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Research Paper

INFLUENCE OF COMMON DEFICIENCIES ON THE CONCENTRATIONS AND GENDER DIFFERENCES OF BONE MARKERS AND THEIR RELATED ANALYTES IN HEALTHY YOUNG ADULTS

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Abstract

Anaemia, with iron, ferritin and vitamin D deficiencies, common in healthy young adults, were found to influence the concentrations of a number of clinical biochemistry analytes. In this study, clinical and subclinical disease states were excluded clinically and by clinical biochemistry methods, respectively, to obtain a healthy bone marker sample (n = 142). During the process of clinical exclusions, the bone marker samples decreased markedly from 600 to 142, and further decreased after exclusion of deficiencies to 40. These deficiencies were more in the female participants. Exclusion of these deficiencies did not alter the concentration of any analyte to a significant level with P value <0.05. But closer examination revealed that mean of serum intact parathyroid hormone and urine NTX decreased, and urine phosphate increased after exclusion of deficiencies. The changes were more in the female sample and could be explained by the increased absorption of phosphate from intestine resulting from increased vitamin D, and by the decreased bone resorption due to decreased intact parathyroid hormone after exclusion of deficiencies. When gender differences in the analytes were evaluated, vitamin D, total calcium and osteocalcin were higher in males, while parathyroid hormone was higher in females. But after exclusion of deficiencies, the gender difference and their significance decreased. It may be concluded that subclinical deficiencies that were more common in female participants, influenced the concentrations of

bone makers and their related analytes, and were better seen by examining the gender differences in the concentrations of these analytes.
Key words: bone markers, parathyroid hormone, NTX, osteocalcin, ostase, vitamin D, anaemia, gender differences.

INTRODUCTION

Healthy adult bone is an actively metabolising tissue and requires continuous turnover to repair damages and replace old bone tissue. The remodeling for repair and for replacement requires three types of bone cells: the osteoclasts that cause bone resorption, followed by osteoblasts that lay down new bone at a site of resorption and osteocytes which support the metabolism of bone. Resorption of old bone is followed by bone formation and they are typically coupled. Bone remodeling is a regulated and localised activity that occurs at different sites known as bone remodeling units. Resorption phase of particular remodeling unit lasts 2 to 4 weeks which is followed by bone formation phase lasting 4 to 6 months. There will be different bone remodeling units at different phases at different locations. This phenomena results in a basal level of bone related markers in healthy young adults [1].

Common subclinical conditions increasing the synthesis, secretion and blood level of intact parathyroid hormone (PTH) are hypocalcemia, vitamin D deficiency, hypophosphatemia, anaemia and iron deficiency, in individuals who are otherwise healthy [1], [2], [3]. PTH regulates calcium and phosphate homeostasis directly through bone and kidney, and indirectly mediated through $1,25(OH)_2D$ or calcitriol by the intestine and bone [4], [5]. The actions of PTH on bone are complex, leading to both bone resorption and bone formation [4], [6].

NTX and CTX are N-terminal and C-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. PTH is directly related to erythropoietin, and inversely to haemoglobin and iron [2], [3].

After resorption ceases, stromal lining cells differentiate to osteoblasts. The development of the osteoblast phenotype has been divided into three consecutive phases, the proliferation, matrix maturation and mineralisation phases [8]. 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. Matrix Gla protein is produced in the second phase and osteocalcin in the third phase. The cytokines involved in regulation of bone resorption are formed in early first phase by the osteoblasts [8].

Earlier it was shown that subclinical deficiencies influence the concentrations and gender differences of analytes related to erythropoietin and PTH [2], [3]. In this study, clinical and subclinical disease states, and deficiencies were excluded to arrive at a reference sample population of healthy young adults aged eighteen to twenty five years. This sample, partitioned according to age and gender, is referred to as the bone marker sample. Concentrations and gender differences of these 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 was done in six stages for exclusion of clinical and subclinical disease states and deficiencies, and for partitioning the sample (Table 1; Stages I to VI). Volunteers 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, hypertension and alcoholism. 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 (Stage I).

Selection of Bone marker 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 (Stage I, Step 2). 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 >11mg/dl (2.75 mmol/l) (Stage II).

