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Establishment of Reference Values of Serum PTH in a Middle-Eastern Healthy Population Using 2nd and 3rd Generation PTH Assays

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Abstract

Background: The purpose of the current study is to determine PTH reference values in vitamin D replete Lebanese adults using a 2nd and 3rd generation of PTH assays, and to look at the factors that affect PTH variations.

Methods: Fasting PTH was measured using 2nd and 3rd generation Diasorin PTH assays in 339 vitamin D replete healthy subjects aged 18 to 63 years (230 men and 109 women) who have normal calcium levels and an eGFR ≥ 60 ml/mn. 25(OH) vitamin D (25(OH)D) was measured using the Diasorin assay.

Results: For the 2nd PTH generation, median (IQR) levels were 48.9 [34.9-66.0] pg/ml and its 2.5th-97.5th percentile values were 19.7-110.5 pg/ml for 25(OH)D values between 20 ng/ml and 30 ng/ml, and 19.7-110.7 pg/ml for 25(OH)D values ≥ 30 ng/ml. For the 3rd PTH generation the median (IQR) values were 23.9 [17.7-30.5] pg/ml and its 2.5th-97.5th percentile values were respectively 9.2 and 50.2 pg/ml for 25(OH)D values between 20 ng/ml and 30 ng/ml, and 8.4 and 45.4 pg/ml for 25(OH)D values ≥ 30 ng/ml. The median (IQR) serum 25(OH)D levels were 27.5 [23.8-32.7] ng/ml. 2nd and 3rd generation PTH values are strongly correlated (r=0.96, p value <0.0001), but poorly concordant (Lin's concordance coefficient 0.365, 95% CI: 0.328-0.401) with observations beyond the 95% Bland-Altman limits of agreement. 2nd and 3rd generation PTH levels did not differ according to gender, and were significantly correlated with age but not with 25(OH)D and serum calcium levels.

Conclusion: Lebanese adult healthy subjects have higher 2nd and 3rd generation PTH levels compared to the reference range provided by the manufacturer. The reference range was not influenced by changing 25(OH)D cut-off the clinical significance of the higher PTH levels in our population should be investigated.

Keywords: Second and third generation PTH; PTH reference; Middle-East

Introduction

In clinical practice, assessing Parathyroid Hormone (PTH) concentration is important in exploring calcium/phosphorus metabolism disorders and in monitoring patients suffering from chronic kidney disease. Unfortunately, this task is complex despite the advent of automated laboratory assays. In fact, there are considerable variations in PTH values obtained from different assays, even when provided by the same manufacturer [1,2]. This variability is mainly related to the assay measurement of different PTH fragments. Older PTH assays, called second (2nd)-generation assays or "intact" PTH are known to measure not only the 84 amino acids molecule but also to cross-react with a truncated PTH fragment called the 7-84 PTH, whereas the more recently introduced assays, named the third (3rd)-generation assays, do not measure this truncated fragment, but may cross-react with another fragment called amino-PTH [3]. This difference explains the higher PTH values obtained with 2nd generation assays compared to the 3rd generation ones in healthy subjects [4,5] as well as in subjects with chronic renal failure [4,6-7].

Establishing a normal reference range for PTH is usually based on the values measured in 95% of healthy individuals after ruling out potential confounding factors since the PTH level is affected by multiple factors, such as vitamin D status, age and renal function [1,8-10]. Therefore, vitamin D deficiency as well as renal failure, both conditions leading to secondary hyperparathyroidism, should be ruled out or taken into consideration when establishing a reference range of PTH [11]. Other interfering factors such as calcium/phosphorus disorders should be also excluded [12]. Reference values for PTH are available in European [13,14], United States [15] and Chinese populations [16]. However, normative PTH values are lacking in the Middle-East, more particularly in Lebanon, a part of the world known for its high prevalence of vitamin D deficiency [17,18]. In addition, reference values were mainly established using either 2nd or 3rd generation PTH assays. Few studies compared both assays in healthy adults [4,5,19], and included only a small number of subjects [4,19]. It is thus unclear if there is a difference in normative values between both assays.

The purpose of the current study is first to determine PTH reference values in Lebanese adults who are vitamin D replete and have a normal renal function using both 2nd and 3rd generation PTH assays; second, to identify factors that may affect PTH variations.

