to calculate the reference intervals for partitions with a sample size of at least 120 participants. For those partitions with fewer participants, a robust method was used. The reference levels corresponding to low and high end-point values were the 2.5th and the 97.5th percentiles, respectively. For each reference interval, the 90% confidence interval was calculated for the end points. Different steps of data analysis are outlined (Fig.).

### Results

The study population consisted of 4800 school students, of which 2400 (50%) were male, with a mean age of 13.8 years. Blood samples without hemolysis were examined; none of the samples was found to have icterus or lipemia. Reference intervals were partitioned into different scenarios using the Harris–Boyd method for statistical calculation. Table shows twelve chemistry analyses partitioned by age and gender, displaying the range of biomarkers of bone, metabolism, nutrition, and obesity between the 2.5th and 97.5th percentiles. Moreover, pediatric reference intervals for 12 biochemical markers (in conventional units) are shown in the supplementary Table. The age of participants ranged from 7 to 19 years. Based on the Harris & Boyd method, significant differences existed only between boys and girls at 18 to 19 years of age for LDL-C. Statistical analysis also showed that among all obtained chemistry results, only the reference intervals for 25(OH)D were similar for all age groups and both sexes (Table). The minimum number of observations required to estimate the 2.5th and 97th percentiles is 39. Therefore, the reference intervals were not calculated for LDL-C and TG of children aged 7-8 years who had a sample size of less than the required number.

### Discussion

In our study, we particularly focused on developing the reference intervals for cardio-metabolic risk factors and liver enzymes, because no universal cut points exist in

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**Exclusion criteria**
- Children with chronic illness
- Metabolic disease
- An acute illness within the previous month
- Use of prescribed medication over previous 2 wk
- Obese children
- Children having severe thinness

**Data quality assessment**
- Sample size selection
  - Iranian children aged 7 to 19 y
- Data inspection for the missing and incorrect values
- Adjusted boxplot at 1-year-interval
- Scatter plots considering trends in age and gender
- Kolmogorov-Smirnov method
- Non-Gaussian-distributed partitions: log-transformed partitions
- Harris/Boyd method
- Sample size not more than 120: Horn and Pesce robust method

**Testing the validity of the results:**
- HIL index
- Identifying temporary partitions
- Determining the normality of each partition
- Determining the possibility of merging partitions considering age or gender
- Gaussian-distributed partitions: Harris/Boyd method
- Sample size more than 120: non-parametric rank method

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**Fig.** Iranian pediatric-specific reference data analysis algorithm based on Clinical & Laboratory Standards Institute guidelines document C28-A3. HIL: hemolysis, icterus, lipemia.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Age</th>
<th>Male (mmol/L)</th>
<th>Female (mmol/L)</th>
<th>Male (mmol/L)</th>
<th>Female (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mmol/L)</td>
<td>7-9 y</td>
<td>7.00</td>
<td>2.15</td>
<td>6.00</td>
<td>1.94</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>7-9 y</td>
<td>4.00</td>
<td>1.61</td>
<td>4.00</td>
<td>1.56</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>7-9 y</td>
<td>3.00</td>
<td>1.00</td>
<td>3.00</td>
<td>1.00</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>7-9 y</td>
<td>2.00</td>
<td>0.80</td>
<td>2.00</td>
<td>0.80</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>7-9 y</td>
<td>20.00</td>
<td>5.00</td>
<td>20.00</td>
<td>5.00</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>7-9 y</td>
<td>15.00</td>
<td>4.00</td>
<td>15.00</td>
<td>4.00</td>
</tr>
<tr>
<td>FBS (mmol/L)</td>
<td>7-9 y</td>
<td>3.00</td>
<td>0.50</td>
<td>3.00</td>
<td>0.50</td>
</tr>
<tr>
<td>Zn (μmol/L)</td>
<td>10-13 y</td>
<td>12.50</td>
<td>2.50</td>
<td>12.50</td>
<td>2.50</td>
</tr>
<tr>
<td>Mg (mmol/L)</td>
<td>10-13 y</td>
<td>0.50</td>
<td>0.05</td>
<td>0.50</td>
<td>0.05</td>
</tr>
<tr>
<td>Cr (μmol/L)</td>
<td>10-13 y</td>
<td>150.00</td>
<td>30.00</td>
<td>150.00</td>
<td>30.00</td>
</tr>
<tr>
<td>Vitamin D (nmol/L)</td>
<td>8-19 y</td>
<td>50.00</td>
<td>10.00</td>
<td>50.00</td>
<td>10.00</td>
</tr>
</tbody>
</table>

* SI: International System of Units; TC: total cholesterol; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; TG: total glyceride; ALT: alanine aminotransferase; AST: aspartate aminotransferase; FBS: fast blood sugar; Zn: zinc; Mg: magnesium; Cr: chromium; Cu: copper. Items in bold: reference intervals for males and females are significantly different. *: the reference low and high end-point values were the 2.5th and the 97.5th percentiles. For each reference interval, a 90% confidence interval calculated for the end points, was shown in the parenthesis.
## Supplementary Table