Participants aged 18 to 25 years were selected after excluding growth phase at <18 years and influence of age at >25 years (Stage III), 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). 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) (Stage IV and V), 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 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 analytes

Inter conversion between SI units and conventional units used in Tables 2-5 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; 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 (1µg/L / 0.1764 = nmol/L.

Assays and analytical control of assays

Immunochemistry autoanalyser Access 2 (Beckman Coulter, USA) and reagents were used for intact PTH, ostase (bone alkaline phosphatase) and ferritin assays using immunometric method with magnetic bead coated anti-PTH, anti-ostase 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 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 PTH 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 PTH was 9.5 ng/l and highest value was 80.5 ng/l for the data in this study. Limit of detection of PTH was taken as the lowest concentration distinguishable from zero (calibrator as 0 ng/l PTH) with 95% confidence was 1 ng/l (0.1 pmol/l) for PTH. It was also far below the lowest linear six point PTH calibrator value (eg. PTH: 10.7 ng/l). Examples each of the actual linear six point calibration values for PTH in ng/l from a particular lot of calibrators were 0, 10.7, 61.2, 303, 1467, 3369.5 [9].

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 variables had Gaussian distribution (before or after transformation) and when there was equality of variance in the groups compared, parametric methods (Student t test) of analysis were used. Otherwise, non parametric method (Mann Whitney U test) was used. Comparisons of gender differences in the sample were by 95% confidence interval of mean (95% CI), two-tailed Student's t test, Mann Whitney U test and maximum-minimum range (max-min) [14], [15].

Reference Intervals and cut off of analytes related to bone markers

Reference intervals used for the healthy limits in this study were PTH: 10 - 65 ng/L [17]; vitamin D: cut off level <20 ng/ml (<50nmol/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) [16]; ferritin: male 29 – 250 ng/ml, female 10 – 150 ng/ml [16], [17]. 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].

RESULTS

Selection of Bone marker reference sample population

Healthy young adult bone marker reference sample population was selected by clinical exclusion criteria, followed by cut off levels of quantitative biochemical analytes, to exclude subclinical disease states (Table 1). There was heavy reduction of sample number from approximately 600 to 142 (Male = 40, Female = 102) during the process of exclusion. Excluded males were more than females in stages I to IV. The range of BMI, waist circumference, serum fasting glucose, ALT, triglycerides, creatinine and hsCRP were within the specified clinical biochemistry cut off levels mentioned under methods and Ref. 3.

Influence of common deficiencies on the analytes in male and female participants

In the male sample, comparison of the concentrations of bone marker and their related analytes were done before and after clinical exclusion of samples deficient in haemoglobin, iron and ferritin. There were no changes in concentration of analytes (Table 2 A&B). This was followed by further exclusion of vitamin D deficient samples (Table 2C). The male sample number reduced from 40 to 35 (A to B) and further reduced to 22 (C), indicating that a number of samples had deficiencies. There were low statistical significances (P value) for the differences in the concentration of the bone markers and related analytes in the male samples before and after exclusions (Table 2B & C). But on closer examination, it was observed in the male sample that mean intact PTH decreased marginally from 26.7±11.6 to 24.6±11.9 while urine phosphate increased from 1.38±0.6 to 1.59±0.64. The explanation for this may be the higher vitamin D levels after exclusion increased absorption of phosphate by the intestine and decreased PTH levels resulting in increase of urine phosphate.

The same exercise was repeated in the female bone marker sample. Comparison of the concentrations of bone marker and their related analytes were done before and after clinical exclusion of samples deficient in haemoglobin, iron and ferritin (Table 3 A&B), followed by exclusion of vitamin D deficient samples (Table 3C). The reduction in the female sample number was much more marked than in males, reducing from 102 to 30 (A to B) and then to 18 (B to C). Statistical significances of the changes in means differences were low. But on closer examination, the mean intact PTH decreased marginally from 35.04±16.31 to 32.64±16.93, while urine NTX decreased from 72.91±38.66 to 64.50±30.99 and urine phosphate increased from 1.44±0.590 to 1.65±0.912. As above for males, the reasons for the changes in the female samples were due to higher vitamin D levels. NTX and PTH decreased due to increase in haemoglobin and vitamin D.

