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ORIGINAL ARTICLE

Determination of Reference Intervals of Some Biochemistry Tests by the Bhattacharya and Hoffmann Methods

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SUMMARY

Background: **Clinical laboratory tests are being evaluated with reference intervals (RI). Therefore, it is important that each laboratory determines and classifies its own reliable RI for each test to ensure an accurate and effective interpretation. The proposed method for determining RI is the "direct" approach, but it is a difficult, troublesome, time-consuming, and expensive method. An alternative approach is the “indirect” approach. In this study, we aimed to compare the RI values determined by the indirect method from the Calcium (Ca), Magnesium (Mg), Phosphate (P), 25-Hydroxy Vitamin D (25(OH)D), and Parathyroid hormone (PTH) test results with the RI provided by the manufacturer.**
Methods: **A total of 1,520,314 Ca, Mg, P, 25(OH)D, and PTH test results, which were studied in our laboratory between January and November 2022, were included in the study. Data cleaning was done for individuals between the ages of 18 - 89, and only one record was allowed. The Tukey method was used to determine and exclude extreme values. Ca and Mg tests were divided into age groups (18 - 59 and 60 - 89 years), P, 25(OH)D, and PTH tests were divided into female - male groups. RI was calculated by using the Bhattacharya and Hoffmann methods. CLIA 19 acceptable limits were used to evaluate the compliance with the manufacturer's RI.**
Results: **The RI results obtained by applying the Bhattacharya and Hoffmann methods were found to be significantly consistent and compatible with each other. According to the manufacturer's RI, Ca and Mg were compatible with RI in both methods, P was considered compatible with PTH and 25(OH)D upper reference limit in the Bhattacharya method, P was considered compatible with 25(OH)D lower reference limit and PTH upper reference limit in the Hoffmann method, while 25(OH)D lower reference limit was found to be different in the Bhattacharya method, and 25(OH)D upper reference limit and PTH lower reference limit were found to be different in the P male group in the Hoffmann method.**
Conclusions: **We believe that it is of great importance for each laboratory to determine the RI specific for the population they serve and to choose the analytical method they use according to age and gender while periodically updating them to interpret the test results correctly.**
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KEYWORDS

Bhattacharya method, Hoffmann method, indirect method, reference intervals

INTRODUCTION

Clinical laboratory tests are an integral part of screening, diagnosis, treatment, and follow-up processes, and clinicians evaluate test results with reference intervals

(RI). Along with improving analytical test quality in medical laboratories, accurate interpretation of results is also of great importance. Laboratory test results can be influenced by uncontrollable factors such as age, sex, geographic location, and ethnicity, as well as controllable factors such as hunger, nutrition, exercise, diet, pregnancy, and laboratory methods. Therefore, it is important that each laboratory determines and classifies its own reliable reference interval for each test to ensure an accurate and effective interpretation [1-5]. In practice, it is almost impossible for each laboratory to establish its own RIs due to the excessive time and cost required to perform additional laboratory tests and to collect an appropriate reference population. Therefore, most laboratory RIs [5] are outsourced. The most common source of RIs is data provided by the test manufacturer. Most clinical laboratories use the RIs contained in the manufacturer's kit. An analysis of the test manufacturers' RI values shows that, although manufacturers declare their procedures to be in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI), reference groups are generally few in number and little attention is paid to many important factors such as race, age group, health status, or body weight. Very rarely, a laboratory procedure is also used to verify the reference intervals provided by the manufacturer according to CLSI recommendations [2].

The proposed method for determining RI is the "direct" approach. In this method, individuals representing the reference population are selected, sampled, and analyzed for this purpose. This process is described in the CLSI document EP28-A3C [2]. An alternative approach is the "indirect" approach, where results obtained from samples collected for routine purposes, such as screening, diagnosis, or follow-up, are used to determine RIs. The direct method is derived from the reference population value distribution, usually by using the central 95% interval and by identifying a given population with a minimum sample size of 120. However, the direct method has some drawbacks. Selecting, communicating, and recording 120 healthy random individuals and finding appropriate reference individuals for age and gender-related tests can be difficult. It also requires a significant amount of time, resources, and costs. Obtaining ethical approval can also be time-consuming.