Materials and Methods

Patients

The study included a total of 339 subjects (230 women and 109 men) aged 18 to 63 years, who presented at the Laboratory of Hôtel-Dieu de France Hospital (one of the biggest university hospitals in the Beirut area) for a routine biochemistry evaluation including calcium, creatinine and 25 hydroxy vitamin D (25(OH)D) measurements. Inclusion criteria were: normal serum calcium (values between 2.10 and 2.56 mmol/L), estimated glomerular filtration rate (eGFR) ≥ 60 mL/min/1.73 m^2 and 25(OH)D levels ≥ 20 ng/ml. The 20 ng/ml cut-off value supported by the Institute of Medicine (IOM) report was used to define optimal vitamin D status [20].

Biochemical measurements

Morning fasting blood specimens were collected in dry tubes then centrifuged within 30 min after venipuncture and analyzed the day of sampling for calcium, creatinine and 25(OH)D measurements. Part of the serum was immediately frozen and stored at -20°C for later PTH measurements. Only samples with a 25(OH)D ≥ 20 ng/ml were subsequently analyzed within one month for both 2nd and 3rd PTH measurements. Measurements of total calcium and creatinine levels were performed using a Vitros 5.1 FS automate (Ortho-Clinical Diagnostics, Inc. Raritan, New Jersey). The eGFR was assessed using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [21].

	Overall sample N=341	Men N=110	Women N=231	Test	p value
Age (years)	43.6 ± 11.8	45.7 ± 12.2	42.5 ± 11.5	T	0.021
Creatinine (µmol/L)	64.3 ± 13.1	76.2 ± 12.6	58.6 ± 9.0	T	<0.001
eGFR (ml/mn)	105.4 ± 13.7	102.5 ± 13.9	106.7 ± 13.4	T	0.008
Calcium (mmol/L)	2.40 ± 0.08	2.42 ± 0.07	2.40 ± 0.08	T	0.047
PTH 2nd G (pg/mL)*	48.9 [34.9-66.0]	46.5 [37.8-66]	51.2 [33.5-68.0]	U	0.788
PTH 3rd G (pg/mL)*	23.9 [17.6-30.5]	23.1 [18.6-30.6]	24.5 [17.2-30.4]	U	0.798
25(OH) D (ng/mL)*	27.5 [23.9-32.6]	27.2 [24-32.6]	27.6 [23.8-32.8]	U	0.84

Data are expressed as mean ± SD or median and its interquartile range (Q1-Q3) PTH and 25(OH)D. (*) denotes variables with a significant departure of normality as detected by Kolmogorov-Smirnov and Shapiro-Wilk test and inspected graphically by Quartile-Quartile plots. T test: Independent samples T test; U test: Mann-Whitney U test

Table 1: Baseline clinical and biological characteristics of the overall sample, men and women.

25(OH)D and PTH assays

PTH and 25(OH)D measurements were done in batches on the LIAISON XL (DiaSorin, Stillwater, MN, USA). Serum 25(OH)D was measured using a direct competitive chemiluminescence immunoassay (CLIA). The observed reference range is 9.3-47.9 ng/ml. The lowest reported value is 4 ng/mL and the inter assay coefficient of variation (CV) <20%. The DiaSorin Liaison PTH 2nd and 3rd generation assays were used to measure PTH values. The PTH 2nd generation assay is a modified 2-step; 2-site sandwich assay using 2 polyclonal antibodies to detect intact PTH, the expected range provided by the kit is comprised between 14.5 and 87.1 pg/ml corresponding to the 2.5th and the 97.5th percentiles. The DiaSorin Liaison PTH 3rd generation or 1-84 PTH assay is also a 2-step, 2-site sandwich assay that uses a first antibody that is highly specific for the N terminus of 1-84 PTH to ensure no cross reactivity with fragments such as 7-84 PTH while the second polyclonal antibody is targeted against the C-terminal region of the 1-84 molecule. The expected reference range provided by the kit is comprised between 6.5 and 36.8 pg/ml. Inter assay CV was less than 15% for both PTH assays.

Percentiles	2nd generation PTH (pg/mL)		3rd generation PTH (pg/mL)	
	20<25(OH)D<30 (ng/mL)	25(OH)D ≥ 30 (ng/mL)	20<25(OH)D<30 (ng/mL)	25(OH)D ≥ 30 (ng/mL)
2.5	19.7	19.7	9.1	8.4
5	23.2	24.9	9.9	12.6
10	28	28.2	13.2	13.4
25	35.5	33.7	17.6	17.7
50	50.7	48.4	24	23.5
75	66	67.5	30.5	30.5
90	88.1	84.8	38.3	37
95	103	92.3	44	41.7
97.5	110.5	110.7	50.2	45.4
p value (U test)	0.696		0.917	
U test: Mann-Whitney U test				

Table 2: Distributions of 2nd generation PTH and 3rd generation PTH according to dichotomized 25(OH) vitamin D values (between 20 and 30 ng/ml and ≥ 30 ng/ml respectively).