Influence of deficiencies on the gender difference of the analytes

Analysis of gender differences showed that in the male sample there were increased concentrations of vitamin D, total calcium and osteocalcin ($P \leq 0.001$) before exclusion of deficiencies (Table 4). Of these, increased vitamin D and total calcium indicate fewer samples with vitamin D deficiency in males when compared to females before exclusions. Higher osteocalcin may be due to increased physical activity in males. In the female sample, there were higher concentrations of PTH ($P < 0.003$) resulting from higher prevalence of anaemia, iron and vitamin D deficiencies.

To analyse the influence of deficiencies in haemoglobin, iron, ferritin and vitamin D, on gender differences in the bone maker samples, Student t test (or Mann Whitney U test) was done after exclusion of deficiencies (Table 5). The statistical significance of the gender difference were decreased: vitamin D ($P = 0.178$), total calcium ($P = 0.020$) and osteocalcin ($P = 0.015$) There were also border line increase in serum phosphate and urine NTX in the male sample, which was absent before exclusion. There was only borderline increase in PTH in the female sample ($P = 0.106$) may be due to lower haemoglobin levels. These results indicated that there were decrease in gender

differences in PTH, vitamin D, total calcium, osteocalcin and urinary calcium. There was also increase in gender difference of serum phosphate. The results indicated that the deficiencies influenced the gender difference of bone marker sample.

DISCUSSION

Vitamin D deficiency decreased the absorption of calcium and phosphate by the intestine, resulting in lower concentrations of serum calcium [1]. The decreasing circulating concentrations of calcium increased the secretion of PTH which in turn increased the release of calcium from bone by bone resorption, resulting in the maintenance of serum calcium. Similarly, erythropoietin senses hypoxia and iron deficiency, resulting in increased erythropoiesis and absorption iron by the intestine [18]. Erythropoiesis required bone resorption to create space for increased erythropoiesis in bone. Earlier this laboratory had shown that in a healthy sample population, there is a direct correlation between EPO and PTH [3], indicating that the role for PTH in erythropoiesis may be clinically detected. Anaemia and iron deficiency should increase PTH in circulating plasma.

Selecting a healthy adult young sample population for erythropoietin and intact PTH resulted in reduction of sample number [3]. In that study, samples were selected with reference to erythropoiesis. In this study, the sample population was selected with reference to bone markers and their related analytes. Here also, the exclusion criteria reduced the sample number markedly from 102 to 18 due to exclusion of deficiencies in vitamin D, haemoglobin, iron and ferritin was much more severe for the female sample (Table 1).

Subclinical deficiencies of vitamin D, haemoglobin, iron and ferritin increased the gender differences in the concentrations of erythropoietin and PTH of a reference PTH sample population [3] and excluding these deficiencies decreased the gender differences of these analytes. As the deficiencies were more in the female participants, PTH and EPO should come down more in the female sample to reduce the gender differences.

Therefore, in this study, the influence of exclusion of deficiencies on bone markers and related analytes were analysed. The male and female samples independently, did not show any significant change in the concentration after exclusion of deficiencies (Table 2 and 3). But on closer examination, in the male and in the female sample mean PTH decreased and urine phosphate increased after the exclusions. This could be explained by the increase in vitamin D, which decreased the concentration of PTH. Increase in vitamin D also increased the absorption of phosphate by the intestine resulting increased excretion of phosphate in urine. Though PTH is a phosphaturic hormone, the small decrease in PTH may not cause significant decrease in phosphate in urine.

NTX is a marker of bone resorption. Haemoglobin and iron deficiency increased bone resorption and bone turnover. It was anticipated that exclusion of haemoglobin and iron deficiencies (A to B) and exclusion of vitamin D deficiency (B to C) would decrease NTX successively from A to C. But in the male sample, the mean NTX did not decrease from A to B and to C (81.88±34.34 to 81.88±36.81 to 79.24±36.13) as the numbers of the deficient samples were few (Table 2). But in the female sample, there were large numbers of participants with deficiencies; The mean NTX decreased from A to B and to C (72.91±38.66 to 67.81±31.60 to 64.50±30.99) (Table 3). In these groups, the statistical significance of the decrease was relatively low with higher P value; may be due to low sample number. But the decrease in the mean could be explained biologically.