Indirect approaches determine RIs using statistical methods based on determining the distribution in the middle of the data rather than evaluating whether individual results in the entire database belong to the reference population. The indirect method has significant advantages over the direct method; one of which is that it is faster and cheaper. It is also based on actual preanalytical and analytical conditions used in routine practices [6]. In indirect studies, methods used in direct RI studies, such as standard parametric methods (mean and standard deviation) or nonparametric statistics (percentages), can also be used.

The first example of the early stages of the indirect method was outlined in a study conducted by Hoffman in 1963 [7]. At this time, computer-based data analysis was still in a simple stage, and there was little theoretical information about the distribution of laboratory data. In 1967, Bhattacharya [8] developed another graphical method to identify one or more Gaussian peaks in the histogram of observed data. This method was applied to the laboratory data, and it was stated that the largest peak represented the reference population, and reference intervals were obtained. The main procedure of the Hoffman and Bhattacharya methods [9,10] is considered to be the conversion of raw data into the Gaussian form and requires advanced knowledge of the distribution of reference values. With the development of medical statistics, Hoffman and Bhattacharya methods, as graphical methods with simple computer operations [3], showed application value and facilitated the calculation method of RIs to some extent. In addition, the fact that the Bhattacharya method can be applied by using software and computer programs [13] has provided widespread use.

This study aimed to compare the RI values determined indirectly from the test results of Calcium (Ca), Magnesium (Mg), Phosphate (P), 25-Hydroxy Vitamin D (25(OH)D), and Parathyroid hormone (PTH) with the RI provided by the manufacturer.

MATERIALS AND METHODS

Study population

This study is a reference interval analysis with an indirect method performed by using patient test result data. The study was performed in the central laboratory of Kanuni Sultan Suleyman Training and Research Hospital, which provides health services in Istanbul, European side. This study aimed to determine the RI by using the Bhattacharya and Hoffman methods and to compare this interval to the RI provided by the kit manufacturer. Our central laboratory serves a large population that resides in the western parts of the city, which has a population of about 5,000,000. On a daily basis, approximately 20,000 samples are received from 16 hospitals and 382 primary healthcare centers, producing 250,000 test results.

Data

The study included patient results from the regional laboratory of our hospital between January 2022 and November 2022. The total number of data was 1,520,314. Two-stage exclusion procedure was applied to this data set as pretreatment. In the first stage, patients under the age of 18 and over the age of 90, pregnant women, patients in the intensive care unit, oncologic patients, and hemodialysis patients were excluded from the study. During the study period, only single and first results of the patients were used to exclude repetitive data in the second stage. In conclusion, the total data size was 206,421. The study included Ca, Mg, P, 25(OH)D, and PTH tests related to calcium metabolism, and there were 62,002, 49,617, 31,549, 34,560, and 28,693 test results for these tests, respectively. All results were obtained through the laboratory information management system (Alis, Ventura Software, Ankara, Turkey) used in our laboratory. During the study period, Ca, Mg, and P levels were measured with Cobas c 702 analyzers (Roche Diagnostics, Mannheim, Germany) by using the photometric method, respectively, Calcium Gen.2, Magnesium Gen. 2, and Phosphate (Inorganic) ver.2 kits (Roche Diagnostics, Mannheim, Germany). 25(OH) D and PTH levels were measured by using the electrochemiluminescence method, using Cobas e801 analyzers (Roche Diagnostics, Mannheim, Germany) and Elecsys Vitamin D total II and Elecsys PTH kits (Roche Diagnostics, Mannheim, Germany).

Statistical methods

Grouping for each test was performed according to criteria established by the kit manufacturer. Accordingly, age groups (18 - 59 years and 60 - 89 years) for Ca and Mg tests and male and female groups for P, 25(OH)D, and PTH tests between the 18 - 89 age group were formed.