Statistical analysis

The distribution of 2nd and 3rd generation PTH values was checked using Kolmogorov-Smirnov (KS) and Shapiro-Wilk (SW) tests with additional visual inspection of quartile-quartile (Q-Q) plots. Logarithmic transformation (natural logarithm) was applied to 2nd and 3rd generation PTH values (labeled Ln (PTH 2G) and Ln (PTH 3G) respectively. The native variables with skewed distribution were expressed as median with its interquartile range (Quartile 1-Quartile 3) (Table 1) and Percentiles 2.5% and 97.5% (Table 2). For the

transformed variables satisfying normality assumptions, the mean and the standard deviation were calculated.

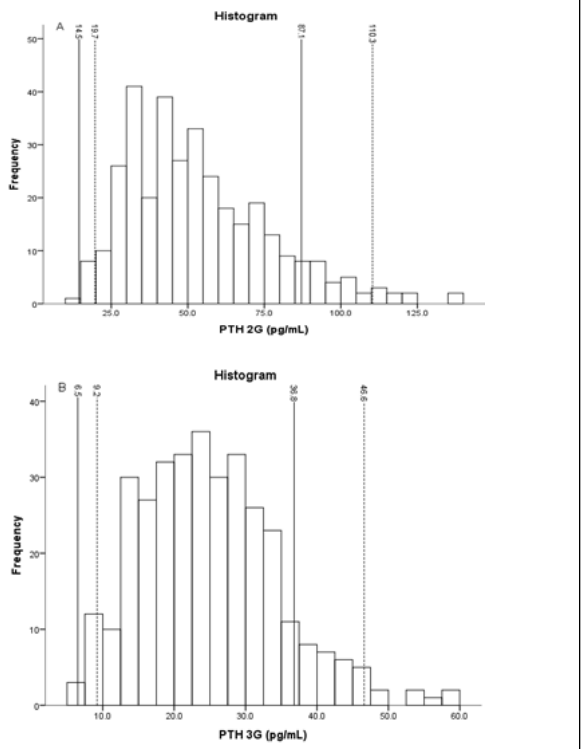

Figure 1: Histograms showing the distribution of 2nd generation PTH values and (A) 3rd generation PTH values (B) Uninterrupted vertical lines represent percentiles 2.5% and 97.5% as provided by the manufacturer. Dashed vertical lines represent actual percentiles 2.5% and 97.5% from the current series.

Correlation between Ln (PTH 2G) and Ln (PTH 3G) was estimated using Pearson correlation coefficient, and its 95% confidence interval was calculated by bootstrapping performed on 1000 samples. R^2 was also calculated. Agreement between Ln (PTH 2G) and Ln (PTH 3G) was studied using Bland-Altman method, and Lin's concordance correlation coefficient was calculated using a macro developed by M. Garcia-Granero (http://gjyp.nl/marta/Lin.sps) and providing asymptotic 95% confidence interval. The distributions of 2nd and 3rd generation PTH values were compared for 25(OH)D values between 20 and 30 ng/ml and values ≥ 30 ng/ml using Mann-Whitney U test. Percentiles 2.5% and 97.5% of PTH 2G and PTH 3G of the current study were further compared with expected values provided with the kits by the manufacturers, respectively, using 95% bias corrected accelerated (BCa) confidence intervals built by bootstrapping on 1000 samples. Correlation between 2nd and 3rd generation PTH values and 25(OH)D, serum calcium levels and CKD EPI eGFR values relied on Spearman's correlation coefficient and its BCa 95% confidence interval. Kappa statistic for agreement between nominal variables was also calculated. The statistical analysis was performed using IBM SPSS (IBM Corp.; SPSS Statistics for Windows v22.0, Armonk, NY, USA).

Results

The baseline characteristics of the sample are shown in Table 1. Female subjects were slightly younger than males (42.5 ± 11.5 vs. 45.7 ± 12.2 years, p=0.021), with a lower serum creatinine (58.6 ± 9.0 µmol/L vs. 76.2 ± 12.6 µmol/L, p<0.001), and marginally higher eGFR (106.7 ± 13.4 vs. 102.5 ± 13.9 mL/min/1.73 m^2, p=0.008) that disappeared after adjusting for age (p=0.135).

