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INFLUENCE OF SUBCLINICAL DEFICIENCIES AND GENDER ON THE CORRELATIONS OF PARATHYROID HORMONE WITH BONE MARKERS AND RELATED ANALYTES IN HEALTHY YOUNG ADULTS

Biochemistry

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ABSTRACT

Introduction: Subclinical deficiencies influence concentrations of related analytes and their gender differences. In this study, influence of common subclinical deficiencies on the correlations of parathyroid hormone with bone markers and their related analytes were evaluated.

Methods: Participants aged 18 to 25 years from rural, central Kerala, who were clinically examined and found to be healthy took part in this observational cross sectional study. They were further evaluated by quantitative biochemical analytes with cut off levels fixed to exclude common subclinical diseases states and deficiencies.

Results: Exclusion of clinical and subclinical disease states reduced sample number from about 600 to 142. There were more males excluded than females. Exclusion of all common deficiencies further reduced sample number from 142 to 40, during which the reduction in the number of females was much more marked than males. In the male sample, before exclusion of deficiencies, there were number of bone markers and related analytes correlating with PTH. But after exclusion of all deficiencies in the male sample, PTH positively correlated with osteocalcin and negatively correlated with bone alkaline phosphatase (Table 4), indicating that when PTH was increased, bone resorption and turnover were higher, and bone formation was low. In the female sample, even osteocalcin which was positively correlating with PTH before exclusion of deficiencies lost its correlation after exclusion.

Conclusion: Exclusion of deficiencies related to parathyroid hormone brought out the gender differences in the correlations of PTH with bone markers and related analytes, indicating gender differences in the metabolism of bone.

KEYWORDS

parathyroid hormone; osteocalcin, bone alkaline phosphatase, NTX, gender differences.

INTRODUCTION

The targets of intact parathyroid hormone (PTH) actions are predominantly on bone, intestine and kidney. PTH regulates plasma ionic calcium and phosphate concentrations directly through bone and kidney. PTH also acts indirectly, mediated through 1,25-dihydroxyvitamin D (1,25$(OH)_2$D), on the intestine and bone[1]. The regulators of PTH secretion are ionic calcium and phosphate. There is an inverse or negative sigmoidal relationship between PTH secretion and free ionic extracellular calcium when the latter is between 1.0 to 1.25 mmol/L ionic calcium[1,2]. Maximal slope of this plot is attained with mild hypocalcemia and mild hypercalcemia, respectively. PTH is directly related to erythropoietin, and inversely to haemoglobin and iron[3,4]. The functions of PTH on bone are complex, leading to both bone resorption and bone formation [5,6]. Earlier this laboratory had shown that deficiencies in haemoglobin, iron, ferritin and vitamin D influence the concentrations and gender differences in the concentrations of erythropoietin, PTH and related analytes[3,4]. The presence of these deficiencies also influenced the correlations and also gender differences in the correlations of erythropoietin and PTH with related analytes[3,4].

Bone markers which are direct products of bone, used in this study were NTX (N-terminal telopeptide cross links), osteocalcin and bone alkaline phosphatase. The bone marker-related analytes used in this study were vitamin D, serum total calcium and phosphate, urine calcium and phosphate. The bone marker-related deficiencies evaluated in this study were deficiencies of vitamin D, haemoglobin, ferritin and iron. NTX is an N-terminal products of degradation of type 1 collagen, the most abundant protein in bone. Their concentrations in plasma and urine reflect osteoclastic resorption function[7], and are, therefore, bone resorption, and turnover or remodelling markers. Bone alkaline phosphatase (ALP) is produced by the osteoblast during the proliferation and matrix maturation phase, when the newly formed collagenous matrix is prepared for the deposition of bone mineral[8]. Therefore, bone ALP, a marker of bone formation, is increased during growth phase (< 18 years) and during severe clinical vitamin D deficiencies. Osteocalcin, the most abundant non-collagenous protein in bone, is produced during bone mineralisation phase which is a part of bone formation. But more osteocalcin is released from bone during bone resorption and, therefore, osteocalcin is a bone resorption and turnover marker.