Gender differences in some of the bone markers and related analytes was markedly influenced by the common deficiencies in haemoglobin, iron, ferritin and vitamin D. Gender difference in vitamin D, PTH, total calcium and osteocalcin were absent or decreased after the exclusion. The influences of deficiencies were better seen in the gender differences of the concentrations of these analytes (Table 4 and 5).

Table 1. Number of participants (n) at various steps of implementation of clinical exclusion criteria for **selection of the Bone marker sample population and partitioning** of samples according to age and gender. The Bone marker sample selected (Stage IV, Step 5; n = 142) was further subjected to **exclusion of deficiencies** (Stage V and VI).

Clinical exclusions at various Stages (I to VI) and Steps (1 to 7) for selection of reference bone marker sample population			**Sample number at various phases of clinical exclusion**		
			Total, n	**Male, n**	**Female, n**
Stage I (1 to 2)	**Step 1.**	Participants before exclusion by **clinical history and examination**	>600	~300	~300
	2.	Participants after exclusion at step 1 and from whom **fasting blood and urine samples** were taken for assays (all age groups).	442	209	233
Stage II (2 to 3)	**3.**	Samples selected after exclusion of subclinical disease states by **Clinical Biochemistry evaluation**, and after exclusion of BMI ≥30 kg/m^2, WC ≥100 cm, F. glucose >126 mg/dl, ALT >60 U/l, TG >200 mg/dl, hsCRP >5 mg/L, creatinine <1.2 mg/dl.	319	125	194
Stage III (3 to 4)	**4.**	Participants after exclusion of growth phase <18 years and the influence of age >25 years (aged 18 to 25 years).	199	71	128
Stage IV	**5.**	**Bone marker samples** selected for this study after excluding ferritin >250 ng/ml,	**142**	**40**	**102**

(4 to 5)		osteocalcin >35 ng/ml, ostase >30 µg/l, NTX >200 nM BCE/mmol urine creatinine.			
Stage V (5 to 6)	**6.**	**Bone marker samples** (18 – 25 years) after excluding deficiencies: haemoglobin <125 g/l, iron <9.85 µmol/l and ferritin <20 ng/ml.	65	35	30
Stage VI (5 to 7)	**7.**	**Bone marker samples** (18 – 25 years) after excluding deficiencies: haemoglobin <125 g/l, iron <9.85 µmol/l, ferritin <20 ng/ml and vitamin D <50 nmol/l.	**40**	**22**	**18**

Table 2. Comparison of the concentrations of bone markers and their related analytes **before (A.) and after (B.) exclusion** of samples deficient in hemoglobin (<125 g/l), iron (<9.85 µmol/l) and ferritin (<20 ng/ml) in the **male samples**. There was further **exclusion of vitamin D deficient** (<50 nmol/l) samples **(C.)** from the samples in B. Comparisons were done by Student t test (parametric) or Mann Whitney U test (non parametric, in brackets), 95% CI of mean and by minimum-maximum range (min – max).

Male sample. Bone markers and related analytes.	**A. Mean±SD Min - Max 95% CI of mean (n = 40)**	**B. Mean±SD Min - Max 95% CI of mean (n = 35)**	**A&B Student t test (or Mann-Whitney U test), P**	**C. Mean±SD (n = 22)**	**B&C Student t test (or Mann-Whitney U test), P**
Age (years)	22.05±1.54 18 – 25	21.97±1.56 19 – 25	----	----	----
Intact PTH (ng/l)	26.72±11.61 9.50 – 59.90 23.01 – 30.44	26.02±11.65 9.50 – 59.90 22.02 – 30.02	0.765	**24.55±11.92**	0.559
Vitamin D (nmol/l)	76.48±46.80 12.46 – 187.20 61.52 – 91.45	76.61±48.98 12.46 – 187.20 59.77 – 93.44	0.917	101.37±45.3	**(0.019)**
Total Calcium (mmol/l)	2.51±0.122 2.20 – 2.75 2.47 – 2.54	2.51±0.118 2.20 – 2.75 2.47 – 2.55	0.754	2.52±0.115	0.955
S. Phosphate (mmol/l)	1.45±0.192 1.07 – 1.91 1.39 – 1.51	1.46±0.20 1.07 – 1.91 1.39 – 1.52	0.944	1.46±0.157	0.830
Osteocalcin (nmol/l)	3.89±1.05 2.26 – 5.92 3.45 – 4.33 (n = 24)	3.82±1.07 2.26 – 5.92 3.31 – 4.32 (n = 20)	0.800	3.88±1.11	0.865
Ostase (µg/l)	9.70±4.64 2.80 – 20.00 7.53 – 11.87 (n = 20)	9.19±3.22 3.00 – 13.50 7.47 – 10.91 (n = 16)	0.981	9.13±3.33	0.942