Indirect RIs were calculated by using the Bhattacharya and Hoffmann methods. In both methods, the outliers were determined by using the Tukey method, one of the M-estimators, and were excluded from the study. The compatibility of the data with the Gaussian distribution was tested by the Kolmogorov-Smirnov test, and variables with Fisher's kurtosis and skewness values between ± 3 were considered to have normal distribution (Figure 1). In the Bhattacharya method, RIs were calculated with the Bellview package program. In the Hoffmann method, the least squares method was used to calculate the regression equation between experimental data with Gaussian distribution and the theoretical quantile of the standard normal distribution [11]. New data points were created within the 95% confidence interval limits of the linear regression equation:

lower reference limit = -1.96 xa + b,

upper reference limit = 1.96 xa + b [11]

Intraclass Reliability Coefficient (ICC) was used to measure the consistency and absolute consistency of the RIs obtained from both methods.

CLIA 19 acceptable limits were used to assess, whether there was a difference between the RIs calculated by the two different methods and the manufacturer's RIs [12]. The difference between two reference values is considered to be compatible if it is less than 1 mg/dL for Ca, 15% for Mg, 10% or 0.3 mg/dL for P, 30% for PTH, and 25% for 25(OH)D (40).

RESULTS

The data size of the LIS dataset before and after data cleaning

The results of 1,520,314 patients, that were initially included in the study, were reduced to 206,421 (13.6% of the initial data) after the exclusion criteria were applied. The Tukey method was used to eliminate extreme values, following the formation of the study groups. A total of 195,781 (12.8% of the first data) test results, 140,053 females (71.5%) and 55,728 males (28.5%), were included in the study after the Tukey method had been applied. The gradual changes in the data size are shown in Table 1.

When the acceptable limits of CLIA 19, the RIs calculated by the Bhattacharya method, and the RIs calculated by the Hoffmann method were evaluated, it was seen that the low reference interval (LRI) and upper reference interval (URI) values for both age groups for Ca and Mg were consistent with each other's values. When the RIs calculated for P were examined, the LRI values of the female and male groups were considered compatible with each other's values, while the URI values were considered compatible for the female group and incompatible for the male group. For 25(OH)D and PTH, while LRI and URI values for females were compatible in the two methods, URI values for males were evaluated as compatible and LRI values as incompatible (Table 2).

When the acceptable limits of CLIA 19, the RIs calculated by the Bhattacharya method, and the RIs of the manufacturer were evaluated, it was discovered that the LRI and URI values for both age groups for Ca and Mg were consistent with the manufacturer's values. When the RIs calculated for P and PTH were examined, it was found that the LRI and URI values of the female and male groups were consistent with the manufacturer's values. While the URI value of the RI calculated for 25(OH)D was compatible with the manufacturer's reference value for males and females, LRI values were deemed incompatible (Table 3).

When the acceptable limits of CLIA 19, the RIs calculated by the Hoffmann method, and the manufacturer's RIs were evaluated, it was discovered that the LRI and URI values for both age groups for Ca and Mg were consistent with the manufacturer's values. When the RIs calculated for P were examined, the LRI values of the female and male groups were considered compatible with the manufacturer's values, while the URI values were considered compatible for the female group and incompatible for the male group. While the LRI value of the RI calculated for 25(OH)D was compatible with the manufacturer's reference value for males and females, URI values were deemed incompatible. When the RIs calculated for PTH was examined, it was found that the LRI and URI values of the female and male groups were consistent with the manufacturer's values. (Table 4).

Table 1. Changes in sizes of laboratory information system (LIS) source dataset of five analytes after data cleaning.