In this study, clinical and subclinical disease states were excluded to arrive at a sample population of healthy young adults aged between eighteen and twenty five years. This sample, partitioned according to age and gender, is referred to as the bone marker sample. Correlations of PTH to certain bone markers and their related analytes were assayed before and after exclusion of deficiencies in hemoglobin, iron, ferritin and vitamin D.

MATERIALS AND METHODS

Study settings and Clinical Case control

Healthy cross section of participants (n = 142) between 18 and 25 years of age, from the near sea level plains of rural Central Kerala, South India, avoiding mountainous regions of Western Ghats, took part in this observational cross sectional study. Study was approved by the Institutional Research and Ethics Committees (IEC. No.05/03/2010/ MCT. Dated 2/07/2010; AIMSIEC/01/2011 dated 9/3/2011 and AIMSIEC/07/2014 dated 31/01/2014). Clinical evaluation of volunteers in this study was done in six stages (Table 1; Stages I to VI of Reference 4) for exclusion of clinical and subclinical disease states and deficiencies, and for partitioning the sample. Volunteers of this study who gave informed oral consent underwent an evaluation by clinical history and examination for exclusion of individuals with disease states, injury, infection, inflammation, allergic reactions, diabetes, hypothyroidism, stressed states and hypertension and alcoholics. Participants included were on regular diet, exercise, rest, sleep, had no drugs for one week and all female participants were in the pre gestational period (Table 1; Stage I in Ref. 4).

Selection of Bone marker reference sample by exclusion of subclinical disease states and deficiencies by Clinical Biochemistry assays

Informed written consent was obtained from each participant who donated blood and urine samples (Table 1, Stage I, Step 2 in Ref. 4). Volunteers underwent Clinical Biochemistry laboratory evaluation for further exclusion of unhealthy individuals at the subclinical level (Stage II). Cut off values of quantitative analytes used as exclusion criteria were as follows: BMI >30 kg/m^2, waist circumference ≥100 cm, fasting glucose ≥126 mg/dl (7 mmol/l), 2 hour glucose challenged or postprandial glucose >180 mg/dl (10 mmol/l), serum triglyceride >200 mg/dl (2.26 mmol/l), serum alanine aminotransferase (ALT) >60 U/l, high sensitivity C reactive protein (hsCRP) >5 mg/l, serum creatinine 1.2 mg/dl (106.08 µmol/l) and total calcium 11 (2.75 mmol/l) (Stage II in Ref. 4).

Samples were selected after excluding growth phase at <18 years and influence of age at >25 years (18 to 25 years) (Stage III in Ref. 4), and after excluding certain bone marker-related analytes outside the following cut off levels: ferritin >250 ng/ml, osteocalcin >35 ng/ml, ostase (Bone alkaline phosphates) >30µg/l, urine NTx (N-terminal telopeptide) >200 nM BCE (bone collagen equivalents) / mmol urine creatinine, total calcium >11 mg/dl (2.75 mmol/l) (Table 1; Stage IV in Ref. 4). These stringent exclusion criteria reduced the sample number from over 600 to 142. After selection of the **Bone marker reference sample population** (n = 142) used in this study, they were again subjected to exclusion of deficiencies in haemoglobin (<125 g/l), iron (<9.85 µmol/l or 55 µg/dl) and ferritin (<20 ng/ml) (Table 1; Stage IV and V in this paper), and vitamin D <50 nmol/l (Stage V to VI), further reducing the sample size (n = 22 males and 18 females).

Unhealthy and higher cut off levels, such as those for BMI, waist circumference, postprandial glucose, triglycerides and others, were designed to include individuals with restricted variations but to rule out individuals with highly abnormal values such as obesity, postprandial glycosuria, high triglycerides and abnormal levels of other analytes.

Sample collection and Preparation

Blood samples were drawn without anticoagulants, after 10 to 12 hours of overnight fast and after two and half hours of waking up from sleep, between 8.00 and 9.00 in the morning. They were centrifuged immediately at 3000 rpm for 5 minutes in plastic tubes to sediment cells before clotting. Plasma was transferred to glass tubes for clotting and clot was separated by a second centrifugation. If clotting was observed after the first centrifugation, then the plasma was allowed to clot in the same tube and then centrifuged. This procedure reduced haemolysis and increased the yield of serum which was preferred over plasma for storage. Hemolysed, jaundiced and lipemic serum samples were excluded. Samples with clot particles were recentrifuged. All assays were done immediately after preparation of serum. Second or third sample of morning fasting urine was collected, centrifuged at 3000 rpm for 5 minutes and assayed immediately for NTx and urine creatinine.