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Age</th>
<th>Male reference interval</th>
<th>Female reference interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dL)</td>
<td>7-9 y</td>
<td>102.00 (101.00, 117.51)</td>
<td>192.32 (182.08, 201.10)</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>7-15 y</td>
<td>69.00 (63.82, 74.45)</td>
<td>69.00 (63.82, 74.45)</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>9-17 y</td>
<td>137.07 (134.79, 138.61)</td>
<td>137.07 (134.79, 138.61)</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>7-9 y</td>
<td>35.29 (32.24, 38.26)</td>
<td>35.29 (32.24, 38.26)</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>7-9 y</td>
<td>31.00 (27.24, 30.76)</td>
<td>31.00 (27.24, 30.76)</td>
</tr>
<tr>
<td>FBS (mg/dL)</td>
<td>7-10 y</td>
<td>67.00 (64.44, 69.56)</td>
<td>67.00 (64.44, 69.56)</td>
</tr>
<tr>
<td>Zn (μg/dL)</td>
<td>10-13 y</td>
<td>86.00 (82.00, 90.00)</td>
<td>86.00 (82.00, 90.00)</td>
</tr>
<tr>
<td>Mg (mg/dL)</td>
<td>10-14 y</td>
<td>6.60 (6.55, 7.10)</td>
<td>6.60 (6.55, 7.10)</td>
</tr>
<tr>
<td>Cr (μg/L)</td>
<td>10-14 y</td>
<td>60.00 (58.00, 62.00)</td>
<td>60.00 (58.00, 62.00)</td>
</tr>
<tr>
<td>Cu (μg/dL)</td>
<td>10-14 y</td>
<td>152.00 (150.00, 154.00)</td>
<td>152.00 (150.00, 154.00)</td>
</tr>
<tr>
<td>Vitamin D (ng/mL)</td>
<td>8-19 y</td>
<td>152.00 (150.00, 154.00)</td>
<td>152.00 (150.00, 154.00)</td>
</tr>
</tbody>
</table>

* TC: total cholesterol; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; TG: total triglyceride; ALT: alanine aminotransferase; AST: aspartate aminotransferase; FBS: fast blood sugar; Zn: zinc; Mg: magnesium; Cr: chromium; Cu: copper. Items in bold: reference intervals for males and females are significantly different. *: the reference low and high end-point values were the 2.5th and the 97.5th percentiles. For each reference interval, a 90% confidence interval calculated for the end points, was shown in the parenthesis.
the pediatric population to set the abnormal levels of these laboratory tests and because ethnic differences in reference values are specially related to these tests.\textsuperscript{[7,21]} For instance, at any given BMI, white adolescents have a greater atherogenic risk profile and are more prone to insulin resistance than black adolescents.\textsuperscript{[22]}

The CLSI/International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) C28 P3 Guidelines recommend having at least 120 specimens in each partition to calculate the reference intervals. In this case, CLSI Guidelines C28-A2 recommend using the non-parametric rank method.\textsuperscript{[23,24]} The robust method as described in the CLSI Guidelines C28-A3 is recommended when the sample size is less than 120. Furthermore, even with as few as 39 data samples, one can use robust techniques to get an estimate of the reference interval\textsuperscript{[25]}  The reference intervals were not calculated for sample size of less than 39 since the minimum number of observations required to estimate the 2.5th and 97th percentiles is 39.\textsuperscript{[20]} As discussed in the CLSI/IFCC C28-P3 document, the reference intervals calculated by both the robust (n=20) and the non-parametric (n=120) analyses are quite similar.\textsuperscript{[25]} The reference intervals for TC (7-9 years old boys/girls) in our study included 47 samples, which is a limitation of our study due to its small sample size for this age group.

The lipid values reported in the main textbook of Pediatrics\textsuperscript{[26]} are still based on data obtained from American youths studied by the Lipid Research Clinic (LRC) in 1980, and are not necessarily applicable to other populations. Comparing the findings of the first CASPIAN study with LRC standards showed that among both sexes and in all age groups, the percentiles of TG were higher and those of HDL-C were lower in Iranian children and adolescents than in the LRC population.\textsuperscript{[27]} Likewise, comparison of the percentiles of HDL-C and TG of Iranian and German children and adolescents revealed similar results.\textsuperscript{[28]}