Analyte	Changes in data size					Male-female ratio after cleaning			
	total data	data after exclusion criteria	%	post-Tukey data	%	male (n)	%	female (n)	%
Ca	350,377	62,002	17.7	59,627	17.0	21,812	36.6	37,815	63.4
Mg	485,849	49,617	10.3	47,541	10.9	11,939	25.1	36,602	76.9
P	298,228	31,549	10.6	30,643	10.3	8,297	27.0	22,346	73.0
25(OH)D	350,375	34,560	9.9	32,862	9.4	7,374	22.4	25,488	77.6
PTH	35,485	28,693	80.9	25,108	70.7	6,306	25.1	18,802	74.9
Total	1,520,314	206,421	13.6	195,781	12.8	55,728	28.5	140,053	71.5

Ca - Calcium, Mg - Magnesium, P - Phosphate, 25(OH)D - 25-Hydroxyvitamin D, PTH - Parathyroid hormone.

Table 2. Comparison of Bhattacharya and Hoffmann method's reference intervals (RIs).

			Bhattacharya		Hoffmann		Difference % between Bhattacharya and Hoffmann RI *	
		n	LRI	URI	LRI	URI	LRI	URI
Ca (mg/dL)	18 - 59 years	41,949	8.65	10.07	8.43	10.06	0.22 *	0.01 *
	60 - 89 years	17,678	8.80	10.23	8.43	10.06	0.37 *	0.17 *
Mg (mg/dL)	18 - 59 years	33,874	1.60	2.62	1.71	2.53	6.8%	3.4%
	60 - 89 years	14,883	1.69	2.47	1.64	2.55	2.9%	3.2%
P (mg/dL)	female	22,346	2.77	4.82	2.60	4.89	6.1%	1.4%
	male	8,297	2.49	4.45	2.69	5.30	8.0%	19.1% ¥
25(OH)D (µg/L)	female	25,488	10.92	51.05	8.66	41.33	20.6%	19.0%
	male	7,374	11.68	51.81	8.66	41.33	25.8% ¥	20.2%
PTH (ng/L)	female	18,802	13.07	73.03	10.56	65.44	19.2%	10.3%
	male	6,306	17.83	77.63	10.56	65.44	40.7% ¥	15.7%

Ca - Calcium, Mg - Magnesium, P - Phosphate, 25(OH)D - 25-Hydroxyvitamin D, PTH - Parathyroid hormone, LRL - Lower reference limit, URL - Upper reference limit.
*** - Absolute difference.**
¥ - Exceeding acceptable limits.

DISCUSSION

Our study was conducted in Istanbul's largest regional laboratory. As far as we know, this is the first RI comparison study to include tests related to calcium metabolism to date. The exclusion criteria were similar to previous multicenter RI studies in terms of initial data size [14-16].

The choice of healthy individuals is one of the most important steps in the RI determination process. In our study, we applied exclusion criteria to determine RIs by using the Bhattacharya and Hoffmann methods to reduce the disease prevalence of the data obtained from LIS. Thus, 86.4% of the data received at the beginning of the study were excluded. In the study by Özarda et al. [14], this rate was 60%. We believe that the reasons for the high number of excluded patients were the high number of repeated admissions in the primary healthcare institution and the inclusion of the first admission data of these patients in the study.

Following the grouping, we applied the Tukey method, which is used to remove extreme values. Thus, we considered avoiding the extreme elimination of physiologically high levels as well as some truly normal 'abnormal

Table 3. Comparison of Bhattacharya method's reference intervals (RIs) with RIs reported by manufacturer.