Inter conversion of Units of variables

Inter conversion between SI units used in Tables 2 to 4 and conventional units are as follows: (Conventional unit) x (conversion factor) = SI unit. haemoglobin: (g/dl x 10) = g/l; glucose: (mg/dl x 0.0555) = mmol/l; Iron: (µg/dl x 0.179) = µmol/l; creatinine: (mg/dl x 88.4) = µmol/l; triglycerides: (mg/dl x 0.0113) = mmol/l; hsCRP: (mg/dl x 10) = mg/l; intact PTH: (pg/ml x 1.0) = ng/l; calcium (mg/dl x 0.25) = mmol/l; vitamin D (ng/ml x 2.496) = nmol/l; osteocalcin = (µg/L x 0.171) = nmol/L; ostase = µg/L; NTx = nmol BCE/mmol creatinine.

Assays and analytical control of assays

Immunochemistry autoanalyser Access 2 (Beckman Coulter, USA) and their reagents were used for intact PTH, ostase (or bone alkaline phosphatase) and ferritin assays using immunometric method with magnetic bead coated anti PTH or anti ferritin antibodies[9,10,11]. The chemistry autoanalyser Vitros 5,1 FS (Ortho Clinical Diagnostics, USA) and their reagents were used for assay of glucose, triglycerides, serum creatinine, total calcium, iron and hsCRP. Vitamin D and osteocalcin assay was done by Diasorin Liaison (Italy)[12]. Haemoglobin assay was done manually by Drabkin's method using colorimeter. There were twice a day uninterrupted internal quality control programs and once a month external quality assurance programs (Biorad, USA). Internal quality control data were analysed by Westgard rules for acceptance or rejection of analyte data[13]. If there was a rejection, appropriate measures were taken to set right errors, if any, in machine functioning, reagents, storage or analyte calibration levels. Interrupted internal quality control data from assays done on the days of intact PTH sample assay gave a mean±SD of 10.802±0.805 and coefficient of variation (CV) of 7.45%. External quality assurance program gave Z scores of below 1.0 in the months of intact PTH sample assays.

The lowest value of intact PTH was 9.5 ng/l and highest value was 80.5 ng/l for the data in this study. Limit of detection of intact PTH was taken as the lowest concentration distinguishable from zero (calibrator as 0 ng/l intact PTH) with 95% confidence was 1 ng/l (0.1 pmol/l) for PTH. It was also far below the lowest linear six point intact PTH calibrator value (eg. PTH: 10.7 ng/l). Examples each of the actual linear six point calibration values for intact PTH in ng/l from a particular lot of calibrators were 0, 10.7, 61.2, 303, 1467, 3369.5[9].

Reference Intervals of analytes related to intact PTH

Reference intervals used for the healthy limits in this study were PTH: 10 - 65 ng/L, Vitamin D: cut off level <20 ng/ml (<50 nmol/l), Total calcium: 8.4 – 10.2 mg/dl (2.10 – 2.55 mmol/l), Hemoglobin: male 133 – 162 g/l, female 120 – 158 g/l; Iron: 7 – 25 µmol/l (41 – 141 µg/dl); Ferritin: Male 29 – 250 ng/ml, Female 10 – 150 ng/ml[11,14,15]. The lowest PTH value was 9.5 ng/L and highest value was 80.5 ng/L for the data in this study. Reference interval of PTH (manufacturer's) was 12 – 88 ng/L (1.3 – 9.3 pmole/L)[9]. Reference interval of ostase (manufacturer's) was 2.59–18.50 U/l[10].

Statistical Analysis

Normality of distribution was estimated by Shapiro-Wilk test. Equality or homogeneity of variances of the groups compared was done by Levene's test. Statistical analysis and calculations were done with SPSS, version 23.0 software. Log_{10} transformations converted most of the positively skewed groups to Gaussian distribution. When at least one of the two correlating variables had Gaussian distribution, before or after transformation, parametric methods of Pearson's correlation was used[16,17].