U. NTX nM BCE/mM creatinine)	81.88±34.34 24.27 – 145.60 65.20 – 95.66 (n = 22)	81.88±36.81 24.27 – 145.60 63.58 – 100.19 (n = 18)	0.955	79.24±36.13	0.845
U. Calcium mmol/mM creatinine	0.140±0.086 0.02 – 0.39 0.112 – 0.169 (n = 38)	0.148±0.087 0.03 – 0.39 0.117 – 0.179 (n = 33)	0.594	0.144±0.079	0.972
U. Phosphate mmol/mM creatinine	1.38±0.611 0.40 – 2.66 1.17 – 1.58 (n = 38)	1.41±0.636 0.40 – 2.66 1.19 – 1.64 (n = 33)	(0.778)	**1.59±0.640**	(0.331)

Table 3. Same as in Table 2 but in the **female sample.**

Female sample. Bone markers and related variables	**A. Mean±SD Min - Max 95% CI of mean (n = 102)**	**B. Mean±SD Min - Max 95% CI of mean (n = 30)**	**A&B Student t test (or Mann-Whitney U test), P**	**C. Mean±SD (n = 18)**	**B&C Student t test (or Mann-Whitney U test), P**
Age (years)	22.28±1.85	22.20±2.16 18.00 – 25.00	----	----	----
Intact PTH (ng/l)	35.04±16.31 10.40 – 80.50 31.83 – 38.24	34.41±16.88 10.40 – 66.30 28.11 – 40.71	0.688	**32.64±16.93**	0.725
Vitamin D (nmol/l)	51.03±25.12 13.20 - 125.1 46.09 – 55.96	61.60±27.11 13.20 – 117.56 51.48 – 71.73	0.057	78.52±20.46	**(0.027)**
Total Calcium (mmol/l)	2.42±0.128 2.13 – 2.70 2.39 – 2.44	2.42±0.134 2.15 – 2.70 2.37 – 2.47	0.804	2.43±0.101	(0.981)
S. Phosphate (mmol/l)	1.43±0.147 1.10- 1.78 1.40 – 1.46	1.43±0.147 1.10 – 1.74 1.37 – 1.48	0.930	1.39±0.158	0.296
Osteocalcin (nmol/l)	2.81±0.956 1.09 – 5.93 2.59 – 3.03 (n = 74)	2.95±1.29 1.09 – 5.93 2.39 – 3.50 (n = 23)	0.912	2.89±1.39	0.855
Ostase (µg/l)	9.43±3.88 1.80 – 18.00 8.53 – 10.34 (n = 73)	8.79±3.65 2.00 – 15.51 7.03 – 10.55 (n = 19)	(0.524)	8.84±4.17	0.905
U. NTX (nM BCE/mM creatinine)	72.91±38.66 21.67 – 190.0 64.19 – 81.63 (n = 78)	67.81±31.60 26.75 – 143.02 54.47 – 81.16 (n = 24)	0.718	**64.50±30.99**	0.747

U. Calcium (mmol/mM Creatinine)	0.135±0.217 0.01 – 2.02 0.092 – 0.179 (n = 98)	0.135±0.127 0.01 – 0.54 0.086 – 0.185 (n = 28)	0.605	0.139±0.134	0.975
U. Phosphate (mmol/mM Creatinine)	1.44±0.590 0.31 – 4.08 1.31 – 1.56 (n = 93)	1.51±0.735 0.37 – 4.08 1.22 – 1.81 (n = 26)	(0.807)	**1.65±0.912**	0.762

Table 4. Comparison of **gender differences** in the concentrations of bone markers and their related analytes **before exclusion of deficiencies** by 95% CI of mean, Student t test (parametric) or Mann Whitney U test (non parametric, in brackets).