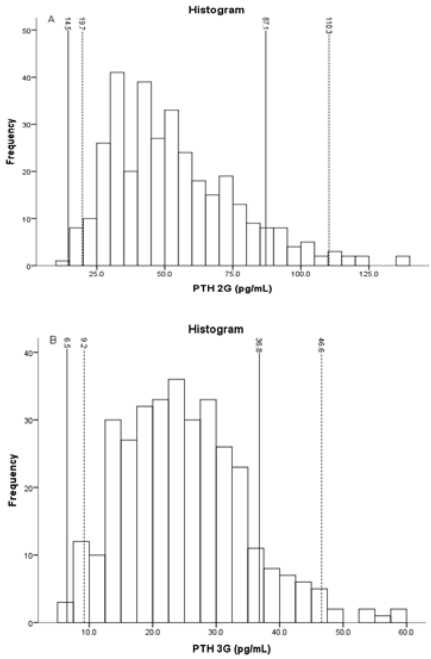

Figure 2: Histograms showing the distribution of log transformed values of 2nd generation PTH values and (A) 3rd generation PTH values (B) Dashed lines represent a superimposed normal distribution.

25(OH)D and PTH measurements and reference range of serum PTH concentrations

The median serum 25(OH)D value was 27.5 [23.8-32.7] ng/ml with no significant difference according to gender (p=0.840). 64.6% of the subjects had 25(OH)D values between 20 and 30 ng/ml and 35.6% had 25(OH)D values ≥ 30 ng/ml. 2nd and 3rd generation PTH showed a significant departure from normality and a right-skewed distributions (Figure 1). Therefore, their natural logarithm transforms (Ln (PTH 2G) and Ln (PTH 3G)), which satisfied normality assumptions, were used in subsequent analysis (Figure 2).

The medians of 2nd and 3rd generation PTH assays were 48.9 [34.9-66.0] pg/ml and 23.9 [17.7-30.5] pg/ml respectively (Table 1). The percentiles 2.5% and 97.5% for 2nd and 3rd generation PTH were [19.7-110.5] pg/ml and [9.2-46.6] pg/ml respectively (Table 2) The distributions of 2nd and 3rd generation PTH according to dichotomized 25(OH)D values (between 20 and 30 ng/ml and ≥ 30 ng/ml), showed no statistical difference between both groups (p=0.696 and p=0.917 respectively) (Table 2). When compared to the reference 97.5% percentile values provided by the manufacturer, respectively 10.0% and 11.2% of the subjects had their 2nd and 3rd generation PTH

value beyond the reference percentile (respective 95% CI: 7.4%-13.0% and 8.1%-14.4%), both comparable but significantly different from the threshold specified by the manufacturer. Out of the 34 subjects with 2nd generation PTH above the reference 97.5% percentile, 4 (11.8%) had normal 3rd generation PTH values. Conversely, out of the 38 subjects with 3rd generation PTH above the reference 97.5% percentile, 8 (21.1%) had normal 2nd generation PTH values (Kappa measure of agreement=0.814 ± 0.052), corroborating the Bland-Altman method findings (see below). None of the subjects had both 2nd and 3rd generation PTH values below the 2.5% percentile reference values.

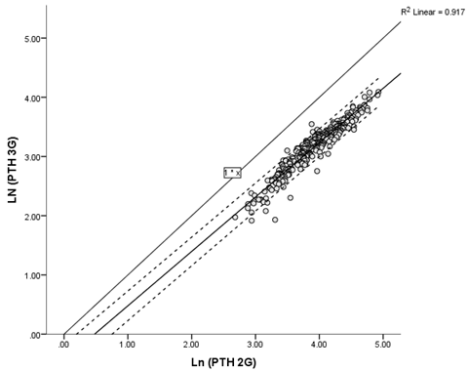

Figure 3: Scatterplot showing the correlation between log (2nd generation PTH) [Ln (PTH 2G)] values and log (3rd generation PTH)) [Ln (PTH 3G)] values. The dashed lines represent 95% confidence limits for correlation. The (1*x) line represents the line of perfect concordance (that is, y=x, where Lin's coefficient equals 1).