RESULTS

Selection of PTH-Bone marker reference sample population

Healthy young adult bone marker reference sample population was selected by clinical exclusion criteria, followed by exclusion of quantitative biochemical analytes, to exclude subclinical disease states (Table 1 of Ref. 4) and deficiencies (Table 1 in this paper). During the process of exclusion of clinical and subclinical disease states, the number of samples reduced from approximately 600 to 142 (Male = 40, Female = 102). There were more male samples excluded than female samples. The range of BMI, waist circumference, fasting glucose, ALT, triglycerides, serum

Table 1. Number of participants (n) at various stages of **selection of the bone marker sample population** (Stage I to IV are in Table 1 of Ref. 4). The bone marker sample selected (Stage IV, n = 142) was further subjected to **exclusion of deficiencies** (Stage V and VI).

Exclusion at various stages for selection of Bone marker sample		Sample number at various phases of clinical exclusion		
		Total sample, Bone markers, n	**Total male, Bone markers, n**	**Total female, Bone markers, N**
Stage IV	Bone marker samples selected for this study after excluding Ferritin >250 ng/ml, Osteocalcin >35 ng/ml, Ostase >30 µg/l, NTX >200 nM BCE/mmol urine creatinine, Age 18 - 25 years.	Total = 142 Osteocalcin = 98 Ostase = 93 NTX = 100	Total = 40 Osteocalcin = 24 Ostase = 20 NTX = 22	Total = 102 Osteocalcin = 74 Ostase = 73 NTX = 78
Stage V	Samples after further exclusion of haemoglobin <125 g/l, iron <9.85 µmol/l and ferritin <20 ng/ml deficiencies.	Total = 65 Osteocalcin = 43 Ostase = 35 NTX = 42	Total = 35 Osteocalcin = 20 Ostase = 16 NTX = 18	Total = 30 Osteocalcin = 23 Ostase = 19 NTX = 24
Stage VI	Samples after further exclusion of haemoglobin <125 g/l, iron <9.85 µmol/l, ferritin <20 ng/ml and vitamin D <50 nmol/l deficiencies.	Total = 40 Osteocalcin = 31 Ostase = 29 NTX = 32	Total = 22 Osteocalcin = 18 Ostase = 15 NTX = 17	Total = 18 Osteocalcin = 13 Ostase = 14 NTX = 15

creatinine and hsCRP were within the specified cut off levels (Table 1 and Methods, in Ref. 3). After exclusion of all deficiencies, the total number of males in Table 1 reduced from 40 to 35 (Stage IV to V) and further decreased to 22 (Stage V to VI). The reduction in the number of female sample was much more marked than in males. Female sample number reduced from 102 to 30 (Stage IV to V) and further decreased to 18 (Stage V to VI).

Influence of deficiencies on the correlations of PTH with bone markers in male and female samples

In the male sample, before exclusion of deficiencies, there were number of bone marker related analytes that were correlating positively and negatively with PTH. In the female sample, only

Table 2. Pearson's correlation (r) and significance of correlation (P) of PTH with bone markers and related analytes in males and in females, **before exclusion of samples with deficiencies.** Parametric (Pearson's, r) method was used as at least one of the two correlating variables had Gaussian distribution after log_{10} transformation.

Bone markers and related analytes	Correlation coefficient, r	Significance of correlation, P	Correlation coefficient, r	Significance of correlation, P
	Male (n = 40)		Female (n = 102)	
Vitamin D	-0.311	0.051	0.166	0.095
Total Calcium	-0.476	0.002	-0.157	0.114
S. Phosphate	0.024	0.884	-0.027	0.784
Osteocalcin	0.374 (n =24)	0.072	0.325 (n = 74)	0.005
Ostase	-0.474 (n = 20)	0.035	-0.087 (n = 73)	0.466
ALP	-0.273	0.088	0.122	0.221
U. NTX	0.138 (n = 22)	0.540	0.038 (n = 78)	0.744
U. Calcium	-0.357 (n = 38)	0.028	-0.043 (n = 98)	0.675
U. Phosphate	-0.029 (n = 38)	0.862	0.161 (n = 93)	0.123

osteocalcin showed a significant positive correlation (Table 2). In the male sample, after exclusion of samples deficient in haemoglobin, iron and ferritin, PTH positively correlated with osteocalcin and negatively correlated with serum calcium, vitamin D and bone alkaline phosphatase (Table 3), indicating that when PTH was increased, bone resorption and turnover were higher, and serum calcium, vitamin D and bone formation were low. In the female sample, after exclusion of samples deficient in haemoglobin, iron and ferritin, there were no significant correlations with PTH.