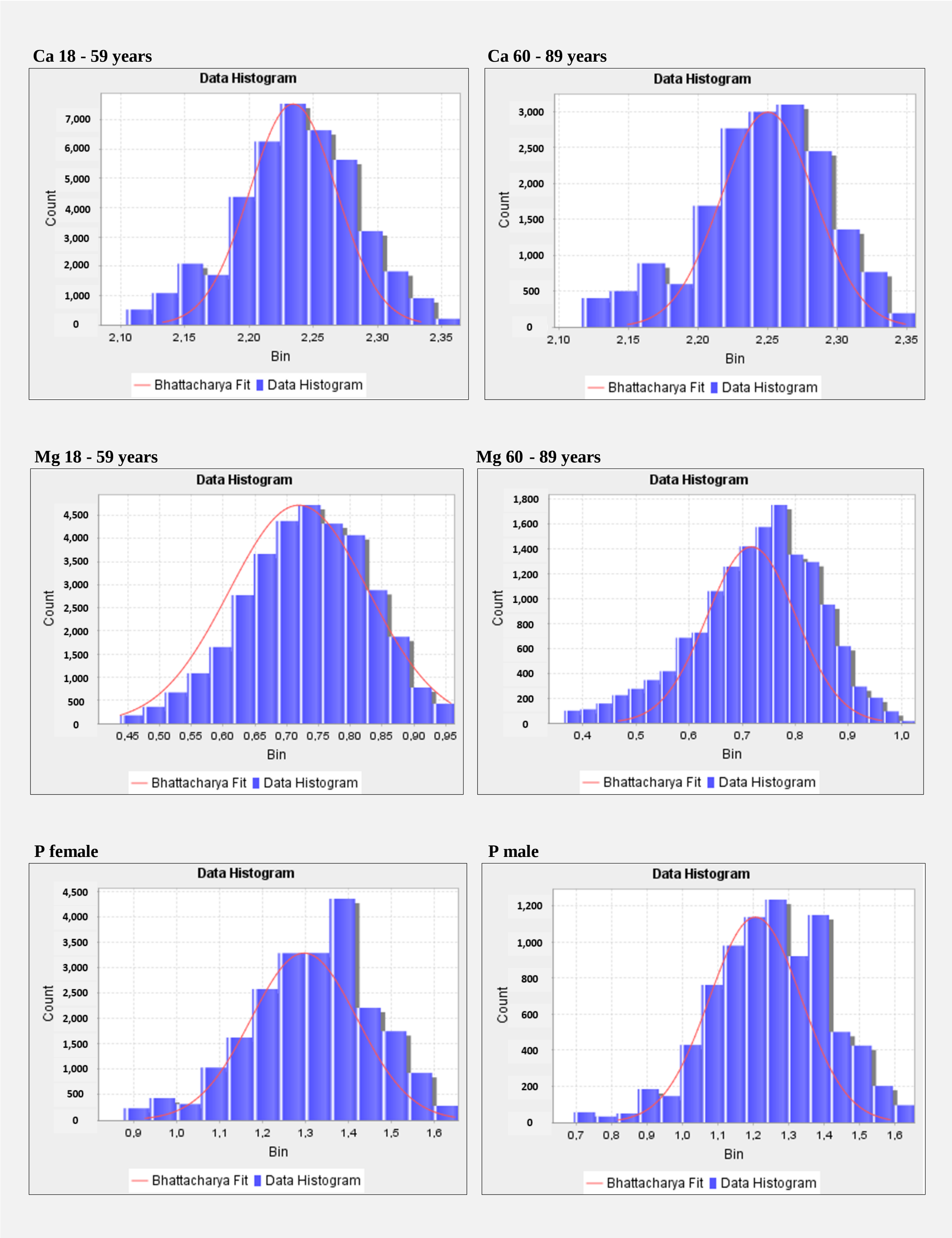
			Bhattacharya		Manufacturer RI		Difference	
		n	LRI	URI	LRI	URI	LRI	URI
Ca (mg/dL)	18 - 59 years	41,949	8.65	10.07	8.6	10.0	0.05 * (mg/dL)	0.07 * (mg/dL)
	60 - 89 years	17,678	8.80	10.23	8.8	10.2	0 * (mg/dL)	0.03 * (mg/dL)
Mg (mg/dL)	18 - 59 years	33,874	1.60	2.62	1.6	2.6	1.2%	0.7%
	60 - 89 years	14,883	1.69	2.47	1.7	2.3	0.5%	7.0%
P (mg/dL)	female	22,346	2.77	4.82	2.6	4.5	6.0%	7.1%
	male	8,297	2.49	4.45	2.6	4.5	4.2%	1.1%
25(OH)D (µg/L)	female	25,488	10.92	51.05	7.61	55.5	43.4% ¥	8.0%
	male	7,374	11.68	51.81	7.61	55.5	53.4% ¥	6.6%
PTH (ng/L)	female	18,802	13.07	73.03	15	65	12.8%	12.3%
	male	6,306	17.83	77.63	15	65	18.8%	19.4%

Ca - Calcium, Mg - Magnesium, P - phosphate, 25(OH)D - 25-Hydroxyvitamin D, PTH - Parathyroid hormone, LRL - Lower reference limit, URL - Upper reference limit.
*** - Absolute difference.**
¥ - Exceeding acceptable limits.