Bone markers and related variables	**95% CI of mean**		**Student t test (Mann Whitney U test) of males and females, P**
	Males (40)	**Females (102)**	
Intact PTH (ng/l)	23.01 – 30.44	31.83 – 38.24	**0.003**
Vitamin D (nmol/l)	61.52 – 91.45	46.09 – 55.96	**0.001**
Total Calcium (mmol/l)	2.47 – 2.54	2.39 – 2.44	**<0.001**
S. Phosphate (mmol/l)	1.39 – 1.51	1.40 – 1.46	0.652
Osteocalcin (nmol/l)	3.45 – 4.33 (n = 24)	2.59 – 3.03 (n = 74)	**<0.001**
Ostase (µg/l)	7.53 – 11.87 (n = 20)	8.53 – 10.34 (n = 73)	(0.963)
U. NTX (nM BCE/mM creatinine)	65.20 – 95.66 (n = 22)	64.19 – 81.63 (n =78)	0.259
U. Calcium (mmol/mM Creatinine)	0.112 – 0.169 (n = 38)	0.092 – 0.179 (n = 98)	0.088
U. Phosphate (mmol/mM Creatinine)	1.17 – 1.58 (n = 38)	1.31 – 1.56 (n = 93)	(0.784)

Table 5. Comparison of **gender differences** as in Table 4 but **after exclusion** of deficiencies in hemoglobin (<125 g/l), iron (<9.85 µmol/l), ferritin (<20 ng/ml) and vitamin D (<50 nmol/l) from the bone marker sample.

PTH and its related variables	95% CI of mean		Student t test (Mann-Whitney U test) of males and females, P
	Male (n = 22)	Female (n = 18)	
Intact PTH (ng/l)	19.26 – 29.84	24.2 2– 41.06	0.106
Vitamin D (nmol/l)	81.28 – 121.47	68.3 4– 88.7	(0.178)
Total Calcium (mmol/l)	2.47– 2.57	2.38 – 2.48	**(0.020)**
S. Phosphate (mmol/l)	1.39 – 1.53	1.31 – 1.47	0.145
Osteocalcin (nmol/l)	3.33 – 4.44 (n = 17)	2.05 – 3.73 (n = 13)	**0.015**
Ostase (µg/l)	7.29 – 10.97 (n = 14)	6.43 – 11.24 (n = 14)	0.650
U. NTX (nM BCE/mM creatinine)	60.66 – 97.81 (n = 16)	47.34 – 81.66 (n = 15)	0.221
U. Calcium (mmol/mM Creatinine)	0.107 – 0.181 (n = 19)	0.068 – 0.210 (n = 16)	0.337
U. Phosphate (mmol/mM Creatinine)	1.29 – 1.89 (n = 19)	1.15 – 2.16 (n = 15)	(0.714)

CONCLUSION

Anaemia and vitamin D deficiencies increased erythropoiesis and bone resorption which may result in alterations in bone markers. Due to stringent exclusion criteria, sample size decreased markedly during the bone marker participant selection from 600 to 142, and further decreased after exclusion of deficiencies to 40. Deficiencies were more in the female participants. Exclusion of deficiencies did not alter the mean of any analyte to a significant level ($P \leq 0.05$). But closer examination revealed that mean serum PTH and urine NTX decreased, and urine phosphate increased. The changes were more in the female sample due to larger number of deficient samples in females. When gender differences in the analytes were evaluated, vitamin D, total calcium and osteocalcin were higher in male participants, while PTH was higher in females. But after exclusion of deficiencies, there were decrease in the gender differences and in their statistical significance. It may be concluded that subclincal deficiencies influence the concentrations of bone makers and their related analytes and were better seen in the gender differences of these analytes.

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