In the female sample, after exclusion of all common deficiencies by excluding samples deficient in vitamin D, haemoglobin, iron and ferritin, there were no significant correlations. But in the male sample, PTH positively correlated with osteocalcin and negatively correlated with bone

Table 3. Pearson's correlation (r) and significance of correlation (P) of PTH with bone markers and related analytes in males and in females, **after exclusion of samples deficient** in hemoglobin (<125 g/l), iron (<9.85 µmol/l) and ferritin (<20 ng/ml). Parametric (Pearson's, r) method was used as at least one of the two correlating variables had Gaussian distribution after log_{10} transformation.

Variables	Correlation coefficient, r	Significance of correlation, P	Correlation coefficient, r	Significance of correlation,P
	Male (n = 35)		Female (n = 30)	
Vitamin D	-0.318	0.063	-0.214	0.256
Total Calcium	-0.459	0.005	-0.195	0.303
S. Phosphate	0.026	0.881	0.152	0.421
Osteocalcin	0.440 (n =20)	0.052	0.288 (n = 23)	0.182
Ostase	-0.457 (n = 16)	0.075	-0.173 (n = 19)	0.478
ALP	-0.232	0.179	0.077	0.686
U. NTX	0.261 (n = 18)	0.296	-0.125 (n = 24)	0.560
U. Calcium	-0.268 (n = 33)	0.131	-0.157 (n = 28)	0.424
U. Phosphate	-0.032 (n = 33)	0.858	0.150 (n = 26)	0.464

Table 4. Pearson's correlation (r) and significance of correlation (P) of PTH with bone markers and related analytes in males and in females, **after exclusion of samples deficient** in hemoglobin (anemia, <125 g/l), iron (<9.85 µmol/l), ferritin (<20 ng/ml) and vitamin D (<50 nmol/l). Parametric (Pearson's, r) method was used as at least one of the two correlating variables had Gaussian distribution after log_{10} transformation.

Variables	Correlation coefficient, r	Significance of correlation, P	Correlation coefficient, r	Significance of correlation, P
	Male (n = 22)		Female (n = 18)	
Vitamin D	-0.094	0.677	0.065	0.799
Total Calcium	-0.279	0.208	-0.133	0.599
S. Phosphate	-0.031	0.892	0.025	0.904
Osteocalcin	0.450 (n = 18)	0.061	0.041 (n = 13)	0.893
Ostase	-0.459 (n = 15)	0.085	-0.244 (n = 14)	0.400
ALP	-0.186	0.407	0.316	0.201
U. NTX	0.290 (n = 17)	0.259	-0.351 (n = 15)	0.200
U. Calcium	-0.153 (n = 20)	0.520	-0.186 (n = 16)	0.491
U. Phosphate	-0.018 (n = 20)	0.940	0.414 (n = 15)	0.125

alkaline phosphatase (Table 4), again indicating that in the male sample when PTH was increased, bone resorption and turnover were higher, and bone formation was low.

The above results were further confirmed by visual examination of the X-Y scatter plot of the correlating analytes after all exclusions including vitamin D deficiency (Fig. 1). The X-Y scatter confirmed the calculations of positive and negative correlations.