Comparison of the same data of Iranian children and adolescents with their counterparts from Brazil and Germany (the BIG study) showed that by considering the definition of the International Diabetes Federation,\textsuperscript{[29]} Iranians had the highest prevalence of depressed HDL-C, followed by Brazilians and Germans. Hypertriglyceridemia was more prevalent in Brazilians, followed by Iranians and Germans.\textsuperscript{[30]} Epidemiologic study\textsuperscript{[31]} has shown a high prevalence of depressed HDL-C level in the Middle Eastern population. A national study in Iran\textsuperscript{[27]} suggested that 80% of adults and 25% of children and adolescents have depressed serum HDL-C levels. A depressed serum HDL-C level of 42.8% was noted among Iranian adolescents in the metropolitan Tehran.\textsuperscript{[32]} Similar ethnic differences and high prevalence of depressed HDL-C levels were found in Turkish youths and adults.\textsuperscript{[33]} The high prevalence of depressed HDL-C level in the MENA region was also observed in normal weight adults and adolescents. Furthermore, the prevalence of depressed HDL-C level was reported to be significantly higher in Middle Eastern immigrants than in Western populations in both youth and adults. The unhealthy lifestyle behaviors in terms of dietary (e.g., breakfast skipping) and physical activities are associated with depressed HDL-C\textsuperscript{[34]} and might explain part of such ethnic differences. These findings show an ethnic predisposition to some lipid disorders, and suggest that different reference values must be defined for these populations.

Serum 25(OH)D levels exhibit large variations in different populations. Very high prevalence of hypovitaminosis D in children of sunny regions\textsuperscript{[35]} may have diverse causes, but the normal levels and the thresholds for defining low levels might be different in various populations. Trace elements play important roles in human health, notably for growing children and adolescents. Their serum levels depend on various factors, and universal reference values are not available. Previous studies\textsuperscript{[36-39]} in Tehran have reported reference values for zinc in adults and pediatric populations that showed somehow wider reference intervals. Our findings are in line with this study showing that serum zinc concentrations were comparable in boys and girls, where the reference interval was reported for boys/girls. Our findings are also consistent with a study on American Caucasian children, in which the reference interval of serum zinc was 12.55-27.54 μmol/L without significant difference between various age groups.\textsuperscript{[37]} Most foods in the USA have been enriched or fortified with zinc since 1987,\textsuperscript{[38]} which is not the case in Iran. However, the similarity of the reference intervals of the two populations might be explained by some daily dietary habits in Iranian families as consumption of foods rich in zinc, e.g., lamb, nuts, and chicken, in addition to the free school milk programs in Iran\textsuperscript{[39]} The reference intervals for serum copper were reported to be age dependent: 11.78-24.02 μmol/L for those aged under 10.3 years, 10.05-20.72 μmol/L for those under 10.3-12.5 years, and 8.95-20.25 μmol/L for those of more than 12.5 years of age.\textsuperscript{[37]} The reference intervals obtained in the current study are higher than these values. The reference intervals for serum copper reported in both studies do not indicate copper deficiency.

Our study showed ethnic differences in comparison with the CALIPER study\textsuperscript{[40,25]} and the other studies in pediatrics in the United States\textsuperscript{[50]} in terms of the number of age groups, in which the reference levels were obtained, with significant differences between reference intervals reported in boys and girls. For instance, TC reference intervals were 2.47-5.58 mmol/L and 2.64-4.98 mmol/L for 7 to 9-year-old boys and girls respectively in our study, but 2.91-5.41 mmol/L
for 1-18 years old boys/girls in Canada.\[9\] The intervals were 3.51-5.20 mmol/L for 2 to 15 years old boys/girls in the United States.\[10\] The other automatically generated reference interval was 2.41-5.15 mmol/L for 10-19 years old boys/girls. TC was significantly higher in girls than in boys (7 to 9 years old), which is similar to that in Tehran.\[40\] The reference intervals for TG were similar for boys/girls in all three main surveys compared, except that three age groups (7-8, 9-16 and 17-19 years old) were generated in our study, but the other surveys reported a universal age-group.

It should be acknowledged that because of limitations in the sample size as well as in financial support, we could study some biochemical markers. Therefore, we selected markers that are commonly used in pediatrics and have variations according to ethnic and demographic factors.

This study presents the first national reference intervals of some biochemical factors in an Iranian pediatric population, and is the first report from the Middle East and North Africa. It underscores the importance of providing reference intervals in different ethnicities and in various regions. Future longitudinal studies may characterize the genetic nature of the ethnic differences documented in this study, and help to determine their clinical importance.

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Ethical approval: This study was approved by the Isfahan University of Medical Sciences (No. 293214).

Competing interest: The authors have no financial relationships or any conflict of interest relevant to this study to disclose.

Contributors: Kelishadi R contributed to the study design, preparation, conducting, and interpretation of findings. Marateb HR contributed to the data analysis and edited the study. Mansourian M contributed to the study design and preparation, and conducted the statistical analysis. Ardalan G and Heshmat R contributed to the manuscript design, conducting, and approving. Adeli K contributed to the study design, conducting, statistical analysis confirming, and editing. All authors read and approved the final version of the manuscript. Mansourian M is the guarantor.

References
18 Linnet K. Nonparametric estimation of reference intervals by

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