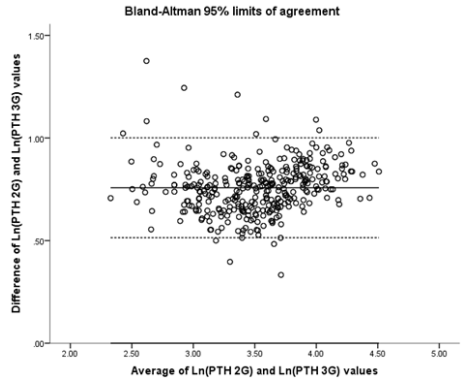

Figure 4: Bland-Altman plot showing the agreement between log (2nd generation PTH) [Ln (PTH 2G)] values and log (3rd generation PTH) [Ln (PTH 3G)] values. Central horizontal line represents the mean bias. The dashed horizontal lines represent 95% limits of agreement.

While the Pearson correlation coefficient between Ln (PTH 2G) and Ln (PTH 3G) was high, reaching 0.957 (95% CI: 0.945-0.968, p<0.0001) as shown in Figure 3, the agreement between Ln (PTH 2G) and Ln (PTH 3G) was low, since Lin's concordance correlation coefficient was only 0.365 (95% CI: 0.328-0.401). The Bland-Altman graphical analysis is consistent with these findings since a significant number of pairs of observations are beyond 95% limits of agreement (Figure 4), with a mean bias of 0.758 on the logarithmic scale (2.13 pg/ml on natural scale).

Relationship of serum PTH level to gender and age

No significant differences in 2nd and 3rd generation PTH levels were observed according to gender (p=0.788 and p=0.798 respectively, Table 1). There was a significant correlation between 2nd generation PTH levels and age (Pearson correlation coefficient for age and Ln (PTH 2G)=0.270 (95% CI: 0.170-0.370, p<0.001), as well as between 3rd generation PTH levels and age (Pearson correlation coefficient for age and Ln (PTH 3G)=0.267 (95% CI: 0.161-0.373, p<0.001).

Relationship of serum PTH level with 25 (OH) D and other biological parameters

There was no significant correlation between 2nd generation PTH levels and 25(OH)D (Spearman's correlation coefficient=-0.030, 95% CI: -0.137 – 0.073, p=0.584), nor between 3rd generation PTH levels and 25(OH)D (Spearman's correlation coefficient-0.002, 95% CI: -0.108 – 0.094, p=0.968).

Likewise, there was no significant correlation between 2nd generation PTH levels and serum calcium levels (Spearman's correlation coefficient=-0.087, 95% CI: -0.192 – 0.024, p=0.109), nor between 3rd generation PTH levels and serum calcium levels (Spearman's correlation coefficient=-0.094, 95% CI: -0.199 – 0.019, p=0.085). There was a weak but statistically significant inverse correlation between CKD-EPI eGFR levels and both 2nd and 3rd generation PTH levels (Spearman's correlation coefficients were -0.107, 95% CI: -0.207-0.001, p=0.049 and -0.111, 95% CI: -0.205 – 0.002, p=0.042 respectively).

Discussion

In this study, we established the reference ranges for PTH in 339 Lebanese subjects using the Diasorin 2nd and 3rd generation PTH assays. These reference ranges were established in vitamin D replete subjects (25(OH)D ≥ 20 ng/ml) after excluding subjects with abnormal calcium levels and low eGFR. We found that the 97.5% percentiles for 2nd and 3rd generation PTH are respectively 110.5 and 46.6 pg/ml, that is, 26.8% higher than the upper limit of normal (ULN) reference values of 87.1 and 36.8 pg/ml provided by the manufacturer.

Different authors evaluated the reference values for PTH in vitamin D replete subjects. As an example, using the Cobas/Elecsys Roche 2nd generation PTH kit, Cavalier et al. [22] found in 240 healthy Belgian subjects, an ULN PTH value of 50 pg/ml, which is lower than the 65 pg/ml value provided by the manufacturer. Similarly, using the same assay Touvier et al. [10] found, that the plasma PTH ULN is of 45.3 pg/ml in 1824 French adults recruited from the SUVIMAX study. At the opposite, two other studies found higher ULN reference values with the same assay. The first one [8] was performed on a Danish population composed of 2316 women, and reported an ULN of 67 pg/ml, while in the second one [16], performed on 1436 healthy Chinese subjects, the ULN reference value was 70 pg/ml. Moreover, using the 3rd PTH Diasorin assay, Souberbielle [13] found in 898 healthy French adults that the ULN value was 28.9 pg/ml, a value that is also lower than the value provided by the manufacturer. Finally, in a United States (US) study [15], the authors compared the performance