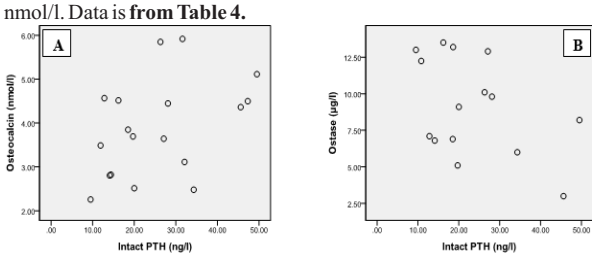

Fig. 1. X-Y scatter diagram of the correlations of PTH with osteocalcin, r = 0.450, P = 0.061 (A) and ostase, r = -0.459, P = 0.085 (B) **in males** of the sample population after excluding hemoglobin <12.5 g/l, ferritin <20 ng/ml, iron <9.85 µmol/l and vitamin D <50 nmol/l. Data is **from Table 4.**

DISCUSSIONS

Common causes of heterogeneity in a sample population are age and

gender. With reference to bone markers, heterogeneity of age can be partitioned into growth phase (<18 years), active young adults (18 to 25 years), adults between 25 and 50 years and above 50 years. All these age-groups are further partitioned into males and females. In this study, the bone marker reference group is considered as the active healthy young adult males and females. Heterogeneity in the composition of a sample can influence the correlations under healthy and disease states. It is important to study, as far as possible, the influence of a single disease or a deficiency state on a healthy, partitioned sample and avoid unknown multiple influences that may even be confounding. Healthy young adult population (18 to 25 years) may have bone markers higher than the less physically active age group of 25 to 50 years. This may help in analysing factors that influence the decrease in bone markers.

The common influences on the correlations of PTH with bone markers, after selection of the bone marker sample, are deficiencies in haemoglobin, iron, ferritin and vitamin D. The influence of these deficiencies on the gender differences were studied after partitioning into gender groups. As the deficiencies were found to influence the gender differences in the correlations of PTH with bone markers, it is important to analyse the correlations after excluding the deficiencies. PTH in the male sample correlated positively with osteocalcin (r = 0.450; P = 0.061; n = 18) and negatively with bone alkaline phosphatase (r = -0.459 ; P = 0.085; n = 15). There was no correlation in the female sample (Table 4). These results indicated that with reference to PTH, there was a strong difference in the bone metabolism in males and females. In males, as PTH increased bone, turnover increased but bone formation decreased. These differences may be due to increase in physical activity in males at young age which required increased bone turnover.

CONCLUSION:

Clinical conditions influence the reference levels of analytes and are excluded during calculation of the reference intervals. It was shown earlier that subclinical deficiencies also influence reference levels of analytes. In this study, it was shown that common deficiencies of haemoglobin, iron, ferritin and vitamin D influence the correlations of PTH with bone markers Exclusion of deficiencies related to PTH brought out the gender differences in the correlations of PTH with bone markers and related analytes, indicating gender differences in the metabolism of bone.

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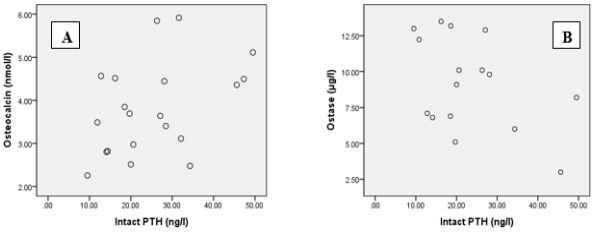

Fig. 2. X-Y scatter diagram of the correlations of PTH with osteocalcin, r = 0.440, P = 0.052 (A) and ostase, r = -0.457, P = 0.075 (B) **in males** of the sample population after excluding hemoglobin <12.5 g/l, ferritin <20 ng/ml and Iron <9.85 µmol/l. Data is **from Table 3.**

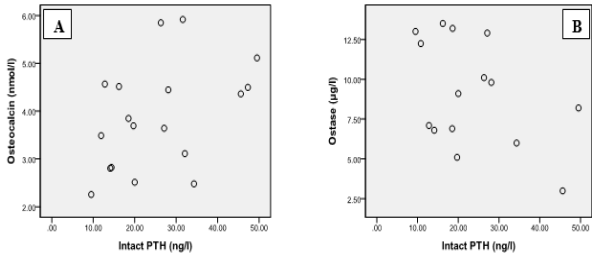

Fig. 3. X-Y scatter diagram of the correlations of PTH with osteocalcin, r = 0.450, P = 0.061 (A) and ostase, r = -0.459, P = 0.085 (B) **in males** of the sample population after excluding hemoglobin <12.5 g/l, ferritin <20 ng/ml, iron <9.85 µmol/l and vitamin D <50 nmol/l. Data is **from Table 4.**

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