Table 4. Comparison of Hoffmann method's reference intervals (RIs) with RIs reported by manufacturer.

			Hoffmann		Manufacturer RI		Difference	
		n	LRI	URI	LRI	URI	LRI	URI
Ca (mg/dL)	18 - 59 years	41,949	8.43	10.06	8.6	10.0	0.17 * (mg/dL)	0.06 * (mg/dL)
	60 - 89 years	17,678	8.43	10.06	8.8	10.2	0.17 * (mg/dL)	0.04 * (mg/dL)
Mg (mg/dL)	18 - 59 years	33,874	1.71	2.53	1.6	2.6	6.8%	2.6%
	60 - 89 years	14,883	1.64	2.55	1.7	2.3	3.5%	10.8%
P (mg/dL)	female	22,346	2.60	4.89	2.6	4.5	0.0%	8.6%
	male	8,297	2.69	5.30	2.6	4.5	2.3%	17.7% ¥
25(OH)D (µg/L)	female	25,488	8.66	41.33	7.61	55.5	13.7%	25.4% ¥
	male	7,374	8.66	41.33	7.61	55.5	13.7%	25.4% ¥
PTH (ng/L)	female	18,802	10.56	65.44	15	65	29.6%	0.6%
	male	6,306	10.56	65.44	15	65	29.6%	0.6%

Ca - Calcium, Mg - Magnesium, P - Phosphate, 25(OH)D - 25-Hydroxyvitamin D, PTH - Parathyroid hormone, LRL - Lower reference limit, URL - Upper reference limit.
*** - Absolute difference.**
¥ - Exceeding acceptable limits.