characteristics of six 2nd PTH assays and found that the ULN values of their population are comparable or higher than the one established by the manufacturers. The differences between the French and Belgian studies [10,13,22] on one side and the other studies on the other is unclear. Since age is a significant determinant of PTH, that difference could be explained by the wider age range of the Danish study [8] (which includes women aged up to 84) compared to the French studies [10,13] in which none or very few of the included subjects were older than 65. Other factors such as a later daytime and a non-fasting blood collection could also contribute to the higher PTH levels in the Danish study. Finally, ethnic differences could explain the higher PTH values observed in the Chinese study [16] as well as in the present study. In our opinion, it is possible that the higher ULN reference values observed in our sample as compared to western countries, is related to the higher prevalence of vitamin D deficiency [17,18]. Consequently, PTH ULN reference values seem to differ between different countries, with the lowest reference values being observed in Belgium and France.

When comparing the 2 different PTH generation assays in our sample, a strong positive correlation was noted, but the agreement between them was low according to Lin's concordance correlation coefficient, thus suggesting that the assays are not interchangeable. Few studies compared the two PTH generations in the same sample: in French healthy subjects [4] PTH concentration did not differ according to the generation of the used kits, except for an overestimation with the 2nd generation assays for values beyond 200 pg/ml. In another study [5], there was poor concordance between the methods; moreover, this worsened in the higher range of measurements.

Because PTH may be increased in patients with vitamin D deficiency and decreased in vitamin D replete subjects, exclusion of patients with vitamin D insufficiency from the reference population for PTH values is mandatory. Since the 25(OH)D cutoff that should be used (20 versus 30 ng/ml) is not clearly established, we compared the reference values based on both 25(OH)D cutoffs. We found that the PTH ULN did not differ no matter the used cutoff, corroborating the US study by La'ulu [15] in which the Diasorin assay was also used. The fact that no significant inverse relationship was found between 25(OH)D and PTH might be due to a vitamin D replete status in our population and suggests that a cutoff of 20 ng/ml might be enough to decrease PTH levels until it is stabilized. This does not exclude the possibility that more higher 25(OH)D levels are requested to ensure this decrease.

We finally searched for a possible relationship between PTH and both age and gender. Similarly to other studies [8] we found a positive relationship between age and PTH without any difference according to gender. Other studies found an increase in PTH values with age in both Turkish [23] and US populations [24]. In addition, in Turkish females, PTH values were higher than those in males [23] while other studies, similarly to ours, did not show this gender difference [10].

The reason why PTH is higher in our population compared to western population is unclear. Since the Lebanese population is at a high risk for vitamin D deficiency [17,18], it is possible that prolonged vitamin D deficiency leads to chronic secondary hyperparathyroidism and subsequently to higher levels of PTH values in normal healthy subjects. As a result, adopting different reference range values in our population may modify our therapeutic decisions. The obvious consequence of this finding is that in clinical practice much less patients will be detected as having normocalcemic primary hyperparathyroidism. Another possibility could be the presence of patients with normocalcemic primary hyperparathyroidism amongst our study population. Here, the only way to rule out early primary hyperparathyroidism would be the follow up of our subjects in time. Further follow-up of our study population might therefore be necessary to give a clearer answer.

One main limitation of this study is the lack of anthropometric and social information (body mass index (BMI), income level). This limits the interpretation of the results since obesity is known to increase PTH levels [10,25]. In addition, dietary vitamin D and calcium intakes and the use of vitamin D supplements were not recorded. However, the above mentioned factors seems to have little impact on the interpretation of the results, since, in a large cross-sectional Danish study, only 16% of the variability of PTH is explained by 25(OH)D, age, BMI and daily calcium intake [8,26,27].

Conclusion

A reference value for PTH was established for the first time in a sample of the Lebanese population using two different generations of PTH assays after excluding vitamin D deficient subjects. With both methods, the Lebanese subjects had higher PTH compared to the reference range provided by the manufacturer. We also confirmed that using a 25(OH)D cutoff of 20 ng/ml instead of 30 ng/ml did not change the reference range. Since several studies have indicated that PTH levels in the upper limit of the normative reference level have a harmful effect on cardiovascular risk and mortality the clinical significance of the higher PTH levels in our population should be further investigated.

Ethical issues

The study was approved by the Ethics Committee of our university hospital (CEHDF 1247).

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