Ca 18 - 59 years
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Mg 18 - 59 years
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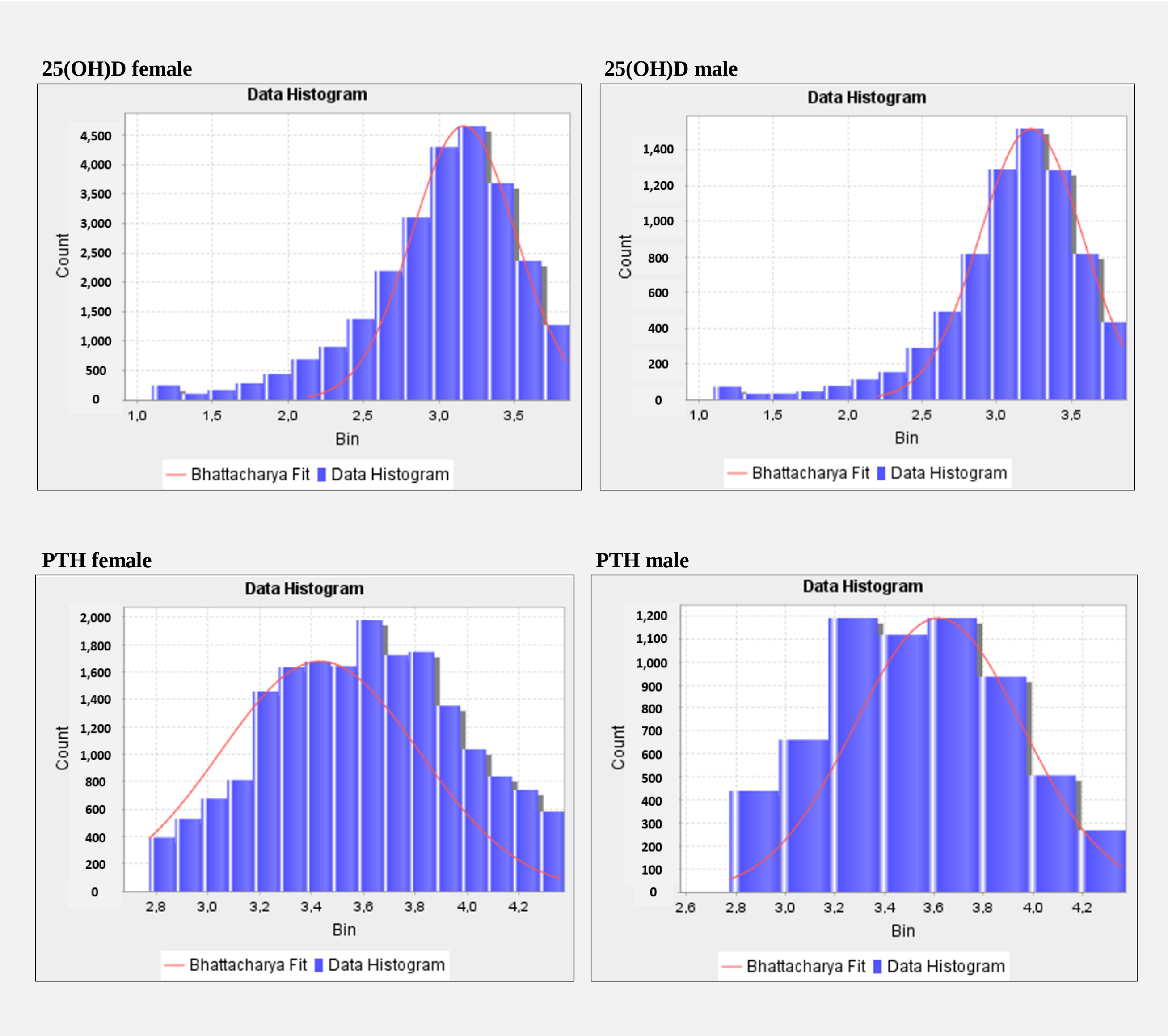

Figure 1. The frequency histograms of the log transformed calcium (Ca), magnesium (Mg), phosphate (P), 25-hydroxyvitamin D (25(OH)D), and parathyroid hormone (PTH) concentrations.

values' that tend to decline with age. The high proportion of females in the patient group (81.5% female, 28.5% male) included in the study was similar to the previous study [14].

In comparison studies, it is recommended to evaluate data grouping in terms of the effects of easily identifiable cofactors such as age and gender [3]. In our study, we created a group of females and males in the 18 - 89 age group by taking the manufacturer's RI group as an example. No female/male grouping was performed for Ca and Mg. In terms of age, the group was divided into two groups: 18 - 59 years and 60 - 89 years. While there was no female-male grouping for Ca and Mg in a previous study [15], the RI results of the female-male group were found to be the same in another study [17]. In our study, the RI value above the age of 60 was found to be higher when the Bhattacharya method was applied, compared to the 18 - 59 age group. In previous studies, it was stated that Ca decreases with age, but this is not the case over the age of 70 [15,31]. We believe that the Ca RI value is high, because patients up to 90 years of age are included in our study.

Although some studies show that PTH and 25(OH)D increase with age [17-21], there are also studies showing that there is no significant difference according to age in some studies [22,23]. In our study, we included the 18 - 89 age group in the same group. Although the debates about whether PTH RI should be classified by age [18,24-27] are still ongoing, most studies have reported that older people have higher PTH concentrations than middle-aged individuals. We believe that a large sample of all age groups and appropriate statistical criteria are required to reach a reliable conclusion in this regard.

In their study, Gong et al. [16] found no difference between 25(OH)D and PTH RI by gender, as did our results. In other studies, conducted in recent years, it has been suggested that there is no need for gender division [17,24]. There are studies advocating gender grouping for 25(OH)D [22,23]. The effect of gender on PTH is still controversial. Males had considerably lower PTH levels than females in all age groups. This is consistent with other previous studies showing higher serum 25(OH)D and Ca levels in males compared to females [29,30]. There is a study stating that males have high PTH levels [31] and another study stating that PTH concentration is not affected by gender [18]. This is probably because the amount of exposure to the sun is different and other factors, such as clothing, that may have affected sun exposure, time spent outside, use of sunscreen or umbrella, and skin color differ between the gender [27].

When the Bhattacharya and Hoffmann methods were applied, Ca and Mg RI values were found to be similar and consistent with the manufacturer's values, and the results of our study were found to be similar to previous studies [15,39]. In line with these results, we concluded that the Ca RI value did not differ directly and indirectly, and did not change much regionally, geographically, and temporally.

The World Health Organization (WHO) also defined 25(OH)D levels below 20 ng/mL as ‘insufficiency’ and levels below 10 ng/mL as ‘deficiency’ [32]. Pediatric Endocrine Association (PES), Endocrine Society (ES), and American Institute of Medicine (IOM) organizations have adopted different 25(OH)D insufficiency and deficiency limit values, ranging from 10 ng/mL to 30 ng/mL [33-35]. In our laboratory, the cut-off value was accepted as 20 ng/mL, and RI was used as 20 - 80 ng/mL. According to this reference interval value, the LRI and URI results obtained by the Bhattacharya and Hoffmann methods remain very low and appear to be incompatible. Although D hypovitaminosis was reported to be lower in the younger age group, many studies reported that 25(OH)D levels were affected by factors such as geographical location, age, gender, race, dressing, diet, cultural habits, physical activity, and inadequate sunlight exposure [36,37].

The manufacturer's RI recommendation for the 25(OH) D test was 7.61 - 55.5 ng/mL. While the LRI was found to be higher in the Bhattacharya method compared to the manufacturer’s RI, it was found to be similar in the Hoffmann method (Tables 3 and 4). The URI values of the Bhattacharya method were found to be similar to the manufacturer’s RI, while the Hoffmann method was found to be lower. Compared to previous studies conducted in Turkey, our RI values found with both methods were found to be higher [22,23]. We believe that this may be due to the non-parametric method used to determine RI [22,23] or the increased use of vitamin D as a supplement in society during the pandemic. A review conducted in India states that there has been a global upward trend in serum 25(OH)D levels over the past few years due to the increased use of common supplements [38].

In the Bhattacharya method, the PTH LRI value was found to be the same as the manufacturer's value, while the URI was higher. In the Hoffmann method, while the LRI was found to be lower, the URI values were found to be the same. These results seem to be consistent with previous studies conducted in Turkey [39]. In a non-parametric study, the LRI value was found to be high, while the URI values were similar [16].

Limitations

The findings of this study should be examined in line with some limitations. Although individuals were carefully and comprehensively selected by using exclusion criteria and statistical methods, potentially ill individuals may also have been included in the study group. We could have also determined the seasonal effects of 25(OH)D, in particular by setting the data time interval to 1 year. Age grouping could have been preferred when determining PTH RI.

CONCLUSION

We found that the Ca, Mg, P, and PTH RI results, which we calculated by using two different indirect methods and by using 10-month data from our laboratory, were acceptable/compatible with the manufacturer’s RIs using CLIA 19 acceptable criteria. The fact that 25(OH)D RIs were found to be high compared to the manufacturer's RI and previous studies may be due to the increased intake of supplements or drugs due to the pandemic, therefore it was concluded that RIs should be redetermined in certain periods. We believe that it may be important for each laboratory to determine its own RIs for PTH according to age groups in terms of correct interpretation of test results.

Ethics Approval:

This study was approved by the Ethics Committee Istanbul Kanuni Sultan Süleyman Training and Research Hospital/2022.12.240.

Declaration of Interest:

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. The article has not been published anywhere and has not been submitted for publication.

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